IOBC / WPRS

Working group "Insect Pathogens and Insect Parasitic Nematodes"

and

COST Action 862
"Bacterial Toxins for Insect Control"

12th MEETING

"FUTURE RESEARCH AND DEVELOPMENT IN THE USE OF MICROBIAL AGENTS AND NEMATODES FOR BIOLOGICAL INSECT CONTROL"

at

Pamplona (Spain)

22 – 25 June, 2009

Editors:

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Preface

Welcome to the 12th meeting of the IOBC/WPRS Working Group "Insect Pathogens and Insect Parasitic Nematodes" held at the University of Pamplona in Navarra, Spain, on 22 to 25 of June, 2009. Pamplona is the city of the famous San Fermin Festival and Bull Running. Furious bulls are released into the city roads and young man with red hankerchiefs around their necks pray to San Fermin and then run in front of the bulls. The route is defined and ends at the "Plaza de Torros". I hope that our route in using pathogens for insect control is not defined by some bulls chasing us down the road. Instead we should take the lead. Therefore, the title of this meeting is: **"Future research and development in the use of microbial agents and nematodes for biological insect control".**

I am sure you all agree that the potential of insect pathogens is not well exploited in the region of the WPRS. One of the reasons is the lack of research funds and support for SMEs (small and medium-sized enterprises). Another is that many of us work isolated and we have little possibilities to obtain funds for international cooperate. Those of you that have once experienced the cooperation within any of the European Framework Programmes know how beneficial and fruitful such a cooperative research can be. However, in the past we all had difficulties to identify our R&D goals in the calls of the European Commission DG Research. Given the urgent need for support for Europe-wide research cooperation, the 12th meeting will particularly gather and discuss future trends in insect pathogens and insect parasitic nematodes with the objective to produce a "white paper" to be submitted to the EU Commission, DG Research, for implementation in the forthcoming calls. With this aim, the two plenary sessions will present the ideas of academia and the private sector, which will be further elaborated in workshops to produce the official working group position paper. I hope for a broad support of this activity, which should not stop after the termination of the meeting.

This Bulletin, Volume 45, was prepared to be handed out at the meeting. Thanks to the support with the review of the manuscripts by the subgroup convenors and by Neil Crickmore (papers on bacteria) and in particular thanks to the support by Horst Bathon for the final review and paging and to Primitivo Caballero for organizing the printing in time. Asking for 4-page manuscripts already for the submission of contributions has significantly increased the quality of the Bulletin and saved us a lot of money.

This meeting is attended by more than 150 colleagues and we have almost the same number of contributions. It gathered all 5 subgroups of the working group. The meeting is held together with the COST Action 862 "Bacterial Toxins for Insect Control" chaired by Neil Crickmore (www.cost862.com). I like to thank Neil for his support and COST for makeing available travel support for participants. I am confident to be able to continue our joint meetings also after the termination of COST Action 862 in 2010.

I also like to thank our sponsors for support, which enabled us to invite experts for the plenary sessions and the IOBC/WPRS to make available travel support to several young scientists. The meeting was organized by Prof. Primitivo Caballero and his team to whom I like to express my special gratitude for the excellent organisation and their hospitality. Muchisimas gracias.

Ralf-Udo Ehlers

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Biological control of plant diseases: Future research goals to make it successful.

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Introduction

In this beginning of the XXI century, there is a need to increase the agricultural production for both food and energy and at the same time decrease the use of fertilizers and pesticides from chemical origin. In this context there is a renewed interest for alternative methods of control. This expression "alternative control methods", which means alternative to chemical methods, covers a lot of different approaches based on agricultural practices, use of "natural products", and beneficial organisms. In this presentation I will only consider biological control. Many different definitions of biological control have been proposed. Eilenberg (2006) defined "biological control as the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be". It was clearly stated that the term "pest applies for insect, mites and vertebrate pests, plant diseases, and weeds". In their book, "The nature and practice of biological control of plant pathogens" Cook and Baker (1983) reviewed the different components of biological control of plant diseases. The organisms which can be used to achieve biological control include (i) avirulent or hypo-virulent individuals or populations within the pathogenic species, (2) antagonistic micro-organisms, and (3) the host-plant manipulated toward greater or more effective resistance to the pathogen. In this presentation I will mainly focus on "microbiological control" of plant diseases, based on the use of living populations of non pathogenic or antagonistic micro organisms. Indeed, the main difference between biological control and other control methods is the use of living populations of beneficial organisms, having several modes of action, involving not only interactions with the target pathogen, but also interactions with the rest of the microbiota and the plant.

Despite the increasing number of scientific papers dealing with biological control, there are still a very limited number of products on the market. In the European Union, only a limited number of micro organisms have been included on Annex I of the directive 91/414. Most of them are bacteria and fungi targeting insects, there are only a few preparations targeting diseases. One can cite a strain of *Coniothyrium minitans* parasitizing the sclerotia of *Sclerotinia* spp., a strain of *Gliocladium catenulatum* targeting various soil-borne fungi, and several strains of *Trichoderma* spp. A few bacteria are also listed on Annex I, a strain of *Bacillus subtilis* aiming at controlling mostly aerial diseases and a strain of *Pseudomonas chlororaphis* for seed treatment against soil-borne pathogens of wheat and barley. It is therefore interesting to review progress and failures in biological control research, to identify the bottle necks which prevent faster success in application of microbial control. One can distinguish several domains in which research is needed: basic research aiming at a better understanding of the modes of action of the biological control agents, technological research aiming at improving the processes of production, formulation and application of biological control agents and also applied research in order to satisfy requirements of the regulation and finally much more field experiments to integrate biological control practices in cropping systems.

Identification of the biological control agent

Many people are complaining about the Directive 91/414, which imposes strong constraints. However, it provides a good framework to write the research plan needed to develop a biological control agent. The first requirement is an accurate identification of the biological control agent. In many cases the identification of a strain at the species level is not easy. Today, in complement to the traditional methods based on morphology, molecular tools are available to place the biological control strains in a phylogenetic tree among strains of known species. It is then useful to develop a method enabling to identify the biocontrol strain itself among other strains belonging to the same species. This is necessary for regulation procedures, and also to track the strain after release in the environment. Research in molecular biology will provide new tools to achieve this goal of perfect identification of biological control agents at the strain level.

Modes of action of the biological control agent

The second requirement is the study of the modes of action of the biological control agent. This is not an easy task even if the strain belongs to a well studied species. As stated above there are always several modes of action based on parasitism, antibiosis and competition. The secondary metabolites potentially of concern have to be identified and their toxicity has to be studied as it is required for a chemical pesticide. This point is one of the most controversial since micro-organisms are able to produce many different secondary metabolites which properties are not known. Moreover the production of these secondary metabolites depends on many factors such as the age of the culture, the growth medium or the plant organ on which the biological control agent is applied. It is quite impossible to predict, which metabolite will be produced, in which quantity and it is economically not possible to analyze all the metabolites present in a culture at trace levels. Thus the production of secondary metabolites is a domain in which research should be developed. Research is also needed in connection to the method of production of the active substance and on quality control. These important aspects are too often neglected. Working with living organisms it is important to develop processes enabling to grow the biological control agent in pure culture without contaminants, to formulate it to ensure a sufficient shelf life and finally to get a commercial product having the requested efficacy. This is necessary to develop quality control procedures and, in most cases, bio-assays have to be designed for this purpose. Specific research efforts should be made in that field.

Effects on human health

To satisfy the regulation requirements, effects on human health have to be studied. In that domain, research is needed to develop methods adapted to micro organisms. Most of the recommended protocols developed for chemical molecules can not be used to study toxicity of micro organisms. For example, all biological control agents are classified as potentially sensitizers since there is no proper method available to test for this risk; similarly, the Ames test aiming at studying the mutagenic activity is not adapted to micro organisms.

In relation to residues, the biological control agent itself has to be considered as the main residue. But again the question rises when the micro-organism produces secondary metabolites that are susceptible to be toxic. As stated above there is a need of research to develop easy procedures to determine if secondary metabolites are produced in situ, and more

generally to propose methods adapted to the study of microbials and addressing important questions in relation to human health.

Behaviour in the environment and effect on non target species

Study of fate and behaviour of the plant protection product in the environment poses quite different questions whether the plant protection product is a chemical or a living micro-organism. There is an unjustified fear that an introduced micro-organism can multiply in the environment and become a pest. This fear is not justified by facts. In the absence of any selection pressure and introduced bacteria or fungus originating from the natural environment will not become dominant when reintroduced in the same environment. To study the behaviour of a BCA in the environment, one must be able to distinguish the introduced strain from the naturally occurring strains belonging to the same species. Thus a molecular marker such as a SCAR which could have been developed to identify the biological control agent at the strain level is required. This type of procedure is time consuming, but we do have the methodology to address this question.

Finally it is also required to study the effect of the biological control agent on the non target organisms. Again the methodology developed to study the non target effects of chemical pesticides is not adapted to microbials. Considering that most of the actual methods were developed by IOBC working groups, should we ask IOBC colleagues to work together to propose methods adapted to test the non target effects of microbials?

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Fungal-based bio-pesticides, multiple solutions for Integrated Pest Management

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Abstract: There are already various examples of fungi used as biopesticides; however, an important number of possibilities are still to be explored. Biodiversity of fungi can provide multiple solutions for pest, disease and also weed control in agriculture, as soon as cooperative research between scientists and biotech companies can take place, for strain isolation and screening. Industries have made significant progress to improve manufacturing processes, resulting in better shelf life, improved efficacy and scale economies. On the other hand, Member State Policies in Europe aim to reduce the load of chemicals in agriculture (France: ECOPHYTO 2018), which represent nice opportunities for biocontrol products in general, and more particularly for fungal-based ones, at least in specialty crops (vine, fruits, vegetables and ornamentals). However, the cost of fungal biopesticides EU registration is relatively high compared to their market size and the general requirements of EU Directive 91/414 are not adapted to the registration of microbials. At this stage of the industry, the companies cannot afford preparing several dossiers at the same time and have to be very selective on resource allocation. Yet, it is possible for the Member States to support the development of such products by funding research projects in biocontrol, guaranteeing accelerated registration procedures and reduced fees. The adoption of REBECA conclusions (www.rebeca-net.de) by the EU Commission would certainly change the whole picture.

Key words: Fungi, biocontrol, *Beauveria*, *Sclerotinia*, *Paecilomyces*, *Metarhizium*, fermentation

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Insect pathogenic fungi in biological control: Status and future challenges

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Abstract: In Europe, insect pathogenic fungi have in decades played a significant role in biological control of insects. With respect to the different strategies of biological control and with respects to the different genera of insect pathogenic fungi, the success and potential vary, however. Classical biological control seem to have little indication of potential, whereas inundation and inoculation of fungi for biological control has several success stories, e.g. within the genera *Metarhizium, Beauveria, Isaria/Paecilomyces* and *Lecanicillium* (previously *Verticillium*). However, the genotypes employed seem to include a narrow spectrum of the many potentially useful genotypes. Conservation biological control with species of the genera *Pandora* and *Entomophthora* have a strong potential, but also *Beauveria* has a potential to be explored further. The main bottleneck for further exploitation of insect pathogenic fungi in biological control is the limited knowledge of host pathogen interaction at the fungal genotype level.

Key words: Insect pathogenic fungi, Hypocreales, Entomophthorales, biological control

Biological control strategies

Biological control includes four complementary strategies: 1) Classical biological control, 2) Inundation biological control, 3) Inoculation biological control and 4) Conservation biological control (Eilenberg *et al.*, 2001). Despite their complementarity, inundation and inoculation are often treated simultaneously (sometimes called 'augmentation'). Here, we treat them together. Below we present an overview of the status of insect pathogenic fungi in these strategies with particular emphasis of new discoveries of relevance for conservation biological control.

Classical biological control

The release of exotic fungal species or strains for insect pest control has never received much attention in Europe. A study in Iceland on Green spruce aphid (*Sitobion avenae*) and its fungal pathogens from Entomophthorales documented that release of the pathogens (*Entomophthora planchoniana* and *Pandora neoaphidis*) could be worthwhile (Nielsen *et al.*, 2001). However, the observational studies have so far not been accompanied by experimental work to prove the hypothesis that a long lasting control can be accieved by a single release of these fungi at selected places.

Inundation and inoculation biological control

Since decades, fungi from Hypocreales have been marketed for biological control of pest insects in Europe. One major bottleneck is the registration. This aspects will be covered by Hermann Strasser (ibid). A second bottleneck is studies to prove efficacy of different

formulations. A third bottleneck is the reluctance of companies to develop and market products for use. European agriculture and forestry is characterized by a variety of crops and climatic conditions, resulting in a variety of pest insects of importance. Thus, the potential of success of a product can be hampered by the small market (regional or even local problems only). The success of fungi in glasshouses can partly be attributed to the limited number of major vegetable crops being common throughout Europe (above all tomatoes and cucumber) and the uniform set of pest insects to be controlled. A fourth major bottleneck is the limited knowledge about ecology of the used fungi. Major improvement have, however, occurred recently by the emplyoment of specific molecular methods to determine host pathogen interaction at the genotype level (Schwarzenbach *et al.,* 2007; Meyling *et al.,* 2009). Such studies will both ease the possibility to study fate and effect of released fungi as well as they will allow us to evaluate the potential for conservation biological control.

Conservation biological control

Conservation biological control have received increasing interest over the last years. Insect pathogenic fungi offer a high potential for usage as part of a conservation strategy. Also, conservation biological control goes well in hand with the recent attempt to develop 'low input' or 'organic' agriculture. The current knowledge of the potential of insect pathogenic fungi is limited. However, recent approaches have given significantly novel insight, for example studies on a) genotype characterization from natural field infections (Jensen *et al.*; 2006, Meyling *et al.,* 2009), b) transmission of disease between different hosts (Baverstock *et al*., 2008b; Jensen *et al*., 2006) as well as c) interactions between fungi, insects and enviroment (Baverstock *et al*.; 2008a; Roy and Cottrell, 2008), also with the inclusion of specific molecular methods of both host and pathogen populations (Jensen *et al*.; 2008, Fournier *et al.,* 2008). A summary of known knowledge and potential of three species is listed in table 1.

Table 1: Comparison of characters of three fungal species with reference to conservation biological control

Fungus species	**Host range at fungus genotype level**	**Suggested conservation strategy**	**References**
Beauveria bassiana sensu lato	Broad (each genotype can naturally infect speciea from several insect orders)	Establish hedges and other semi-natural habitats to enhance genetic diversity of fungus	Meyling *et al.,* 2007; Meyling *et al*., 2009
Pandora neoaphidis sensu lato	Semi-narrow (each genotype can naturally infect several aphid species)	Establish habitats for alternative aphid hosts for population build-up of the fungus	Ekesi *et al.,* 2005, Enkerli *et al.,* 2008 Baverstock *et al*., 2008a
Entomophthora muscae sensu lato	Narrow (each genotype can naturally infect only one host species)	Establish possibilities for the fungus to develop in host population	Jensen *et al.,* 2006

Conclusion

In summary we can characterize the potential of the main insect pathogenic fungal genera as presented in Table 2. We recommend future research projects to include studies at species and genotype level and also studies which include elucidation of several biological control strategies. Through such approaches we may get more benefit from insect pathogenic fungi.

Table 2: Main insect pathogenic fungal genera with potential for biological control in the EU

Fungus genus or species	**Hosts**	**Main environment**	**Inundation/ inoculation**	**Conservation**
Metarhizium	Various arthropods, including weevils	Soil, insects and lower parts of vegetation	Products in EU for a number of years	Limited documented potential
Beauveria	Various arthropods, including scarabs	Soil, insects and vegetation	Products in EU for a number of years	Studies indicate strong potential
Isaria/ Paecilomyces	Various arthropods and nematodes	Soil, insects and vegetation	Products in EU for a number of years	Limited documented potential
Lecanicillum (previously *Verticillium*)	Aphids and whiteflies	Insects and vegetation	Products in EU for a number of years	Limited documented potential
Aschersonia Aleyrodis	Whiteflies	Insects and vegetation	Product in EU has disappeared	No documented potential
Pandora neoaphidis	Aphids	Insects, vegetation, top soil layer	No product have been marketed	Studies indicate strong potential
Entomophthora muscae s.l	Flies, including root flies	Insects, vegetation, top soil layer	No product have been marketed	Studies indicate strong potential

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Insecticidal proteins from *Bacillus thuringiensis*: their use in conventional and transgenic plant biotechnology

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Abstract: The protection of plants against the attack of insects is an old problem that has been partly alleviated by the introduction of conventional resistance genes into crop plants and partly solved by the use of chemical insecticides for about half a decade. However, with the discovery of the insecticidal proteins from *Bacillus thuringiensis* and the introduction of their genes into plants (the so-called Bt-crops) we are witnessing a revolution in the field of plant protection. With the current trend of reduction of chemical insecticides and the growing demand of organic food, it is anticipated that use of *B. thuringiensis*-based insecticides will grow. Bt-crops are a different alternative to the use of chemical insecticides in plant protection; the global area planted to Bt-crops is steadily growing and this trend is expected to continue in future years. The main threat to the long term efficacy of this type of products (both sprays or transgenic crops) is the emergence of resistance in pest populations. The use of strategies for resistance management is therefore crucial to preserve this technology.

Key words: Bioinsecticides, Bt-crops, transgenic plants, mode of action, insect resistance

Introduction

Some insects have become pests since the very beginning of plant domestication. Traditionally, various cultural procedures have been introduced that tried to limit the damage produced by insects, but their success has been relative. The discovery and use of synthetic chemical insecticides was a crucial step in plant protection which contributed to the development of modern agriculture and allowed to reach the current levels of food production.

The extensive use of these products led to emergence of resistant insect populations, forcing the development of new types of synthetic insecticides to replace those that became ineffective. In addition, increased tolerance to insecticides has led to an intensive use of them, with an increase in both the frequency of applications and the dose. The abuse of these treatments has produced a strong impact on ecosystems and, in some cases, even on human health.

Awareness towards these issues has led to the reduction or elimination of certain pesticides with serious side effects and has led to the increased use of bioinsecticides. They are more friendly to the environment because they do not accumulate in food chains and are harmless to humans, wildlife and beneficial insects. Furthermore, they have become the only alternative in the cases of resistance to chemical insecticides, because their mechanism of action is different from conventional insecticides. For these and other reasons the biopesticide market is growing and it is expected to do so in the coming years, though their market share is still very low (Fig. 1). Among bioinsecticides, bacterial biopesticides claim about 74% of the market, and the vast majority are based on different strains of the bacterium *Bacillus thuringiensis.* The demand for chemical insecticides has been declining mainly due to general environmental and health concerns. However, their market is not so much replaced by bioinsecticide sales, but mainly by the rapid adoption of Bt-crops.

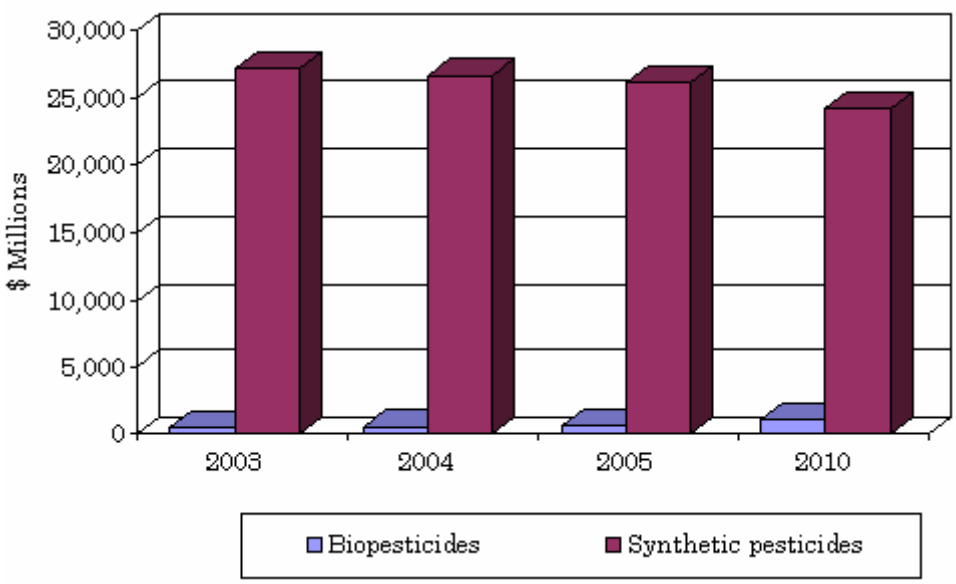

Figure 1. Estimated and predicted global market for biopesticides and synthetic pesticides (after Thakore, 2006, http://www.bccresearch.com/report/CHM029B.html).

Bacillus thuringiensis as a source of insecticidal compounds

B. thuringiensis insecticidal properties were described for the first time in the early twentieth century, first in Japan (1901) and then in Germany (1911), as infectious agents isolated from insect larvae which cause them a quick death (Caballero and Ferré, 2001). *B. thuringiensis* is found distributed in almost any habitat. *B. thuringiensis*, like other species of the genus *Bacillus*, can produce large amounts of virulent factors, both in the vegetative phase and in the stationary phase, including phospholipases, proteinases, chitinases, Vip proteins, δ-endotoxins, β-exotoxins, etc. However, each strain can only produce some of these compounds and different strains produce different combinations of them. A common feature to all *B. thuringiensis* strains is that they produce δ-endotoxins during sporulation which are accumulated in proteinaceous crystals, the so called insecticidal crystal proteins (ICPS) or Cry proteins. Each ICP has a different sequence and has a characteristic spectrum of insecticidal activity (Schnepf et al., 1998). ICPS and Vip proteins are classified based on their phylogenetic relationships with respect to their amino acid sequence (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt). In the insecticidal strains, it is mainly the proteins in the crystal that is responsible for the insecticidal properties. However, depending of the preparation method, the commercial formulations can contain other additional insecticidal compounds that are secreted to the culture media during the vegetative growth.

B. thuringiensis genes expressed in plants: "Bt-crops"

The most innovative and successful use of Cry proteins for crop protection has been their expression in transgenic plants by introduction of their genes into the plant genome (Nester et al., 2002). In this way the plants are protected against the major pests and the number of insecticidal treatments needed is reduced substantially, which are thus limited to control other insects or diseases for which the plant is not protected. Bt-crops are especially effective against borer insects, difficult to control by non-systemic insecticides. They solve the problem

of the general low persistence of Cry proteins since these are synthesized during all or nearly all the life cycle of the plant and remain protected from environmental adverse conditions (UV radiation, rain, degradation by bacteria, etc.).

Bt-crops are the most widely planted GM crops after those with herbicide tolerance. In 2008, the global area of transgenic crops for commercial planting was 125 million hectares, of which 79 were planted to herbicide tolerance crops, 19.1 to crops containting only the insect resistance trait, and 26.9 to crops combining insect resitance with herbicide tolerance (James, 2008) (Fig. 2). The largest proportion of Bt-crops is planted in the U.S. Bt-maize is the insect resistant largest crop planted worldwide, followed by Bt-cotton. In the European Union only Bt-maize is allowed for commercial planting and Spain has the largest planted area, with almost 80,000 ha planted in 2008.

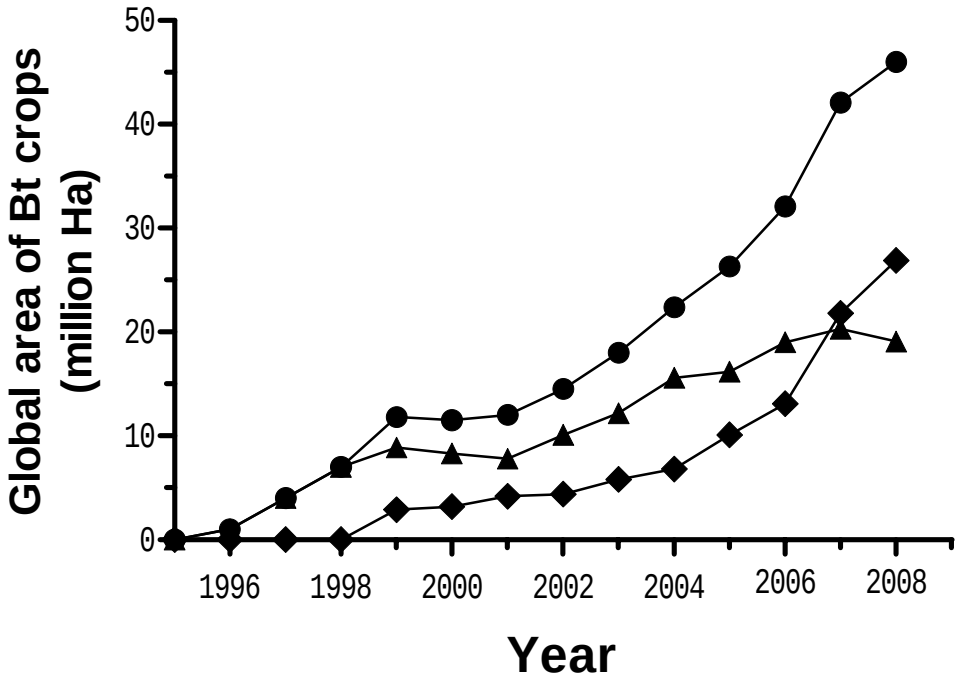

Figure 2. Area planted to Bt-crops. ●, Total area of insect resistance crops; ▲, only with the insect resistance trait; ◆, the insect resistance trait combined with herbicide tolerance (compiled from James, 2008).

Maize has been transformed with *B. thuringiensis* genes that encode proteins active against their major pests. Thus, Bt-maize has been transformed with the genes *cry1Ab*, *cry1Ac* or *cry9C* to confer protection from corn borers *Ostrinia nubilalis* and *Sesamia nonagriodes*, with the *cry1F* gene to confer protection against *Spodoptera frugiperda*, and with the *cry3Bb* gene to confer protection from rootworms of the genus *Diabrotica*. Crops in the pipeline against *Diabrotica* express the binary toxin Cry34Ab/Cry35Ab or a modified Cry3A protein.

Most of the Bt-cotton which is grown commercially is Cry1Ac cotton. Cry1Ac is highly active against Lepidoptera larvae that attack the cotton. *cry2Ab* and *cry1Ac* genes have been combined in the plant, resulting in what is known as second generation of Bt-cotton, which is the only type of Bt-cotton that is planted in Australia and has also been adopted in the the U.S. Another combination of genes that has been recently marketed combines the *cry1F* and

cry1Ac genes, and confers additional protection against *Spodoptera* spp. The current trend is to combine different traits (i.e., herbicide tolerance and insect resistance) as well as different genes within each trait. Stacked trait plants are under development which combine several *cry* genes, or *vip* and *cry* genes. The reason for combining two toxins is not only to widen the spectrum of action, but also for the purpose of delaying the evolution of resistance.

Table 1. Insect species that have developed resistance in the laboratory (except *P. xylostella*, that has developed resistance in the field) to Cry proteins from *B. thuringiensis* and the year in which the first report was published (Ferré and Van Rie, 2002; Ferré et al, 2008).

Insect species	Order	Year
Plodia interpunctella	Lepidoptera	1985
Heliothis virescens	Lepidoptera	1989
Plutella xylostella	Lepidoptera	1990
Trichoplusia ni[1]	Lepidoptera	1994
Spodoptera exigua	Lepidoptera	1995
Leptinotarsa decemlineata	Coleoptera	1995
Spodoptera littoralis	Lepidoptera	1997
Culex quinquefasciatus	Diptera	1997
Chrysomela scripta	Coleoptera	1998
Ostrinia nubilalis	Lepidoptera	1999
Pectinophora gossypiella	Lepidoptera	1999
Helicoverpa zea	Lepidoptera	1999
Helicoverpa armigera	Lepidoptera	2000
Sesamia nonagrioides	Lepidoptera	2004
Diatraea saccharalis	Lepidoptera	2007

[1] The first report on resistance to *T. ni* was under laboratory selection. Resistance in greenhouses was published in 2003.

Risk of insect resistance to *B. thuringiensis* toxins

The major threat to the long term efficacy of *B. thuringiensis*-based insecticides and to Bt-crops is the emergence of resistance to *B. thuringiensis* insecticidal proteins in insect populations. The potential of insecticide resistance development by the insects is very high, as it has been widely corroborated with conventional insecticides. *B. thuringiensis*-based bioinsecticides have been used for more than 40 years without the emergence of resistance, except for some field populations of *Plutella xylostella* and greenhouse populations of *Trichoplusia ni*, both which had been continuously exposed to crystal/spore formulations. Although these are the only species that has developed resistance to Cry proteins outside the laboratory, artificial laboratory selection has shown that all insect species have the potential to develop resistance (Table 1). The large-

scale use of Bt crops and the increased adoption of *B. thuringiensis*-based insecticides will accelerate the emergence of resistance to Cry proteins in field populations (Tabashnik, 2009).

The study of resistant populations has revealed that the most common mechanism of resistance is the modification of the binding to target sites in the membrane (Ferré and Van Rie, 2002; Ferré et al., 2008). Insects with an alteration in a binding site may become resistant to one or a closely related set of proteins that bind to this site and still be highly susceptible to other proteins which bind to other sites. This is an important concept, because Cry proteins that do not share binding sites can be considered insecticidal agents with different modes of action.

Possibly, the use of strategies to prevent resistance to *B. thuringiensis* toxins has been the major reason why resistance to Bt crops has not been detected yet, although some recent reports have indicated that resistance may be building in some populations (Tabashnik, 2009). Among the various strategies that have been proposed, the most widely adopted one combines the expression of high doses of toxin in the transgenic plants with the use of refuges with non-transformed plants. This strategy is the one recommended by the U.S. EPA and the USDA and has become mandatory in the U.S. (http://www.epa.gov/pesticides/biopesticides/).

A second strategy to delay insect resistance is to combine the use of two *B. thuringiensis* toxins effective against the same pest and with different modes of action. Variants of this second strategy may include the temporary rotation of crops expressing different toxins or the use of a crop variety that simultaneously expresses two different toxins. The combination of the high dose/refuge strategy with the second generation Bt crops combining two or more toxins, would provide greater protection against insect resistance and will contribute to the long term use of this technology which can bring many benefits to farmers, consumers and the environment.

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The promise, practice and prospects of baculoviruses in biocontrol

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Abstract: Baculoviruses are an insect-pathogenic group of dsDNA viruses, which have obtained considerable attention as biological control agents of insect pests. Baculovirus based biocontrol agents promise high efficiency, unequalled specificity for target pest insects and no negative environmental impact. Baculoviruses are rapidly inactivated in UV light, limiting their environmental persistence. On the other hand, baculoviruses have to be ingested by the target insect and the development of infection along with killing of the target insects takes several days. This delay is often long enough to allow some damage on the crop. Though some progress was made in genetic engineering baculoviruses in order to accelerate their speed of killing, correct virus timing and the control of early larval stages are pivotal to optimize their efficacy in the field. Another aspect of baculovirus efficacy is the sustainable control of insects. As known from codling moth control using *Cydia pomonella* granulovirus (CpGV) there is an apparent effect on the reduction of target population resulting in a reduced infestation pressure over the time. This effect makes CpGV an ideal partner in integrated pest management strategies. Recent observations of field resistance against CpGV products made it obvious that resistance management strategies need also to be developed in cases where baculovirus products are intensively applied. However, due to the plasticity of baculovirus genomes and the diversity of naturally occurring baculoviruses, even among different isolates from the same baculovirus species implies that the development and use of baculovirus products is not static, but can be modulated. The exploitation of the diversity of baculoviruses will be an important aspect of optimizing baculovirus application. This will allow managing the virulence of naturally baculovirus products by selecting the most efficient isolates. New legislation on baculovirus registration is promising but needs to be implemented in to practice. Further research will be necessary to fully understand virulence factors and virus host interaction on an organism and population level. Improving environmental stability of the viruses will be a key step to make baculoviruses even more attractive in biological crop protection,

Key words: Granulovirus, nucleopolyhedrovirus, biological control, host range, registration

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General perspective of the biocontrol industry

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Abstract: Regulation and policies can strongly influence the opportunities for biological control agents to be used as plant protection products. There are a number of driving factors that can favour their use in alternation with or in addition to synthetic agrochemicals in IPM programmes.

Key words: Regulation, biocontrol products, integrated pest management, organic farming

Introduction

Pesticides are active substances or mixtures of active substances developed to control and/or kill pests such as insects, plant pathogens, weeds, molluscs, nematodes. Although there are benefits to the use of pesticides, there are also drawbacks, such as potential toxicity to humans and other animals. Pesticides can cause undesiderable adverse effects on non target organisms, human health and the environment, and for this reason they are subject to regulation in the European Union and its Member States.

Over the years, an elaborated system has been developed for evaluating the risks of pesticides to human health and the environment. Despite the existing regulatory framework, undesiderable amounts of certain pesticides are still found in the environment and residues exceeding the regulatory limits are still detected in agricultural products. In the 6th Environmental Action Programme, the European Parliament and the Council recognised the need to achieve a more sustainable use of pesticides through a full implementation and revision of the relevant legal framework and developing a Thematic Strategy on the Sustainable Use of Pesticides. In fact, the existing legal instruments are not able to achieve all the objectives outlined in the 6th Environmental Action Programme.

Regulatory framework

In a plenary session held on 13th January 2009, the Members of the European Parlament approved a new legislative package on pesticides (European Parliament, 2009a, 2009b). The package contains several novel measures such as the framework Directive for a Sustainable Use of Pesticides (COD/2006/0132) and a Regulation on the placing of plant protection products on the market (COD/2006/0136). The new Regulation, in contrast with the present Directive 91/414/EEC, introduces the concept of cut-off criteria for the approval of active substances based on hazards rather than risks, and the application of the substitution principle.

Impact on crop protection

During the ongoing EU re-registration process under Directive 91/414 /EEC, sixty percent of the plant protection products have already been withdrawn from the market in Europe. With the recently approved new Regulation, which will replace Directive 91/414/EEC, the Parliament added criteria (cut-off criteria and comparative assessment), which could contribute to a further loss of between 20-65% of the remaining active substances.

Diverging opinions have been expressed on the impact of this regulation on crop protection and agricultural produce in Europe. Many researchers objected that, from a resistance management point of view, a reduction in the number of active substances and an increased use of any pesticide with its specific mode of action will enhance the risk of resistance development. Furthermore, the whitdrawal of active substances due to the criteria of the Regulation could result in increased difficulties in controlling insect pests on a broad range of major and minor crops. Under these circumstances, biocontrol agents (BCAs) are considered valuable tools to reduce the risk of development of resistance and to improve the value and life span of agrochemical products. Within the newly approved Regulation, the Biocontrol Industry could thus play an important role.

Low-risk active substances and basic substances

The new Regulation takes into consideration a number of aspects related to BCAs. For example, the Commision has introduced the categories Low-Risk Active Substances (Art. 22) and Basic Substances (Art. 23).

The first category comprises low-risk active substances. It may be expected that plant protection products containing these substances will pose only a low risk to human and animal health and the environment. Biocontrol products perfectly fit into this category, since the exclusion criterion defined according to Directive 67/548/EEC (carcinogenic, mutagenic, toxic to reproduction, sensitizing chemicals, etc.) does not refer to biocontrol substances. Low-risk active substances shall be approved for a period not exceeding 15 years instead of the normal 10-year approval, and Member States shall decide within 120 days on whether to approve an application for authorisation of a low-risk plant protection product. This will allow for longer market protection of the substance despite its smaller market size, and defintely result in faster access to the market.

The Regulation also includes the category Basic Substances. A basic substance is an active substance which is not predominantly used and not marketed as a plant protection product, but nevertheless has some use as a plant protection product. The substance must have neither an immediate or delayed harmful effect on human or animal health nor an unacceptable effect on the environment. Following application to the Commission, the substance may be approved for plant protection use for an unlimited period of time. In order to obtain approval, any available information and evaluations that prove and ensure coherence with other Community legislation, such as Regulation EC N° 178/2002, laying down the general principles, procedures and requirements of food law and in matters of food safety, must be provided.

The approval criteria for Low-Risk Active Substances and Basic Substances descibed above may be considered as an illustrative example on how the objectives of the new Regulation concerning the placing of plant protection products on the market are in line with those of the legal framework on the Sustainable Use of Pesticides. In fact, one of the specific objectives of the Thematic Strategy is to reduce the level of harmful active substances also by substituting the most dangerous substances with safer (including non chemical) alternatives.

National Action Plan

The Directive on the Sustainable Use of Pesticides, among other things, requests Member States to adopt National Action Plans (Art. 4) to identify crops, activities or areas, for which risks are worrying, and these should be addressed in priority. National Action Plans, aiming at setting objectives for the reduction of risks, including hazards and dependency of pesticide use, and at promoting non chemical plant protection, should be used by Member States in order to facilitate the implementation of this Directive.

An example of a National Action Plan is represented by Ecophyto 2018. In particular France has adopted a PIRRP, a risk reduction plan related to pesticides. The objective is to reduce by 50% the sales of the most hazardous active substances on the market. Furthermore, a list of active substances, represented by very toxic to toxic and noxious substances having an adverse effect on the environment, which will be banned in France, has been identified.

The adoption of a National Action Plan by the Member States is of sound importance for a sustainable use of pesticides, but strong differences among Member States in acknowledging these National Action Plans may emerge.

Protection of human health and the environment

Member States shall furthermore ensure that the use of pesticides is minimised or prohibited in specific areas such as parks, protected areas, public gardens, sports and recreation grounds, school grounds and playgrounds and in the close vicinity of healthcare facilities. In these areas, the use of pesticides can be particularly dangerous, and it shall be reduced as much as possible, or eliminated where appropriate. Appropriate risk management measures shall be taken and the use of low-risk plant protection products, as defined by the Regulation concerning the placing of plant protection products on the market, and biological control measures shall be considered in the first place.

Particular attention shall also be paid to the protection of the aquatic environment through measures such as buffer strips and hedges along water courses. The aquatic environment is especially sensitive to pesticides. It is therefore necessary that particular attention is paid to avoid pollution of surface water and groundwater, and to reduce exposure of water bodies to spray drift. Member States shall ensure that, when pesticides are used in the vicinity of water bodies, preference is given to products that are not dangerous for the aquatic environment.

Implementation of IPM

Article 13 of the new Directive on the Sustainable Use of Pesticides refers to the necessary measures in order to establish the conditions for implementation of Integrated Pest Management. Member States should encourage the use of crop specific standards of IPM by changing the approach in the management of pest control: increased use of biocontrol agents in integrated pest and disease management programmess, alone and together with new, environmentally friendly chemical pesticides. In 2014, IPM will become mandatory for all Member States.

Perspectives for the Biocontrol Industry

The Biocontrol Industry is becoming aware of the important role that it can play in the European agricultural system in improving food quality, protecting public health, and preserving and/or creating better environmental conditions. Biological pest control products will become increasingly important for achieving a more sustainable agriculture.

The concerns of the large-scale organized distribution and consumers with regard to residue and ecotoxicological issues (especially regarding honey bees) may definitely be of help for the implementation of IPM strategies in agriculture. Over the last five years, super-maket chains became increasingly aware of toxicological and ecotoxicological issues due to the growing demand of consumers for fruit and vegetables with limited chemical residues. In Italy, for example, in many cases fruit and vegetables designated for export (mainly to Germany and UK) must meet production requirements that do not permit more than four

different chemical residues, and residue levels of these four substances must not exceed 25% of their MRLs.

The recent development of IPM in Almeria (Spain) shows that unnecessary restrictions to trade can be avoided: biocontrol agents and beneficials can definitely be extremely valuable tools for helping growers to reduce residue levels in the final produce (Benuzzi, 2008). Furthermore, by integrating the limited number of available agrochemicals and BCAs into IPM strategies, growers will be able to develop efficient resistance management programmes, and to comply with other limitations, such as re-entry time, pre-harvest interval, and safety measures for workers.

Nowadays many growers already consider biocontrol products a realistic option. The quality of the biocontrol products, available on the market today, has been considerably increased due to the adoption of thorough and scrupolous quality control methods by the Biocontrol Industry. Essential information on side effects of agrochemicals on BCAs is available, enabling the integration of biological and chemical plant protection products. The biocontrol industry is able to provide reliable alternatives for “real” IPM, and it should therefore no longer be considered a niche market.

However, numerous Biocontrol Industries are still small and medium-sized enterprises, which are not acting on a large scale (globally). Most of these companies have access only to the local market, and their financial resources for marketing and registration are limited. This is severely affecting the availability of biocontrol products on the European market. Specific actions are needed to overcome these difficulties, and to help biocontrol industries in the area of research and registration (GENOEG/ Biopesticide scheme) at a European and Member State level (National Action Plan).

In conclusion, if also in Europe registration and regulatory rules will become more flexible and faster (as in the USA) than they used to be, the development of biocontrol products and biocontrol industries will have an ineludible brilliant future.

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Host factors that may affect susceptibility to *Bacillus thuringiensis* crystal toxins

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Abstract: Diverse physiological factors have been identified that have the potential to affect the susceptibility of lepidopteran larvae to the pesticidal crystal (Cry) toxins of *Bacillus thuringiensis* (*Bt*). Here, we review the results of comparative studies using both laboratory- and field-selected populations that have highlighted factors that may contribute to tolerance of, and resistance to, *Bt* Cry toxins.

Key words: *Bacillus thuringiensis*, resistance, tolerance

Introduction

In addition to the relatively well-characterised Mode 1 pattern of resistance, a number of host factors have been proposed to affect the susceptibility of lepidopterans to *Bt* Cry toxins. Factors have been identified that could each conceivably affect one or more of the commonly accepted stages in Cry toxicity such as ingestion, crystal dissolution, proteolytic processing, degradation and receptor binding as well as more controversial stages such as opportunistic septicaemia (Table 1).

Mode 1 resistance

The most common type of resistance found in both laboratory- and field-selected populations appears to be Mode 1 resistance (Heckel et al., 2007) which is inherited as a recessive trait and is characterized by resistance to (and reduced binding of) at least one Cry1A toxin with negligible cross-resistance to Cry1C (Tabashnik et al., 1998). There appears to be a common genetic basis for Mode 1 resistance in *Heliothis virescens, Pectinophora gossypiella* and *Helicoverpa armigera* where mutations in genes encoding 12-cadherin-domain proteins are genetically linked to the resistance phenotype (Jurat-Fuentes et al., 2004, Morin et al., 2003, Xu et al., 2005). The exact role of the 12-cadherin-domain proteins in toxicity is unclear although it has been suggested that they mediate a processing step, which promotes subsequent binding of the toxin to aminopeptidase receptors (Bravo et al., 2004). The genetic basis of *Plutella xylostella* Mode 1 resistance is currently unknown but is not genetically linked to mutations in the 12-cadherin-domain protein (Heckel et al., 1999, Baxter et al., 2005).

Altered proteolysis

Reduced proteolytic activation has been implicated in resistance to Cry1Ac in *Plodia interpunctella* (Oppert et al., 1997). The presence of a Cry1Ac-hydrolyzing protease, T1, in larval guts was negatively correlated with resistance and further analysis demonstrated cosegregation of T1 expression with Cry1Ac susceptibility. Comparative analysis of larval gut protease

indicated that reduced activation may affect Cry toxin susceptibility in *H. virescens, Ostrinia nubilalis* and *H. armigera* (Karumbaiah et al., 2007, Li et al., 2004, Rajagopal et al., 2009).

Table 1. Factors that may affect lepidopteran susceptibility to Cry toxins.

Mechanism	Mediator	Species	Reference
Selective feeding	Avoidance behaviour	*H. armigera* *H. zea* *H. virescens* *S. litura*	(Singh et al., 2008) (Gore et al., 2005) (Gore et al., 2005) (Singh et al., 2008)
Reduced dissolution	Gut pH	Hypothetical	(Heckel et al., 2007)
Precipitation	Multi-protein complex	*C. fumiferana*	(Milne et al., 1998)
Reduced activation	Protease	*P. interpunctella* *O. nubilalis* *H. virescens* *H. armigera*	(Oppert et al., 1997) (Li et al., 2004) (Karumbaiah et al., 2007) (Rajagopal et al., 2009)
Sequestration	Peritrophic matrix	*B. mori*	(Hayakawa et al., 2004)
	Carboxylesterase	*H. armigera*	(Gunning et al., 2005)
Coagulation	Immune proteins	*H. armigera* *E. kuehniella*	(Ma et al., 2005) (Rahman et al., 2004, Rahman et al., 2007)
Reduced binding ("Mode 1")	Altered receptors	See reference	(Heckel et al., 2007)
Pore blockage	?	Hypothetical	(Heckel et al., 2007)
Degradation	Protease	*H. armigera*	(Shao et al., 1998)
Altered microflora	Facultative pathogen	*L. dispar*	(Broderick et al., 2006)
Epithelial repair	?	*H. virescens*	(Martinez-Ramirez et al., 1999, Forcada et al., 1999)

Larval avoidance behaviour

Larvae of several lepidopteran species have been shown to exhibit selective feeding when allowed to choose between contaminated or control meridic diets (Table 1). Furthermore, *H. armigera* and *Helicoverpa zea* were reported to exhibit a concentration response selecting diets with lower concentrations of Cry toxins (Gore et al., 2005, Singh et al., 2008). Thus it is possible that avoidance behaviours could affect larval susceptibility in the field where insecticide coverage may be non-uniform. Research on a number of species have shown both oviposition and neonate feeding initiation to be unaffected by the presence of Cry toxins in transgenic crops (Liu et al., 2002). However, it is possible that larvae of some species, such as *H. zea* which is more mobile on Bt cotton (Gore et al., 2002), may selectively feed on transgenic plant structures with lower toxin concentrations thereby increasing their survival.

Precipitation

Milne et al. (1998) highlighted robust precipitation of Cry1Aa by a protein complex derived from *Choristoneura fumiferana* larval guts. Precipitation was independent of serine protease and metalloprotease inhibition, and was unaffected by boiling or by chelation of divalent cations. Moreover, precipitation proceeded when gut extract was diluted 32 fold. It was suggested that precipitation could prevent Cry1Aa from diffusing across the semi-permeable peritirophic matrix (PM) and reduce the concentration of soluble active Cry1Aa in larval gut.

Sequestration

Degradation of the PM has long been known to potentiate Cry toxicity. Recently, the presence of specific binding sites on the PM has been proposed to exert a trapping effect, reducing the ability of specific toxins to diffuse across the PM and interact with the gut epithelium (Hayakawa et al., 2004). *Bombyx mori* larvae from a population that was susceptible to Cry1Aa and tolerant to Cry1Ac were shown to possess PMs that were significantly less permeable to Cry1Ac. Binding of Cry1Ac to PM proteins was inhibited by pre-treatment with *N*-acetylgalactosamine (GalNAc), and co administration of Cry1Ac and GalNAc significantly increased the rate of diffusion of Cry1Ac through dissected PM. *In vitro* inhibition of PM trapping without PM degradation is now required to assess any involvement in susceptibility to Cry toxins.

Overproduced carboxylesterases are frequently implicated in insecticide resistance and have been shown to sequester or degrade a wide range of synthetic insecticides including organophosphates, carbamates and pyrethroids (Li et al., 2006). Esterase-based sequestration has been reported as the mechanism of Cry1Ac resistance in the silver selected strain of *H. armigera* where resistant larvae were shown to possess increased gut esterase activity which was inhibited by both *in vitro* and *in vivo* addition of Cry1Ac (Gunning et al., 2005). Surface plasmon resonance (SPR) was also used to demonstrate a direct interaction between esterase and Cry1Ac. However, the authors provide no evidence of co-segregation of Cry1Ac-binding esterases with resistance (Heckel et al., 2007). Additionally, SPR data describing the "strong affinity" of Cry1Ac for esterase isozymes from resistant insects lacks comparison to a negative control (SPR using isozymes from susceptible insects). Thus the report is inconclusive and the data incomplete.

We have investigated the possibility of an esterase-mediated sequestration resistance mechanism in the *P. xylostella* SERD4 population which shows polyfactorial resistance to Cry toxins (Sayyed et al., 2005) with cross-resistance to the pyrethroid deltamethrin (Sayyed et al., 2008). Notably, a modified form of piperonyl butoxide with specific inhibitory activity against esterases synergizes the activity of deltamethrin and Cry1Ac against both cognate selected subpopulations. Using a combination of extensive isozyme profiling, interference assays and ligand blotting we found no evidence that midgut esterases sequester Cry1Ac or that the observed cross resistance was due to a single esterase-based mechanism.

Coagulation

Elevated phenoloxidase (PO) activity, suggested to be indicative of heightened immunity, has been associated with tolerance to Delfin and purified Cry1Ac in *Ephestia kuehniella* (Rahman et al., 2004) and *H. armigera* (Ma et al., 2005), respectively. In both cases, trans-generational immune priming was identified as the most likely mode of inheritance although more complex genetic explanations should not be rejected. In both cases, tolerance was selected over multiple generations and could be increased by exposing larvae to a sublethal concentration of formulation or toxin. In the case of *H. armigera*, it was speculated that heterotetramers

of 60- and 69-kDa Cry1Ac fragments bind sugar moieties on the storage protein and pro-coagulant, hexamerin, leading to a coagulation reaction (Ma et al., 2005). The concentration of hexamerin in larval haemolymph was shown to be increased by sublethal exposure to Cry1Ac and an additional isoform was found in the midguts of tolerant larvae.

Subsequent work with *E. kuehniella* used parasitoid- and tropolone-mediated suppression of PO to demonstrate that elevated PO activity is linked to, but not responsible for, Cry1Ac tolerance (Rahman et al., 2007). During low-density gradient centrifugation a small proportion of Cry1Ac molecules were found to co-fractionate with the storage lipoprotein and pro-coagulant, lipophorin, It was tentatively suggested that lipophorin, which is known to be secreted from the haemocoel into the gut lumen, could potentially sequester Cry1Ac in a coagulation reaction (Rahman et al., 2007). Comparison of the immune parameters of a DiPel-resistant *Trichoplusia ni* population from greenhouses revealed lower PO activity and fewer inducible antimicrobial peptides in a DiPel-selected subpopulation when compared to an unselected subpopulation (Ericsson et al., 2009). In contrast to *E. kuehniella* and *H. armigera*, only susceptible *T. ni* larvae exhibited an induction response whereby sublethal exposure to DiPel resulted in an 8.6-fold increase in tolerance. It was suggested that the general decrease in immunity and lack of induction in the resistant subpopulation could be a fitness cost of DiPel resistance or that resistance was preventing damage to larvae which might be necessary for immune induction. We investigated the possibility that elevated immunity was partly responsible for the curious pattern of inheritance in the *P. xylostella* SERD4 population where the progeny of Cry1Ac-resistant females are nine-fold more Cry1Ac-resistant than the progeny of Cry1Ac-resistant males. Various immune parameters were screened including both cell-free and haemocyte-mediated responses. We concluded that there was no evidence that heightened immunity contributed to Cry1Ac resistance in SERD4.

Midgut bacteria & Cry toxicity

Multiple lepidopteran midgut censuses have highlighted the presence of Lactic Acid Bacteria such as lactococci, enterococci and streptococci that could potentially lower gut pH and thus affect the solubility of Cry toxins (Broderick et al., 2004, Xiang et al., 2006, Takatsuka & Kunimi, 2000). Controversially, it has been reported that intestinal Enterobacteriaceae are absolutely required for toxicity of both DiPel and Cry1Aa towards *Lymantria dispar* (Broderick et al., 2006). This raises the possibility that the absence of facultatively pathogenic members of the midgut microflora could drastically reduce susceptibility to *Bt* toxins in other species.

The diet-adapted Gen-88 strain of *P. xylostella* has been used to demonstrate that midgut bacteria are not required for the toxicity of *Bt* kurstaki HD1 or HD73 and that removal of the microflora does not reduce Cry1Ac toxicity to Gen-88 (Raymond et al., 2009). Additionally, we have shown that toxicity of Cry1Ac alone, or in synergism with Cry$^-$ *Bt* spores, is not reduced by removing the intestinal microflora of *Manduca sexta*. However, it is entirely conceivable that facultative pathogens within the midgut microflora could synergize Cry toxicity or that such microbes could rescue the toxicity of avirulent pleiotropic *Bt* mutants. We assert that an obligate requirement for intestinal bacteria is not a general feature of the toxicity of *Bt* or its Cry toxins.

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Resistance to Cry toxins and epithelial healing

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Abstract: Resistance to Cry toxins can develop by alterations in any of the steps in the Cry toxin mode of action. Most characterized mechanisms of resistance to Cry toxins involve alterations in enzymatic toxin processing or toxin interaction with receptors in the insect midgut epithelium. Previous reports have suggested an alternative mechanism of resistance to Cry1Ac toxin in *Heliothis virescens* larvae involving enhanced midgut regeneration after toxin-induced injury. Our current hypothesis is that as midgut mature cells interact with Cry1Ac and undergo injury, stem cells divide and differentiate to replace damaged mature cells. Considering this hypothesis, resistant *H. virescens* larvae may display a more effective regenerative mechanism that prevents compromising epithelial integrity. We report the detection of differences in the proteins secreted by mature cells upon Cry toxin treatment and an efficient regenerative response to intoxication in midgut cells from Cry1Ac-resistant *H. virescens* larvae.

Key words: Cry toxins, resistance, *Heliothis virescens*, midgut regeneration, stem cells, flow cytometry, proteomics.

Introduction

Cry toxins produced by the bacterium *Bacillus thuringiensis* (Bt) are used in insecticidal mixtures or expressed in transgenic plants (Bt crops) for environmentally-sound and effective insect control (Crickmore 2006). Upon ingestion by the insect, Cry toxins are solubilized and enzymatically processed in the insect midgut to an active toxin core. According to the model of Bravo et al. (Bravo et al. 2004), Cry toxin monomers bind to cadherin proteins on the midgut epithelium and undergo a conformational change that results in toxin oligomerization and an increased binding affinity towards aminopeptidase-N (APN) (Pardo-Lopez et al. 2006), and possibly alkaline phosphatase (ALP) (Jurat-Fuentes et al. 2004). Binding of toxin oligomers to these proteins results in accumulation of oligomers on lipid rafts, oligomer insertion, pore formation, and cell death by osmotic cell lysis. In an alternative model (Zhang et al. 2005), binding of toxin monomers to cadherin activates an intracellular oncotic signaling pathway resulting in cell death. Once midgut epithelium integrity is compromised, bacteria proliferate and invade the insect hemocoel causing septicemia and ultimately insect death (Broderick et al. 2006).

Even though insect resistance to Cry toxins can develop by alteration in any of the steps in the toxin mode of action, research efforts have focused on the most common mechanisms (Ferre et al. 2002): alteration of toxin proteolysis or changes in interactions between toxin and midgut receptors. In comparison, little is known on the role of epithelial regenerative mechanisms in resistance to Cry intoxication. This type of process would help explain cases of resistance to Cry toxins for which no mechanisms have been identified (Wang et al. 2007; Anilkumar et al. 2008). Enhanced midgut cell sloughing and regeneration has been proposed to result in resistance to baculoviral infection in *H. virescens* larvae (Hoover et al. 2000). Direct correlation between midgut regeneration and resistance to Cry1Ac was previously

reported in larvae from the CP73-3 and KCB strains of *H. virescens* (Forcada et al. 1999; Martinez-Ramirez et al. 1999). Clearly, further characterization of the regenerative response after Cry intoxication is needed to establish its potential role in resistance to Cry toxins.

In lepidopteran larvae, since differentiated cell types do not divide, midgut epithelium regeneration depends on stem cells (Hakim et al. 2001). Midgut stem cells respond to specific growth factors by proliferating and/or differentiating to mature cell types as needed. Isolation of primary midgut mature and stem cell cultures from *H. virescens* larvae (Loeb et al. 1996), has allowed the identification of several bioactive factors inducing a response in midgut stem cells in vitro (Loeb et al. 1999; Loeb et al. 2002; Blackburn et al. 2004). When primary midgut cell cultures from *H. virescens* larvae were treated with Cry toxins, a fast increase in the number of differentiating cells in response to dying mature cells was observed (Loeb et al. 2001a). Upon removal of Cry toxin from the culture, the proportion of each cell type returned to initial levels, suggesting a tightly regulated response to intoxication (Loeb et al. 2001b). Based on these observations from assays using isolated midgut cell cultures, our current hypothesis is that specific growth factors are produced during exposure of mature cell to Cry toxins that result in activation of the stem cell-mediated regenerative response. This process may be improved in Cry-resistant larvae resulting in complete recovery after intoxication.

The main goal of our project is to characterize the midgut regenerative response to assess its potential role as a mechanism of resistance to Cry toxins. In this report, we present preliminary data for the work that will be presented during our keynote presentation. Using primary midgut cell cultures and differential proteomics, we have been able to detect proteins differentially secreted in primary midgut cell cultures from *H. virescens* upon exposure to Cry1Ac. Primary midgut cell cultures from Cry1Ac-resistant larvae, but not from susceptible larvae, recovered from Cry1Ac intoxication.

Material and methods

Insect rearing and bacterial strains

H. virescens eggs were purchased from Benzon Research Inc. (Carlisle, PA). After hatching, larvae were reared on tobacco budworm artificial diet (Bio-Serv, Frenchtown, NJ) with a 8:16 (D:L) photoperiod at 26°C. Cry1Ac and Cry3Aa protoxins were purified from cultures of *B. thuringiensis* HD-73 and var. *tenebrionis*, respectively.

Isolation of primary midgut cell cultures

Larval midguts were dissected following methods described elsewhere (Loeb et al. 2001b; Loeb et al. 2003). Briefly, dissected guts were incubated in media (Grace's media (Gibco) diluted 3:1 in Ringer's solution) for 1 hour, then homogenized by pipetting. Tissue debris was discarded using 70µm filters. Mature and stem cells in the filtrate were separated by centrifugation (600 x *g* for 15 min.) in Ficoll-Paque (GE Healthcare). Recovered cells were washed in media twice by centrifugation (400 x *g* for 5 m). Final cell pellets were resuspended in media and incubated in surface-treated 12-well plates (NUNC, Rochester) at 26°C.

2D gel electrophoresis of midgut cell culture secretomes

Midgut cell cultures were treated with buffer (20 mM carbonate buffer, pH 9.8), or 0.06 µg/ml of purified Cry1Ac or Cry3Aa protoxin for 1 day at 26°C, then collected by centrifugation (400 x *g* for 5 min.). Supernatants were processed and quantified for 2D electrophoresis as described elsewhere (Krishnamoorthy et al. 2007). Proteins (20 µg) were loaded on 7 cm, pH 3-10 Immobiline DryStrips (GE Healthcare) and resolved using isoelectrofocusing (IEF) as first dimension followed by SDS-8%PAGE as second dimension. After electrophoresis, gels were silver stained.

Response to intoxication in midgut cell cultures from Cry1Ac-susceptible and resistant H. virescens larvae

Midgut cell cultures from larvae of susceptible (YDK) and Cry1Ac-resistant (CXC) strains. Cell cultures were treated with a dose of Cry1Ac protoxin representing a sublethal concentration for CXC larvae (6.7 μg /ml) and incubated at 26°C. Cultures were observed at days one and four after intoxication. To determine cell mortality, sub-cultures were stained with trypan blue before microscopic observation.

Results and discussion

Discrimination of H. virescens midgut mature and stem cells

While mature cells had a complex morphology with the presence of brush border on some cells, stem cells were round and semi-translucent (Fig. 1). Separation of stem and mature cells allowed us to test for the expression of ALP and cadherin. Alkaline phosphatase has been described as a stem cell marker in mammalian systems (Nunomura et al. 2005), while cadherin use as stem cell marker depends on the tissue (Watt, 1998). As shown in Fig. 1, stem cells had much lower levels of both ALP and cadherin than mature cells. Lower expression of an adhesion protein such as cadherin would be expected for midgut stem cells, which are loosely attached to the epithelium. In contrast, the low levels of ALP in midgut stem cells may suggest differences in protein expression between mammalian and invertebrate stem cells. These data identify both cadherin and ALP as negative markers for insect midgut stem cells. Additionally, since both ALP and cadherin are proposed functional Cry1Ac receptors, our data help explain lower susceptibility of midgut stem cells to Cry1Ac toxin (Loeb et al. 2001b).

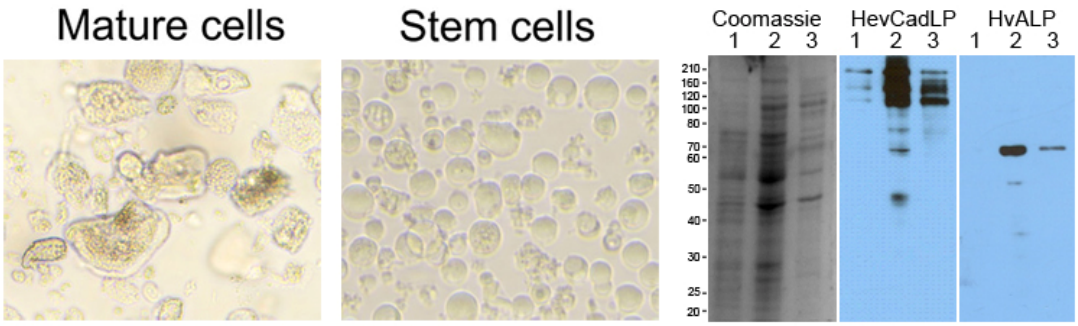

Figure 1. Morphological characteristics of mature and stem cells (left and center panels). Detection of expression of cadherin (HevCadLP) and alkaline phosphatase (HvALP) in stem cells (1), mature cells (2), or brush border membrane vesicles (3).

H. virescens midgut cells secrete specific proteins during Cry1Ac intoxication

Our current hypothesis is that upon exposure to sublethal levels of Cry1Ac protoxin, dying cells express and secrete specific growth factors that activate stem cell proliferation and/or differentiation. A prediction from this hypothesis would be that mature cells treated with Cry1Ac would secrete proteins to induce stem cell-based epithelial regeneration. We treated *H. virescens* midgut cell cultures with buffer or a sublethal concentration of purified Cry1Ac or Cry3Aa, the later being inactive against *H. virescens*. After 1 day, we collected secreted proteins by centrifugation and compared the secretome pattern induced by each treatment

using 2D electrophoresis. Several protein spots appeared to be specifically expressed after treatment with Cry1Ac (Fig. 2B). These proteins were not observed in control cultures treated with buffer (Fig. 2A) or with Cry3Aa (Fig. 2C). A group of proteins between 120- to 160-kDa in size were preferentially expressed when midgut cells were treated with Cry1Ac. Additionally, a protein spot smaller than 30-kDa was not detected in cultures treated with Cry1Ac, evidence that treatment with this toxin induces complex alterations in the secretome.

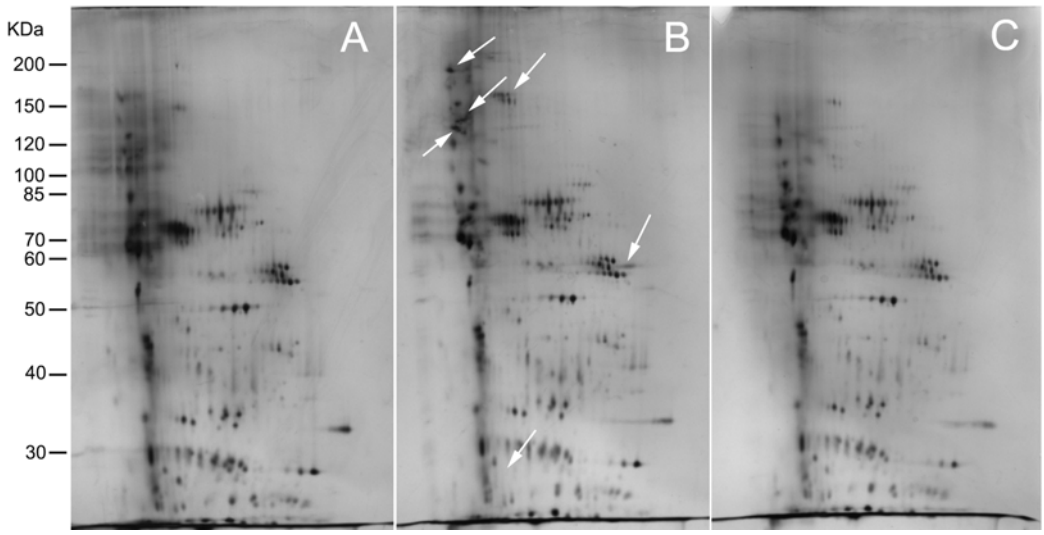

Figure 2. Secretomes of primary midgut cells treated with buffer (A), Cry1Ac (B), or Cry3Aa (C) as indicated. White arrows indicate some of the differentially expressed proteins.

Response to Cry1Ac intoxication in midgut cells from susceptible and resistant H. virescens larvae

To test whether this enhanced regenerative response could be reproduced in our in vitro system, we treated midgut cell cultures prepared from larvae of a susceptible (YDK) and Cry1Ac-resistant (CXC) strain of *H. virescens*. Cell cultures were treated with a dose of Cry1Ac protoxin representing a sublethal concentration for CXC larvae (6.7 μg /ml). After staining sub-cultures with trypan blue to detect dead cells, we observed the cultures after one and four days (Fig. 3). In YDK cultures, mostly ghost and dead cells were observed at both time intervals. In contrast, while most cells appeared dead in CXC cultures during the first day of incubation, some live cells were also detected. After 4 days, no live cells were detected in YDK cultures, while live cells were observed in CXC cultures among some dead and ghost cells. These observations suggest midgut regeneration by a more effective regenerative response in CXC larvae. This mechanism would explain the cross-resistance phenotype to Cry1Ac and Cry2Aa toxins observed in this strain (Jurat-Fuentes et al. 2003).

Summary

Our data suggest the expression and secretion of specific proteins when midgut cells are exposed to an active Cry toxin. These proteins are not observed when the Cry toxin is inactive against the cells. Regeneration of primary cell cultures from CXC larvae suggest the presence of an enhanced regenerative mechanism in larvae of this strain, which may contribute to resistance. Additional data on the characterization of the regenerative response as well as the role of enhanced regeneration in resistance will be displayed during our presentation.

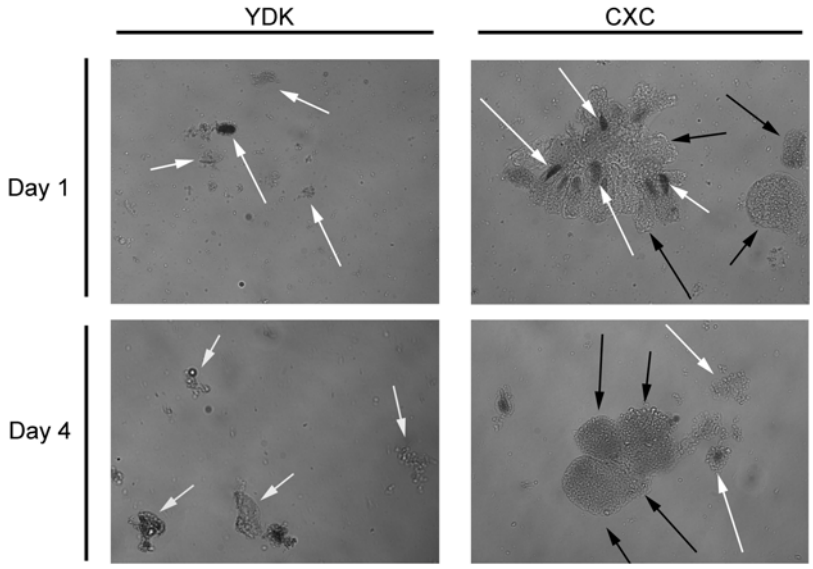

Figure 3. Effect of Cry1Ac treatment on midgut cells from susceptible (YDK) and Cry1Ac-resistant (CXC) larvae. White arrows indicate dead cells, black arrows indicate live cells.

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Bacillus thuringiensis Cry34Ab1/Cry35Ab1: development in stacked *Bt* gene corn products for control of *Diabrotica* spp.

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Abstract: The larvae of western corn rootworm (WCRW, *Diabrotica virgifera virgifera* LeConte), northern corn rootworm (NCRW, *Diabrotica barberi* Smith and Lawrence) and Mexican corn rootworm (MCRW, *Diabrotica virgifera zeae* Krysan and Smith) are major coleopteran pests of corn. Each year damage from CRW infestations results in $1 billion or more in lost farm income. *Bacillus thuringiensis* (Bt) binary insecticidal crystal proteins Cry34Ab1 and Cry35Ab1 (Cry34/35Ab1) when combined together have oral activity against *Diabrotica* species (Ellis et al. 2002). Cry34/35Ab1 exhibit highly selective biological activity for larval stages of Coleoptera and have no activity on Lepidoptera or non-target beneficial insects. Upon ingestion by WCRW, Cry34/35Ab1 appears to function by disrupting the WCRW midgut epithelium (Moellenbeck et al. 2001). Protein sequence and structural comparisons suggest a unique mode of action for Cry34/35Ab1. Cry34Ab1 is one representative of a family of 14 kDa proteins that have no sequence homology beyond the Bt Cry34 group (Schnepf et al. 2005). Cry35Ab1 is a member of a family of Bt 44 kDa proteins. Cry35Ab1 has low sequence homology to Bt Cry36Aa1, *Bacillus sphaericus* mosquitocidal binary proteins BinA and BinB (Ellis et al. 2002) and a recently discovered *B. sphaericus* mosquitocidal binary protein, Cry49Aa1, that by contrast functions in combination with a 3-domain protein, Cry48Aa1 (Jones et al. 2007). The protein crystal structures of Cry34Ab1 and Cry35Ab1 are distinct from one another, and from the three-domain coleopteran-active Bt Cry proteins including Cry3Bb1. Cry34/35Ab1 has been developed for in-plant protection against CRW feeding damage. Maize event DAS-59122-7 expressing Cry34/35Ab1 is the basis for corn rootworm control in HERCULEX® XTRA from Dow AgroSciences. Recently, event DAS-59122-7 has been combined by conventional corn breeding as a component of *SmartStax*™, the first combined (stacked) trait corn product to provide multiple modes of action for above- and below-ground pest control in corn. *SmartStax*™ combines Dow AgroSciences' Cry34/35Ab1 event DAS-59122-7 with Monsanto's Cry3Bb1 event MON 88017 for corn rootworm control, along with events TC1507 and MON 89034 for Lepidopteran control. Comparative binding data indicate that Cry34/35Ab1 and Cry3Bb1 proteins bind to different midgut brush border membrane proteins and are therefore highly unlikely to be cross resistant. Efficacy data indicated that the coleopteran-active proteins in the stacked trait product function in an additive manner resulting in 99% reduction in adult CRW emergence and 99.9% mortality of CRW larvae. Dow AgroSciences field trials conducted during 2007 and 2008 show that *SmartStax*™ consistently provides better control than single mode-of-action traits. Simulation models demonstrate that Cry34/35Ab1 and Cry3Bb1 in *SmartStax*™ combined with a 5% non-transgenic conventional refuge is durable and superior to single trait products with a 20% refuge, including scenarios where parts of corn acreage continue to be planted with the corresponding single insecticidal protein products. Laboratory and field data support the combined use of Cry34/35Ab1 and Cry3Bb1 Bt proteins for CRW resistance management. These proteins are structurally distinct, appear to have different modes of action, and are highly efficacious. Simulation models predict that stacked Bt products based on a combination of MON 89034 × TC1507 × MON 88017 × DAS-59122-7 combined with a 5% refuge are durable.

Key words: *Bacillus thuringiensis*, *Diabrotica*, insect resistance management

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Research needs for entomopathogenic nematodes - An industry's perspective

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Abstract: Many disciplines from fundamental and applied sciences contributed to the development of entomopathogenic nematodes as a biocontrol industry. Studies on the nematode taxonomy, the biology of the pest insects and the symbiotic association with bacteria were as indispensable as the product development involving mass production techniques, formulation and application. The (incomplete) industry's wishlist for future research includes fundamental research on the ageing of the infective juveniles, on how to expand their "shelf-life", and on how to trigger infection behaviour. Solving these questions would decrease the cost of nematode production and the rates used per area and would open new markets. The exploration of new pest targets, also in the context of advances in application technology and reduced nematode prices is part of this wishlist as well as the exploration of whether pest associated rhabditid nematodes can be used as vectors for pathogenic micro-organisms. Recent advances show that two-party partnerships of public research institutes and industry were highly successful in the development of for instance the slug killing nematode or the liquid mass production of *Heterorhabditis* sp.. It is argued that within the EU framework programmes less stringent requirements for the formation of research consortia would enable the project initiators to choose the best suited partners and ultimately contribute to a more efficient use of EU-funding. Often, substantial discoveries are made by chance and outside specific public-private research projects (like the discovery of *Aplectana kraussei*). Fundamental and independent research from scientists with broad interest is most likely to delivery such innovations and should therefore be maintained. In industry the view tends to become short-termed and narrow since all activities are rated against profits made per time unit. While indispensable for a quick product development, this is not a good environment for the emergence of new ideas.

Key words: Research funding, entomopathogenic nematodes

Introduction

Science activities are commonly categorized in fundamental and applied research. As the name suggests, biological contol belongs to applied research. Within the applied research there are longer term research goals and short term goals. If companies are asked for their research needs, they usually have rather clear-cut short term goals in mind which are likely to increase their profit by expanding their market. But is this really what the companie's business is based on? What can we learn from the development of nematodes as biocontrol agents in the past about how science and industry should work together? Which problems should we focus on? This contribution attempts to answer these questions.

The history of entomopathogenic nematode industry

The term entomopathogenic nematodes is used here for those bacteria feeding nematodes of the order Rhabditida which are associated with bacteria and due to this association are highly virulent to insects (the genera *Steinernema* and *Heterorhabditis*) and slugs (*Phasmarhabditis*

hermaphrodita). They can be mass produced with standard biotechnology equipment on artificial media and be applied in the same way as chemical pesticides. To a certain extent, they actively seek out their prey and kill suitable host insects within a few days.

Less than 10 years after the discovery of the first *Steinernema* species by Krausse (Steiner, 1923), the first biocontrol product based on *S. glaseri* was applied in the USA to control the larvae of the invasive Japanese beetle (*Popillia japonica*) in turf (Steiner, 1929,Glaser & Farrell, 1935). A beautiful example for a rapid commercial exploitation of a scientific discovery! The symbiotic relation to bacteria was not yet discovered at this time and so the mass production on artificial media soon failed to provide infective nematodes. With a more profound study on the nematode's biology before commercialisation this failure could have been avoided. After a long gap caused by wars and the heavy reliance on chemical pesticides the renaissance of nematodes started in the 1980s together with the development of beneficial arthropods which were needed to control insect pests in greenhouses where bumblebees started their duty and many insecticides could no longer be used. Meanwhile, the symbiotic bacteria of *Steinernema* (Poinar & Thomas, 1966) and *Heterorhabditis* (Poinar, *et al.*, 1977) had been identified, many nematode species had been discovered and the efficacy against potential target insects proven. The method for mass production was developed by the industry itself (e.g. Biosys founded in 1983) but also by public research entities (CSIRO in Australia, Christian Albrechts University in Germany).

The basis of today's nematode industry

Today's nematode industry is obviously based on specific discoveries in the mass production, formulation, application and field development process. A more fundamental prerequisite, however, was the discovery of the entomopathogenic nematodes themselves, their classification and the elucidation of the symbiotic relationship with bacteria. This "scientific infrastructure", despite being indispensable for industry development is often neglected when industry-academia partnerships are discussed. Often, new species are discovered during ecological projects which are not linked to any industry development. Likewise, taxonomy is usually not financially linked to industry. Still, without these public funded sciences, the development of entomopathogenic nematodes as an industry would be impossible.

On the side of the pests targeted with nematode products today, industry surely benefit from the wealth of knowledge that classical entomology collected on important pest insects. In-depth studies on the life cycle and biology of pest insects are needed for an optimisation of nematode application in space and time. Despite being slightly "old-fashioned", this discipline of classical entomology is still alive and often legitimately funded within private-public research projects.

Finally, the nematodes need to be produced at a compatible price in order to develop an industry. There are currently three techniques for mass production: The production in living insects (in vivo), the propagation on artificial solid substrate (Solid state culture) and the production in fermentors in submersed culture (liquid culture). The highest level of industrialisation is implemented in the liquid culture, mainly because it is based on the developments in the chemical and biotechnological industry. Hence, the nematode industry is to a significant extent based on the development in a different discipline. Likewise, the application of nematodes to the field has long simply been covered from the liquid application of chemical pesticides. Only recently, with the need for a reduction of nematode application rates new and more efficient ways for nematode application are developed.

Examples for recent advances

A good example for a recent product development traceable from the nematode discovery to the product introduction is the slug-control product based on *Phasmarhabditis hermaphrodita* (Wilson & Grewal, 2005). The parasite had been known since 1859 and was first lab-cultured in 1900 but its pathogenicity to slugs was described only in the early 1990s. The nematode is on sale since 1994. The rapid product development was certainly supported by the involvement of the company Microbio Ltd. and, subsequently, of the close and focussed collaboration with the public research entity. Besides, it was supported by the scientific background from entomopathogenic nematodes. Without the background knowledge on pathogenic associations of nematodes and bacteria a focussed evaluation of the role of bacteria associated with *P. hermaphrodita* on its reproduction and pathogenicity would probably not have been undertaken.

Another more recent product development is the use of *Steinernema* species to control codling moth (*Cydia pomonella*) overwintering larvae. The pathogenic action of nematodes to these pests is known since the discovery of *Steinernema carpocapsae* on a cadaver of codling moth in 1955 (Dutky & Hough, 1955). However, the idea of controlling these insects with nematodes had not been popular as long as cheap chemical and biological alternatives were available. Only when the problems with resistance to chemical pesticides became pressing and when nematodes were available at a compatible price, research was intensified in the early 2000s by the USDA-ARS and companies became interested in these results, spurred by the alarming cases of codling moth resistance to baculovirus-products in Europe in 2006. Since 2007, an increasing number of nematodes are sold to control codling moth larvae.

An incomplete list of industry's research needs

The ultimate objective of industry is to expand the use of entomopathogenic nematodes in biocontrol and there are several ways for achieving this. Fundamental aspects of the **shelf-life** of entomopathogenic nematodes and possibilities for improvement remain to be a key issue. The production costs for a longer lasting nematode product would decrease since larger bio-reactors could be used. Another general aspect is the **reduction of the nematode application rates** by increasing their efficacy in the field. Modifying the application techniques from the simple drench approach to more focussed strategies bringing pest insects and nematodes together are of interest. One example is the idea to include high doses of nematodes in baits attracting poorly susceptible arthropods like woodlice or wireworms. More fundamentally, infectivity of the nematodes and how it changes with time should be elucidated with the aim to switch on infectivity artificially in the whole population applied to the field.

Whereas these aspects aim at the overall performance of nematodes in established markets the **exploration of new pest targets** is important. New pests are constantly emerging and public research institutes should be kept aware of the possibility of using nematodes against insect larvae in moist environments. Target pests which have been neglected in the past, should be re-assessed regularly under the aspect of decreasing costs for nematodes and improved application techniques. The use of nematodes against the western flower thrips (*Frankliella occidentalis*), first proposed in the beginning of the 1990s has been established only in the beginning of 2000 when nematodes were sufficiently cheap to apply them biweekly Wardlow, *et al.*, 2001.

The support of public research entities is especially needed for research which is "risky" in the sense that the feasibility of the concept is unknown and hence commercial exploitation can not be predicted. The idea of **using nematodes as vectors for pathogens** is an example

of such a concept. The example of the slug killing *Phasmarhabditis hermaphrodita* shows that it is possible to alter a nematode's pathogenicity by combining it with different bacteria. A systematic search for rhabditid nematodes associated with pest insects which could be turned into pathogens by combination with bacteria could expand the use of nematodes substantially.

Finally, it should be pointed out that public research should not only listen to industry when defining their research objectives. Often, substantial discoveries are made by chance (like the discovery of *Aplectana kraussei*). We need fundamental free research from scientists with a broad interest. The training of scientist should be kept fairly broad for this purpose so they are able to think outside their subject. In industry the view tends to become short-termed and narrow since all activities are rated against profits made per time unit. While indispensable for a quick product development, this is not a good environment for the emergence of new ideas.

Patterns of public-private research projects

A large variety of disciplines is involved in the development of products based on entomopathogenic nematodes starting with the scientific infrastructure, the taxonomy of the nematodes, the symbiotic bacteria and the pest insects or slugs to be controlled and (probably not) ending with sauce-economics and ethical sciences. The scientific world is highly specified and **communication** between branches is very limited. Industry must identify the multiple research needs and gather the information necessary. With respect to entomopathogenic nematodes, industry in Europe greatly benefit from the intensive exchange of information within the COST programme in the years 1990 to 2006. Beyond communication, there are several levels of partnerships between public and private research, ranging from simple toll research contracts, where specific research is sourced-out by the companies to huge multinational concerted actions which have become popular in the last two framework programmes of the EU. Two-party partnerships between universities and industry proved to be quite successful as evidenced by the development of the slug-killing nematode (Long Ashton Research station & Microbio Ltd) or the liquid culture of *Heterorhabditis bacteriophora* (Christian Albrechts University & Ecogen Inc. / e-nema GmbH). The policy of the EU-research schemes to limit financial support to projects with a certain number of research- and industry partners from different member states often results in collaborations of partners, which did not know each other before and which might fail to collaborate efficiently. Too often, the main interest of the single partners is to finance their ongoing research and not to solve the specific questions. Less stringent requirements for the formation of research consortia would enable the project initiators to choose the best suited partners and ultimately contribute to a more efficient use of public EU-funding.

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Concepts and visions to overcome problems with microbial biocontrol agent registration

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Abstract: On January, 13th 2009 the European Parliament voted on a new EU pesticides regime. A compromise had been reached between the European Commission and the European Parliament which now helps to evaluate all plant protection products following a stringent evaluation system. Although all relevant stakeholder groups welcome the new legal registration regime, the proposals of the EU-funded project REBECA still have to be considered in order to improve primarily the registration of bio-control organisms. The purpose of this presentation is to discuss visionary approaches and concepts which could be implemented rapidly by EU member state rapporteurs and registration authorities to overcome the very diverse problems with microbial bio control agents.

Key words: Communication, biological control, pre-submission meeting, registration, regulation, risk index system

Introduction

In Europe the Council Directive 91/414/EEC governs the regulation of plant protection products (PPP) and their active ingredients, including microbial biocontrol agents (mBCAs), plant exstracts and semiochemicals (EU 2001). A nearly infinite story had been brought to an end by defining a compromise between negotiators from the European member states and the European Parliament in January 2009. This agreement was based on the two legislative proposals from the European Commission: the revison of the EU agrochemical registration directive 91/414 and the thematic strategy on sustainable pesticide use (EurActiv 2008). The new European Council regulation 2006/0136 (COD) will read that the European Union is divided into three zones (north, south and central) inside which mutual recognition of pesticides will become the rule. Highly toxic chemicals, namely those which are genotoxic, cancerogenic or toxic to reproduction will be banned in the near future. Officials vow that more than 20 chemical pesticides will be phased out of use in European market. The new pesticide regulation will also contain separate paragraphs relating to “low risk substances”, “basic substances”, and “substances of concern”. These categories, however, do not meet the standards for natural occurring substances and micro-organisms. The criteria for low risk substances are only clearly defined with chemical active substances to allow for a reduction of the timelines for the authorisation of plant protection products.

Despite this new pesticide regulation it is the author’s opinion that most of the REBECA proposals should have been impelmented. The REBECA project was carried out from 2006 to 2007 with the objective to identify key risks and problems, to elaborate proposals for improvement, and to make suggestions on how to simplify the registation procedure which will help shorten the timeline drastically for the registration process. However, as a high priority all existing saftey standards have been kept in place without any compromise. The complete REBECA proposals can be found on the REBECA website (REBECA 2008).

The focus of this presentation is to provide visionary approaches and concepts which can be implemented rapidly by EU member state rapporteurs and registration authorities (i.e. EFSA, OECD, US EPA, PMRA). Aside from the concept of pre-sumission meetings, a scheme for the assessement of potential relevant metabolites of mBACs is presented to find a sound basis for the verification of the safety of these products. Furthermore, a risk indicator (RI) system is proposed, which enables the assessment of both conventional and biological pesticides, but also may serve to define low risk and reduced risk pesticides in the future.

Concepts for microbial BCAs registration

Pre-submission meeting

Pre-submission meetings are an indispensible tool for any successful registration. The meetings have proven to be very effective in a number of countries and are recommended to be held as part of a routine. Data requirements vary depending upon what a pesticide looks like and how it is used. The applicant and the Rapporteur MS evaluators must come together prior to the submission of a dossier to be able to identify the most relevant data requirements for a successful registration of the new microbial BCA. Moreover, any information on the identity of the micro-organisms relevant to open literature information on the active agent/organism should be made available to the evaluators.

It is important that the identification method used always represents the state of the art approved by a specialist for the specific taxonomic group. A correct taxonomic identification of the micro-organism is the prerequisite for the correct attribution of published scientific data in the subsequent risk assessment process.

Table 1. Information check list for pre-submission meeting (Strauch et al. 2008).

Relevant data	**Source available**
Identification and taxonomic position of the mBCA	√
Natural distribution of the species in particular on food and feed and in agriculture environments	√
Modes of action and host range	√
Toxicity data	√
Metabolites produced by the mBCA	√
Intended use of the product (target organisms)	√
Formulation of the product	√
Site and method of application	√
Health and medical reports	√
Absence from the list provided in Dir. 2000/54 EC concerning worker's protection from micro-organisms	√
Maximum growth temperature	√
List of available effective antibiotics	√

The applicant is encouraged to provide information if an already tested/registered strain is similar/comparable to a production strain. It is the applicant's decision whether to exchange letters of access to protected data, or to use open literature data as well as risk assessment documents performed in other countries. The decision on the relevant data, which can be derived from the applicant's data and/or published literature (Table 1), to be provided to registration authorities shall be based on the following information.

Based on an improved communication between regulators and applicants, the formal data requirements should be adapted to the real needs in assessing the safety and the efficiency of the very heterogeneous mBCA. The vision is that by improving the communication between applicants and regulators, as well as amongst regulators from different EU member state countries, all relevant points which regulators consider as important, will be addressed in a concise and short timeline. Through identifying waivers and accepting open literature information, the introduction of low risk profiled products on the market will be eased and accelerated.

Metabolite toxicology of microbial agents

The EU-approach to microbial metabolites is still under discussion because guidance documents are missing. It is understandable that regulatory authorities want to have a stringent procedure in place for the assessement of potentially toxic metabolic by-products by candidate micro-organism. In general, little information is available on relevant toxins on developed or beeing developed biocontrol agents. The applicant's knowledge of that information very often depends on either what already has been published in scientific literature, or, in few cases, has become apparent by chance from high throughput screening programmes during the product development.

Being confronted only with the need to meet the standard validation criteria for sample preparation techniques and analytics, it has to be obvious that the data requirements under the present Directive and New Regulation, respectively, cannot be met, particularly by the European small-sized industry.

Not to hinder the marketing of potential microbial products, REBECA experts propose a decision scheme to be used to assess the risks of metabolites secreted or accumulated by mBCAs (Figure 1, Strauch et al. 2008). The author recommends that if available (or open literature) information demonstrates the absence of relevant (toxic) metabolites, which are produced by the micro-organims in relevant amounts or if no exposure to relevant metabolites will occur, no data on metabolites should be required. Natural background levels and the related natural exposure to the micro-organisms fungus should always be taken into account (see Figure 1: step 1 and 2). The data provided at the pre-submission meetings should help the Rapporteur MS evaluators to propose a waiver, especially if no hazards are known from a regular exposure to humans and other non-targets to the micro-organisms.

If case questions (1) and (2) cannot be answered in the negative then, in the first instance, it should be investigated if relevant amounts of toxins are contained in the product, and the toxigenicity of the micro-organisms should be evaluated (step 3). At this stage the toxicity assessment of defined toxicants should be carried out using at least a 10 times higher concentration than the maximum expected environmental exposure (see user recommenadtion of the active ingredient). The toxigenicity under different conditions should be evaluated through culture supernatants and crude extracts, which is a mixture of all possible metabolites, which are produced under different growth conditions (Strasser et al. 2008).

Growth conditions (temperature, substrate) after the application of the mBCA should also be taken into consideration, as well as the fact that metabolites are biodegradable. First sensitive, high throughput and cost effective standard bioassays for the cyto- and genotoxicity

assessment are already available (Strasser et al. 2008, Strauch et al. 2008). Therefore, if no relevant amounts of toxins can be detected in a product or in the toxigenicity assessment, no further data requirements on metabolites should be necessary. Otherwise, a complete metabolite profiling must be assured as proposed by Strauch et al. 2008.
The vison for future registration procedures is not to treat mBCAs and their metabolites like synthetic chemicals. A need for different approaches for registration purposes is still necessary and should be realiesed consequently. In 2005 the EU Commission and OECD has already displayed political willingness in defining all microbial biological control agents (BCAs) as "generally to pose little or no risk to man and the environment". This statement still applies even if these agents secrete produce toxic metabolites. Let us reflect on today's practice: There is still no official "lesson learned" document available which was expected to be published on the experience of the judgement of the 4th list substances (2229/2004/EC). Nevertheless, for the first time, the Commission gave a clear indication that all already notified old active biocontrol agents do not have any harmful effects on human or animal health, nor on ground water, nor do they have any unacceptable influence on the environment. Otherwise, these mirco-organims would not be listed in Annex I of EU Directive 91/414/EEC. (EU 2008).

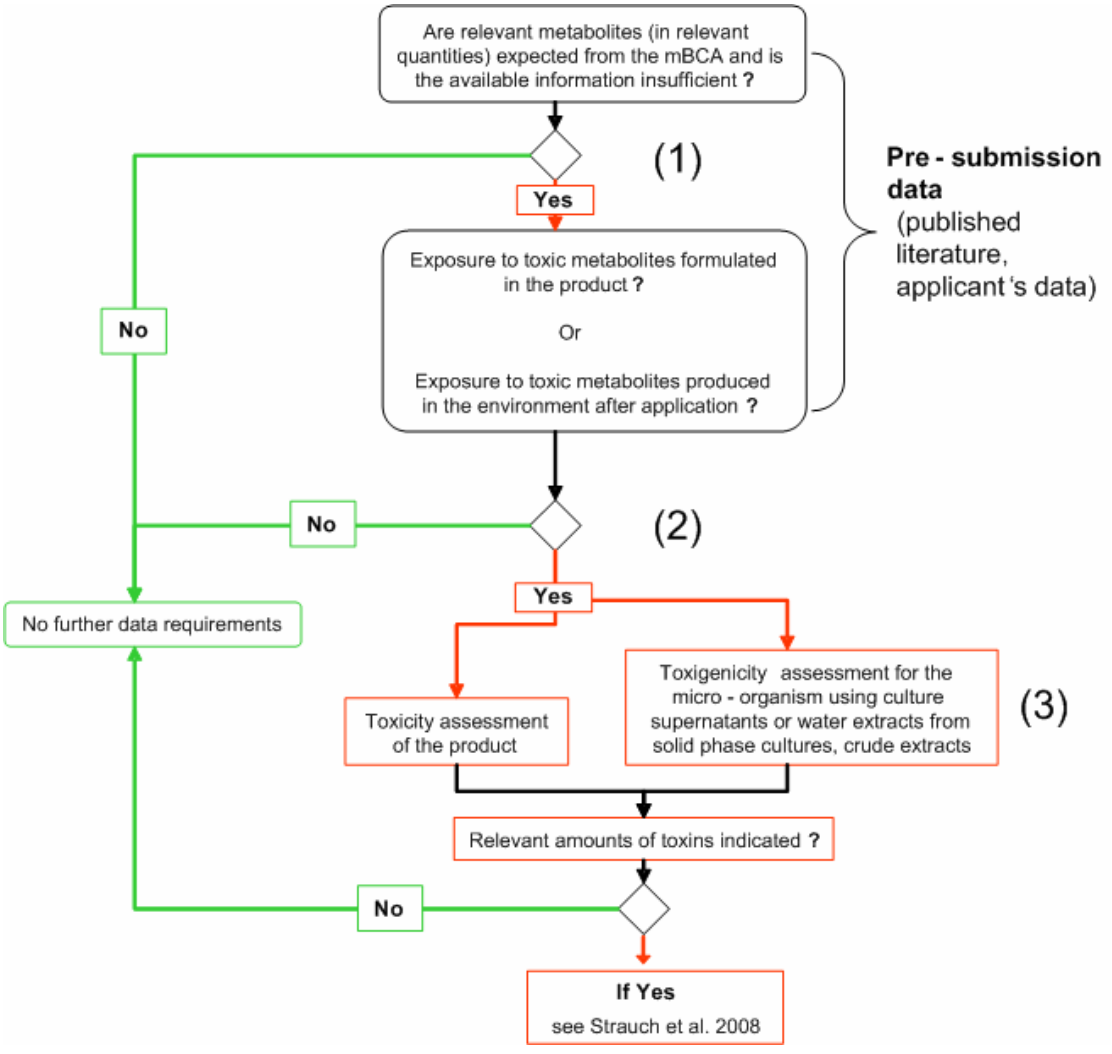

Figure 1. Scheme for assessment of potential relevant metabolites of microbial biocontrol agents (Strauch et al. 2008, modified).

Identification of low risk products

A proposal for an environmental 'risk indicator' system was developed in the framework of the REBECA project, allowing a comparative assessment of biological and chemical plant protection products and the identification of low risk products (Längle & Strasser, re-submitted). This risk indicator system is a refinement of earlier models. However, the proposed model is the first indicator allowing a direct numerical comparison of relative environmental risks posed by microbials and conventional chemical pesticides. The suitability of this model system was demonstrated by calculating the risk scores for seventeen selected, well studied mBCAs and chemical plant protection products used for similar puposes (i.e. *Bacillus thuringiensis*, *Beauveria* spp., *Metarhizium anisopliae*, *Coniothyrium minitans*, *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Trichoderma harzianum*, Antrazine, Benomyl, Chrorpyrifos, DDT, Methyl bromide, Phorate, Pyrethrins, Streptomycin).

Five basic components have been proposed for the calculation of the overall environmental risk indicator: These are the following: (1) the persistence of the substance, (2) the dispersal potential, (3) the range of non-target organisms that are affected, (4) the direct and (5) the indirect effects on the ecosystem. Each of the component values consists of a "likelihood" multiplied by a "magnitude" factor, and both values are scored on a scale from 1 to 5. The direct effect score is multiplied by a weighting factor if vertebrates or other groups of specific importance are affected. Chemical plant protection products and mBCAs may score in a range from 6 to 5.000. Indices are calculated using open literature and published regulatory documents.

The overall environmental risk score varied for the seventeen selected biological control agents and conventional pesticides from a very low risk score of 24 (*Coniothyrium minitans*, soil application) to a near maximum risk score of 4.275 (high risk reference DDT, foliar spray). The authors concluded that the score of low risk products should not exceed 100, whereas a threshold of 500 seemed justified for the term "reduced risk". While these cut-offs must be discussed on a broader basis, regulatory implications related to defining such categories include related data requirements or the acceptance of data waivers upon the submission of a well documented risk score. REBECA experts believe that the proposed system will help stakeholder groups to facilitate discussions regarding the regulatory approaches to microbial and other pest control agents.

Conclusion

The current registration system for mBCAs in the EU is costly, time-consuming as well as dependend upon the expertise of the registration authority evaluators. This practice, however, will apply even if Europe will adopt its new EU regulation regime.

REBECA experts encouraged the European Commission to develop a guidance document which would describe a pre-submission procedure which would establish a routine within all EU member states. Such a guidance document would include a detailed description of the information which would be required in a pre-submission information package. Based on a risk index system, the EU member state rapporteurs and registration authorities would establish a web-based database on open literature and published regulatory documents. The ranking of risks posed by diverse PPP would help accept data waivers upon low risk products.

In the future it should be possible to evaluate micro-organisms differently rather than simply as chemical-synthetic products. Also, it is not always necessary to gather new emperical data, especially if there is either already plenty of information regarding type and effectiveness of the active substance „micro-organism" available, or the use of the active substance has shown no negative side effects during usage for many years.

For this reason, the demands of some to eliminate the microbial control agents from the binding EU-Directive 91/414/EEC have to be understood as an attempt to help realise that only relevant data which is needed for an evaluation of the safety of the product should be requested.

In order to achieve this, important tools and ideas have been introduced in this presentation. The vision of a „whole“ approach, based upon already exisiting information, would lead to a significant reduction of studies necessary for biocontrol organisms. The risk-indicator system, which has been developed and tested by professionals, should become the accepted means of evaluation. There is no lack of legal recommendations and rules; however, there is political unwillingness to correctly evaluate already known substances, as well as new substances, based upon generally valid criteria. The concept of zoning is an important and correct step toward insuring that safe PPP are offered on the market in Europe. Applicants, however, can only profit from this system if the communication between the member states within Europe is significantly improved. Rethoric about already existing „Uniform Principles” alone is insufficient.

The constant quest for more data on product safety can result in a never ending collection of empirical data which ultimately, however, does not neccesarily improve the evaluation of an active substance. On the contrary, the search for more information, methods, and tools could be interpretated as misuse, especially when there is already sufficient verifiable information/knowledge available in order to answer a posted question.

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Research needs and promising approaches for the biological control of *Diabrotica* and other emerging soil insect pests with pathogens or nematodes

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Abstract: This paper suggests priority areas for Europe-wide research cooperation on biological control of newly emerging soil pests, such as the invasive maize pest *Diabrotica v. virgifera* LeConte (Coleoptera: Chrysomelidae), with insect pathogens or insect parasitic nematodes. The conclusions presented in this paper were derived from discussions by international experts. Discussions revealed that insect pathogens and nematodes will be useful elements of a strategic approach to the control of soil pests. Although biological control may not always be competitive with soil insecticides or transgenic crops in conventional production, an integrated or biological pest management strategy is likely to incorporate application of biological control products, classical releases of biological control agents, attract-dissemination-kill methods, and modifications of cultural techniques to enhance natural enemy populations. Overall, there is good evidence that accelerated exploration of biological control options may provide the advances in soil pest management we urgently need.

Key words: Inundative, classical, conservation biological control, integrated control, attract-dissemination -kill methods, *Diabrotica virgifera* subsp. *virgifera*, western corn rootworm

Background

Each year, a significant proportion of crops is destroyed by various insect pests. More than 90% of those pests have a stage in the soil (Klein, 1988). Naturally occurring enemies of insect pests have been known for a long time and are consciously used to control insects. Only in the middle of the 20th century did people increasingly believe in chemically synthesized control agents (Peters, 2008). In the following decades, resulting environmental problems became evident and subsequently, the most persistent pesticides were banned (e.g. Phillips, 2009). The chemical control of soil living pest insects is therefore facing a dilemma: On one hand, the active ingredient must reach the soil and be taken up by the insect; on the other hand it must not enter the ground water. Therefore, quick degradation of the poison in the soil is required. These contradicting requirements for pesticides in the soil are hardly fulfilled by any pesticide (Peters, 2008). Insect pathogens or nematodes can replace some of the resulting gaps in control of soil insect pests.

One of the major emerging soil pest problems in Europe is the invasion of the western corn rootworm (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) into maize production areas (Kiss et al., 2005). The three larval instars of this North American pest feed almost exclusively on maize roots (Moeser & Hibbard, 2005), often causing plant lodging and economically significant yield losses (Chiang, 1973). *D. v. virgifera* belongs to the 100 worst invading species as described by the European invasive species database DAISIE (EU project SSPI-CT-2003-511202). Although its spread is still ongoing, control

costs and yield losses have been calculated to already exceed 150 000 000 Euro per year (for 7 European countries analysed by the EU Project DIABROTICA (QLK5-CT-1999-01110). This still does not include non-countable costs of biodiversity reductions due to heavily implemented chemical control measures (EU Project ALARM GOCE-CT-2003-506675).

In an effort to prevent yield losses, European farmers began either to rotate their crops, thereby interrupting the life cycle of *D. v. virgifera*, or to subject the larvae to granular soil insecticides or insecticide-coated maize seeds (Ward et al., 2005). Throughout North America, transgenic *Diabrotica*-active *Bacillus thuringiensis*- maize is used together with insecticide-coated seeds (Ward et al., 2005). However, the current ban of seed coatings with Clothianidin, Thiamethoxam, or Imidacloprid + Methiocarb in several countries (Deutsches Maiskomitee), the likely ban of Tefluthrin-based soil insecticides, the evolution and spread of resistance of *D. v. virgifera* to insecticides (Ciosi et al., 2009; Parimi et al., 2006) and to crop rotation (Levine et al., 2002), the possible resistance development to transgenic maize (Meihls et al., 2008), and the conflicts between the chemical control of *D. v. virgifera* and the practiced biological control of *Ostrinia nubilalis* (Hübner) (Lepidoptera, Pyralidae) with *Trichogramma* wasps have converged to generate a sense of urgency for developing biological control options against *D. v. virgifera*. To date, biological control strategies including inundative or classical biological control were not considered potentially competitive with control achieved by insecticides, crop rotation or transgenic maize. Currently few small and medium-sized enterprises deal with aspects of biological control of *Diabrotica* pests globally. Given the increasing market for sweet and seed maize, together with the above-mentioned concerns, the interest of companies to offer products for the biological control of *D. v. virgifera* adults or larvae will increase in the near future. For several Western and Central European countries, a biological control product could even be developed before the major invasion front of *D. v. virgifera* arrives and pest populations build up. Therefore, currently two biological control products are being developed (Babendreier et al. 2006): one based on entomopathogenic fungi (Pilz et al., 2007) and another on entomopathogenic nematodes (Toepfer et al., 2005). Virulent and fast growing strains of *Metarhizium anisopliae* (Metsch.) Sorok. (Hypocreales: Clavicipitaceae) with high spore production are currently prioritized and tested under field conditions. Moreover *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) is currently prioritised due to its efficacy against *D. v. virgifera* larvae in the field (Toepfer et al., 2008), its persistence in the field (Kurtz et al., 2007) and its classification as a cruise forager that can actively search for host larvae in or on maize roots (Lewis et al., 1992). Finally, specific parasitoids of *D. v. virgifera* are being surveyed in its native ranges in order to find effective and safe classical biological control agents for releases in Europe (Kuhlmann et al., 2005).

Many other soil insect pests are relatively long-known problems to European agriculture, such as Scarabaeidae larvae (e.g. *Phyllopertha horticola* (L.), *Amphimallon solstitiale* (L.), *Melolontha melolontha* L.), Elateridae larvae (e.g. *Agriotes* spp. in maize potatoes, ornamental crops and small fruits, Furlan & Burgio, 1999), Cucurlionidae larvae (e.g. *Otiorhynchus sulcatus* (F.) in strawberries, shrubs, conifer nurseries, Peters, 2008), Sciaridae larvae (e.g. *Lycoriella* sp., *Bradysia* sp. in seedlings and in mushroom production, Peters, 2008), Noctuidae larvae (e.g., *Agrotis* (*Scotia*) *ipsilon* (Hufnagel) in maize fields, Furlan et al., 2001), Hepialidae larvae (e.g. *Hepialus (Korscheltellus) lupulina* (L.) in garden vegetables and tree nurseries, Edwards & Dennis, 2007), or Tipulidae larvae (e.g. *Tipula* spp. on lawns and golf courses, Peters, 2008). Some pests, like *Gryllotalpa gryllotalpa* (L.) (Orthoptera: Gryllotalpidae) are even decreasing pest problems (Sheppard, 1995).

Only a few soil pests, like *D. v. virgifera*, can be considered as emerging pests. For example, *Melolontha melolontha* was nearly eradicated in the middle of the 20th century

through broad-spectrum pesticides; however, it is re-gaining its historic importance as a soil pest in, for example, plantations of young fruit trees, and maize and beet production (Lakatos & Toth, 2006). Other Scarabeidae recently became pest problems, such as *Hoplia philanthus* Fuessly in lawns and gardens, and *Aphodius* spp. in turf and tree nurseries (Peters, 2008). Also, wide-spread problems with the larvae of *Agriotes* spp. have recently increased, particularly in potatoes (Porteneuve, 2008). Moreover, the larvae of *Otiorhynchus sulcatus* regained importance as pests in garden shrubs and strawberries due to horticultural intensification, banning of persistent insecticides, and the adoption of husbandry techniques favourable to the pest such as the use of mulches (Moorhouse et al., 2008). Finally, larvae of *Sitona lineatus* (L.) (Coleoptera: Cucurlionidae) are an increasing pest problem in organic legume production (larvae destroy the N-fixing legume root knots) (Vankosky et al., 2009).

Most of the above-mentioned soil pests have potential to be controlled by insect pathogens or nematodes; and some are already biologically controlled. Commercial fungal products are already successfully used. For example, *M. anisopliae* is used against *Amphimallon* spp., *Phyllopertha horticola* and *Otiorhynchus sulcatus*, while *Beauveria brongniartii* (Sac) Petch (Hypocreales: Cordycipitacea) is used against *Melolontha* spp. (Keller, 2004; Keller & Schweizer, 2007; Strasser et al., 2006; Zimmermann, 1992). Commercial nematode products are similarly available. *Heterorhabditis bacteriophora* is used against *Otiorhynchus sulcatus*, *Hepialus lupulinus*, *Phyllopertha horticola* and *Hoplia philanthus* (Peters, 2008). *Steinernema carpocapsae* (Weisser) (Rhabditida: Steinernematidae) is used against *Gryllotalpa gryllotalpa* and *Agrotis* spp. *Steinernema feltiae* (Filipjev) is used against Sciaridae and Bibionidae larvae. These examples clearly show the potential of using fungal pathogens or nematodes for controlling soil insect pests.

However, in order to utilize insect pathogens and insect parasitic nematodes to their full potential, we need to fill the gaps in our knowledge. This paper therefore gathers and discusses information on knowledge gaps and recent trends in the development of biological control strategies against recently emerged soil pests. Results were derived from discussions by international experts from universities, industry, farmer associations, international organisations, governmental or private extension services and farmers (see acknowledgements), as well as from literature surveys (Anonymous, 1913 - 1973; Anonymous, 1973 - 2006). Subsequently, the most promising biological control strategies against *D. v. virgifera* and other emerging soil pests were determined for European agricultural systems. Priority areas for Europe-wide research cooperation on the biological control of *D. v. virgifera* and other emerging soil pests are proposed.

Research needs and promising approaches for the biological control of *D. v. virgifera* with pathogens or nematodes

To address above-mentioned concerns of *D. v. virgifera* control, decision-makers at the European Union level are advised to **take action** in supporting the development of biological control options. There are several small but promising biological control research initiatives in Europe, and it is suggested that the European Union supports these activities. National legislators and decision-makers are encouraged to connect the existing subsidy for maize production with a requirement for integrated or organic farming, as it is, for example, already practiced on a regional-scale for the use of *Trichogramma* wasps against *Ostrinia nubilalis*.

Here we prioritize **research needs** for the following most feasible biological control options of the *D. v. virgifera* pest:

Approach 1. Inundative biological control: The periodic application of biological control products, such as formulations with bacteria, fungi, viruses or nematodes, has considerable potential. Species of **nematodes** and **fungi** are already registered for use against soil dwelling beetle pests in many countries (Babendreier et al., 2006; Copping, 2004), making them more feasible research subjects than the poorly studied **protista**, **microsporidia**, **viruses** and **bacteria**. Registration of new nematode-based biological control products is generally more feasible than of pathogen-based products (EU project on "Regulation of biological control agents, REBECA SSPE-CT-2005-022709 see Hunt (2008)). Key factors behind the success or failure of nematodes or fungi in pest control still need to be investigated:

(1a) Insect parasitic **nematodes** have potential in the biological control of *D. v. virgifera* larvae. The development of products using insect parasitic heterorhabditid and steinernematid **nematodes** against *D. v. virgifera* larvae is facilitated by the increasing number of commercially mass-produced nematode species and strains (Ehlers, 2001). Critical interactions between field crop, soil, beetle larvae and nematodes must be better understood. For example, the influence of soil structure and composition on nematode movement, orientation and persistence warrants further detailed investigation, particularly in view of possible applications in field crops. Furthermore, nematode application techniques need to be optimised, since knowledge on the large-scale use of nematodes in field crops is limited (reduce doses, develop optimal carrier materials, etc.) (Cabanillas et al., 2005). Means by which nematode population levels are maintained during critical periods should also be investigated. For example, how nematodes persist during periods of host absence or environmental extremes should be elucidated; nematodes may require non-target hosts of optimal size and population density in the soil. Ecological studies may also elucidate the influence of management practices on indigenous nematodes in the maize agro-ecosystem. A breeding program could enable the selection of nematode strains for propagation, locomotion through the soil, host virulence and/or ability to locate the host plant (Barbercheck et al., 2003; Turlings et al., 2009). Being short-lived and highly fecund, nematodes are feasible candidates for such selective breeding. Finally, plant breeders should be encouraged to select for maize hybrids that emit sufficient amounts of nematode-attracting volatile plant signals upon feeding by *D. v. virgifera* larvae (Rasmann et al., 2005).

(1b) Insect pathogenic **fungi** may have potential in the biological control of both *D. v. virgifera* adults and larvae. Little is known about fungi outside the genera *Beauveria* and *Metarhizium* with regard to *D. v. virgifera* control. However, strains in both genera that are highly virulent to *D. v. virgifera* may allow the development of biological control products. Commercial fungal formulations are already widely and successfully used against many soil dwelling pests; for example, *Metarhizium anisopliae* against *Amphimallon* spp., *Phyllopertha horticola* and *Otiorhynchus sulcatus*; as well as *Beauveria brongniartii* against *Melolontha* spp. (Keller, 2004; Keller & Schweizer, 2007; Strasser et al., 2006). Isolates may exist that show adaptations, such as partial or complete specificity, to *D. v. virgifera*, and molecular techniques could prove valuable in distinguishing them (Schwarzenbach et al., 2007). Commercial formulations for pest control need to be optimized for long-term storage, as can be achieved with many isolates of *Beauveria* and *Metarhizium* spp. Efficient application techniques must be developed, such as seed coating, water dispersible powders, granules, baits, and auto-dissemination methods. Furthermore, the ecology of insect pathogenic fungi in the soil is still poorly understood, including the phenomena of endophytism, host repellence, seasonal persistence, non-target effects, and interactions with the maize rhizosphere.

Ecological studies may also elucidate the influence of management practices on indigenous fungi in the maize agro-ecosystem.

(1c) Despite being a lower priority than nematode and fungal products, further exploration of the occurrence and ecology of **other pathogens** in *D. v. virgifera* may reveal potential biological control options. Although transgenic *Bacillus thuringiensis* - maize is widely used, no conventionally formulated **bacteria-**based products are used against *D. v. virgifera* or many other beetle pests in field crops. Bacterial research should focus on the isolation and characterization of indigenous *Bacillus thuringiensis* Berliner strains with specific activity against *D. v. virgifera*. *Bacillus thuringiensis* strains may be isolated from soils and characterised using degenerative PCR primer sets, specific for various *Cry* gene families (Bravo et al., 1998; Uribe et al., 2003). *Cry*34 and *Cry*35 proteins have shown high activity against larvae of *Diabrotica* species, but toxicity tests of conventional *Bt* products based on *Cry*34 and *Cry*35 are still lacking. Furthermore, application strategies for using conventional *Bt* products against soil dwelling *D. v. virgifera* larvae do not exist, and it is still questionable how larvae could be brought to feed on *Bt* in the soil (Klein, 1988). Models exist from other systems and should be explored, including *B. thuringiensis japonicum* against *Popillia japonica* Newman (Coleoptera: Scarabaeidae), and *Serratia entomophila* Grimont (Proteobacteria: Enterobacteriaceae) against *Costelytra zealandica* White (Scarabaeidae) (Toepfer et al., 2009). Some Rickettsiaceae may also show potential for the control of *D. v. virgifera* (Floate et al., 2006). The rapid expansion of *Wolbachia* research may offer new directions for the study of *D. v. virgifera* population dynamics (Floate et al., 2006; Giordano et al., 1997). The development of molecular screening of *D. v. virgifera* guts may identify promising new bacteria species.

Protista and **microsporidia** in *D. v. virgifera* also require further exploration, as some of these pathogens have been used successfully for the control of other pests. For example the grasshopper pathogen, *Paranosema (=Nosema) locustae* Canning (Microsporidia), is registered and commercially available for biological control (Copping, 2004; Lacey et al., 2001), although it tends to act as a chronic control agent for the control of populations over time rather than a quick acting control agent of a pest outbreak. Baculoviridae offer the greatest potential among **viruses** for development of pathogen-based biopesticides; Nucleopolyhedroviruses and Granuloviruses ought to be surveyed in Diabroticina pests in their American areas of origin (Miller et al., 1999). Techniques from existing successful biological control systems may be adopted. Examples include the use of the *Helicoverpa zea* - nucleopolyhedrovirus against the Lepidoptera *Heliothis* spp. and *Helicoverpa* spp. (Copping, 2004), and the augmentative control of the Scarabeidae *Oryctes rhinocerus* (L.) using *Oryctes*-viruses (Huger, 2005).

Approach 2. Attract- dissemination -kill methods: Environmentally-friendly attract-dissemination -kill methods against adult *D. v. virgifera* are promising management options, because several semiochemicals are known to attract Diabroticina (Chandler et al., 1995; Guss et al., 1984; Guss et al., 1982; Hammack et al., 1999; Metcalf et al., 1995). Attractive semiochemicals, such as like sex pheromones, floral baits or feeding stimulants of certain Diabroticina (Cabrera Walsh et al., 2008; Hesler & Sutter, 1993; Hoffmann et al., 1996), could be laced with **pathogens** that would kill adult beetles (Levine & Oloumi, 1991). *D. v. virgifera* has a strong preference for cucurbitacins, bitter triterpenoids that function as plant chemical defences primarily in the family Cucurbitaceae (Howe et al., 1976; Metcalf & Lampman, 1989a, b). Baits containing cucurbitacin-rich juices or powders are commercially available under at least five brand names and several formulations (Behle, 2001; Chandler,

2003). Moreover, these baits have shown no negative impact on non target arthropods (Ellsbury et al., 1996; McKenzie et al., 2002), making them compatible with other biocontrol programmes (Lewis et al., 2005). The results obtained with the application of these baits laced with insecticides to control *D. v. virgifera* have been variable (Chandler, 2003; Gerber et al., 2005). Three explanations have been proposed for these irregular results: resistance to toxicants (Parimi et al., 2003), inadequate residual activity of the bait (Gerber et al., 2005), and a distinct male bias in response of *D. v. virgifera* to cucurbitacin sources (Cabrera Walsh et al., 2008). These three factors indicate that current formulations require further improvements to serve as effective pest management tools.

Approach 3. Conservation biological control: The conservation and enhancement of naturally occurring insect **pathogens** or insect parasitic **nematodes** may reduce pest damage on a local scale. This option is generally promoted by guidelines and legislation for IPM or organic production worldwide, particularly in Europe (Boller et al., 1997). However, the impact of pathogens or nematodes on beetle population dynamics remains largely unknown. Life table analyses under different cultural practices could help to identify practice-related biotic mortality factors in the area of origin of *D. v. virgifera* (Toepfer & Kuhlmann, 2006). These could be compared with life table analyses from areas where *D. v. virgifera* are invasive to elucidate the differences in mortality factors and population dynamics. Once key natural enemies or natural mortality factors are identified, cultural practises could be adapted to maximize predation or natural mortality factors in an economically feasible way.

Approach 4. Classical biological control: Classical biological control should be considered against the invasive *D. v. virgifera* (Kuhlmann & Burgt, 1998; Kuhlmann et al., 2005) by inoculative release of host-specific natural enemies of Diabroticina beetles, such as parasitoids or viruses, bacteria and other pathogens, from the beetle's areas of origin in Mexico and surrounding countries (Krysan & Smith, 1987). Successful introduction and establishment of exotic natural enemies could provide long-term reduction in *D. v. virgifera* damage on a regional scale (Waage & Hassell, 1982). Pathogens, such as **viruses** or **bacteria,** are often host-specific and may have potential as classical biological control agents, but remain largely unexplored. Natural enemies of the soil dwelling stages of many *Diabrotica* pests have also yet to be explored. Surveys of Diabroticina in northwestern South America and of immatures throughout temperate zones of the New World are suggested. The latter is particularly crucial since pests introduced from subtropical climates to temperate zones often adapt to the new cooler environment better than their natural enemies (see *Leptinotarsa undecimlineata* (Say) in (O'Neal et al., 2002)).

Research needs and promising approaches for the biological control of other emerging soil insect pests with pathogens or nematodes

In terms the biological control of larvae of emerging soil pests, including *Agriotes* spp., *M. melolontha, H. philanthus, Aphodius* spp., *O. sulcatus,* or *S. lineatus,* the above-described approaches and **research needs** for **inundative biological control** and **conservation biological control** are also largely valid.

In contrast to *D. v. virgifera*, commercial **fungal** products of *Beauveria brongniartii* are already successfully used against *Melolontha* spp. (Zimmermann, 1992), and *Metarhizium anisopliae* is used against *Amphimallon* spp., *Phyllopertha horticola* and *Otiorhynchus sulcatus* (Booth & Shanks, 1988; Keller, 2004; Keller & Schweizer, 2007; Strasser et al., 2006). However, the above-mentioned research needs for improving commercial formulations

and application techniques still remain relevant, as well as the need for a better understanding of the ecology of insect pathogenic fungi in the soil. In particular, the control of *Agriotes* spp. with fungi remains a challenge (Ester & Huiting, 2007; Furlan, 2007).

Also in contrast to *D. v. virgifera*, commercial **nematode** products of *Heterorhabditis bacteriophora* are already successfully used against *Otiorhynchus sulcatus*, *Hepialus lupulinus* and *Phyllopertha horticola; Steinernema carpocapsae* is used against *Gryllotalpa gryllotalpa* and *Agrotis* spp.; and *Steinernema feltiae* is used against Sciaridae and Bibionidae larvae (Peters, 2008). Again, the above-mentioned research needs in terms of gaining a better understanding of nematode movement, orientation and persistence, as well as optimising nematode applications and selectively breeding nematodes, remain relevant. The control of *Melolontha* spp., *Amphimallon solstitale* and *Agriotes* spp. with nematodes remains a particular challenge (Lakatos & Toth, 2006), and more detailed research on nematode strain specificity, strain adaptations to local conditions (Campos-Herrera & Gutiérrez, 2009), pest defence mechanisms, and target oriented application techniques is required.

Research needs with regard to insect pathogenic **bacteria** and **viruses** are, as described above for *D. v. virgifera*, also relevant for controlling other emerging soil pests, such as for *Agriotes* spp., *M. melolontha, O. sulcatus* or *S. lineatus* (Klein, 1988). Besides the already described research needs connected with *Bacillus thuringiensis, Bacillus popilliae* Dutky also requires further consideration for its potential control of Scarabaeidae. Research should concentrate on improving mass production methodologies for different *Bacillus popilliae* strains that are specific to certain Scarabeidae species, as well as on the population ecology of this bacteria in the soil (relationship among bacteria, pest and soil; selection for activity at low temperatures; etc.) (Klein, 1988).

The above described **attract- dissemination -kill methods** and connected **research needs** may also be valid for pests with known attractive semiochemicals, such as the sex pheromones of several *Agriotes spp*. (Furlan et al., 1997; Toth et al., 2002), or the sex pheromones and food lures of some Scarabeidae (Jackson & Klein, 2006).

Conclusion

Overall, there is good evidence that accelerated exploration of biological control options may provide the advances in soil pest management we urgently need.

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Microbiological insecticides against lepidopteran pests in greenhouse horticulture in Almeria, Spain

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Abstract: Biological control has recently become widely established in greenhouse horticulture in Almeria. During the crop season of 2008-2009, the application of beneficial arthropods is the basis for all pest control measures in approximately 50% of the total of 27.000 ha. This offers a good solution for most pest problems, but is insufficient against others, especially lepidopteran pests. Since the presence of beneficial insects and mites sets a severe limit to the use of chemical pesticides, the available selective microbiological insecticides are widely used to resolve remaining problems. In this paper, the current use of microbiological insecticides, as well as new developments relating this issue, will be discussed.

Key words: Biological control; Baculovirus; *Bacillus thuringiensis*; *Spodoptera exigua*; sweet pepper.

Introduction

The province of Almeria, Spain, houses approximately 27.000 ha of plastic covered horticultural crops, probably representing the most densely concentrated greenhouse area in the world. Due to the proximity of the greenhouses and the overlap of different crop cycles, the area is extremely vulnerable to pests and diseases. Insect transmitted viruses, such as TSWV, transmitted by *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), and TYLCV, transmitted by *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), can be considered as the main phytosanitary problems. Low tolerance for these pests has led to intensive chemical control programs, as a result of which the population of various pests have developed resistance against the applied active ingredients. This holds in particular for thrips, *Frankliniella occidentalis* (Espinoza et al., 2002 a, b), and also for pests like *Bemisia tabaci* and *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). Because of this, chemical pest control became totally unsustainable in several crops, particularly in sweet pepper.

Biological control has been applied on small scale since over 15 years (van der Blom *et al*., 1997), initially with rather unpredictable results. However, due to the availability of new biological control agents and to the grown experience in the implementation of the IPM system, the system became technically viable and economically feasible (van der Blom, 2002, van der Blom *et al*., 2008). Figure 1 shows that biological control has recently been implemented in virtually all sweet pepper crops in Almeria. Key beneficial species in this system are *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) and *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae).

In spite of the successful control of most of the pest, others still need correction by means of phytosanitary products. This holds especially for *Spodoptera exigua* and other lepidopteran pest that might affect the crop. Given the importance of the beneficial insects and mites, all products that are used should be harmless to these natural enemies. Especially *Orius laevigatus* is delicate and cannot be combined with many active ingredients based on Insect Growth Regulators (IGR's). Therefore, the use of specific microbiological insecticides is

attractive and, within the constraints of the legislation for the registration of these products as insecticides, widely applied. These products generally have no side effects on the beneficial fauna and do not leave residues. The absolutely natural character of these products also makes them compatible with organic, or ecological, crop systems.

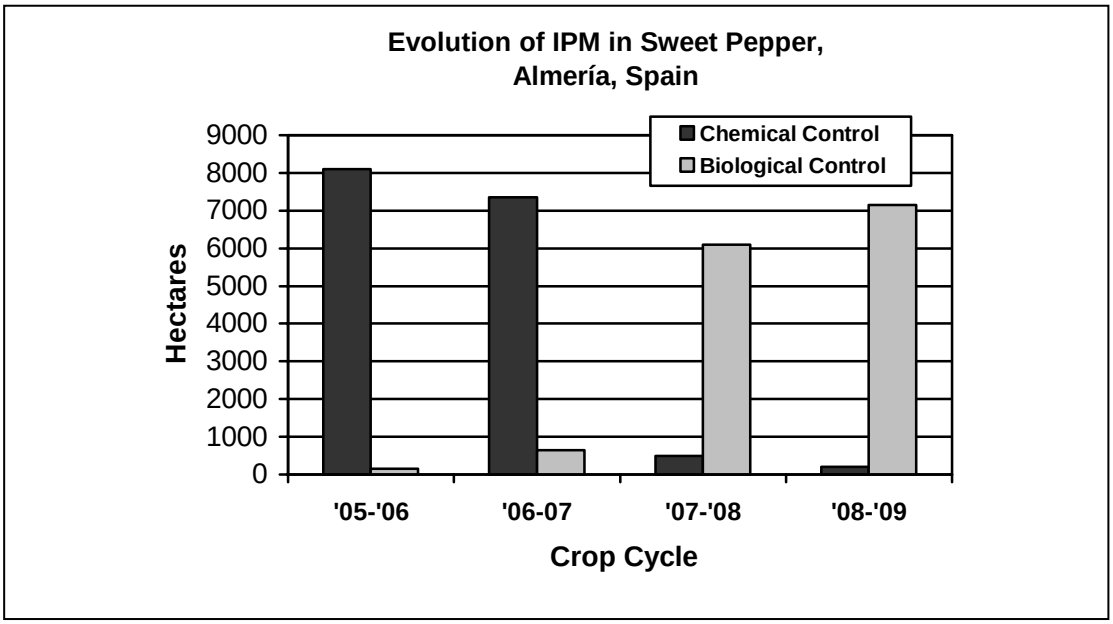

Figure 1. Evolution of the surface of sweet pepper greenhouse crops where biological control is applied as main 'tool' for pest control. (Data: COEXPHAL)

Control of lepidopteran pests

Lepidopteran pests form an important problem in sweet pepper and, to a lesser extent, in cucurbit crops. By far the most important species is *Spodoptera exigua*, accounting for more than 95% of the caterpillar damage in sweet pepper. Other noctuid species, with a more anecdotic presence, are *Helicoverpa armigera* Hübner; *S. littoralis* Boisduval; *Autographa gamma* L. and *Chrysodeixis chalcites* Esper. Lepidopteran pests cause problems at the beginning of the crop cycle, at the end of summer, i.e. when polyphagous predators have not yet reached big populations. From the other pepper production area in Spain, Murcia-Alicante, where the crops are planted in winter and the lepidopteran pests appear when biological control is well on its' way, it is known that *Orius laevigatus* can contribute a lot to the control of *Spodoptera*, as well as *Chrysoperla carnea* (Neuroptera, Chrysopidae) and a range of spontaneously appearing spiders.

Bacillus thuringiensis

After the implementation of biological control, growers have applied large quantities of *Bacillus thuringiensis* (*B.t.*) in order to control *Spodoptera exigua*. This mostly concerns the strain *aizawai*, although it is frequently alternated with *kurstaki.* It was estimated that, in the last two crop cycles, an average of 8 to 10 treatments with *B.t.* have been carried out in the total of 7.500 ha of sweet pepper (data: COEXPHAL). In most greenhouses, this means that the applications were weekly from the transplantation onwards, until the pest pressure stopped in autumn. Some relatively selective chemical products, like spinosad, methoxyfenozide,

tebufenozide and indoxacarb, are also used, on average 3 to 4 times. It is obvious that *B.t.* only offers a limited control and that the extremely high frequency of the treatments implies an important cost factor for the growers.

Baculoviruses

Recently, good results have been obtained with products based on Multiple Nuclear Polyhedrosis Virus (SeMNPV), belonging to the family of Baculoviruses. Two products have been used experimentally on a sufficiently wide scale to evaluate their effect and are currently in process of registration: 1. SPOD-X®, based on a SeMNPV strain from Florida (U.S.), previously registered in The Netherlands; 2. VIR-EX®, product based on autochthonous strains from Almeria. The development of the latter product is the result of collaboration between research institutes in Pamplona (UPNA) and Almería, who isolated the virus, isolated and sequenced the different genotypes that were present and established the most active composition of different genotypes for pest control (Belda et al., 2000; Murillo et al. 2006, 2007). In order to make this control solution available for the growers, COEXPHAL took the lead in the registration of the product (VIR-EX®) as a microbiological insecticide. A very high efficacy was proven by Lasa et al. (2007) and later confirmed in trials in commercial crops. In 2007, no further applications were necessary against *S. exigua* during the rest of the season in 40% of the crops where VIR-EX® was applied[1]. In 2008, this was the case in 46% of the cases[2].

The selectivity of the SeMNPV, thus far, offers important advantages for the sweet pepper production in Almeria. It is effective and has no side effect on other organisms. Nevertheless, the strict selectiveness also sets limits to the use in practice. In a survey realised by COEXPHAL in December 2008, data concerning 381 greenhouses were collected, all with *S. exigua* problems. The survey was carried out among the field advisors of cooperatives that belong to COEXPHAL. In addition to *S. exigua*, presence of *H. armigera* was reported in 73% of the greenhouses, *S. littoralis* in 18% and Plusidae in 9% of the crops. These data may not be completely accurate, because it was found not all field technicians were able to identify the larvae of different Noctuidae adequately. Nevertheless, it may indicate that the presence of noctuids other than *S. exigua* is not punctual anymore and that a more important damage of other noctuids may be expected in future. It is obvious that SeMNPV offers a solution against only part of a problem which, by the growers, is usually considered to be one single pest: caterpillars. In order to target applications with SeMNPV correctly, growers will have to identify the pest carefully and, in many cases, will have to apply additional products against other noctuids. In crops where mixed populations of noctuids are frequent, it may therefore be more profitable to apply a more broad-spectrum product.

Conclusions

The massive implementation of IPM in greenhouse crops is a big step forward in order to guarantee the sustainability of the greenhouse production in Europe in general and in Almeria in particular. Microbiological pesticides form a vital part of the IPM system, complementing the macro-biological pest control agents. It is highly desirable that, at short terms, many more related products become available. Baculoviruses are known, and experimentally available, for most lepidopteran pests. Their selectiveness, and minimum environmental impact, has been exhaustively documented all over the world. Nevertheless, very little baculoviruses have been

[1] VIR-EX was applied two times, with an interval of a week, in a dosage of 5×10^9 inclusion bodies, suspended in 1.000 l water, per hectare.

[2] These applications were part of a research program that was carried out under the responsibility of IFAPA, the agricultural research institute of the Government of Andalucía, in collaboration with COEXPHAL.

registered in Europe, due to the fact that the process of registration is still costly and slow. Within the EU, this process is now simplified compared to the registration of synthetic chemical insecticides, largely thanks to the work of the EU policy support commission REBECA (www.rebeca-net.de). Nevertheless, it still requires an investment that may be difficult to justify for products that, due to their selectiveness, will only serve a very limited market and, on the other hand, are difficult to protect commercially. Further simplifications in the legislation of microbiological pesticides, as well as a better understanding of this matter at national level in most EU countries, are necessary in order get to an agile development of IPM in all crops (Alvarado Aldea, 2009).

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The mode of action of *Bacillus thuringiensis* Vip3A and its applications for transgenic insect control

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Abstract: The vegetative insecticidal protein, Vip3A, from *Bacillus thuringiensis* (*Bt*), has great value to agriculture due to a broad lepidopteran spectrum and a unique mode of action compared to that of *Bt* insecticidal crystal proteins. Further observations comparing studies used in determining Vip3A mode of action are shared, as well as recent bioassays used to examine potential interactions of Vip3A with Cry1Ab. Data indicate no interactions, in agreement with a novel mode of action for Vip3A. Individual component Vip3A and full-length (FL) Cry1Ab transgenic cotton events and the stacked, VipCot™ product, are also examined for high dose status and discussed in the context of insect resistance management (IRM) practice.

Key words: Vip3A, mode of action, high dose, insect resistance management, VipCot™, Agrisure Viptera™ trait

Introduction

Forty-three separate Vip3A gene sequences have been identified, but approximately three-fourths of those represent identical amino acid sequences, or at most differ by less than five percent, when the *Bt* Cry toxin nomenclature system has been applied (Crickmore *et al.*, 2009). Though the Vip3A sequences themselves are highly-related, they share no homology with the *Bt* Cry toxins. In addition, Vip3A shares no sequence homology with other identified classes of proteins. Vip3A exists in a significant amount of *Bt* isolates (Estruch *et al.*, 1996; Espinasse *et al.*, 2003), which is interesting when one considers its impact on the insecticidal properties of *Bt*. Like the *Bt* Cry proteins, however, there is no clear role established in nature for Vip3A protein when the *Bt* spores or cells *do not* go through a susceptible insect.

Vip3A's complex mode of action parallels, but differs, from that of *Bt* insecticidal crystal proteins (Lee *et al.*, 2003). Vip3A undergoes proteolytic activation and binds to the surface of the insect midgut, subsequently forming pores in the midgut epithelium (Yu *et al.*, 1997, Lee *et al.*, 2003). Importantly, Vip3A did not show an interaction with the known Cry1 receptors. Though it possesses broad lepidopteran activity, Vip3A does demonstrate various potencies toward lepidopteran species and has non-detectable activity to *O. nubilalis* (Hübner) or *D. plexippus* (Linnaeus), for example. This differing potency is likely due to receptor binding differences. Although Vip3A can form stable ion channels in receptor-free membranes, this was measured at concentrations above that needed for bioactivity; receptor interactions are likely critical to manifest toxicity at bioactive concentrations. This is supported by *M. sexta* midgut voltage clamp experiments, where 15nM Vip3A caused pore-formation, whereas typically 100-fold more protein (1 to 2 μM) was required in the receptor-free planar lipid bilayers. A role for receptors is also supported by the observation that Vip3A did not produce pores in the midgut of the non-susceptible insect, *D. plexippus* (Lee *et al.*, 2003).

In recent years, Syngenta has examined Vip3A in diet bioassay and *in planta* with transgenic Vip3A material and breeding stacks of component events. The transgenic cotton, termed "VipCot™", is a pyramid of two genes combined through conventional breeding, *cry1Ab* (from the event COT67B) and *vip3A* (from the event COT102). Bioassays designed to look for any potential interaction of the isolated toxins has been another tool to consider in contrasting the mode of action. For example, an absence of an antagonistic interaction in such a mixed toxin bioassay, might be expected in agreement with the existence of different receptor binding interactions *in vivo* (Lee *et al.*, 1996; Liao *et al.*, 2002).

During the registration of transgenic traits, the high dose status against *Heliothis virescens* (Fabricius), and *Helicoverpa zea* (Boddie), has also been examined. IRM experts have supported a strategy of pyramiding toxins, combined with an appropriate refuge, to minimize resistance to *Bt* toxins (Gould, 1986; Roush, 1998). Relevant to this strategy is determination of whether the plant material presents a "high dose" to the target pest(s). A US EPA subpanel set the definition of high dose as “25 times the toxin concentration needed to kill susceptible larvae”, based on empirical data. They determined at least five ways to assess this, with a cultivar considered as being high dose if two of the five approaches so indicated (US EPA, 1998). Other IRM considerations for Vip3A *in planta* application in cotton and maize are briefly discussed for Syngenta's VipCot™ and the Agrisure Viptera™ trait, respectively.

Materials and methods

Vip3A and FLCry1Ab interaction experiments

Purified Vip3A and FLCry1Ab were diluted either independently or as mixtures for toxin bioassay. Five dilution series were prepared for each test, comprising one series each of Vip3A and FLCry1Ab alone, and three dilution series of ratios of the two proteins together. Sterile 96-well plates were prepared with 150 µl of artificial multiple species lepidopteran larvae diet (Southland Products, Inc., Lake Village, AR) and respective dilutions applied to the surface of the diet in 20 µl and allowed to dry. Each well was infested with a single neonate *H. zea*, and mean percent mortality across 3 replicates of 20 larvae recorded for each treatment rate recorded at day 6. Mortality was corrected using Abbott’s correction (Abbott, 1925). The entire test was conducted independently three times.

The presence of any synergistic or antagonistic interactions between toxins was assessed by comparing the observed mortality when the proteins were mixed with the predicted responses based on bioassay of each protein individually. In view of the evidence showing that Vip3A and Cry1Ab act at different binding sites, predicted responses were calculated based on an assumption of independent action, using the Colby formula (Colby, 1967).

U.S. EPA Method 1: Bioassays using lyophilized tissue

Greenhouse-grown Isoline (Coker 312), COT102, COT67B, and VipCot™ cotton plants which had terminal leaves (third leaf from top) harvested four weeks after planting were used to assess high dose status following guidelines of U.S. EPA high dose method 1 (U.S. EPA 1998). Leaves were stored at -80°C then, lyophilized gradually over a step-wise cycle (VirTis Wizard 2.0, SP Industries, Gardiner, NY); material was then ground to a fine powder and stored at -80°C. One and one-half ml of artificial multiple species lepidopteran larval diet (Southland Products, Inc.) was dispensed into individual wells of 24-well plates and allowed to set. Suspensions of lyophilized leaf tissue at 4% and 0.8% (wt/vol) were prepared in 0.2% bactoagar and dispensed (0.45 ml per well) into each well of separate treatment plates, allowed to dry and infested with a single neonate *H. virescens* or *H. zea* (eggs from North

Carolina State University). Mortality for each treatment was recorded and adjusted using Abbott's method (Abbott 1925) and four experiments conducted.

U.S. EPA Method 5: Bioassays of 1st and 4th instar larvae

Following guidelines of U.S. EPA high dose method 5 (U.S. EPA 1998), bioassays with Cry1Ab and Vip3A were performed using neonates and fourth instar larvae verifying this older instar of *H. zea* as at least twenty five-fold more tolerant to the toxins than neonates (data not shown). Four weeks after planting, the youngest leaves with a width of at least four cm were harvested for fresh leaf bioassay. For neonate *H. zea* larvae, two leaf disks were cut and placed on top of a pre-wetted Millipore ™ glass fiber pre-filter (Millipore Corporation, Billerica, MA) in each well of a 12-well plate, and one neonate placed into each well. Twenty four wells were used for Coker312, COT102, COT67B, and VipCot™ cotton and the plates were covered with a layer of Breathe-Easy™ (Diversified Biotech; Boston, MA; USA) film. Old leaf discs were removed and fresh water and new leaf discs added every two days. Percent mortality was recorded until 100% mortality was observed in the transgenic samples. For fourth instar *H. zea* larvae, six to eight 15 mm squares of leaf tissue were cut and placed into each well of plates prepared as described above, then one fourth instar larva placed into each well. Surviving larvae were removed from each well and reset into newly prepared wells every two to three days. Percent mortality was recorded until 100% mortality was noted in the transgenic samples. Three independent experiments were conducted.

Results and discussion

Vip3A and FLCry1Ab interaction experiments

In experiments I, II, and III, observed mortality was greater than expected mortality in 8 out of 19, 8 out of 20, or 14 out of 22 cases, respectively (Fig. 1), affording no clear case for synergism or antagonism of FLCry1Ab with Vip3A. Overall, observed mortality was greater than the expected mortality in 30 out of 61 cases. In addition, the magnitude of the differences in mortality (positive or negative) was less than 20% in 52 out of the 61 cases. If an interaction exists, the mixture should result in synergism or antagonism in all or nearly all of the cases. Differences seen are likely the effects of random variation. An absence of antagonistic interaction agrees with other biochemical data supporting two modes of action.

U.S. EPA Method 1: Bioassays using lyophilized tissue

This method involves the bioassay of lyophilized transgenic tissues. Dilution of tissue in an agar suspension which is overlaid on artificial insect diet, provides reduced exposure relative to the living plant. A result of 99.9% or greater mortality of neonate insects demonstrates high dose. For *H. zea*, the 25-fold dilution of tissue of VipCot™, COT67B and COT102 caused 100, 100 and 71% mean mortality, respectively (Fig. 2). At 125-fold dilution, VipCot™ and COT67B tissue still caused high mortality. These data show VipCot™ and COT67B as high dose toward *H. zea*. Similar data were obtained for *H. virescens*, with VipCot™, and COT67B causing 100 % mean mortality at a 25-fold dilution and high mortality even at a 125-fold dilution (Fig. 2). COT102 caused 65 % mean mortality at 25-fold dilution. COT102 alone displays significant, but not high dose activity towards either pest using this method.

U.S. EPA Method 5: Bioassays of 1st and 4th instar larvae

This method involves identification of an instar that is at least 25-fold more tolerant to the toxin than neonates and determining whether fresh transgenic material shows at least 95% mortality of the older instar compared to neonates (U.S. EPA, 1998; U.S. EPA 2001).

Exposure to either COT102 or COT67B component event leaf material or the VipCot™ stack leaf material resulted in 100% mortality for both neonate and fourth instar *H. zea* (Fig. 3), supporting both component events and the VipCot™ stack as high dose using this method.

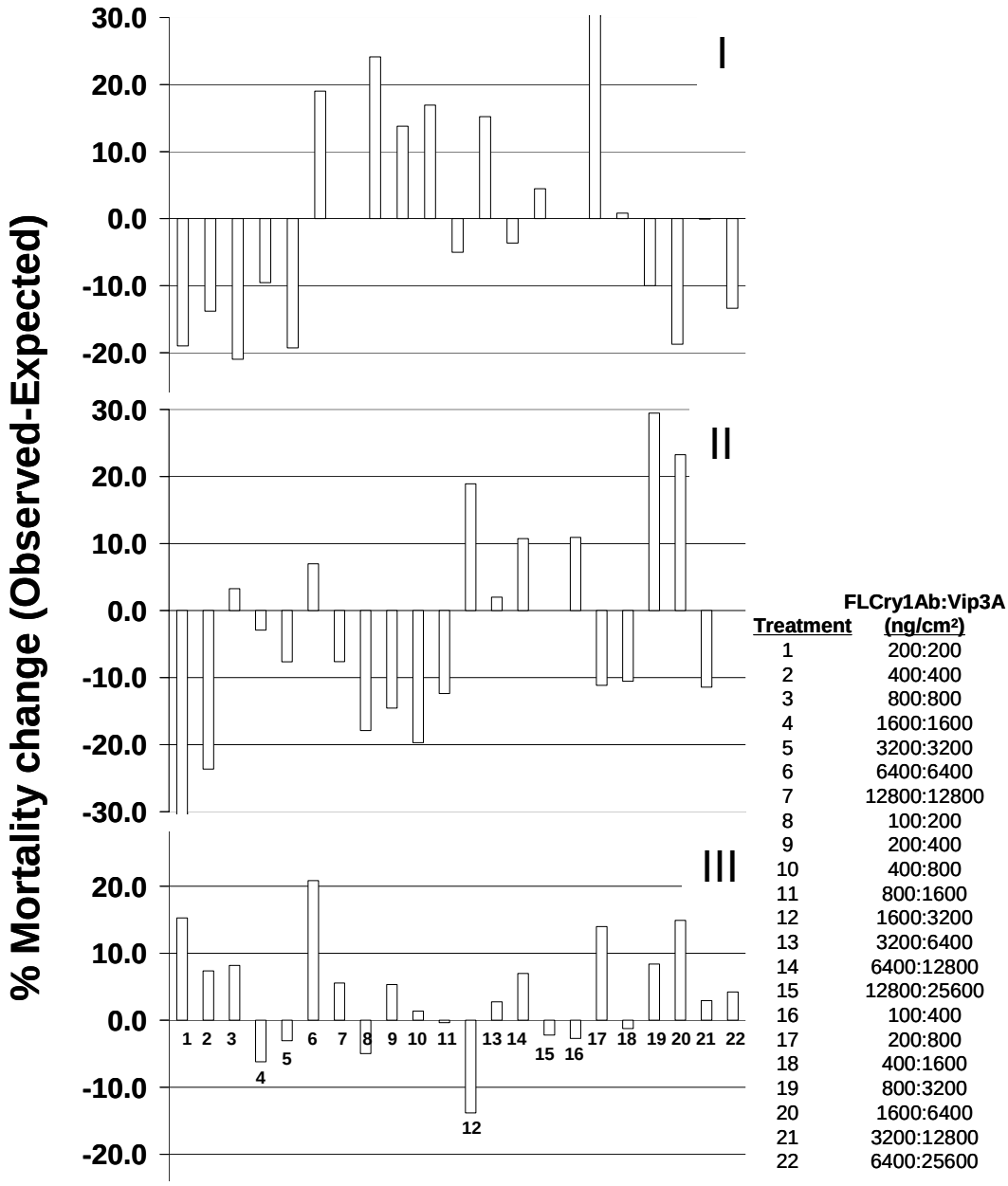

Figure 1. Assessment of synergistic or antagonistic interactions between Vip3A and FLCry1Ab toward neonate *H. zea* in three independent bioassay experiments.

Clearly, the evaluation of a high dose status for transgenic toxin traits is a challenging and imperfect task (U.S. EPA, 1998; U.S. EPA 2001). At times the differing high dose methods may not be in full agreement (*e.g.*, as seen with the method 1 vs. method 5 study here for the COT102 event). Previous data supported the stacked VipCot™ as being high dose against *H. virescens* and *H. zea* using 2 or 3 separate EPA methods, respectively, (Kurtz *et al.*, 2007), but the present data also consider the role of the individual component events.

Importantly, data establishing a significant level of pest control after use of these methods can support the development of IRM strategies. That is, a dual mode of action, pyramided, high-dose product strategy should prove valuable for the durability of either trait. Recently, EPA method 1 was used to investigate high dose status of Syngenta's transgenic Vip3A trait in maize named "Agrisure Viptera™". This trait alone was found to be high dose against S*podoptera frugiperda* (J.E. Smith), and to provide significant control (73% corrected mortality) against *H. zea* at the 25-fold dilution. In addition, the breeding stack of Bt11 maize (expressing Cry1Ab) with the Agrisure Viptera™ trait was determined to be high dose against both pests plus *O. nubilalis* (data not shown). The novel mode of action that Vip3A provides for lepidopteran pest control should continue to be advantageous for future crop IRM strategies.

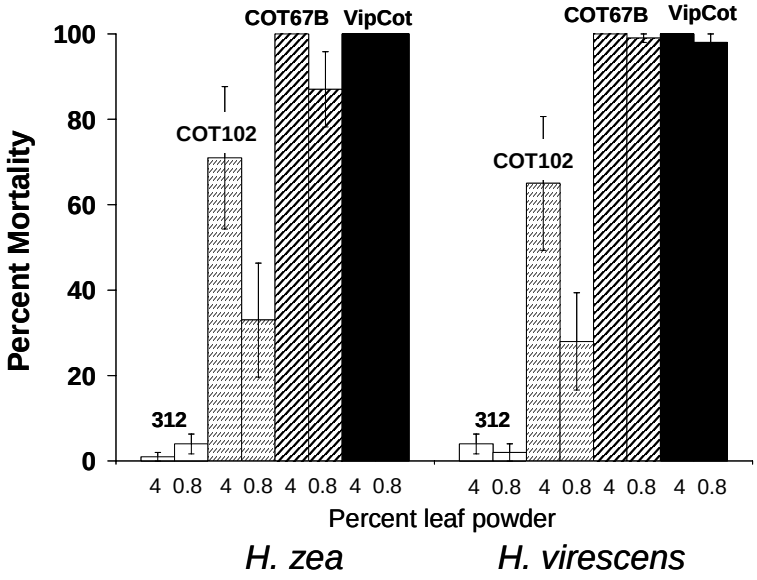

Figure 2. EPA Method 1 high dose bioassays with *H. zea* and *H. virescens*. Percent mortality at the respective dilutions of lyophilized leaf tissue shown; error bars represent mean ±S.E.

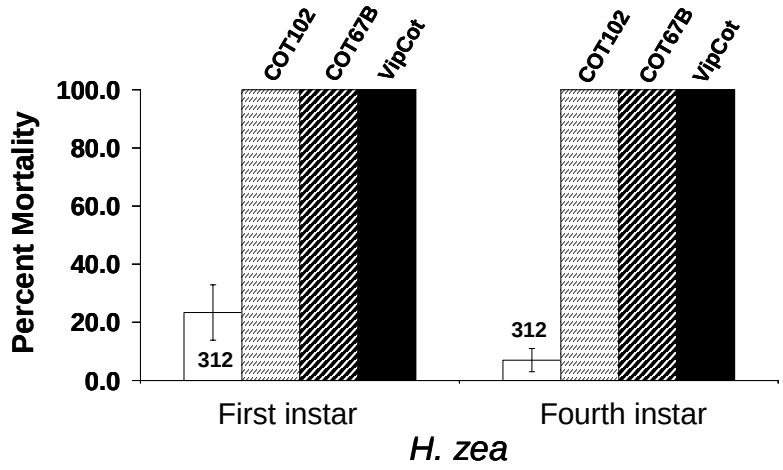

Figure 3. EPA Method 5 high dose bioassays with *H. zea*. Data for three bioassays are summarized as the mean mortality for all bioassays. Error bars represent mean ±S.E.

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Baculoviruses as biopesticides: Need for further R&D

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Abstract: Baculoviruses (BV) are the most frequently used viruses as biopesticides. Many studies were carried out on this virus-family, making broad knowledge accessible to science and industry. So far, this resulted in at least 20 commercially available BV products worldwide. However, a lot has to be done to extend the product range and to allow these products to compete with chemical pesticides. The most important fields for further investigation are: viruses for key pests, isolation and selection of new virus isolates, optimization of insect and virus production, development of new formulations, improvement of application, and insect resistance against virus The following article focuses on the need for further research and development, from an industrial point of view.

Key words: Baculovirus, biopesticide, research & development, needs, industry, formulation, resistance, production, isolate selection, application, new viruses

Introduction

Baculoviruses (BV) have many fields of application, e.g. as model organisms in ecology & evolution (host-parasite interaction) or as an expression system for proteins. Because they occur exclusively in arthroprods and generally have a narrow host range, BVs are very interesting for use as biopesticides. This is reflected in the fact that some BV products are well-established on the market.

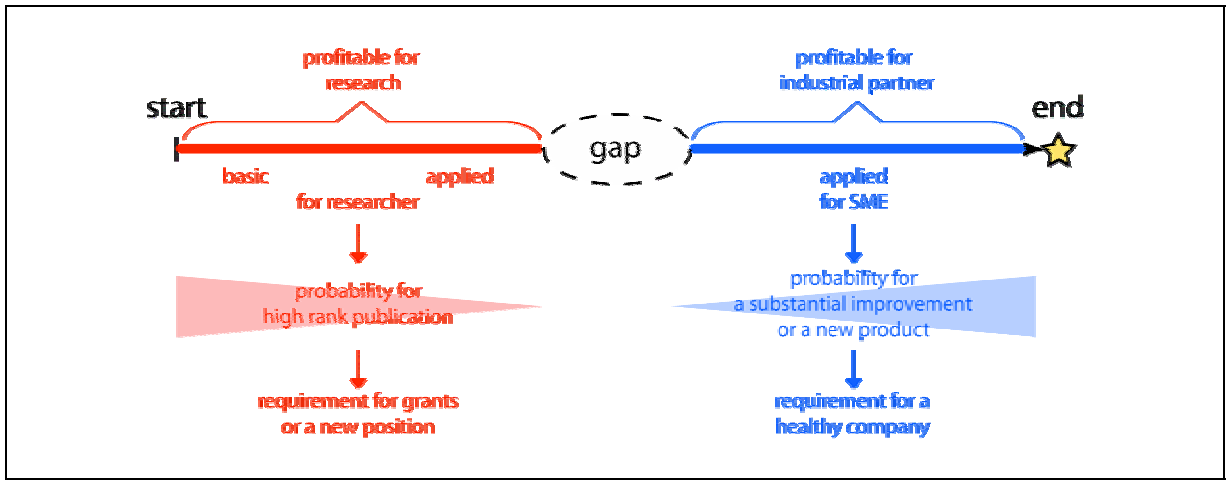

Figure 1. During the research & development of new findings/products, a gap often results from the fact that the profitable phases for research and SME do not overlap.

However, the development of a new product and subsequently the maintenance is labour- and cost-intensive. Many aspects have to be fulfilled for a successful product. From a

producer's point of view that means: a pest insect that can be mass reared, a virus with a high occlusion body (OB) production (maximum yield, minimum cost), virulent isolates, successful formulation with a long shelf life, stable production and good field performance (quality management), resistance strategy, high demand for the product on the market, a competitive product, and of course, the product has to be profitable.

To achieve all these requirements, (basic) research is necessary on a big scale, and this is not something that a small or medium-sized enterprise (SME) can carry out alone. Therefore, SMEs are dependant on a wide range of scientific findings from research institutions. Despite a lot of research effort in the field of BVs, far more studies are needed. Unfortunately, there is a conflict of interest between research institutes and SMEs. The lucrative phases for each of the two parties during research and development of new findings and products do not overlap (Figure 1).

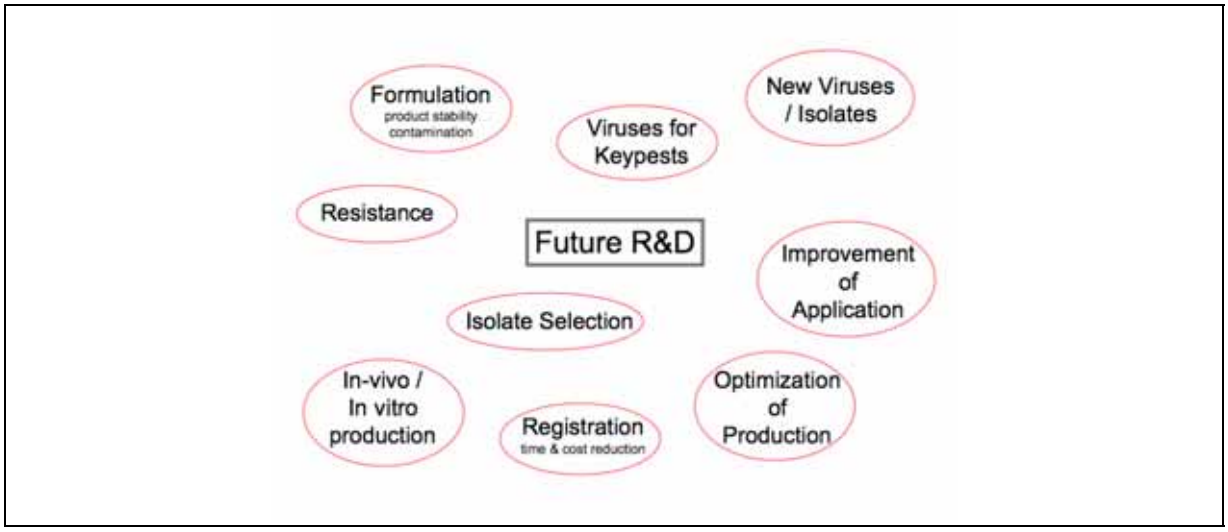

Figure 2. Overview of the most important needs concerning BVs, from an industrial point of view.

The aim of the present article is to point out the most important needs (Figure 2) for further research from an industrial point of view, that will allow the successful development and commercialisation of BV insecticides.

Specific topics concerning BV products and their research needs

In the following, various aspects of the development and maintenance of BV products are described in more detail:

Viruses for Key Pests

More than 20 virus products for different pest species (e.g. *Cydia pomonella, Adoxophyes orana, Cryptophlebia leucotreta, Helicoverpa armigera, Spodoptera litura/littoralis/exigua*) are available on the market. Many of them are well established and have become even more important due to an increasing development of resistance against chemical pesticides, the more rigorous residue situation and the banning of several chemical substances.

Although BVs are found in many different hosts, no virus products exist for many pest species. Especially in the case of key pests, the development of virus products would have a

high economic potential. One possible reason for the absence of such products could be the fact that research is mostly conducted with common host-pathogen systems, where the basic principles of the interaction are known

Closely associated with the lack of new products is the problem of isolating new viruses. Effective methods for finding new viruses in the field are needed (see "Finding / Selection of new virus isolates").

Examples for key pests are: *Lobesia botrana*, *Tuta absoluta*, *Cydia molesta*, *Eupoecilia ambiguella*, *Cameraria ohridella*, *Grapholita funebrana*

Finding / Selection of new virus isolates

Insect-pathogenic viruses are very effective natural antagonists of lepidopterans. However, if BVs are commercially used as biopesticides, selection in the host-pathogen system can become a problem. The occurrence of resistance in the codling moth – CpGV system (see "Insect Resistance against Virus") revealed that it is extremely important to have a large number of different virus isolates. A broad genetic basis is a prerequisite for developing new virus products that can break existing resistance. In the future, there will be a need for various virus isolates for all BVs used on a large scale as biopesticides.

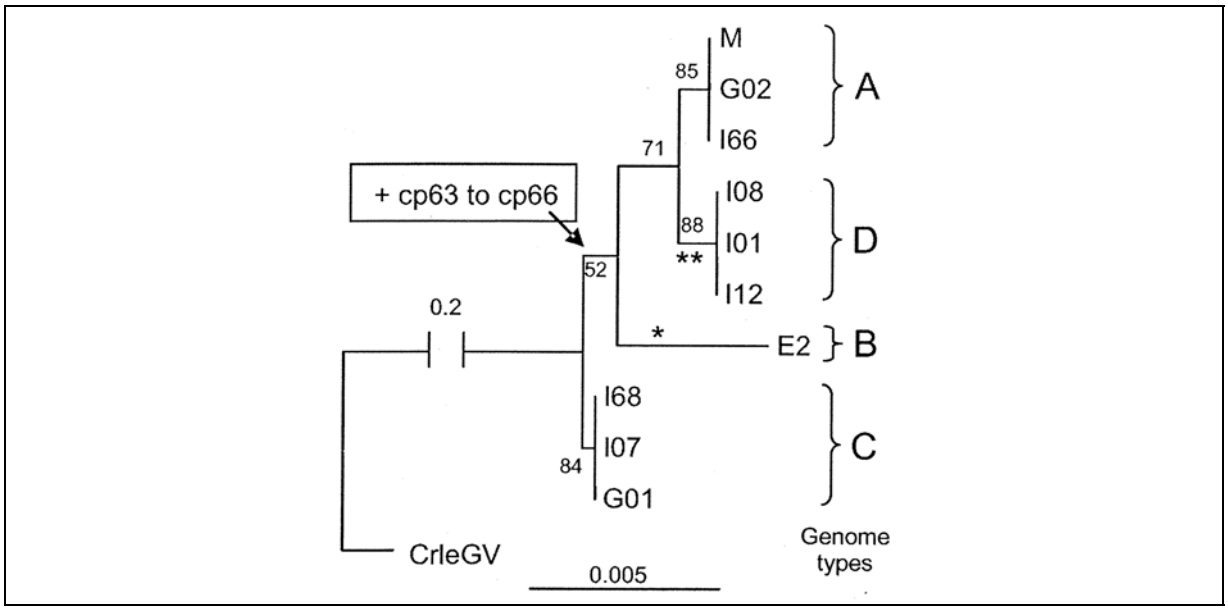

Figure 3. Minimum-evolution tree of nine CpGV isolates and CpGV-M (Source: Eberle et al. 2009).

Therefore, research projects are urgently needed that will help find more viruses or virus isolates worldwide. Efficient methods for finding and isolating viruses in the environment, especially if they only exist in small amounts, are important.

Optimization of Insect & Virus Production

Virus producers have a large know-how concerning the production of BVs, that is based on the results of scientific projects of research institutes, e.g. about the biology of insects/viruses or small-scale rearing methods. The extension to a large scale production is done by the companies themselves and is subject to non-disclosure. Unfortunately, it is becoming more and more difficult to finance projects dealing with fundamental biological or rearing questions

(the justification is an absence of high tech and innovation). This leads to a significant decline in the number of research groups catering to such important topics. In Europe, this problem is more critical than in the USA, where several universities and institutes have competence centers for insect rearing (e.g. Mass-rearing research units in Arizona & Mississippi, Insect Diet and Rearing Institute).

The following table shows the most important topics with respect to production optimization:

Table 1. Hot topics for further investigations concerning the process of product optimization.

Insect	Diet optimization with regard to palatability, insect survival rate, insect quality & quantity, costs
Codling moth	Microclimatic conditions for each insect stage
	Timing & synchronization through light & temperature
	Development characteristics: Mortality, reproduction rate, etc
	Inbreeding & Pathogens: Symptoms, impact, avoidance
Virus	Microclimatic conditions for virus multiplication
SlNPV	Infection time & OB concentration
	Oral infectivity & latent infections
	Purification for large amounts (large-scale)
	Differences GV – NPV

Formulation

The choice of the right formulation is crucial for a successful plant protection product. The general function of the formulation is to protect the active ingredient from adverse environmental conditions, to make the active ingredient palatable to the target pest and to allow a user-friendly handling of the product. This includes many requirements: The formulation has to be harmless to the environment, sprayable and cheap, it may not negatively affect the active ingredient, it shows good acceptance by the target (possibly increased by phagostimulants), the bacterial contamination has to be below the legal limit, small application rate, and the formulation allows a long shelf-life at different temperatures and protection after spraying in the field against UV radiation and rain.

A general weakness of virus products compared to chemical substances is their short half-life in the field due to UV radiation, and the fact that they are relatively slow in killing off their host. This calls, for example in the case of *Cydia pomonella* control, for a spraying interval of 7 to 10 days given sunny conditions. Over the last years, no significant improvements were achieved with any of the available CpGV products.

Worthwhile topics for research concerning formulation of BV products are screening for additives such as UV-protectors, phagostimulants, stickers and surfactants, whereas the substances used have to be in accordance with the requirements described above. New formulations

could have better properties such as longer shelf life, better UV protection and / or user-friendliness. It is generally important to consider the difference between laboratory and field.

Improvement of Application

The problems concerning BV pesticide application are similar to those for the formulation. The experience acquired with chemical pesticides is far greater than with virus products and this drawback still exists. Many application techniques were developed for chemical pesticides and are thus only limitedly applicable for virus products.

Such fundamental topics as application method, application rate and time of application need further research. Furthermore, BV products are often used in anti-resistance strategies and therefore their application together with chemical pesticides and mating disruption has to be investigated. An important prerequisite is the compatibility in tank mixes. The use of BVs for different crops and pest species needs to be optimized and their potential in population management studied. The phenomenon of a significant variety dependence, e.g. for the efficacy of CpGV products, also raises several questions.

Insect Resistance against Virus

The continuous use of a BV as a control agent can lead to increasing development of resistance on the part of the target insect. One prominent example for this is the codling moth – CpGV system. At least 40 codling moth populations, showing resistance against certain CpGV isolates exist in Europe – according to the current state of knowledge. Up to now, a monogenetic dominant resistance mechanism is hypothesized (Asser-Kaiser et al. 2007). However, the mechanism has not yet been fully clarified.

Table 2. Summary of the main results from the EU-Project “Sustain CpGV”.

The resistance is effective against the Mexican Isolate of the CpGV (CpGV-M).
Dominant, sex-linked inheritance of the resistance.
Viruses are still able to overcome the peritrophic membrane.
Virus replication is blocked in a very early stage.
The resistance occurs in Germany, France, the Netherlands, Italy, Austria and Switzerland, so far restricted to organic farms.
About 10 CpGV isolates, which overcome the resistance, were either found in the field or selected in the laboratory.
About 30 proven cases of resistance in Germany.

In the case of CpGV, several resistance breaking virus isolates were found and naturally selected (without genetic modification). Some of them are already commercially available. It is crucial to clarify the resistance mechanisms to develop and accelerate anti-resistance strategies, e.g. with new virus selections. Future research could build on the results of the recently ended EU-Project “Sustain CpGV”, which is an excellent example for a successful cooperation between research and industrial partners. The most important results are summarized in Table 2.

In addition to the CpGV products, all BV products that are commercially in use since several years (e.g. AoGV, ClGV, HaNPV, SlNPV, SeNPV) run the risk of resistance problems. Therefore, it is also essential to push resistance research for these insect-virus systems.

Conclusions

Baculoviruses have been successfully established as biopesticides worldwide. Many gaps in our knowledge concerning BVs still exist, partially due to the large differences between BVs and chemical pesticides and to the conflict of interest between research institutes and SMEs. The main areas of interest when it comes to reasearch and development do not overlap. For a SME a lot of basic information is needed to develop a profitable product.

Required research areas for BVs are discovery of new viruses for key pests, isolation of new viruses and isolates, optimization of insect & virus production, virus isolate selection, formulation, improvement of application and insect resistance against virus. Producers and researchers need to intensify the applied BV research, and national and international research projects can help accelerate success.

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Basic research to widen the use of nematode-based biocontrol agents

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Abstract: The most important aspect of designing an applied research project is choosing the right questions. In terms of use of nematode-based biological control agents, improved performance could be obtained by a greater understanding of mode of action, production, resistance to stress and understanding the behaviour of nematodes in soil following application. Recently developed scientific techniques, particularly related to sequencing of genomes and transcriptomes have much to offer, but in some cases, "low-tech" research methods may provide greater insight. All research is limited by the availability of funding, and different funding agencies have different priorities. Entomopathogenic nematodes and slug parasitic nematodes are useful models to study evolution, physiology and ecology of soil organisms. Agencies that fund research on basic biology may fund hi-tech, high-cost projects on our nematodes if they believe the outcomes will be sufficiently broad to interest biologists working with a range of organisms. Conversely, many funding agencies are interested in funding short term "problem solving" grants e.g. to control newly emerging pests. In such cases, problems can often be solved using low-tech solutions. The ability to maintain long term research programmes on nematode-based biological control agents is likely to be secured by combining both approaches.

Key words: Entomopathogenic nematodes, *Phasmarhabditis hermaphrodita,* DNA sequencing, integrated control

Overview

Nematodes are now well established as biological control agents (Gewal *et al.,* 2005) but the areas of land treated with nematodes is still very small. Factors often cited for the low uptake of these technologies include high costs and the perception that the products are unreliable. The first factor can be overcome by research, whereas the second will need some research, but mostly grower education.

Many people believe that costs can only be lowered through more efficient mass production. However, any research that substantially improves the performance of individual nematodes, increases the percentage of the applied population that survive, or improves the ability of the nematode population to recycle once applied has potential to reduce costs. In addition, the short shelf life of most nematode products means many nematodes are not used. Thus, extending the shelf life of formulated nematode products also has potential for reducing costs.

Second generation (and the promised third generation) sequencing technologies offer unprecedented opportunities for studying nematode (or EPN bacteria) host interactions (Morozova & Marra, 2008). For example, transcriptomics could be used to identify key effector molecules in the interactions. These effector molecules could either be over-expressed or cloned for uses in transgenic plants. The same approach could be used to identify key factors nematodes use to protect themselves against the stresses associated with formulation or application.

We still have a poor understanding of nematode behaviour and survival in soil following applications. Soil is often referred to as a “black box” but new soil analysis techniques, combined with laboratory experiments and mathematical modelling may help to predict how nematodes will move in the soil. Furthermore, we now have better ways to quantify nematodes in the soil that rely on real-time PCR (Macmillan *et al.*, 2006).

The adoption of modern biological techniques to study nematode based biological control agents appeals to many funding agencies. However, as securing such funding becomes more competitive, chances of success are likely to increase if such research asks general biological questions that will be relevant to a broad range of organisms, rather than only to nematodes that parasitise invertebrates.

Other funding agencies are more interested in solving problems for growers, reducing pesticide use or promoting use of IPM. Nematode biological control agents have much to offer in such systems. Research in these areas can provide interesting answers using more traditional techniques. For example we do not know how nematodes behave when applied to tree trunks for codling moth control. In such cases, researchers must work closely with growers and nematode producing companies to clearly identify knowledge gaps that truly have potential to enhance performance.

Ultimately, both types of research complement one another and both will feature prominently in future research on nematode based biological control agents. There is a tendency for scientists to adopt one approach or the other, but ideally both should be combined. Lack of facilities (e.g. field sites or high-tech lab equipment) often prevent the two areas of research from being conducted by the same group. However, in such circumstances, close collaboration between complementary groups should be encouraged.

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Virus

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Investigations on the mechanism of CpGV resistance in *Cydia pomonella*

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Abstract: After the successful use of CpGV in control of codling moths in organic and integrated farming for decades, some populations of *Cydia pomonella* developed resistance against CpGV products. Resistant populations were detected in several orchards in Germany, France, the Netherlands, Italy and Switzerland. The resistance is dominant, monogenic and sex-linked inherited. Beside the knowledge of heredity, it is mandatory to know the mechanism of resistance for the development of successful resistance management strategies. Three different experimental approaches were followed to investigate the mechanism involved in CpGV resistance: First, virus replication in three different tissues of susceptible (CpS) and resistant (CpRR1) insects was estimated by quantitative PCR. Second, humoral and cellular immune responses as a reason for resistance were investigated by transfusion of haemolymph from resistant into susceptible insects. Third, by-passing the midgut budded virus was directly injected into the insect's haemocoel in order to investigate whether resistance is present in others than midgut cells.

Key words: *Cydia pomonella*, codling moth, CpGV, resistance, baculovirus, resistance mechanism

Introduction

The *Cydia pomonella* granulovirus (CpGV, Baculoviridae) is the most important biocontrol agent of the codling moth in apple production. In the last four years, CpGV resistant codling moth populations been observed in Germany, France, the Netherlands, Italy and Switzerland (Fritsch et al., 2005; Asser-Kaiser et al., 2007; Jehle unpublished) The resistance is inherited by a single dominant gene which is located on the Z-Chromosome (Asser-Kaiser et al., 2007). Here we report investigations on the mechanism of CpGV resistance in *Cydia pomonella*.

Material and methods

Insect rearing

The insects used for the experiments derived from the insect rearing of the Agricultural Service Centre Palatinate (DLR Rheinpfalz), Neustadt. The susceptible codling moth strain, designated as CpS was reared at the DLR for the last 10 years. The CpGV resistant codling moth strain, CpRR1, was produced by homogenisation of a heterogeneous resistant field strain in 2007 (Asser-Kaiser et al., 2007). The insects were reared under controled conditions at 26°C, 60% humidity and 16h:8h light:dark.

Virus
The CpGV isolate used for the experiments was the so called “Mexican isolate” (CpGV-M) Here we report investigations on the mechanism of CpGV resistance in *Cydia pomonella* (Tanada, 1964; Eberle et al., 2009). This virus was propagated in fourth instar CpS larvae. The viral occlusion bodies (OBs) were purified following Jehle et al. (1992). The number of OBs per µl of the stock solution was counted using a Petroff-Hauser counting chamber (depth 0.02 mm) in the dark field optic of a Leica light microscope (DMRBE). Budded virus (BV) was produced by oral infection of fourth instar larvae of the susceptile strain with 1000 OBs and haemolymph extraction four days post infection. The amount of virus copies in haemolyph was determined by QPCR using granulin specific primers.

Tissue specific virus replication
To compare the spread of CpGV infection in codling moth larvae, both resistant and susceptible, codling moth larvae (L5) were orally infected with 1,000 CpGV OBs or intrahaemocoelar by injection of budded viurs into the haemocoel. Control larvae obtained virus-free food, or were injectied with haemolymph free from virus. 24, 48, 72 and 96 hours post inoculation (hpi) samples of the midgut, haemolymph and fatbody were taken from 5 larvae per treatment. DNA of these samples was isolated and the number of CpGV genomes was estimated with QPCR using granulin specific primers.

Transfusion of haemolymph
Humoral and cellular immune responses as a reason for resistance were investigated: Haemolymph of CpS larvae (L4), as well as of infected and not infected resistant CpRR1 larvae (L4) were injected into CpS larvae (L4). Sf900 medium was injected to control larvae. Afterwards these larvae were fed with a piece of diet either contaminated with 1,000 OBs or free of virus. Mortality was scored nine days later.

Intra-haemocoelar infection
In order to determine whether resistance is located in other than midgut cells, per oral infection was by-passed by injecting BV of CpGV into the haemocoel of CpS and CpRR1 larvae. First, CpS larvae (L4) were infected orally with 1000 OBs. Five days later haemolymph of these larvae was extracted and the concentration of BV was measured using QPCR. 10^5 BVs in 2 µl Sf900 were injected into the haemocoel of 20-25 CpS and CpRR1 larvae (L4). 2 µl Sf900 medium was injected into control animals. Mortality was scored after 7 days. Five replicates were performed.

Results and discussion

After both, oral and intrahaemocoelar infection, the amount of CpGV copies detected in all three tissue types of CpS increases with time elapsed. In none of the isolated tissue types of CpRR1 virus replication could be detected. This indicates that CpGV infection is already blocked at the midgut and not further spread, or resistance is present in each of the cell types.

Injection of CpRR1 haemolymph into CpS could not convey resistance in CpS. Thus, no immune response was observed and no factor in the haemolymph wich induces resistance could be identified. Injection of haemolyph from inoculated CpRR1 larvae into CpS larvae did not cause viral infection. Hence, the injected haemoplymph of inoculated CpRR1 larvae did not contain infectious budded virus.

In CpS injection of budded virus caused 100% mortality after 7 days, but no mortality was observed in CpRR1. Intrahaemocoelar injection of BVs did not overcome resistance. Accordingly, resistance is not restricted to the midgut but also to secondary infection.

Based on these results it is proposed that virus replication is affected in all cell types, suggesting a virus-cell incompatibility in resistant codling moths. An early block of vius replication is proposed as resistance mechanism.

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A *Phthorimaea operculella* granulovirus (PhopGV) containing several genotypes is highly efficient on *Tecia solanivora*

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Abstract: The granulovirus of *Phthorimaea operculella* (PhopGV) is becoming an alternative for biological control of potato moth pests. A PhopGV isolate from *P. operculella* in Peru, multiplied for several years on the alternative host *Tecia solanivora* in Colombia (named #Col6) appears to successfully control this pest, in spite of previous studies showing the inefficacy of PhopGVs on other hosts including *T. solanivora.* In this work, we studied the possible reasons for the high performance of this PhopGV. Restriction profiles and PCR analyses were performed on #Col6 and a reference PhopGV isolate (#1346). Submolar bands were found in #Col6 particularly with *Sma* I and *Nru* I endonucleases. PCR amplification of the 90-91 gene of #Col6 gave two bands, one of 767 bp and another of 619 bp, corresponding to a deleted gene found previously in viral isolates from *T. solanivora.* This indicates that #Col6 is a mixture of several genotypes, one of them probably coming from a local latent GV infection in the *T. solanivora* laboratory colony used for virus multiplication. The LC_{50} was assessed on *T. solanivora* with #Col6, #1346, the original Peruvian isolate and a PhopGV isolated from *T. solanivora* in Colombia (#VG003). #Col6 exhibited the highest virulence, probably because of the presence of several genotypes coming from *P.operculella* and *T. solanivora*.

Key words: PhopGV, Biological control, *Tecia solanivora*, *Phthorimaea operculella*

Introduction

The Guatemalan potato moth *Tecia solanivora* (Povolny) (Lepidoptera:Gelechiidae) is the most important potato pest in Colombia. For the control of *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae), the International Potato Center (IPC), recommends the use of a granulovirus (PhopGV) isolated from *P. operculella* in Peru. As no granulovirus was previously isolated on *T. solanivora*, the IPC suggested the use of PhopGV to control this pest. However, previous studies have shown the inefficacy of granulovirus isolates from *P. operculella* on alternative hosts, especially *T. solanivora* (Pollet *et al.,* 2003; Rebaudo *et al.,* 2006). The virus was able to replicate, but its efficacy was very low on the alternative hosts.

In 2002, the Colombian Corporation for Agricultural Research (CORPOICA) started manufacturing a PhopGV isolate provided by IPC on a laboratory colony of *T. solanivora*. The original isolate was thus multiplied for several years on this alternative host. The product successfully controls *T. solanivora* larvae under storage of potato seeds (Villamizar *et al.,* 2008a). This virus isolate was called #Col6. This work tried to elucidate the reason for the high performance of #Co16 on *T. solanivora* and to analyze the differences between the original isolate provided by IPC and #Col6.

Material and methods

Characterization of the viral isolates

Four different PhopGV isolates were used. PhopGV from *P. operculella* (gift from IPC) originated from La Molina (Peru), named "#Peru original" and "#Col6" provided by CORPOICA, which is the result of the multiplication of "#Peru original" on *T. solanivora* for ten passages. These isolates were compared to PhopGV isolate #1346 (gift from Dr. R. EL Bedewy, IPC, Egypt), characterized by Léry *et al.* (1998) and completely sequenced (Gene-bank accession: NC004062) and isolate "#VG003", isolated from *T. solanivora* in Funza (Colombia), by CORPOICA (Villamizar *et al.,* 2008b).

After occlusion bodies (OBs) purification and DNA viral extraction (Taha *et al.,* 2000), DNA concentration was determined by measuring the absorbance at 260 nm. The restriction profiles with 10 endonucleases (*Bam* HI, *Hind* III, *Sma* I, *Mlu* I, *Hpa* I, *Dra* III, *Nde* I, *Bst* EII, *Nru* I and *Nsi* I) that shown differences on PhopGV isolates were used (Léry *et al.,* 2008). Amplification by PCR of the five variable regions of the PhopGV (Léry *et al.*, 2008) using specific set of primers was performed.

Biological test

Bioassays to determine the mean lethal concentration (LC_{50}) were carried out with neonate larvae (L1) of *T. solanivora* using purified OBs. The OBs concentration was evaluated using the formula: $6.8x\ 10^8x\ OD_{450}$ x dilution = Number of OBs/ml (OD_{450}=optical density at 450 nm) (Zeddam *et al.,* 2003). Dilutions of purified virus stock in distilled water were used ($1.5x10^5$ to $5x10^9$ OBs in 2 ml of water). The final concentrations on the potato surface were 0.15, 0.5, 1.5, 5, 15, 50, 500 and 5,000 OBs/mm^2. They were applied homogeneously using a nebulizer apparatus (Carrera *et al.,* 2008). Two tubers were used for each concentration. Three replicates per dose, each consisting in 15 neonate larvae per tuber, were set up. Infected and dead larvae were collected. LC_{50} was calculated using Polo PC (LeOra Software, 1987).

Results and discussion

Restriction profiles of the viral DNA were different between isolates #1346 and #Col6. With *Sma* I a 5,830 bp submolar band could be detected in #Col6. Using *Nru* I, two submolar bands not present in #1346 could be detected at 12,016 bp and 5,450 bp respectively. One band at 6,100 bp is present as submolar in #Col6, whereas it is normally detected in #1346. Finally, a new band of 5,511 bp was detected in #Col6 (Fig. 1A). These results indicated that #Col6 is a mixture of several genotypes.

Previously results on the genetic characterization of #Peru original and #VG003, indicated that #1346 and #Peru original could be considered identical and correspond to one of the genotypes of #Col6 whereas #VG003 corresponds to one of the other genotypes. To verify the hypothesis of a combination of several genotypes in #Col6, PCR analyses were carried out. Gene 90-91 presents a deletion of the gene at 148 bp that appears to be a specific modification only found in viral strains obtained from *T. solanivora* in Colombia (Léry *et al*., 2008). PCR amplification indicates that #Col6 has two bands of 767 bp, corresponding to the band of #1346, and 619 bp, corresponding to the deleted gene (Fig. 1B), confirming the presence of at least two different genotypes in a proportion of 60-40% in the genome of #Col6.

The commercial production of PhopGV in Colombia used a viral infection of eggs from a laboratory colony followed by potatoes infestation with infected larvae. The infection of eggs for the next batch was made from these larvae in a successive passage model (unpublished data). Presence in #Col6 of at least one genotype from #Peru original and another probably

from #VG003, coming respectively from *P. operculella* and *T. solanivora,* could be explained. There was a permanent introduction with insect field population made after 8 to 10 generations in the production process. It is possible to imagine that some larvae with a local latent GV infection were added to the rearing. Because of these new data on the genetic characteristic of #Col6 and results of biological assays previously obtained (Villamizar *et al.* 2008a), we compared the biological activities of #Col6 and its two genotypes (#Peru original and #VG003).

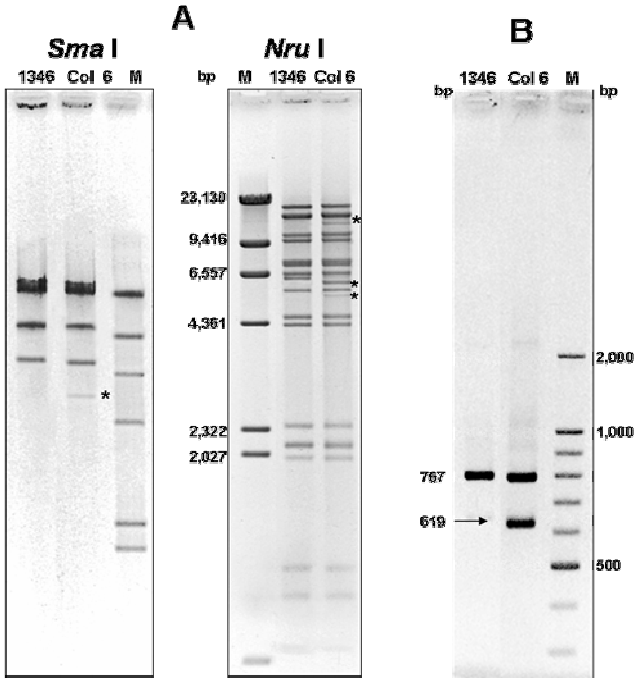

Figure 1. (A) Restriction DNA profiles of PhopGV #1346 and #Col6 isolates obtained with *Sma* I and *Nru* I. Asterisks indicate differences in band patterns. (B) Electrophoresis of PCR fragments amplified from the genomic DNA of #1346 and #Col6 with primers amplifying a region of 90-91 genes.

Biological test

The PhopGV #Col6 isolate exhibited the highest virulence with a LC_{50} of 3.39 OBs/mm^2. In comparison, the LC_{50} of #Peru original and #1346 were respectively 30.11 and 60.67 OBs/mm^2, when the LC_{50} of #VG003 was 6.93 OBs/mm^2 (Table 1). These results reinforce the previously studies (Pollet *et al.,* 2003; Rebaudo *et al.,* 2006) which indicated the lower virulence of the PhopGV isolates from *P. operculella* when tested on *T. solanivora.*

Curiously #Col6, which is a mixture of at least two genotypes (one from *P. operculella* and one from *T. solanivora*), is more efficient that #VG003, adapted to *T. solanivora*. This confirms results obtained by Villamizar *et al.* (2008a), which demonstrate the high virulence of #Col6 against *T. solanivora.* One hypothesis, which was demonstrate on another baculovirus model (NPV), is that a mixture of two different genotypes could be more efficient than the cloned genotypes alone (Simon *et al.,* 2006). This could explain the results obtained with #Col6. To verify this hypothesis, it would be necessary to run test with experimental mixtures of different viral isolates with different genetic and biological properties. The biological activity of such mixtures could be improved, in order to propose a more active and universal biopesticide against *T. solanivora.* The same work must also be performed on *P. operculella*.

Table 1. LC_{50} values of four PhopGV isolates on *T. solanivora* (in OBs/mm^2).

Isolate	LC_{50}	95%	
		Lower limit	Upper limit
#1346	68.67 a*	39.03	99.44
#Peru original	30.11 a	15.333	63.481
#VG003	6.93 b	4.895	9.808
#Col6	3.39 b	1.992	5.497

*LC_{50} followed by the same letter is not significantly different

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Isolation and characterization of a Colombian *Spodoptera frugiperda* nucleopolyhedrovirus

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Abstract: A native *Spodoptera frugiperda* nucleopolyhedrovirus was isolated after analyzing 652 larvae collected from the department of Córdoba (Colombia). It was codified as NPV003. This isolate presented a mean particle size of 2.0 ± 0.5µm and a LC_{50} of 2.3 x 10^5 occlusion bodies (OBs)/mL determined over neonatal larvae. The sensitivity of the isolate to different temperatures (30°C, 40°C and 50°C) and to UV radiation was also determined, obtaining a viral inactivation of 32.1% when OBs were exposed to 50°C during four hours and UVC light caused a significantly viral inactivation after six hours of irradiation.

Key words: Biological control, *Spodoptera frugiperda*, nucleopolyhedrovirus, UV-radiation

Introduction

Larvae of the armyworm, *Spodoptera frugiperda* (J.E. Smith) cause important economical losses (García y Del Pozo, 1999). An alternative for controlling *S. frugiperda* is the use of baculoviruses (Caballero *et al.*, 2001). The NPV of *S. frugiperda* has been successfully used in various countries (Valicente *et al.*, 2008). However, this biocontrol agent has been poorly studied in Colombia and no biopesticide based baculovirus is available. The work aimed to prospect for SfMNPV isolates and characterize them for their potential as biocontrol agent.

Materials and methods

Larvae collection

S. frugiperda larvae were sampled from corn and pasture located in the department of Cordoba (North of Colombia). Larvae were placed individually in plastic glasses containing artificial diet (Valicente *et al.*, 2008). Symptomatic and dead insects were individually placed into Eppendorf tubes with 1 ml of saline solution. Samples were transported to the laboratory for their analysis.

Detection and identification of native nucleopoliedrovirus isolations

Each larva was classified and codified according to the place of sampling. The larvae were reared in the plastic glasses with diet and were maintained in quarantine at 26°C ± 2°C. They were daily checked to detect symptomatic or dead larvae, which were transferred to 1ml of saline solution. Dead larvae were triturated and the suspensions were observed in the light microscope (40x) to detect viral OBs. To confirm the infection, larvae suspensions were used to contaminate groups of 30 third instar *S. frugiperda* larvae by the droplet feeding method (Hughes & Wood, 1981).

Morphological characterization
The mean OBs size was determined by direct observation, using a light microscope by averaging the individual diameters of 100 OBs (Hirech *et al.*, 2003). The OBs morphology and shape were examined by scanning electron microscopy (SEM) and internal structure was determined by transmission electron microcopy (TEM).

Determination of lethal concentrations
Bioassays were carried out with neonate larvae by peroral droplet technique (Huges and Wood, 1981). Viral suspensions were prepared by serial dilutions of purified virus and concentrations were adjusted from 2 x 10^4 to 2 x 10^8 OBs/mL. Thirty larvae were used for each treatment, and placed individually in a cup with artificial diet. Control treatment consisted in neonatal larvae without ingestion of any treatment. Cups were maintained at 28°C and 70% RH. Percentage of mortality was recorded 7 days after larvae exposition to treatments. Bioassay included two repetitions. Dosage response data were analyzed by using Probit test (Finney, 1971) to determine the LC_{50} and $LC_{90.}$

Viral susceptibility to UV radiation
Samples of viral suspensions adjusted to concentrations from 2 x 10^4 to 2 x 10^8 OBs/mL were irradiated for 6 hours with monochromatic UV light type A (365nm), B (302nm) and C (254). After irradiation, samples were evaluated for viral activity by using the bioassay described above.

Viral susceptibility to temperature
Samples containing 20 µL of viral suspensions adjusted to 2 x 10^7 OBs/mL were incubated for 2, 4 and 6 hours at 30°C, 40°C and 50°C. Control treatment consisted in untreated larvae. After temperature exposition viral suspensions were used to evaluate biological activity. For both, UV and temperature experiments, mortality data were used to determine efficacy by the Schneider Orelli equation (Zar, 1999).

Results and discussion

Larvae collection
Alter sampling five different towns located in the department of Cordoba (North of Colombia), 652 *S. frugiperda* larvae were collected. However, only in one place larvae showed viral infection symptoms, consisting in a white colour abdomen.

Detection and identification of native nucleopoliedrovirus isolations
From 652 collected samples, 41 larvae (6.3%) presented typical symptoms of nucleopolyhedrovirus infection, but all these samples were collected in the same place, so they were considered as the same isolate which was codified as NPV003. Granulovirus infection was also observed in 3.7% of the samples (24 larvae). Mortality due to other biocontrol agents as nematodes and parasitoids was observed in 119 larvae (18.3). Four hundred and sixty-eight larvae successfully developed into adults (71.8%).

Valicente *et al*. (2008) collected *S. frugiperda* larvae in Brazil during 1989 isolating 22 NPV from 14,000 larvae collected in different places, this result corresponding to the 0.15% of the samples. In the present work, only one isolate was obtained from 652 sampled larvae, result that also corresponds to the 0.15% of the evaluated samples.

Morphological and biological characterization: Lethal concentrations

The OBs of NPV003 isolate presented irregular shape and diameters rearing from 1.5 to 2.5 µms with an average value of 2.0 ± 0.5 µms, which were confirmed by SEM micrograph (Figure 1a). NPV003 OBs contain multiple nucleocapsids per envelope allowing classifying this native strain as multiple enveloped nuclear polyhedrosis virus of *S. frugiperda* (SfMNPV) (Figure 1b).

The value of χ^2 was not significant at the 95% probability level, indicating no systematic heterogeneity of response. A dose response effect was observed. Mean lethal concentrations LC_{50} and LC_{90} were 1.6 x 10^5 and 1.2 x 10^6OBs/mL respectively.

The LC_{50} determined for the native virus codified as NPV003 is in the same range as that obtained for other SfNPV isolates. For example, when Barreto *et al.*, (2005) analyzed 22 Brazilian isolates of *S. frugiperda* NPV LC_{50}, obtaining values from 0.029 to 6 x 10^5OBs/mL.

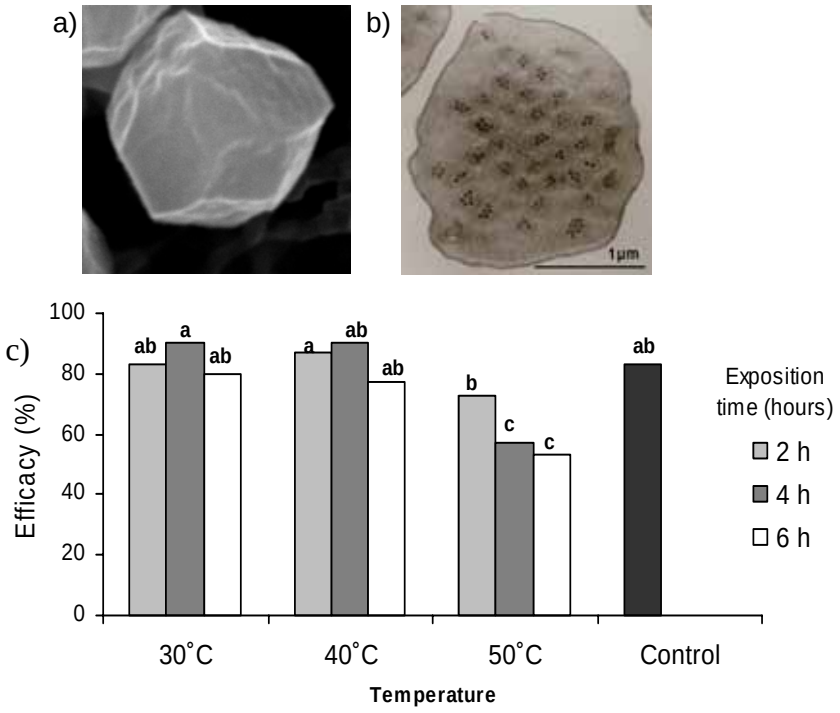

Figure 1. (a) SEM and (b) TEM micrographs of NPV003 OBs. (c) Effect of temperature on NPV003 isolate efficacy. Treatments with the same letter are not significantly different (Tukey test, 95%)

Viral susceptibility to UV radiation and temperature

Mean lethal concentrations of the irradiated virus are shown in Table 1. When the virus was irradiated with low energy radiations (UVA and UVB), no significant differences were observed in the LC_{50} among irradiated OBs as compared with non-irradiated OBs, suggesting that this isolate was not inactivated for these wavelengths. Nevertheless, when OBs were irradiated with UVC (254 nm), the LC_{50} significantly increased from 1.6 x 10^5 to 2.5 x 10^7OBs/mL after 60 minutes, being the final mean lethal concentration 156 times the initial concentration. Obtained results allowed concluding that NPV003 isolate was only inactivated by UVC radiation, possibly due to the highest energy, which causes damage over proteins and DNA (Griego *et al*, 1985).

Table 1. Effect of the UV radiation over NPV003 LC_{50} (OBs/mL). Numbers with the same letter are not significantly different according to Probit confidence limits comparison.

Exposition time (min)	UV radiation		
	UVA	UVB	UVC
0	1.6×10^5 a	$1,6 \times 10^5$ a	$1,6 \times 10^5$ a
15	5.4×10^4 a	$4,3 \times 10^4$ a	$6,7 \times 10^5$ b
30	2.7×10^4 a	$1,4 \times 10^5$ a	$1,5 \times 10^6$ abc
45	$3,9 \times 10^5$ a	$1,8 \times 10^6$ a	$4,9 \times 10^6$ abc
60	$3,7 \times 10^5$ a	$4,9 \times 10^5$ a	$2,5 \times 10^7$ c

Native isolate NPV003 was not significantly inactivated when OBs were exposed during 6 h to 30 and 40°C (Figure 1c). However, the virus presented a significant reduction of the biological activity after been maintained during 4 h at 50°C, with a final efficacy of 68% (total inactivation of 32.1%). This is important information for downstream processing and formulation, where temperatures higher than 40˚C are not allowed, in order to maintain viral activity.

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Using African armyworm NPV as a strategic biological control agent: Will studying pathogen ecology provide the answers?

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Abstract: The African armyworm *Spodoptera exempta* (Lepidoptera: Noctuidae) is one of the most devastating agricultural pests in sub-Saharan Africa, destroying staple crops such as maize, wheat, sorghum, millet, rice and pasture grasses. We are interested in the ecology of a natural disease, *S. exempta* nucleopolyhedrovirus (SpexNPV), and examining how this virus may be utilised in a strategic control program. In this report, we describe preliminary findings from a single field-season, exploring the structure of natural overt SpexNPV populations; and the transmission strategies of the virus, specifically covert infections.

Key words: African armyworm, *Spodoptera exempta*, nucleopolyhedrovirus, biocontrol, transmission strategies

Introduction

One of the most devastating crop pests in Africa is the larval stage of the African armyworm moth, *Spodoptera exempta*, a major pest of maize, wheat, sorghum, millet, rice and pasture grasses. Armyworm have been reported throughout sub-Saharan Africa, however the vast majority of outbreaks occur in the eastern half of the continent, and especially in Tanzania, Kenya and neighbouring countries. In East Africa, armyworm outbreaks are considered a serious problem in nine out of ten years (Rose *et al.,* 2000), and in major outbreak years armyworm can affect millions of hectares, causing massive losses to staple grain crops, as well as more widespread damage to pasture and livestock production (Rose *et al.,* 2000).

Armyworms play host to a specific baculovirus: *S. exempta* nucleopolyhedrovirus (*Spex*NPV). Larvae become infected when they ingest vegetation contaminated with virus occlusion bodies (OBs). Although extensive epidemics of *Spex*NPV often cause armyworm populations to crash (causing <98% mortality; Rose *et al.,* 2000), these natural disease outbreaks are usually far too late in the armyworm seasonal cycle to prevent major crop damage.

We are investigating the ecology of natural *Spex*NPV infections and its wider role in the population dynamics of armyworm, with a view to determining how best to exploit the virus as a strategic biocontrol agent. In this report, we describe preliminary findings from a single field-season, examining the population structure of overt viral disease; and looking at the transmission strategies of the virus, specifically covert infections. We will address two hypotheses:

(i) As *overt* virus (virus causing the death of its host) becomes more prevalent in late-season outbreak populations, so the proportion of genotypically variable infections increases, leading to opportunities for more pathogenic strains to out-compete less pathogenic ones. We hypothesise that late-season outbreaks will contain more genotypically-variable and genotypically-mixed viral isolates.

(ii) As the seasonal rains end, and the supply of green vegetation diminishes, so larval densities decline and high density larval outbreaks no longer occur, limiting the opportunities for density-dependent horizontal virus transmission between larvae. We hypothesise that virus persists during the dry season primarily as persistent non-lethal, vertically transmitted *covert* infections (i.e. infections that exhibit no external symptoms).

Material and methods

Assessing overt NPV prevalence and population structure

During the 2007-08 armyworm season (between December 2007 and April 2008), we compared early-, mid- and late-season larval armyworm outbreaks throughout Tanzania for virus prevalence. Quadrat counts were undertaken to establish densities of both healthy and NPV-killed larvae. Viral cadavers (single dead larvae) were collected and returned to the laboratory, where viral DNA was isolated. Genotypic characterization of the virus was based on *Eco*RV restriction fragment length polymorphism (RFLP) of single cadavers, providing an indication of the diversity of individual isolates.

Assessing covert NPV prevalence

Presence of covert NPV infection was examined in both larval outbreaks and from adult moths. Healthy larvae were collected from outbreaks during the 2007-08 armyworm season. In addition, adult moths were collected at the beginning of the 2008-09 armyworm season, from a network of pheromone and light traps established throughout Tanzania. Insect samples were stored in 100% ethanol, and returned to the laboratory for analysis. DNA and RNA were isolated using the AllPrep DNA/RNA Mini Kit (Qiagen). Polymerase Chain Reaction (PCR) was undertaken on samples using universal host *28S* gene primers as a control, and NPV-specific *Polyhedrin* primers to identify viral infections (Vilaplana *et al.,* in press). Reverse transciptase-PCR (RT-PCR) was developed with the same primer sets to determine whether the *Spex*NPV genome was actively transcribing. The PCR products were analysed using agarose gel electrophoresis techniques.

Results and discussion

Investigation of overt NPV populations

Twenty-one larval outbreaks of armyworm were sampled throughout Tanzania during the 2007-08 season. Overt NPV was discovered in 9 (43%) of the populations sampled, causing up to 17% mortality. DNA was isolated from 208 individual viral cadavers, and *Eco*RV restriction enzyme used to quantify genotypic variation. 54 genetically-distinct virus isolates were identified. An investigation of the viral population structure at each site indicated that early-season (January-February) outbreaks were dominated by a single genetic isolate, causing over 60% of the mortality. Late-season (April) outbreaks had a more mixed population structure with no one isolate dominating and out-competing the others. Late-season cadavers were found to contain more mixed-genotype infections than early-season populations (data not shown).

Covert NPV prevalence

Healthy larvae collected during 2007-08 armyworm season were investigated for the presence of covert NPV infections. DNA was isolated, and PCR used to look for the SpexNPV specific gene, *Polyhedrin*. From 255 samples, 96% were found to be infected with covert infections. RNA is currently being investigated to distinguish whether the covert infections are persistent (i.e. replicating) or latent (i.e. dormant).

However, we are aware that these data may be open to criticism; due to the fact that the presence of NPV in larvae may just be a result of early developing overt infections, and not covert infections at all. For this reason, we have also looked at adult moths caught in pheromone and light traps. This is a life stage where NPV cannot be acquired and NPV overt disease does not occur. Also, we wanted to investigate moths from early in the armyworm season, before larval outbreaks occur. Fifteen early-season moths were caught between October and November 2008 from 3 pheromone traps, and PCR used to look for covert NPV infections. Covert infections were detected in 100% of adult moths tested (Figure 1). These moths are most likely derived from solitary-form larvae, with their larval development occurring during part the dry season. Therefore, this suggests that NPV most likely persists over the dry season as a covert vertically-transmitted infection; a period when larval densities are extremely low and horizontal transmission is negligible.

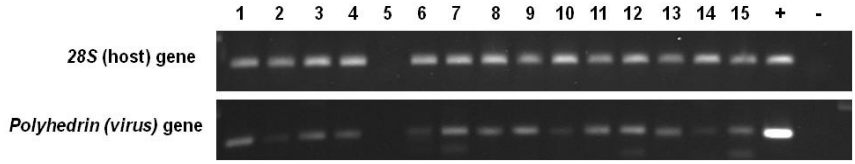

Figure 1. PCR of healthy early-season adult moths collected from pheromone and light traps. The gel illustrates the presence of the host gene *28S* (control, to assess quality of DNA) and the presence of the NPV gene *Polyhedrin*. 1-15: adult moth samples; +: positive control (NPV DNA); -: negative control (dH_2O).

Our current belief is that covert infections are somehow triggered into overt disease and that horizontal transmission then establishes as larval densities increase in early-season populations. Spontaneous virus deaths, most likely from triggered covert infections, were observed in our laboratory cultures. Two genetic isolates were detected from the triggered viral deaths, labelled isolates A and B. Both isolates were different to those seen in the field, and different to any used in the lab, and therefore unlikely to be caused by contamination. Both A and B are genotypically unlike isolate VAR1, the dominant early-season isolate (the isolate we hypothesise most likely appeared from early-season triggered covert infections).

Vertical transmission of NPV in armyworm was first recorded in the 1960s, when laboratory populations spontaneously developed NPV disease (Brown and Swaine, 1965; Swaine, 1966). In more recent experiments examining vertical transmission, both solitary and gregarious forms of larvae were sub-lethally infected with NPV, and allowed to develop to adults. Offspring were subsequently investigated for prevalence of NPV mortality, with solitary-form armyworm more susceptible to vertically-transmitted virus than gregarious-form (Vilaplana *et al*., 2008). Further evidence that insect populations harbour covert virus infections come from other lepidopteran systems. In *Pieris brassicae*, host insects yielded genetically-distinct viral progeny after challenge with a different virus (Smith and Crook, 1993); in addition, when *M. brassicae* larvae produced MbMNPV after challenge with

Panolis flammea (Pafl) NPV, an analysis of host DNA from uninfected insects using PCR showed that they contained MbMNPV DNA (Hughes *et al.,* 1993). Studies on field populations of *M. brassicae* have subsequently shown covert infections to be prevalent (Burden *et al.,* 2003). For future studies, we will conduct a series of bioassays using multiple virus isolates collected from early-, mid- and late-season outbreaks, quantifying the following virus attributes: LD_{50}/LC_{50}, ST_{50}/LT_{50} and OB yield. These bioassays will allow us to test the hypothesis that late-season isolates are more virulent than early-season isolates. In addition, we will try to identify what generates and maintains genetic diversity, and whether it plays an important role in virus epizootics.

Acknowledgements

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Dual reprogramming of the AcMNPV chitinase (*chiA*) and cathepsin (*v-cath*) expression profiles

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Abstract: The co-dependent function of the baculoviral CHIA and V-CATH enzymes is to aid viral progeny dissemination by promoting host tissue liquefaction. Mis-expression (earlier or increased) of these endogenous viral enzymes may increase the virulence of the baculovirus infection, and might accelerate host debilitation, and thereby might enable enhancement of a baculovirus-based biopesticide. Recombinant AcMNPV with *chiA* transcription reprogrammed by AcMNPV *polh* or *p6.9* promoters adjacent to the native intergenic *v-cath* promoter had *chiA* expression reprogrammed as expected, but lacked detectable *v-cath* transcription or cathepsin enzyme activity. Dual reprogramming of the adjacent *chiA/v-cath* genes with AcMNPV-derived *p6.9/polh* promoters, respectively, simultaneously modulated the transcription profiles of both genes. The reprogrammed transcription profiles of the two genes were characteristic of the alternate *p6.9/polh* promoter sequences and resulted in a 1.5 and 1.15 fold increase in the relative chitinase and cathepsin activities at 40 hours post infection.

Key words: baculovirus, endogenous enzymes, chitinase, cathepsin protease, gene mis-expression

Introduction

Baculoviruses are popular as species-specific biopesticides (Moscardi, 1999) 0 but their slow replication allows for extensive pest insect feeding damage after infection. Baculoviruses can be engineered to produce toxic heterologous proteins such as Bt toxins, insect neurotoxins or, insect-derived protease or chitinase enzymes. However, these raise environmental and marketing concerns. To avoid using foreign genes, we sought to generate enhanced baculoviruses by inducing mis-expression of two viral genes, chitinase (*chiA*) and cathepsin protease (*v-cath*).

The *chiA/v-cath* gene locus (Figure 1) is conserved in many baculoviruses, especially the group I alphabaculoviruses such as *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) (Slack et al., 2004) and share a common intergenic promoter sequence. Chitinase and cathepsin enzymes are dispensable for virus replication, and thus blocking or modulating their temporal expression should not adversely affect virus production. Hodgson et al. (2007) showed that substitution of the native *chiA* promoter with *p6.9* or *polh* promoters resulted in enhanced *chiA* expression but *v-cath* mRNA transcription was lacking. Chitinase activity was detected about 6 hours after *chiA* mRNA was first detected for native and the chiA-reprogrammed viruses. Using the *p6.9* and *polh* promoters there was 2.5x and 3.5x higher expression respectively, of chitinase at 48 hpi. However, the expression of active V-CATH enzyme is required for efficient cell lysis and concomitant release of both enzymes. Therefore, the *p6.9* and *polh* promoters were used to reprogram expression of both enzymes in one dual-reprogrammed virus.

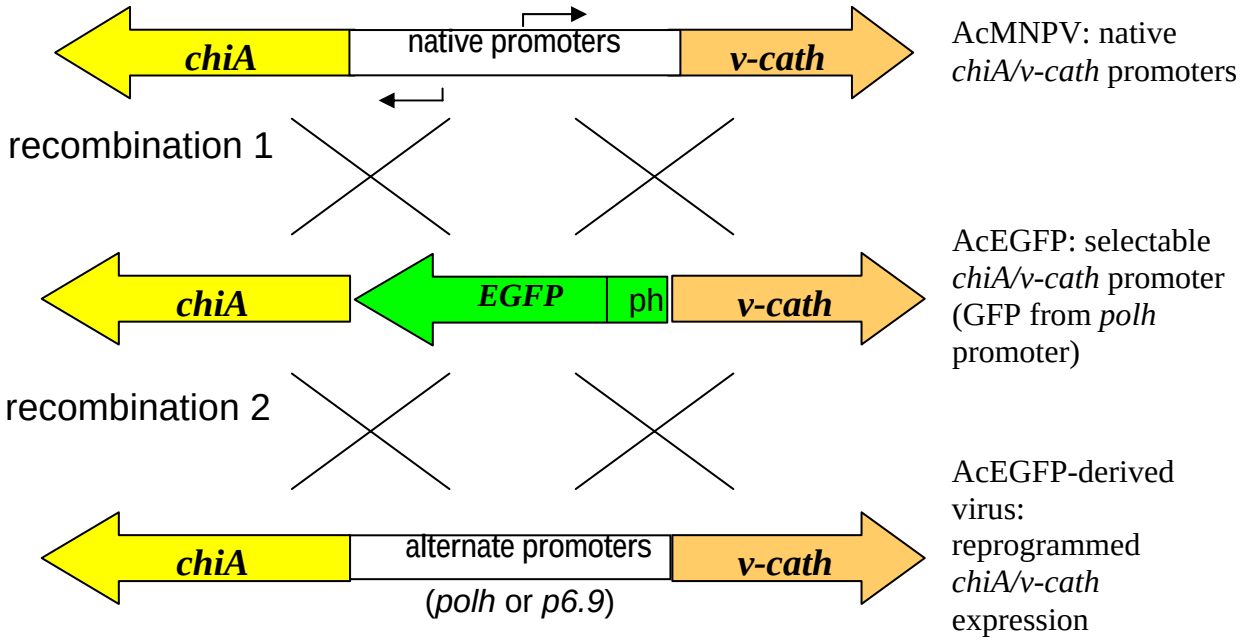

Figure 1. Structures of viral *chiA/v-cath* loci showing the intergenic promoter region and indicating the methodology for generating reprogrammed viruses by homologous recombination. and highlighting the organization of genes and promoters.

Materials and methods

Virus and cell culture

98 Monolayers of Sf21 cells were infected with AcMNPV strain E2 and used as a base to make recombinant viruses. All expression work was with Sf21 cells infected with the different reprogrammed viruses.

Generation and isolation of reprogrammed viruses

98 AcMNPV genomic DNA was isolated from budded virions and liposome-transfected (Cellfectin) with *polh-EGFP* plasmid DNA into Sf21 cells to generate the intermediate AcEGFP. The EGFP was subsequently replaced with the modified alternate promoters and reprogrammed viruses generated as described by Hodgson et al., (2007). Restriction enzyme analysis and Southern blotting of viral genomic DNA was used to verify the expected genomic changes.

Characterization of reprogrammed virus chiA and v-cath expression

98 Sf21 cells were infected with the different virus constructs to analyse relative *chiA* and *v-cath* mRNA and chitinase enzyme production. Total RNA was extracted using TRIzol (Invitrogen), electrophoresed in denaturing (2.2 M formaldehyde) 1.3 % agarose MOPS gels, blotted to (+)nylon membrane and probed with a DIG-labelled ssDNA strand-specific probe. Relative chitinase activities were determined by digestion of carboxymethylchitin-RBV substrate (Loewe). Relative amounts of cathepsin (V-CATH) protease activity were determined by an Azocoll (Sigma) substrate-digestion assay (Ohkawa et al., 1994).

Results and discussion

ChiA and v-cath RNA expression of native and reprogrammed viruses

Northern blot analysis showed identical temporal RNA expression by the native and repair viruses (not shown). Transcripts expressed from the repaired native late *chiA* and *v-cath* promoters yielded overall expression profiles resembling that of native AcMNPV. The profile of the repair virus was compared to that of the dual reprogrammed virus (Figure 2). The *p6.9* promoter-derived *chiA* mRNAs are much more abundant at 9 hpi than those from the native promoter, and are produced in higher amounts through to 48 hpi. The very late *polh* promoter-expressed *v-cath* mRNA is detectable from 9 hpi, but as is typical of very late baculovirus transcription patterns, *polh*-mediated *v-cath* transcription rapidly increases after 18 hpi to levels that far exceed that expressed from the native (repaired) *v-cath* promoter. The native sizes of the 2.6 kb *chiA* and 1.5 kb *v-cath* transcripts were not affected by dual reprogramming with the *p6.9* and *polh* promoters in the intergenic region. This data demonstrates that it is possible to reprogram both *chiA* and *v-cath* transcription simultaneously from alternate, AcMNPV-derived promoters (*p6.9, polh*) within the intergenic *chiA/v-cath* promoter region. The similar *chiA* and *v-cath* mRNA expression patterns of AcMNPV and the repair virus suggested that *v-cath* transcription from its native promoter adjacent to the *p6.9* or *polh* promoters in the chiA-reprogrammed viruses (Hodgson et al., 2007) might have been blocked because of a critical rearrangement of the *cath* promoter due to insertion of either of the alternate *chiA* promoters.

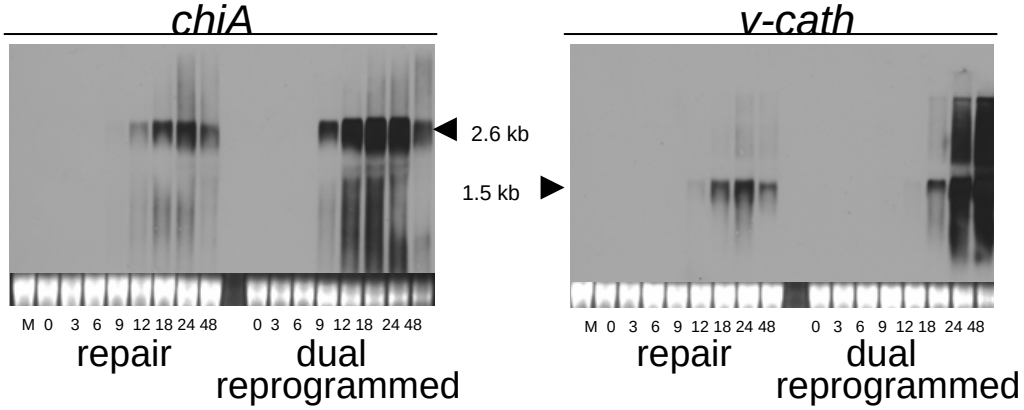

Figure 2. Northern blot analysis of *chiA* and *v-cath* mRNA (10 µg/lane) temporally produced by the *chiA/v-cath* repair virus and the dual (*p6.9/polh*) reprogrammed virus. Indicated sizes are based on an RNA ladder. The ethidium bromide stained rRNA bands below lanes indicate equivalency of the relative amounts of different RNAs loaded.

Enzyme expression of native and reprogrammed viruses

The amount of CHIA and V-CATH enzymes produced by the parental AcMNPV and the repair virus was not considerably different (not shown), and was consistent with their *chiA* mRNA expression pattern. Unlike native AcMNPV, the single p6.9-chiA reprogrammed virus did not express detectable amounts of the viral cathepsin protease, as expected based on their lack of *v-cath* mRNA expression (Hodgson et al., 2007). However, the dual reprogrammed (p6.9-chiA/polh-v-cath) virus expressed increased amounts of both the chitinase and cathepsin enzymes at 40 hpi relative to that from the repair virus (Figure 3). The dual reprogrammed virus expressed 1.5 fold more chitinase activity and 1.2 fold more cathepsin activity than did

AcMNPV or the repair virus at 48 hpi. Therefore, the dual reprogrammed expression of *chiA* and *v-cath* mRNA leads to an increase in the amount of both the CHIA and V-CATH enzymes. Using this approach we expect to generate more efficacious viruses without resorting to use of foreign toxin genes.

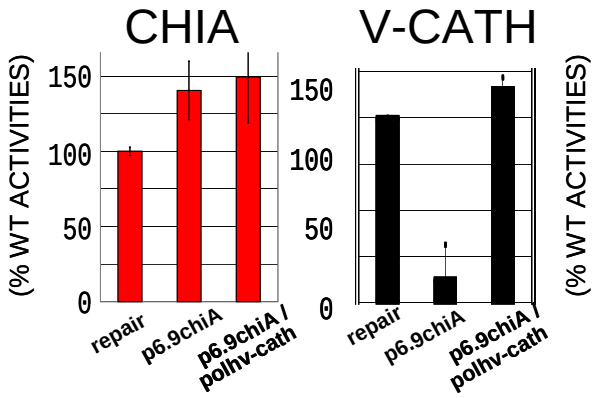

Figure 3. Relative amounts of chitinase (CHIA) and viral cathepsin (V-CATH) enzyme activity produced by the chiA/v-cath repair virus, the single p6.9-chiA and dual p6.9-chiA/polh-v-cath reprogrammed viruses at 40 hpi.

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Spodoptera exigua nucleopolyhedrovirus is not infectious for *Agrotis segetum* larvae *per os*, but only after intrahemocoelic injection

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Abstract: *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) and *Agrotis segetum* NPV are close relatives, but distinct baculovirus species. Their genomic organization is remarkably similar. The cross infectivity of these two viruses for *S. exigua* and *A. segetum* larvae has been analyzed. AgseNPV was able to infect *S. exigua* orally, although the dose needed was high (LD_{50} of 8,3 x 10^5 OBs/larvae) and the yield of progeny polyhedra very low. In contrast, SeMNPV appeared to be non-infectious for *A. segetum* larvae when administered *per os*. The course of SeMNPV infection in *A. segetum* was monitored by real-time PCR in a time course experiment and showed that SeMNPV was able to infect and produce polyhedra in *A. segetum* larvae by intrahemocoelic injection. Infection of *A. segetum* with a mixture of AgseNPV and SeMNPV, only resulted in AgseNPV polyhedra. So, in the case of SeMNPV the midgut is the major barrier for infection. The possible mechanisms of *A. segetum* resistance for SeMNPV *per os* are discussed.

Key words: *Spodoptera exigua* multiple nucleopolyhedrovirus, *Agrotis segetum* nucleopolyhedrovirus, cross-infectivity, specificity, genetic co-linearity.

Introduction

It is often claimed that baculoviruses in general have a narrow host range, though experimental studies on virus specificity are rather limited (Gröner, 1986). Moreover cross-infectivity studies often lack verification, to determine if the progeny virus produced due to cross-infection is actually the one used for the cross infection experiments or a resident or latent baculovirus in the challenged host (McKinley et al., 1989; Cory, 2003). Several studies have shown that in these experiments progeny virus may result from contamination or the induction of a latent virus (Cooper et al., 2003). In the current study we investigated the specificity of two NPVs, SeMNPV and AgseNPV versus *S. exigua* and *A. segeum* larvae by oral ingestion as well as by intrahemocoelic injections.

Materials and methods

Insects, cells and viruses

A. segetum and *S. exigua* larvae were as described by Hinks and Byers (1976) and Smits et al. (1986), respectively. *A. segetum* nucleopolyhedrovirus (AgseNPV) was isolated from *A. segetum* larvae collected in a cabbage crop in Poland (Jakubowska et al., 2005). *S. exigua* MNPV was SeMNPV-US1 and SeMNPV-SP2. Viruses used for intrahemocoelic infections were collected from hemolymph of infected larvae. Infectious BVs titer was determined by

endpoint dilution assays (Vlak, 1979) in AiE1116T and Se301 cell lines, respectively for AgseNPV and SeMNPV.

Per os and intrahaemocoleic infections

To determine the infectivity of SeMNPV in the heterologous host *A. segetum* L2 larvae were infected orally by the droplet feeding method with high doses of 10^8 OBs/ml of the respective viruses. The same infection procedures were used to preliminarily determine the infectivity of AgseNPV in *S. exigua* larvae. In order to calculate LD_{50} values of AgseNPV for both species L2 larvae were infected with 5 doses of AgseNPV (10^1 – 10^5 OBs/larvae for *A. segetum* and 10^3 – 10^8 OBs/larvae for *S. exigua*). The dose-mortality data were analysed by probit analysis, using the computer program POLO (Russel *et al.*, 1977).

For detection of viral transcripts *A. segetum* larvae were orally infected with SeMNPV (10^7OBs/larvae) or with a mixture of SeMNPV and AgseNPV. In parallel, *A. segetum* larvae were injected 10µl SeMNPV BVs (10^8 $TCID_{50}$/ml) as well as with a mixture of SeMNPV and AgseNPV BVs. Orally infected or injected larvae were reared individually until material collection for RNA isolation. Also midguts were isolated from randomly selected larvae.

Detection of transcripts – quantitative RT-PCR

Total RNA was extracted from insect larvae according to standard protocols using TRIpure Isolation Reagent (Roche). Quantitative real-time RT-PCR was employed to determine the presence or absence of SeMNPV and AgseNPV DNA polymerase (*DNApol*) transcripts in cross-infectivity studies. Following DNase I (Invitrogen) treatment, cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. All reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in duplets. Purified SeMNPV and AgseNPV DNA dilutions (10^{-1} – 10^{-8}) were used for standard curves. The DNA was quantified with a BioPhotometer (Eppendorf) and the number of target gene copies was calculated based on the DNA concentration and the average DNA basepair weight. ATP synthase gene expression was used as intrinsic reference. The PCR reactions were performed in the ABI PRISM 7000 from Applied Biosystems. All primer sets were checked for specificity. Moreover the cross reaction of the SeMNPV and AgseNPV specific primer sets was checked by performing reactions with AgseNPV and SeMNPV DNA as templates. Real-time PCR data were visualized and analyzed using 7000 System Sequence Detection Software version 1.2.3 (Applied Biosystems).

Results and discussion

Per os infectivity of SeMNPV and AgseNPV against S. exigua and A. segetum larvae

AgseNPV appeared to produce a fatal infection in *S. exigua.* However the rate of infection was very low. A high dose of AgseNPV virus, 10^8 OBs/ml, caused only 30% mortality in second instar *S. exigua* larvae infected using the drop-let feeding method. OBs were present in the tissues of infected and dead insects. The restriction pattern of virus resulting from cross-infection was identical with the pattern of input virus (not shown). We conclude that *S. exigua* is a semipermissive host for AgseNPV. For comparison of LD_{50} values of AgseNPV for both insect species a bioassay was conducted. The 50% lethal dose for AgseNPV in *A. segetum* and *S. exigua* were 83 OBs/larvae and 8,3 x 10^5 OBs/larvae, respectively.

In contrast, SeMNPV did not produce a fatal infection in *A. segetum* larvae by oral inoculation, even not at the highest tested doses.

Intrahemocoelic infection of A. segetum larvae with SeMNPV BVs

Given that SeMNPV is not orally infectious for *A. segetum* larvae we performed intrahemocoelic injections of SeMNPV into *A. segetum* hemocoel. More than 90% L3 larvae injected with high dose of SeMNPV BVs (10^7 $TCID_{50}$/larvae) died from virus infection. The restriction pattern of the recovered virus was identical to the input virus SeMNPV (not shown). Injections of SeMNPV BVs, in contrast to oral infections, readily caused mortality in *A. segetum* larvae suggesting that midgut was the main barrier for infection with SeMNPV.

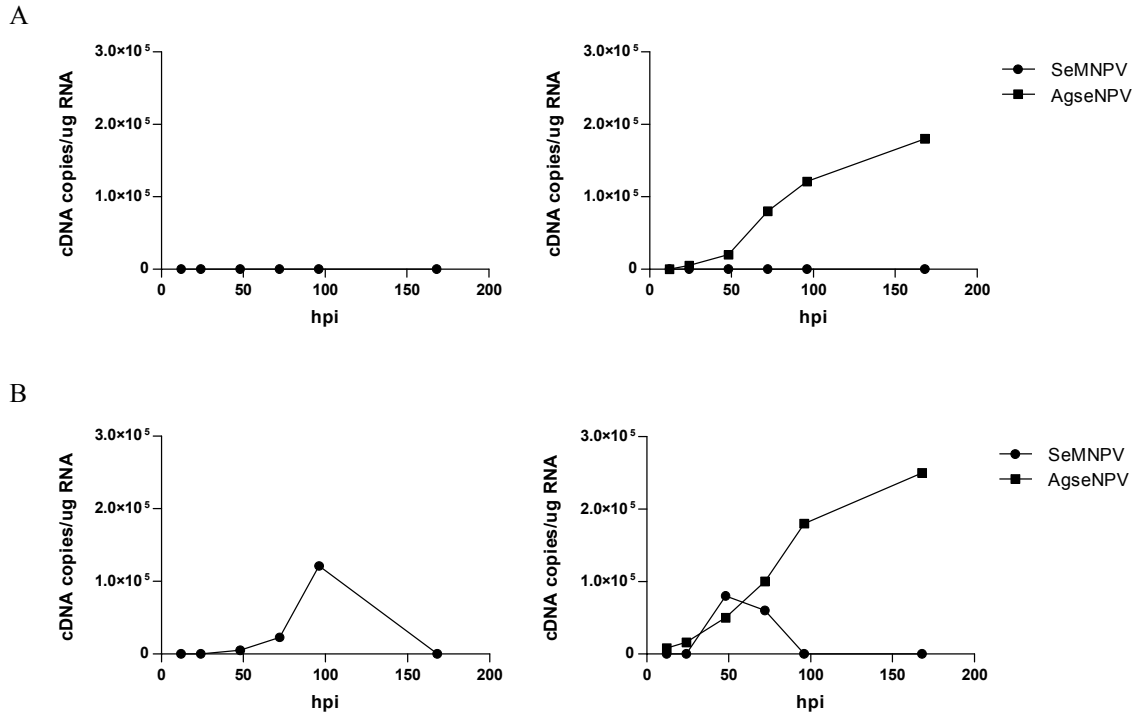

Figure 1. SeMNPV and AgseNPV *DNApol* gene expression in *A. segetum* larvae infected with SeMNPV only (left panel) or the mixture of both viruses (right panel) monitored by real-time qPCR. A. *Per os* infections; B. Intrahemocoelic injections.

Detection of SeMNPV and AgseNPV specific transcripts at different times post infection

Quantitative real-time RT-PCRs were performed on the RNA isolated at different times post infection from *A. segetum* larvae infected and injected either with SeMNPV alone or in a mixture with AgseNPV. In *per os* single infections with SeMNPV no *DNApol* transcripts of this virus were detected at any time post infection and the larvae continued to show regular growth and pupation. However a few examined midguts showed the presence of SeMNPV transcripts suggesting that virus entry into midgut cells is not *a priori* impossible.

In mix infected larvae only AgseNPV transcripts were detected at 24 hpi. The transcript level increased until 168 h p.i. (Fig. 1A). A certain level of mortality of the larvae was observed due to infection with AgseNPV.

In single intrahemocoelic injections with SeMNPV the transcripts of this virus were detected at 48 hpi and increased till 96 hpi (Fig. 1B). At 168 hpi no SeMNPV transcripts were detected anymore, probably due to the collapse of insect cells and their death. In mixed injections the AgseNPV transcripts were detected as early as 12 hpi and continued to increase until 168 hpi. In mixed infections, the transcripts of SeMNPV were detected at 48 hpi, decreased at 72 hpi and then disappeared at 96 hpi, whereas Agse NPV transcripts continued

to increase in level. This suggests that AgseNPV does not support, complement or sustain SeMNPV infection, but instead out-competes SeMNPV.

Conclusions

1. AgseNPV causes lethal infections in the examined insect species *A. segetum* and *S. exigua.*
2. SeMNPV does not cause a lethal infection in *A. segetum* larvae when administered orally.
3. SeMNPV is able to enter midgut epithelial cells as shown by the presence of transcripts, but does not pass the infection to other tissues.
4. SeMNPV infects *A. segetum* by intrahemocoelic injection suggesting that the lack of efficient entry into midgut cells and/or replication in epithelial cells may be the main barriers for infection.
5. A low level of SeMNPV *DNApol* transcripts was observed in infected *A. segetum* larvae midguts (results not shown), thus we conclude that midgut entry and replication barriers are not the only determinants of a successful SeMNPV infection in *A. segetum* and other mechanisms like immune response and/or apoptosis, may be involved.
6. SeMNPV and AgseNPV and their native hosts represent an attractive model for studying the determinants of NPVs host range.

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Covert infections in *Spodoptera exigua* laboratory culture

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Abstract: A laboratory colony of *Spodoptera exigua* was investigated to assess the role of covert (latent or persistent) baculovirus infections on spontaneous disease outbreaks. Two nucleopolyhedrovirus (NPV) species were found to be reactivated into fully lethal forms and were identified as SeMNPV and *Mamestra brassicae* by REN and PCR-sequencing. By use of q-PCR, both NPVs species have been quantified in asymptomatic individuals over several generations to elucidate the fluctuating trends of latent or persistent infections.

Key words: NPVs reactivation, persistent/latent infections, SeMNPV, MbNPV.

Introduction

Spontaneous baculovirus outbreaks have been found to occur frequently in laboratory colonies which have been reared strictly under virus-free conditions. Karpov (1979) reported *Bombyx mori* nucleopolyhedrovirus (NPV) outbreaks as a problem in practical sericulture. Only few studies have been conducted to elucidate the underlying phenomena (Fuxa et al., 1999; Hughes et al., 1997). Very little is known about the molecular mechanism(s) involved in the maintenance of NPV latency or persistence or how the virus is triggered into a lethal overt infection (Cory & Myers, 2003). A lack of reliable and accurate techniques for detecting low levels of viral DNA in the presence of host DNA is that which hampers our understanding. Recent advances in molecular techniques have improved the detection of viral DNA and its transcripts in assymptomatic insects (Burden et al 2002, 2003). Real-time or quantitative PCR (q-PCR) has been demonstrated to be extremely useful for studying infectious disease processes (Mackay et al., 2002).

Repeated spontaneous baculovirus overt infections in late instar larvae of our laboratory colony of *Spodoptera exigua* suggested a persistent or latent virus might be present. From this colony two NPV species resembled the *Spodoptera exigua* (Se) MNPV and the *Mamestra brassicae* (Mb) NPV by REN analysis. In this study, q-PCR was used to quantify both NPVs species in asymptomatic individuals to elucidate the fluctuating trends of latent or persistent infections.

Material and methods

Insect rearing

S. exigua insects in continuous culture were used in this study and reared on artificial diet (Hunter-Fujita et al, 1998). The colony was maintained at 25°C, 16:8 light: dark photoperiod, and 60 – 70 % humidity in a dedicated room at CEH Oxford.

Persistent viral level over* S. exigua *development

Fifty fourth instar *S. exigua* were sexed at pupation after being individually reared on virus-free diet in a UV-sterilized incubator that provided the standard rearing conditions. Twenty couples were mated in paper bags and provided with water feeding pots. The females were allowed to lay eggs for 2-3 days, and both adults were frozen for subsequent analysis. Egg batches were placed in 300 ml polypots containing artificial diet. When neonate larvae hatched, 25 insects were individually reared through to adults (offspring F1) in 25-compartment plates with artificial diet. Insects were observed daily for NPV symptoms. The survivors in lines from which some F1 offspring died of overt but spontaneous NPV infections were allowed to breed and produce the F2 offspring. Three more lines, in which no deaths were observed, were also allowed to generate a F2 in the same conditions. Ten asymptomatic individuals at each development stage of F2 were frozen to subsequent DNA analysis by q-PCR.

Detection of covert infections by Q-PCR.

A SYBR Green based PCR was developed for the quantification of persistent viral DNA. Highly specific primers were designed and targeted to the unique genes VP80 and P87 for *S. exigua* and *M. brassicae* genomes, respectively (table 1). A housekeeping gene was designed targeting the *S. exigua* Elongation Factor Alfa gene sequence. All reactions were performed using Platinum SYBR Green q-PCR SuperMix-UDG. Standard curves were performed using 7 × $\log_{10}$ dilutions of a CsCl - purified viral DNA ($3 – 3\times10^{-6}$ ng / µl). Six water (no template controls) controls were included in each run of a total of 72 samples. Melting curves were generated by fluorescence readings over ramped temperatures at the end of cycling between 50 to 99 °C. In order to asses the specificity of primers, each primer set was tested for cross reactivity against either MbNPV, SeMNPV, or host DNA (Tab. 1).

Table 1. Oligonuleotides used in this study for quantification of SeMNPV and MbNPV by Q-PCR. The specificity of the primers was indicated by the generation of a single amplicon with a specific melting temperature and no cross reactivity to the heterologous virus.

Primer Name	Primer Sequence (Fw / Re)	Range of detection		Amplicon melting temperature for specific template (°C)		
		ng / µl	viral genomes/µl	MbNPV	SeMNVP	*S. exigua*
SeVP80	5'-CGAGCGCTGTTGATGAAATAG-3' 5'-GAATTTAACGGCCATCAACG-3'	$3 - 3\times10^{-6}$	$6\times10^{5} – 60$	n.p.	86	n.p.
MbP87	5'-GCATTAGGGTGTCGTTATCG-3' 5'-CCATCGTTATTAGCCTCGACA-3'	$3 - 3\times10^{-6}$	$6.8\times10^{5} – 68$	83	n.p.	n.p.
SeEF	5'-GGCTGGTATCTCGAAGAACGG-3' 5'-GCTTGACACCGAGTGTGAAAGC-3'	$10 - 1\times10^{-6}$	n.a.	n.p.	n.p.	82

n.p.= no detectable product for the template range tested

Results and discussion

Spontaneous NPV-induced mortality was recorded in the parentals (F1) of couples A, B, and C, but no longer in the first two lines (Tab. 2). Levels of persistent virus of the progeny (F2) was significantly higher than the background level in water controls ($8.99\pm0.33\times10^{-7}$ ng/µl) (t-test pairwise, $p>0.05$) across the six lines selected (Tab. 2). Remarkably, the offspring from couple A recorded the highest viral levels of $1.98\pm 0.07\text{x}10^{-1}$ ng/µl with 45 positive out of 80 tested insects (t-test pairwise, $p < 0.05$).

Table 2. Frequency of spontaneous NPV-mortality in F1 and F2 offspring, and Q-PCR quantification of Se-like persistent DNA for F2 offspring across six lines of *S .exigua* insects generated by breeding two generation.

Breeding line (F2)	Number of spontaneous overtly Infected insects		Se-like persistent viral DNA	
	[1]F1 (parent line)	[2]F2	[3]Mean (ng / ul) ± se	N (q-PCR positives)
Lines in which spontaneous NPV infections were recorded in F1				
Couple A	5	10	$1.98 \pm 0.07 \times 10^{-1}$ a	45
Couple B	2	0	$3.29 \pm 0.12 \times 10^{-5}$ b	5
Couple C	2	0	$6.97 \pm 0.12 \times 10^{-5}$ b	4
Lines in which spontaneous infections were not detected				
Couple D	0	0	$8.65 \pm 0.26 \times 10^{-5}$ b	6
Couple E	0	0	$1.51 \pm 0.18 \times 10^{-5}$ b	6
Couple F	0	0	$1.49 \pm 0.18 \times 10^{-5}$ b	8
Background levels in water controls			$8.99 \pm 0.33 \times 10^{-7}$ c	72

[1]N=25, [2]N=75, [3]Average of q-PCR positive insects (N) data pooled from different development stages. Different letters denoted significant differences by the t test ($p < 0.005$).

Vertical transmission was reported in *S. exigua* derived offspring from adults fed on virus as L_5 larvae (Smits & Vlak, 1988) but no studies have investigated thus far the natural levels of persistent virus DNA in non challenged insects. For the first time persistent viral levels have been successfully detected and quantified over the *S. exigua* life cycle, demonstrating fluctuating trends and the correlation between spontaneous NPV deaths with the virus DNA levels.

Persistent/latent viral DNA levels monitored throughout the host development cycle were further studied in offspring from couple A. When viral readings were standardized to host DNA (SeEF primer set) and log transformed, maximum viral levels were recorded in eggs and in first larval instars. Spontaneous NPV-induced death trends previously reported described that the latent virus is rarely reactivated in early instars but more commonly in mature caterpillars (Karpov, 1979). Consistent with this study, the viral levels of persistent DNA dramatically increased with larval develop up to 10^4 folds, suggesting an extremely active viral replication period that eventually caused deaths at L_4. In contrast, when viral levels were normalised to a house keeping gene very low viral levels were found at late instars, pupae and adults that were similar to the general background level (when the virus may not be actively replicating) (Fig. 1). Thus, the eggs and first larval instars seem to be the best time to test individuals for latent infections by PCR-based techniques. In this context, large scale NPVs production methods could benefit from incorporating this step to confirm viral-free nature of the eggs before they are used for seed multiplication purposes.

The offspring from couple A (F2) were also tested for MbNPV-like virus. Fifty percent of the eggs, and the very earlier stages (L_1 and L_2) sampled were positive for MbNPV. Therefore both NPVs species covertly infect these individuals at the same time, although Se like virus was always far predominant (Fig 1). Nevertheless, the lack of a robust negative control compromises the consistency and reliability of measures when viral levels dropped close to the detection limit.

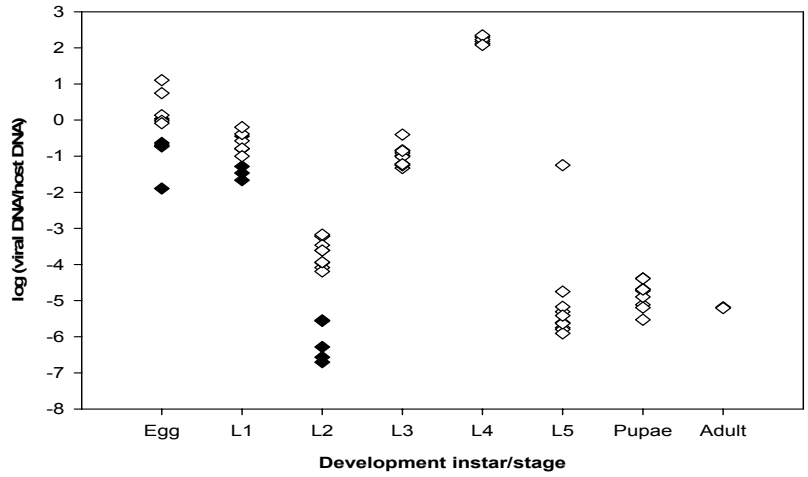

Figure 1. Log latent SeMNPV-like DNA (SeVP80, open dots) or MbNPV-like (MbP87, solid dots) DNA standardized by host DNA as measures in asymptomatic insects from sampled as eggs, L_1, L_2, L_3, L_5, pupae, and adult of couple A (F2 offspring). Viral DNA levels for fully lethal infected L_4 larvae were included as a reference assuming a value of 1 for host DNA.

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Stability of *Spodoptera frugiperda* multicapsid nucleopolyhedrovirus and Sf29null bacmid genomes after sequential rounds of *per os* infection: implications for virus transmission

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Abstract: The *Sf29* gene of the *Spodoptera frugiperda* nucleopolyhedrovirus is a viral factor that determines the number of virions within the occlusion bodies. We investigated the stability of the SfMNPV bacmid (Sfbac) and Sf29null bacmid after serial rounds of *per os* infections. The bac replicon was lost in both viruses. In addition, the Sf29null virus was able to recover the *Sf29* gene. We hypothesize that deletion of the *Sf29* gene supposes a selection pressure, favouring recombination between the Sf29null virus and a covert nucleopoly-hedrovirus infection already present in the host insect. Sf29null virus recovers this gene to improve the likelihood of transmission.

Key words: *Spodoptera frugiperda*, nucleopolyhedrovirus, bacmids, *Sf29*, stability

Introduction

Baculoviruses are potential biological control agents (Moscardi, 1999), but their slow speed of action has limited their use. Considerable attention has been focused on the possibility of improving their efficiency by genetic manipulation (Bonning and Hammock, 1996). Bacmid-based systems have reduced the time taken to obtain pure recombinants (Luckow et al., 1993). This system was used to determine the role of *Sf29* gene in the structure and replication of wild-type *Spodoptera frugiperda* MNPV (SfWT). The *Sf29* gene is a viral factor that determines the number of virions in occlusion bodies, playing an important role in SfMNPV transmissibility (Simon et al., 2008). In the present study we investigated the stability of the SfMNPV bacmid (Sfbac) and the Sf29null bacmid after serial rounds of *per os* infection in host larvae.

Materials and methods

SfWT occlusion bodies (OBs) were obtained by *per os* infection of *S. frugiperda* larvae, and Sfbac and Sf29null bacmid OBs by intrahemocoelical infection of DNAs. These OBs were considered to be passage 0. The virus populations were followed for five rounds of *per os* infection. Different genotypic and phenotypic aspects were evaluated. The genomic stability of SfWT, Sfbac and Sf29null bacmid viruses was determined by restriction endonuclease analysis of the DNAs extracted from samples of $5x10^8$ OBs. DNA quantity per OB was estimated by reading the optical density at 260 nm. Mean virion titre per OB, representing the ODV infectious units, was determined by endpoint dilution by releasing the ODVs from $5x10^8$ OBs and using the resulting suspension to infect 10^4 Sf21 cells. The relative proportion of genomes containing the kanamycin or *Sf29* gene in OBs sampled at each passage was

estimated by densitometric analysis of the PCR products amplified from the DNAs using primers that differentially amplify for kanamycin cassette and *Sf29* gene. Finally, the relative proportion of genomes containing the bacmid replicon and the kanamycin cassette was determined by growing colonies in chroramphenicol and chloramphenicol+kanamycin plates after transformation of DH510B GeneHogs electrocompetent cells with 1 µl of Sfbac and Sf29null purified DNAs.

Results and discussion

Genomic stability and DNA content

When absolute amounts of DNA were analysed, the restriction profile of Sf29null bacmid was clearly less intense at P0, however throughout successive passages the intensity increased. The fragment representing the bac replicon decreased in intensity in Sfbac and Sf29null viruses throughout successive passages. No significant differences were found in the mean amount of DNA in OB samples between SfWT and Sfbac at each passage ($P>0.05$). However, the DNA content in Sf29null bacmid OBs at P0 was ~6 times less than that observed in SfWT OBs. The DNA content within Sf29null OBs after three rounds of *per os* infection was similar to that found in the viruses carrying the *Sf29* gene.

ODV infectivity

Samples of $5x10^8$ OBs of SfWT and Sfbac viruses produced similar titres of infectious units (IU)/ml at each passage. In contrast, ODV infectivity of Sf29null OBs at P0 was ~9 times lower than that of SfWT, but after three rounds of *per os* infection ODV infectivity was similar to that of SfWT or Sfbac.

Relative proportion of Sf29 gene containing genomes

In Sf29null OBs sampled at P0, the proportion of the genomes containing the *Sf29* gene was 0. However, over successive rounds of *per os* infection this proportion increased. After four passages the frequencies of genomes containing the *Sf29* gene and kanamycin cassette converged to a common ratio comprising approximately 50% of each.

Number of colonies produced by Sfbac and Sf29null DNAs

The number of colonies grown in chloramphenicol plates decreased throughout successive passages in both Sfbac and Sf29null viruses, suggesting the lost of the bac replicon. In addition, the number of colonies grown in chloramphenicol+kanamycin plates in Sf29null virus also decreased during passages, suggesting that the kanamycin cassette was lost during serial passage.

The *Sf29* gene of SfMNPV is an important gene that determines the number of ODV per OBs. Sf29null virus recovered this gene after serial rounds of *per os* infection. Sequence analysis of the recovered gene demonstrated that this gene was 100% homologous to the SfMNPV *Sf29* gene. Vertical transmission and covert infections have been described in *S. frugiperda* (Vilaplana et al., 2008). We hypothesize that deletion of *Sf29* gene favours recombination between Sf29null virus and a covert nucleopolyhedrovirus infection present in the experimental insects. By recovering the *Sf29* gene, the Sf29null virus obtains important gains in the likelihood of transmission.

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Infuence of diet composition on mortality of *Cydia pomonella* larvae infected with CpGV

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Abstract: Various diets and methods of virus application can be used in bioassays to test the efficacy of *Cydia pomonella* granulovirus (CpGV) biocontrol agents against codling moth (*Cydia pomonella* L.). Usually, diet incorporation of virus occlusion bodies (OBs) or application of the OBs on the diets surface is used in bioassays. Here, we compared the effect of three diet compositions, the Ivaldi-Sender diet, Guennelon diet and Manduca Premix-Heliothis Premix, on the mortality of codling moth larvae when CpGV OBs were incorporated into the diets. Statistical differences were found between the mortality of codling moth on the Premix diet and Ivaldi-Sender and Guennelon diets, with a significantly higher median lethal concentration (LC_{50}) value of CpGV-M on the Premix diet. The impact of the diet composition on bioassay results is discussed.

Key words: Codling moth, *Cydia pomonella* granulovirus, bioassay, median lethal concentration, diets.

Introduction

Cydia pomonella granulovirus (CpGV) biocontrol agents are currently commercially available in many European countries, in North America, Argentina, Australia, New Zealand, and South Africa. In the Czech Republic, CpGV products are just being registered. Recently, populations of codling moth resistant to CpGV-M strain occurred in several European countries (Asser-Kaiser et al., 2007) and susceptibility testing of codling moth populations from the Czech Republic to CpGV-M have been recently initiated by determining ther median lethal contentration in laboratory bioassays (Zichová et al., 2008). Different diets and methods of application can be used in bioassays to test the efficacy of CpGV against codling moth. For application of the virus, incorporation of virus into the diet or surface treatment of the diet with virus OBs is usually used (Hunter-Fujita et al., 1998). In our experiments, the commercially available diet (Manduca Premix-Heliothis Premix, Stonefly Industries, USA) with incorporated CpGV was used (Zichová et al., 2008). The aim of the study was to evaluate the effect of three diets on bioassay results of codling moth larvae infected with CpGV incorporated into the diet.

Material and methods

Diets

Three different diets were prepared. The diets according to Ivaldi-Sender (1974) and Guennelon (1981) are based on water, agar, corn meal – polenta, wheat germ, brewer's yeast, ascorbic acid and nipagin (hydroxybenzoic acid methyl ester) and differed from each other in

the relative amounts of the particular components. Benzoic acid and 30% formaldehyde are usually added in the Guennelon diet to allow longer storage of the diet. In our experiment, formaldehyd was not used. For the Ivaldi Sender diet, water, agar and the corn meal mixture were autoclaved for 20 min at 120°C. Then, wheat germ, brewer's yeast and nipagin (dissolved in 70% ethanol) were added. For Guennelon diet preparation, water and agar were boiled in a microwave oven and then other components (corn meal polenta, wheat germ, brewer's yeast, nipagin a benzoic acid dissolved in 70% ethanol) were added. A hand-held mixer was used to blend the ingredients. After cooling both diets to 60°C, ascorbic acid (dissolved in water) was added. CpGV OBs suspended in water were added after the diets were allowed to cool to 45°C. Further cooling of the diets in open air of a safety hood allowed drying of thediets and evaporation of ethanol resdues. Instant diet Manduca Premix-Heliothis Premix (Stonefly Industries, USA) is based on wheat germ and soya. This diet was prepared from dry mixture and water.

Bioassasys

Before the diets were prepared, CpGV-M (Mexican isolate prepared at the DLR Rheinpfalz, Germany) was quantified in Petroff-Hauser couting chamber (depth 0.02 mm) using dark field optics of a Leica light microscope (DMRBE). 50-well autoclavable plates were used for the bioassays. 45 ml of Ivaldi-Sender or Guennelon diets mixed with 5 ml of water-virus suspension were introduced into the plates. Instant diet Manduca Premix-Heliothis Premix (Premix) was prepared from 12.5 g of the premix and 37.5 ml water-virus suspension (final volume 50 ml) was introduced into the plates. In total, six virus concentrations were tested with each of the diet: $3x10^2$, $1x10^3$, $3x10^3$, $1x10^4$, $3x10^4$ and $1x10^5$ OBs/ml. Control diet was without the virus. A highly susceptible codling moth laboratory colony (CpS) reared in DLR Rheinfalz (Germany) was used in bioassays. 35 neonate larvae (L1) were used for each concentration. The untreated control contained 70 larvae. Plates were incubated at 26 °C and at a photoperiod of 16 hours light to 8 hours darkness. One day post infection, larvae killed from handling and transfer were eliminated from the bioassay. Larval mortality in the bioassays was determined after 7 and 14 days. Two independent replicates were performed per each concentration of the virus.

Statistical analysis

Probit analyses was performed from virus induced mortalities corected against the mortalities in the untreated control (Abbott, 1925) using the software package ToxRat Version 2.09 (ToxRat Solution GmbH, 2005). The slope of the probit line, the median lethal concentrations (LC_{50}s), and their 95% confidence intervals were determined. Comparison of LC_{50} values of CpGV-M on different diets was carried out using the overlapping of the confidence intervals as a criterion.

Results and discussion

Bioassay

The first bioassay evaluation was carried out seven days post infection, as it is the most frequently used time-point for evaluation of codling moth mortality caused by CpGV incorporated into the diet (Asser-Kaiser et al., 2007; Eberle et al., 2008) or applicated on the surface of the diet (Lacey et al., 2005). The LC_{50} values determined for each of the tested diet are given in Table 1. At seven days post infetion, the LC_{50} obtained from Premix diet was more then two-times higher than those on Ivaldi-Sender diet and significanty differed from the Ivaldi-Sender and Guennelon diet. A similar effect, though not that pronounced was

observed in the 14-day evaluation. The 14-day evaluation is usually used for evaluation of resistance of field codling moth populations to CpGV-M, because at the earlier term, low mortality about 20% is observed despite high virus concentration $1x10^7$ OBs/ml (Fritsch et al., 2005).

Table 1. Median lethal concentrations (LC_{50}) of CpGV-M determined 7 and 14 days post infection in particular diets.

	7 days			14 days		
Diet	LC_{50} (OBs/ml)	95 % conf. limits (OBs/ml)	slope	LC_{50} (OBs/ml)	95 % conf. limits (OBs/ml)	slope
Ivaldi-Sender	$1.12x10^3$	$0.88 – 1.42 x10^3$	1.71	$4.64x10^2$	$3.43 – 6.29 x10^2$	1.76
Guennelon	$1.61x10^3$	$1.28 – 2.01 x10^3$	1.82	$3.94x10^2$	$2.93 – 5.31 x10^2$	1.56
Premix	$2.83x10^3$	$2.26 – 3.54 x10^3$	1.70	$7.18x10^2$	$5.58 – 9.23 x10^2$	1.92

Pairwise comparisons of the LC_{50} values and their confidence intervals at day 7 showed a significantly higher LC_{50} on Premix diet ($2.83x10^3$ OBs/ml) than on Ivaldi-Sender ($1.12x10^3$ OBs/ml) and Guennelon ($1.61x10^3$ OBs/ml) diet. On day 14 there was still a difference between Guennelon and Premix but not between Ivaldi-Sender and Premix. The determined LC_{50}s on the Premix diet were at day 7 two-times lower to LC_{50}s $5.42x10^3$ OBs/ml (95 % conf. limits 3.55-8.96x10^3 OBs/ml) and at day 14 two-times higher to $3.47x10^2$ OBs/ml (95 % conf. limits 1.73-5.69x10^3 OBs/ml) determined for laboratory colony reared in Czech Republic (Zichová et al., 2008). The observed difference might be due to difference in counting of OBs using different microscopes and/or difference in the susceptibility of tested larvae used in the different laboratories. Asser-Kaiser et al. (2007) found similar LC_{50} values of $1.4x10^3$ OBs/ml (7 days) and $5.0x10^2$ OBs/ml (14 days) on Ivaldi-Sender diet using the same origin of test animals and microscope for counting as in this study. In addition, Eberle et. al (2008) reported LC_{50} values of CpGV-M on the Ivaldi-Sender of $1.9x10^3$ OBs/ml (7 days) and $1.4x10^2$ OBs/ml (14 days).

Possible reasons for the observed differences in the LC_{50} values were the differences in the uptake rates or larval development rates on the different diets. However, we could not find any statistical difference of larval weight, developmental time, pupal weight of the control animals on the different diets (data not shown). Therefore it is conceivable that the diet itself directly influenced the larval susceptibility to the virus.

In conclusion, Premix diet has been be successfully used for codling moth bioassays, however, the LC_{50}s were 2- to 2.5-fold higher than those observed on other diets.

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Virulence management as a long term strategy to overcome virus resistance of codling moth

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Abstract: Many virus isolates which can overcome virus resistance have been found in the field or through a selection process on resistant codling moth populations. Differences of efficacy of resistance breaking virus isolates on highly resistant codling moth populations lead to the strategy of virulence management by switching to new isolates every three to five years.

Key words: Codling moth, *Cydia pomonella*, granulovirus, CpGV, resistance, new isolates, virulence management

Introduction

Since 2004 codling moth (*Cydia pomonella*) populations with resistance towards the Mexican isolate of *Cydia pomonella* granulovirus (CpGV-M) have been found in Austria, France, Germany, Holland, Italy and Switzerland. In the following years Andermatt Biocontrol developed the already commercialised products Madex Plus, Madex I12, Madex Max and the test products ABC V06 and ABC V08 which contain either new virus isolates found in the field or new isolates selected on resistant codling moth populations in the laboratory.

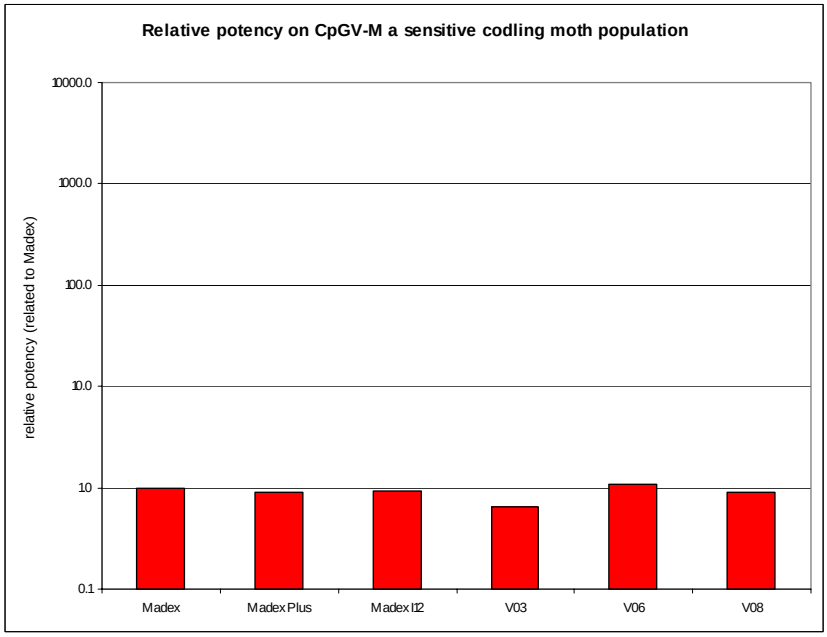

Figure 1. Relative potency of different CpGV isolates tested on a virus sensitive codling moth population compared to Madex (CpGV-M) in bioassays. Evaluation after 12-14 days.

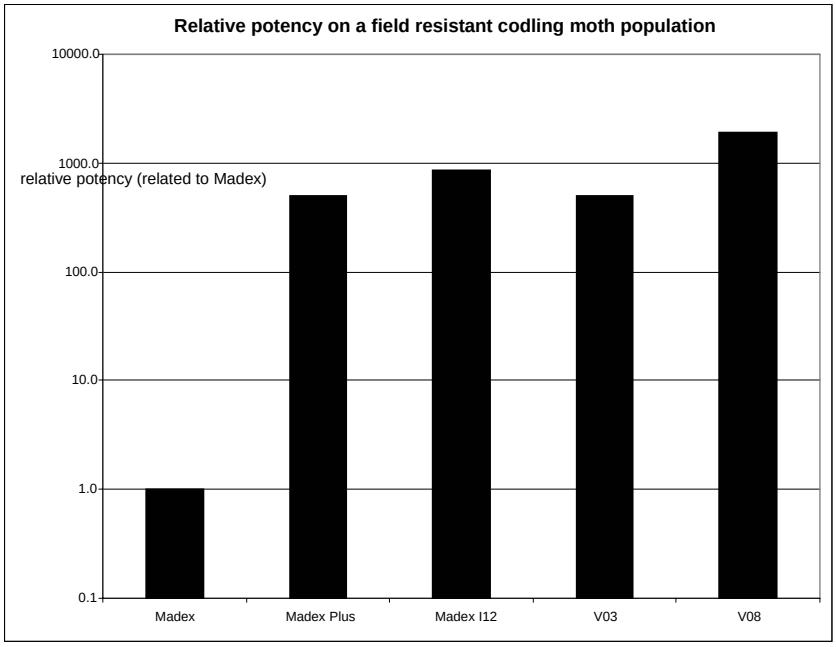

Figure 2. Relative potency of different CpGV isolates tested on a field-resistant codling moth population compared to Madex (CpGV-M) in bioassays. Evaluation after 11-13 days.

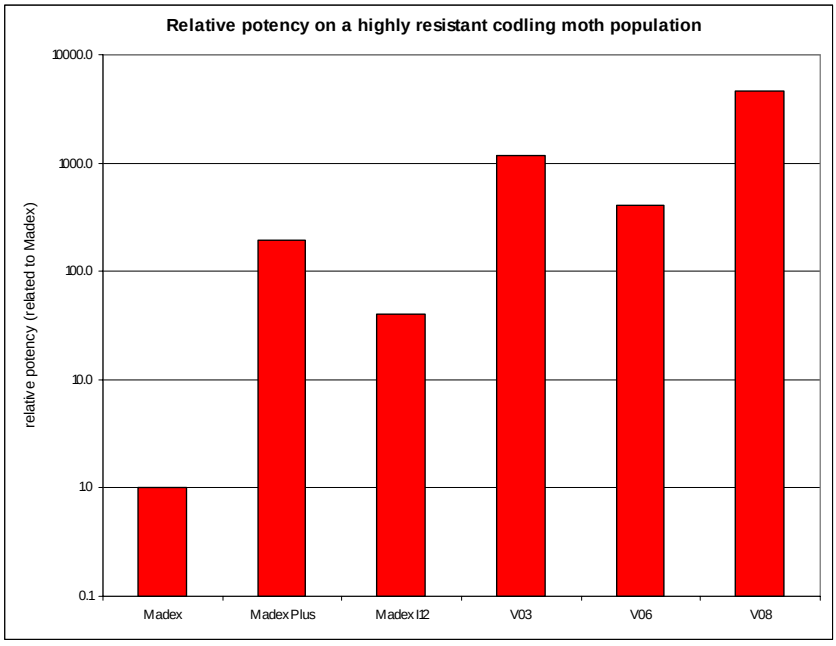

Figure 3. Relative potency of different CpGV isolates tested on a highly virus-resistant codling moth population compared to Madex (CpGV-M) in bioassays. Evaluation after 11-13 days.

Results and discussion

The virus isolates were tested on a virus-sensitive and on two virus-resistant codling moth populations. The results are shown in Figures 1-3. All virus isolates were formulated in the same way as Madex with $3x10^{10}$ OB/ml.

All tested new virus isolates showed a good efficacy on sensitive codling moth larvae comparable to that of the Mexican isolate CpGV-M. All these new virus isolates also had a good efficacy when tested on a Mexican isolate-resistant codling moth population from the field. However, significant differences in the efficacy of the different virus isolates were found when they were tested on a highly resistant laboratory-developed codling moth population. The virus isolate selected on the highly resistant codling moth population (ABC V08) was more effective than the others. This fact shows the potential of virulence management when the most resistant codling moth populations are used in the selection process.

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Curriculum Vitae and Scientifc Accomplishements of Dr. Juerg Huber

Jürg Huber, born in Zurich in Switzerland, studied zoology and entomology at the Swiss Federal Institute of Technology (ETH), Zurich. In 1973, he received his PhD after working with Prof. Benz at the ETH on "Selection of resistance against peroral infection of a granulovirus of a laboratory stock of the codling moth, *Laspeyresia pomonella*)"

Then he joined the Institute for Biological Control of the Federal Biological Research Centre for Agriculture and Forestry (today Julius Kuehn Institute) in Darmstadt, where he focused on the development and application of insect viruses for control of insect pests in agriculture and forestry; production, registration and commercialization of microbial and viral pesticides. From 1989 to 1991 he was Commissioned Director of the Institute and from 1991 until today he has served as the Institute´s Director.

Juerg has been member of numerous national and international scientific organizations, e.g. the Swiss Entomological Society and the German Society of Phytomedicine. He was Vice President and President of the Society of Invertebrate Pathology (1996-2002). He was founding member of the IOBC/WPRS Working Group on Insect Pathogens and Insect Parasitic Nematodes in 1985. Since then he served in different functions for the IOBC, as Convenor of the IOBC/WPRS Working Group on Insect Pathogens and Insect Parasitic Nematodes (1989–1991), Treasurer of the West Palaearctic Regional Section (IOBC/WPRS) (1989–1997), Vice President (1997–2005) and Council Member (2005–2009).

For his research on the development of microbial control agents Juerg was awarded the Karl-Escherich-Medaille of the German Society of General and Applied Entomology (2005).

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More than 20 years of CpGV-Commercialization

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Abstract: Products against the codling moth based on CpGV were first mainly used by organic growers. But as resistance of the codling moth against most of the chemical insecticides became more and more obvious, it was soon regarded as an interesting tool for IPM, especially as it has a long lasting effect on the codling moth population and as it allows producing residue free fruits. CpGV is now well accepted in all important apple producing countries where the codling moth is a key-pest.

Key words: CpGV, commercialisation, population management

20 years ago organic apple producers had no tools available to fight against their key-pest, the codling moth. Often more than 50 % of the fruits were damaged. But in the same time comprehensive literature about the *Cydia pomonella granulovirus* (CpGV) was already available in the libraries. Some companies started to think about the commercialization of a CpGV based product, but no product appeared on the market. As a student in Agronomy doing my M.Sc. in biocontrol, I was aware of the literature on CpGV and of the problems of the organic fruit-growers on the other side. I naively contacted the Swiss registration authority and asked why they registered only chemical products against the codling moth and not environmentally friendly products such the CpGV. The answer was simple: "Bring us a registration file, we will check it, and if everything is fine, we will register such a product". With the help of Prof. Dr. Jürg Huber, JKI Darmstadt, Germany, I put together a registration file, which was mainly based on literature. It was submitted in January 1987 to the registration authority in Switzerland. In the spring of the same year, the first batches of a CpGV product, which was called MADEX, was produced in our small student flat. Field trials conducted in 1987 gave good results. A provisional registration for MADEX in Switzerland was granted in December 1987. This was a time when reasonably thinking people made reasonable decisions in a reasonable period of time! And all this without any fee!

Early in 1988 the company Andermatt Biocontrol was founded and more than 500 ha-units were produced. The product was sold to organic apple producers in Switzerland, but also to organic producers of neighbouring countries which combined a visit to Switzerland with some "shopping". During the following years the demand was growing slightly, but in the early 90's, MADEX was only used by organic farmers. Attempts were made in some European countries and in New Zealand to register MADEX, but as the registration file was rather limited and the registration authorities were not used to registering such products, it took several years to get these registrations. In the late 90's the organic production boomed in Western Europe. At the same time, the resistance of the codling moth against most of the chemical insecticides became more and more obvious. Furthermore, many old active ingredients disappeared from the market. Suddenly, CpGV became interesting also for conventional producers, especially, after Kienzle et al. (2002) demonstrated that even a single MADEX application early in spring could reduce the overwintering codling moth populations by more than 50 %. Apple growers accepted that a codling moth may enter a fruit before dying, but they also accepted that ultimately the larval mortality would be much higher than

with many chemical insecticides. Charmillot and Pasquier (2002) concluded after a seven years study in Switzerland that only combination of CpGV and mating disruption can sustainably maintain codling moth populations at low levels. With only CpGV treatments, these results can be achieved but 4-6 treatments per season over several years are required (Pasquier and Charmillot, 1998). For the general acceptance of CpGV as an important component in the fight against the codling moth, it was crucial to convince the growers and their advisers, that CpGV is not mainly a damage control tool but rather an insect population control tool. During the last few years another important aspect became more and more important: residues on the fruits. CpGVs alone or in combination with mating disruption are ideal to produce residue-free fruits.

MADEX, as well as other registered CpGV-products, are now important IPM tools in almost all apple producing countries worldwide. In total four companies manufacture CpGV-products with a total yearly production capacity of more than 500,000 ha-units.

Due to the high acceptance of CpGV in the market and the continuous and frequent use in many orchards, codling moth populations showing a highly reduced susceptibility to CpGV have been first found in Germany (Fritsch et al., 2005) and later also in some other European countries. Some thought that the life-cycle of the CpGV-products was already over. But new isolates from different countries in the world and also new selected isolates show that not only codling moth is able to make evolutionary steps but of course also CpGV. This ensures that the interesting story of the CpGV commercialization will continue.

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Genomic and proteomics of hymenopteran baculoviruses

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Abstracts: To date, a total of 48 baculovirus genomes have been sequenced including three of hymenopteran baculoviruses that are, so far, the smallest and appear to be more ancient than lepidopteran nucleopolyhedroviruses. The genomes of hymenopteran baculoviruses lack a number of genes that were thought of as essential for the replication of lepidopteran baculoviruses. Most notably are those encoding for IE-1 protein and membrane fusion proteins needed for budded virus formation. Hence, the latter phenotype may not play a role in the biology of hymenopteran baculoviruses. Comparative genomic analyses of three hymenopteran baculoviruses revealed co-linearity in most of the genome but an obvious non-syntenic region that contains many genes and ORFs with closer identity to insect genes than those of viral origin. Proteomics analysis by LC-MS/MS has so far demonstrated the presence of 18 proteins associated with the virion, three of which are totally novel and do not exist in any other sequenced baculovirus genome.

Key words: Hymenoptera, baculovirus, proteomics

Introduction

Several hymenopteran baculoviruses have been developed and registered in North American and Europe as successful biological control agents against sawflies. Two reasons can be attributed to their success; the strategy of virus replication and the gregarious habitat of the larval host. It has been know that sawflies infected with a baculovirus develop infectious diarrhea that disseminates the virus in high density insect colonies. Indeed, the introduction of one infected insect into a healthy colony results in infection of almost all larvae that eventually die within 4-7 days. Interest in the genomes of these viruses stemmed from the fact that they were relatively few highly efficacious viruses infecting a much more ancient insect order than Lepidoptera. Apart from the hypothesis that the budded virus phenotype does not play a role in the biology of the virus, a number of previously identified essential proteins were absent from the hymenopteran baculoviruses. Proteomics analysis by LC-MS/MS has so far showed the presence of 18 virion associated proteins, three of which are totally novel and not present in other baculoviruses or in the *Bombyx mori* EST data base.

Materials and methods

Viruses and insects

Since tissue culture is not available for the hymenopteran baculoviruses, they were replicated in larvae and upon death that happened without larval melting, they were harvested, homogenized and the occlusion bodies were purified by standard procedures. Occlusion derived virus (ODV) was purified by dissolving the occlusion bodies in an alkaline solution followed by rate zonal centrifugation through sucrose gradients. The viruses used in the studies were those infecting *Neodiprion lecontei* (NeleNPV, Lauzon *et al*, 2004), *Neodiprion sertifer*

(NeseNPV, Garcia-Maruniak *et al*., 2004) and *Neodiprion abietis* (NeabNPV, Duffy *et al*., 2006).

DNA sequencing and proteomics analysis

Purified viral DNA from the *N. lecontei* baculovirus was nebulized, repaired at the ends and cloned into a sequencing vector. Sequencing was carried out by the combination of shot-gun, primer walking and PCR to generate gap-spanning fragments and to give a total coverage of 12X. The sequence data were analyzed by DNA Star and McVector softwares.

ODV proteins were separated on 12% PAGE, stained with Lumitein and bands or regions of the gel were excised and subjected to in-gel digestion by trypsin following provider instructions (Pierce, Rockford, IL). The digests were analysed by LC-MS/MS technique using a triple quadrupole ion trap instrument at Yale University Proteomics laboratory. The data were analysed by Mascot software and compiled into a scaffold.

Results and discussion

Comparative genomics

It became apparent from the three genomes analysed in the three independent laboratories that the hymenopteran baculovirus genomes have common features and they differed markedly in their genomics arrangements from those of lepidopteran and dipteran baculoviruses. For example, repeated regions were found in all three genomes but they did not have the typical structures of homologous repeated regions in other baculoviruses. In the latter viruses, these regions have been shown to act as enhancers of gene expression and the proximity of some seemed to have undergone rearrangements.

Approximately 30 genes and ORFs that were thought to be conserved in all baculoviruses were absent from the genomes of hymenopteran baculoviruses. Similarly, many genes that were thought of as essential for virus replication (ex. *ie-1*) were also absent. Most notably was the absence of genes encoding proteins essential for BV structure such as membrane fusion proteins. This, along with other evidence, made us conclude that the BV phenotype may not play a role in hymenopteran baculovirus biology.

A comparison of linearised maps of NeseNPV and NeleNPV shows that to the largest extent the two genomes are collinear except for a large region on the left side of the genomes where the maps become non-syntenic. In this non-syntenic region there appears to be a number of insertions and deletions and has been called the Indel region (Fig. 1).

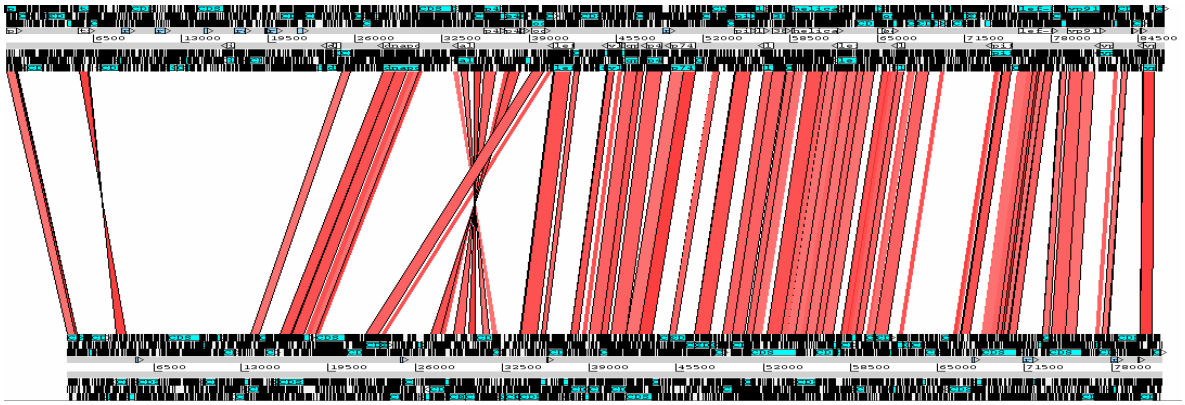
In the Indel region, the NeseNPV genome contained 15 ORFs that did not exist in the NeleNPV genome. Similarly the latter genome had 8 ORFs that were absent from the genome of NeseNPV. A number of ORFs had closer identity to insect ORFs rather than baculovirus ORF. For example, nese17 and nele11 are *iap* genes that showed top BLAST matches to insect iap genes rather than baculovirus *iap* genes. This strengthens the hypothesis that baculovirus iap genes may have insect origins.

Interestingly, the NeleNPV and NeseNPV genomes contain an ORF that potentially encodes a trypsin-like serine protease, which is the first of such to be reported in baculoviruses. Three dimensional modeling of the predicted protein clearly shows a trypsin-like structure with an intact catalytic triad of histidine, aspartic and serine as well as the six conserved cysteines (Fig. 2). Both ORFs share BLAST matches to similar insect proteins.

It has been previously reported that the NeseNPV occlusion bodies lack an alkaline protease that has been found and characterized in other baculoviruses. Perhaps a trypsin-like serine protease may play a role in the dissolution of the occlusion bodies in the larval midgut but this is yet to be demonstrated.

--------***Indel region***-----

***Neodiprion sertifer* baculovirus**



***Neodiprion lecontei* baculovirus**

Figure 1. Comparison of two hymenopteran baculovirus genomes, Neodiprion sertifer baculovirus and Neodiprion lecontei baculovirus.

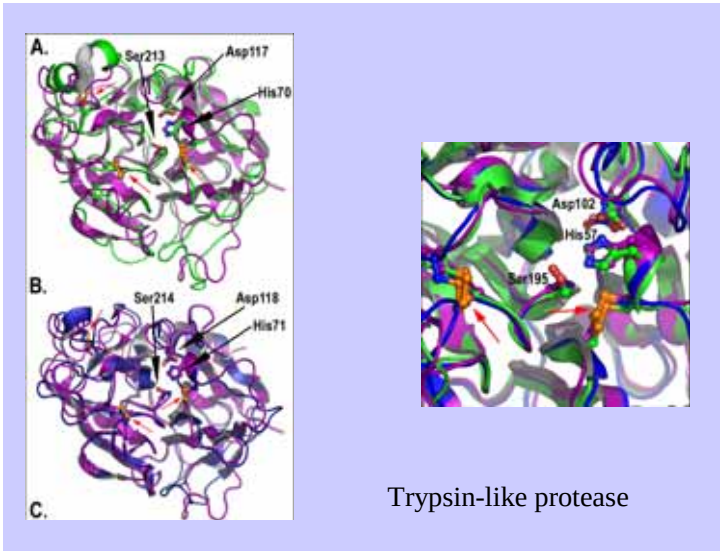


Figure 2. Predicted tridimensional structure of the trypsin-like protease from hymenopteran baculovirus

Mass Spectroscopy

Due to logistic reasons, mass spectroscopy analysis was conducted on ODVs from NeabNPV. The virions were purified on sucrose gradients and the proteins were separated on 12% PAGE. Bands and regions were excised, subjected to in-gel digestion and analysed by LC-MS/MS technique using a triple quadrupole ion trap instrument. So far, a total of 18 proteins were found to be associated with the virions and most of which are homologous to other baculovirus ODV proteins. Three totally novel proteins encoded by neab13 (81 kDa), neab89 (8 kDa) and neab90 (36 kDa) were found. They had absolutely no homology to any baculovirus proteins and searches against the *Bombyx mori* database did not show any homology to insect proteins. Clearly, these are new proteins associated with the virion structure and their function is yet to be elucidated.

Acknowledgements

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Resistance to baculoviruses - new answers to an old question

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Abstract: The *Cydia pomonella* granulovirus (CpGV) is one of the most important commercialized baculovirus biocontrol agents. It has been successfully used for codling moth (CM) control in Europe since more than 20 years. This success was threatened when first reports on CM populations became available in 2005, demonstrating an up to 1000-times reduced susceptibility to CpGV products. Intensive research supported by the European Union and national funding agencies in Germany and France were initiated to understand the prevalence, the molecular mechanism and the genetics behind this phenomenon. New CpGV strains overcoming the resistance were identified or selected from existent isolates. This contribution will provide an overview on recent developments in resistance research of baculoviruses.

Key words: *Cydia pomonella* granulovirus, biological control, resistance monitoring, inheritance

Introduction

The codling moth (CM, *Cydia pomonella* L.) is one the most important pests of apple, pear and walnut and causes severe fruit damage and high losses of marketable fruits. CM control based on the *Cydia pomonella* granulovirus (CpGV) obtained an important role in organic and integrated fruit production, because it is effective and environmental friendly.. CpGV products have been registered and are frequently used in many European countries but also in North and South America, as well as in South Africa and New Zealand.

Since 2004, single orchards where CpGV products failed to control CM infestation were observed. CM populations in these orchards in Germany were found to be about 1000-times less susceptible to CpGV (Fritsch et al., 2005). Further orchards with reduced susceptibility were also found in France (Sauphanor et al., 2006). As these findings were threatening the successful CM control using CpGV products different research activities were initiated to understand the resistance phenomenon and to overcome this problem. Presently, research consortia, sponsored by the Bundesprogramm Ökologischer Landbau (BÖL) and the European Commission (Craft Project SustainCpGV), deal with this problem (Jehle et al., 2006): The main focus of these projects are: (i) analysis of the distribution of CpGV resistance in different growing areas and European countries, (ii) analysis of the inheritance and mechanism of CpGV resistance, (iii) analysis of new CpGV isolates for their effectiveness on resistant CM populations, and (iv) development of alternative CpGV products.

Results and discussion

Susceptibility testing using reared populations originating from more than 40 orchards in Central and South Europe has been performed at the JKI Insitute for Biological Control in Darmstadt. These orchards were selected because there was evidence for CpGV failure. In fact, in more than 35 orchards in Germany, France, Italy, Switzerland, the Netherlands, and

Austria CM of CpGV resistance were found in Germany. Though the CpGV resistance has a wide geographic distribution it appears only locally and is concentrated in single orchards or even parts of single orchards. This occurrence pattern provides some evidence that the CpGV resistance is effectively selected under CpGV selection pressure.

Indeed, crossing experiments demonstrated that the CpGV resistance can be rapidly selected (Asser-Kaiser et al., 2007). The unusual inheritance of this resistance (the resistance allele shows a concentration-dependent dominance and is located on the sex (Z) chromosome) favours a fast and efficient selection. The resistance ratio (based on the mean lethal concentration for neonate larvae) exceeded a factor of 100,000 in both resistant females (genotype Z^RW) and homozygous males (Z^RZ^R) when compared to susceptible CM larvae; heterozygous males (Z^RZ^S) are about 1000 times less susceptible compared to susceptible CM larvae.

After both, oral and intrahaemocoelar infection, the amount of CpGV copies detected in different tissue types of susceptible CM (CpS) increased over time. In none of the isolated tissue types of highly resistant CM (CpRR1) virus replication could be detected. This indicates that CpGV infection is already blocked at the midgut and not further spread, or resistance is present in each of the cell types.

First insights into the mechansim of resistance have been established in our laboratory and are covered by the contribution of Asser-Kaiser & Jehle (this volume). Briefly, infection experiments of resistant CM larvae gave clear evidence that there is a cellular virus-host-incompatibility which impedes virus replication in the cells. Apparently the virus is able to enter the cell for infection but it cannot replicate. By these experiments, a mutated midgut receptor or the involvement of the immune system as the sole resistance mechanism could be excluded (Asser & Jehle, this volume).

Significant progress has been made in the identification and characterization of novel CpGV isolates able to overcome the resistance. Among several CpGV field isolates originating form Iran we have identified one isolate CpGV-I12, which swhowed superior efficacy against CpGV resistant CM larvae (Rezapanah et al. 2008; Eberle et al., 2008). Meanwhile several new CpGV isolates, e.g. MadexPlus, NPP-R1, overcoming CpGV resistance have been selected and tested in the field (Zingg, 2008, Berling et al., 2009). MadexPlus, which was selected in resistant CM larvae by passaging CpGV-M, is the first of these new isolates which became registered in Switzerland in 2008. Hopefully, other new isolates overcoming CpGV resistance will follow soon.

We are still at the beginning of understanding the phenomenon of CpGV resistance in CM. However, the finding of different CpGV isolates overcoming CpGV resistance put the future into a brighter light than a few years ago.

Acknowledgements

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Further information can be found on www.sustaincpgv.de and www.apfelwickler.de.

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An overview of the molecular biology of *Cydia pomonella granulovirus*

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Abstract: From the middle of the 1980s and early 1990s research on the molecular biology and genetic manipulation of granuloviruses (GVs) lagged behind that of nucleopolyhedroviruses (NPVs), in particular *Autographa californica* NPV, *Bombyx mo*ri, *Orgyia pseudotsugata* NPV. The rate limiting step was the absence of GV permissive cell lines that yielded high virus titres. Previously, homologous recombination in *C. pomonella* cells was used to produce recombinant CpGV but this was both protracted and unreliable. Recently, considerable progress has been made in the ease of producing recombinant CpGV, using a CpGV bacmid. The bacmid will allow the studies on the function of specific genes and promoters and will advance understanding in important areas, such as host resistance to CpGV. The genome organisation of CpGV was published, followed later by the analysis of the complete genome sequence. This presentation will cover progress in the *in vitro* propagation, molecular biology and genome organisation of CpGV.

Key words: *Cydia pomonella, Cydia pomonella granulovirus*, *granulovirus*, cell culture, recombinant granulovirus.

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Comparative analysis of a granulin fragment of Colombian granulovirus isolated from *Tecia solanivora*

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Abstract: A 410bp fragment of the granulin gene was sequenced from a granulovirus (GV) obtained from *Tecia solanivora* larvae sampled in Cundinamarca, Colombia. This isolate has been obtained as part of a project to control the Guatemalan potato moth (*T. solanivora*) in Colombia. Degenerate primers were designed to amplify the granulin gene. The PCR product was cloned in a plasmid vector and sequenced. The deduced amino acid sequence was aligned with 16 granulin/polyhedrin sequences obtained from Genbank. A dendogram displaying genetic similarities was constructed using UPGMA method. The granulin of Colombian isolate showed 99% of identity with *Phthorimeae operculella* (PhopGV) gene. The topology of the dendogram was congruent with the previously described division of lepidoteran granulovirus and nucleopolyhedrovirus (NPV).

Key words: Potato tuber moth, *Tecia solanivora*, granulin, baculovirus

Introduction

The Guatemalan potato moth, *Tecia solanivora* (Povolny) (Lepidoptera: Gelechiidae) is a widespread pest in Colombia and other Andean countries (Vargas *et al.* 2004). In potato, loses due to this insect can represent up to 100% in stored tubers and to 56% under field conditions (Sotelo, 1997). Viruses belonging to the Baculovirus family are characterized by an infective particle (*virion)* protected by protein coat (granulin or polyhedrin). They are considered a promising alternative to control this pest, due to the absence of undesirable secondary effects on the environment. In South America, the potato moth has been frequently controlled with a Peruvian PhopGV isolated from *Phthorimeae operculella* (Alcázar & Ramán, 1993). As part of a project to develop and implement biological control for potato pests, the Corpoica research group on biological control has isolated a granulovirus from *T. solanivora* larvae collected in Colombia. This granulovirus presents high biocontrol activity. Previously, this and other PhopGV isolates from hosts captured in different locations were characterized by using specific restriction endonucleases and by amplification of four variable regions. This characterization found a variable region previously described as 90-91, where a 630 bp band distinguished the Colombian isolate from the other PhopGV strains (Léry *et al.*, 2008).

On the other hand, previous studies have suggested that analysis of highly conserved genes (i.e. late expression factor 8, late expression factor 9 and polyhedrin/granulin) is a powerful tool to identify and study the diversity of lepidopteran-specific baculoviruses (Lange *et al.,* 2004; Jehle *et al.,* 2006). The polhyedrin/granulin (polh/gran) locus is one of the most conserved genes in lepidopteran-specific baculoviruses. This gene has been previously used for phylogenetic studies and for simple molecular identification (Lange *et al*, 2004). In order to continue the molecular characterization of the Colombian *T. solanivora* GV isolate, the present work compared the granulin sequences of the native isolate with other GV and NPV sequences available in the GenBank database.

Materials and methods

DNA extraction

One Colombian and one Peruvian GV were isolated from *T. solanivora* and *P. operculella*, respectively. These isolates were multiplied in larvae of the original host species. Granules were purified by using a sucrose gradient according to Caballero *et al*., (2001). Virions were released by alkaline treatment. Briefly, one volume of Na_2CO_3 (0.5M) was mixed with an equal volume of purified granules and heated at 50°C for 15 min. DNA was extracted from virions by incubation with SDS (10%) and proteinase K (10mg/ml), followed by phenol-chloroform extraction and alcoholic precipitation. DNA concentration was estimated by using a nanodrop ND-1000 equipment (Biorad).

Design of degenerate PCR primers

Available granulin genes from fully sequenced granulovirus genome were downloaded from Genbank database. Multiple alignments were performed using Clustal W (Thompson *et al,* 1994). Degenerate primers were designed from highly conserved regions and tested for hairpin and primer – primer interactions by using the Jellyfish program 3.3.1. The sequence of primers was Gran-F: 5´-ATGGGATAYAAYAAAWCDYT-3´ and Gran-R: 5´-TYARTANGCBGGDCCVGTRAA-3´. Amplification was carried out using the following conditions: an initial denaturation step of 95°C for 4 min; 35 cycles of 95ºC for 30 sec, annealing at 52°C for 1 min, extension at 72ºC for 90 sec, and a final extension step 72ºC for 10 min. Each 25 µl PCR reaction contained 50-100 ng of genomic DNA, dNTPs 200 µM (Pharmacia Biotech 27-1850, 1860, 1870, 1880), 0.5 µM of forward and reverse primers, MgCl2 2.0 mM and buffer 10X (50 mM KCl, 10 mM Tris HCl pH 9.0 0.1% Nonidet), 2U of Taq polimerase (Promega M1665). The amplification products were loaded in agarose gel (1%) and stained with SYBR safe (Invitrogen).

Cloning and sequencing

The PCR products were directly ligated into the pCR 2.1-TOPO plasmid vector, using TOPO TA Kit (Invitrogen K4500-01). Briefly, 2 µl of the ligation reaction were mixed gently with chemically competent *Escherichia coli* cells Top10 (Invitrogen K4500-40). Bacteria were incubated for 30 seconds at 42°C without shaking and for one hour at 37°C in SOC medium. Finally, transformed cells were spread into LB plates containing ampicillin (50 µg/ml) and incubated at 37°C for 14 hours. The ampicillin resistant colonies were screened by PCR using Gran-F and Gran-R primers. Positive colonies were used for plasmid DNA isolation using standard techniques. DNA sequencing was performed using universal primers of both strands.

Sequence alignment and genetic similarity analyses

The protein sequence deduced from granulin gene sequenced fragment of the *T. solanivora* GV, was aligned with 11 GV sequences reported in Genbank database (accession numbers NC005038, NC005839, NC008168, NC005068, NC002816, NC10240, NC004062, NC002593, AB290316, NC003102, NC002331) and 5 NPV (accession numbers NC004778, NC003084, NC005906, NC005905, NC001875), in Clustal W (Thompson et al 1994). Unrooted trees were computed by using UPGMA analyses (1000 bootstrap replicates) in MEGA program version 4 (Tamura *et al.*, 2007).

Results and discussion

The designed degenerate primers amplified successfully the granulin gene from the Peruvian PhopGV and Colombian GV isolates. In both cases, product sizes were 746 bp (Figure 1A). Previous studies have report that this gene is one of the most conserved genes in lepidopteran-specific baculoviruses (Lange *et al.*, 2004). Native *T. solanivora* GV granulin gene sequenced had a partial length of 410pb. Alignment of the sequenced fragment with homologous genes reported in Genbank database, showed 99% of identity with PhopGV granulin gene (NC004062). The differences between these two sequences were 3 substitutions at positions 205, 729 and 735 of the sequenced gene. The base change A for G at nt. position 205 produced a non-synonym aminoacid substitution from aspartate to asparagine. The results presented here confirm the high genetic relatedness between the isolates from both potato tuber moths that differ in their biological activities. These virus isolates can, however, be distinguished both by point mutations at the granulin gene or by PCR analysis of other variable regions (Léry *et al*., 2008).

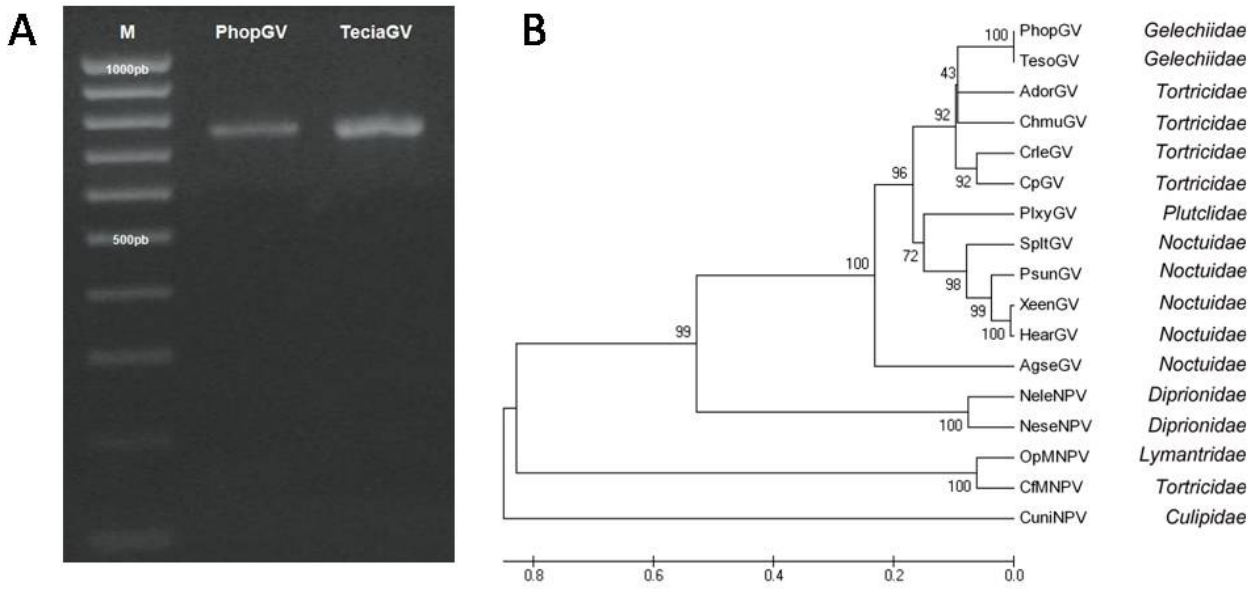

Figure 1. (A) SYBR stained agarose gel of amplified granulin gene in Colombian *T. solanivora* isolate and Peruvian PhopGV. (B) Baculovirus dendogram based on granulin gene. The tree was constructed using the UPGMA method and the numbers at the nodes indicate the bootstrap value (1000 replicates).

A dendogram was constructed with the deduced aminoacid sequences of gran/polh fragments obtained from Colombian *T. solanivora* GV and 15 published sequences (11 GVs and 4 NPV) (Figure 1B). The tree topology shows two principal viral clusters congruent with the virus genera in Baculoviridae family (NPVs and GVs). The granulovirus isolated from *T. solanivora* (TesoGV) grouped within the cluster of virus infecting the Gelechiidae and Tortricidae insect families, separated from viruses infecting the Noctuidae hosts. These results were supported with high bootstrapping value (96 and 92 respectively). The topology of the tree obtained from the granulin gene was consistent with previously reports developed by using three conserved genes (Jehle *et al*, 2006)

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The *Sf32* gene of *Spodoptera frugiperda* multicapsid nucleopolyhedrovirus influences occlusion body yield

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Abstract: The recently sequenced *Spodoptera frugiperda* nucleopolyhedrovirus genome from the Nicaraguan isolate (SfNIC) contains ten unique ORFs, among which is *Sf32*, of unknown function. A PCR and a bacmid-based recombination system was used to delete *Sf32* from the SfMNPV genome that had been previously inserted into a bacmid (Sfbac). Deletion of *Sf32* had not apparent effect on SfMNPV pathogenicity in terms of OB lethal concentration or speed of kill, but a significant decrease was observed in OB production per larva in the *Sf32* mutant compared to the wild-type. This indicates that the *Sf32* gene product influences OB yield.

Key words: SfMNPV, bacmids, *Sf32*, pathogenicity, virulence, OB production.

Introduction

The fall armyworm, *Spodoptera frugiperda* is an important pest of maize and sorghum in much of Latin America. An isolate of *S. frugiperda* NPV (SfMNPV) from Nicaragua with potential for the control of *S. frugiperda* was selected for formulation and field trails in Honduras and Mexico (Williams *et al*., 1999). The identification of factors determining the performance of this isolate such as pathogenicity (expressed as mean lethal concentration, LC_{50}), speed of kill or occlusion body (OB) productivity would facilitate the development of recombinant viruses with improved insecticidal characteristics. With that aim, the SfMNPV genome has been recently sequenced (Harrison et al., 2008; Simon et al., 2008a). The broad majority of genes common to all baculoviruses are structural or involved in transcription or DNA replication (Herniou et al., 2003). In contrast, those unique to different virus species may play an important role in virus specificity and/or virulence. Ten ORFs have been identified in the SfMNPV genome as unique genes that do not have homologues in other baculoviruses sequenced to date. In the present study, we investigated the role of a unique SfMNPV gene, *Sf32*, on the insecticidal properties of SfMNPV. A PCR and a bacmid-based recombination system was used to delete *Sf32* from the SfMNPV genome.

Material and methods

Insects and viruses

Larvae of *Spodoptera frugiperda* were obtained from a laboratory colony maintained at 25 ºC, 75% RH, and 16 h light:8h dark photoperiod on a semi-synthetic diet. OBs of the Nicaraguan wild-type isolate of SfMNPV (SfWT) were amplified by *per os* infection of *S. frugiperda* larvae. The genome of the most abundant genotype of SfWT and previously plaque-purified was inserted into a bacmid (Sfbac) (Simón et al., 2008b) and used for the construction of the recombinant virus.

Construction of recombinant bacmid and OB production
The *Sf32* gene was deleted from Sfbac using Red/ET recombination (Gene Bridges GmbH, Heidelberg, Germany). Sfbac and Sf32null bacmid DNAs were purified from *E. coli* colonies by alkaline lysis. To produce Sfbac and Sf32null OBs, *S. frugiperda* fourth instars were injected with a suspension containing bacmid DNAs (100 μg/μl) and lipofectin reagent (Invitrogen). SfWT OBs were amplified and collected.

Determination of DNA content, pathogenicity, virulence and yield
The DNA content of OBs was estimated by reading the optical density at 260 nm after DNA extraction from samples of $5x10^8$ OBs. The mean lethal concentration (LC_{50}) and mean time to death (MTD) of Sfbac and Sf32null were determined by insect *per os* bioassay following the droplet feeding technique. All statistical analyses were performed using the Generalized Linear Interactive Modelling (GLIM) program. OB production was determined in second instars that had been orally infected with an OB concentration estimated to result in 90% mortality. OB yields per insect were estimated by OB counting using a Neubauer hemocytometer. The results were subjected to analysis of variance using the SPSS v10 program.

Results and discussion

DNA content within OBs
REN profiles of DNA obtained from SfWT, Sfbac and Sf32null OBs confirmed their identities. Five DNA extractions were performed for each virus sample containing $5x10^8$ OBs and the yields subjected to Kruskal-Wallis and Mann-Whitney non-parametric analyses. No significant differences in the mean (±S.D.) amount of DNA in OB samples were detected among SfWT (132 ± 82 ng/μl), Sfbac (100 ± 82 ng/μl) and Sf32null (231 ± 82 ng/μl).

Table 1. Logit regression and mean time to death (MTD) analysis for Sfbac and Sf32null in second instar *S. frugiperda* larvae.

Virus	LC_{50} (OBs/ml)	Relative potency	95% fiducial limits		P	MTD (h)	95% fiducial limits	
			Low	High			Low	High
Sfbac	$1.8x10^4$	1	-	-	-	161a	157	165
Sf32null	$1.8x10^4$	0.997	0.663	1.500	>0.005	161a	157	166

Pathogenicity and virulence
No significant differences in LC_{50} values were detected between Sfbac and Sf32null (Table 1). No differences were either observed in mean time to death values between Sfbac and Sf32null, indicating that *Sf32* deletion has not substantial effects on pathogenicity or speed of kill.

OB production
SfWT, Sfbac, and Sf32null produced significantly different OB yields. Sf32null produced an average of 5.3×10^7 OBs/larva which was the least productive of the three viruses. OB yields were 1.6 and 2.2-fold greater in insects infected by the Sfbac and SfWT viruses, respectively, compared to those infected by Sf32null.

In conclusion, deletion of the *Sf32* gene from SfMNPV had no significant effects on OB lethal concentration or speed of kill. However, OB production was significantly reduced in the *Sf32* mutant. This phenotype is similar to that of the few polyhedra (FP) mutant phenotype found in cell culture after serial passages of SfMNPV (Pedrini et al., 2004). The FP mutants produce fewer polyhedra and high levels of budded viruses (BVs). With further experimentation we plan to determine the effect of *Sf32* gene over BV production and the precise role of this unique gene in SfMNPV infectivity.

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Do vertically and horizontally transmitted variants of *Spodoptera exigua* multiple nucleopolyhedrovirus differ in their insecticidal characteristics?

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Abstract: The coexistence of a wide range of genotypic variants within different *Spodoptera exigua* nucleopolyhedrovirus populations has led us to examine the hypothesis that genotypes may be specialized for different transmission routes: vertical and horizontal. To test this, we analyzed NPV-killed larvae originating from the progeny of field-collected adults, as well as from laboratory reared adults for the presence of vertically-transmitted genotypes. Seven different genotypes were identified among the progeny of laboratory adults (Se-OX1, Se-OX2, Se-OX3, Se-OX4, Se-OX5, Se-OX6 and Se-OX7), two of which were identical to those found among the progeny of field-collected adults (Se-AL1, Se-AL2). Three of these isolates, Se-AL1, Se-AL2 and Se-OX4, and another three that had been previously reported from soil isolates (Se-G24, Se-G25 and Se-G26), were selected to determine their insecticidal characteristics. We conclude that transmission route and insecticidal properties do not appear to be correlated in this virus.

Key words: SeMNPV, vertical transmission, genotypic diversity, biological activity.

Introduction

Baculoviruses are transmitted horizontally when insects eat plants that are contaminated with viral occlusion bodies (OBs). However, these viruses can also be transmitted vertically, from parents to their offspring (Kukan, 1999). Vertical transmission is believed to play an important role in the survival of NPVs when opportunities for horizontal transmission are limited (Burden et al., 2003) or following the invasion of new areas via host dispersal (Cooper et al., 2003).

The *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) (Baculoviridae: *Alphabaculovirus*) is commonly found in the environment and frequently causes a high prevalence of infection in *S. exigua* populations in southern Spain (Almeria). Genetic and phenotypic studies have revealed the high genotypic heterogeneity present within wild type isolates that originated from a pool of NPV-killed larvae (Muñoz *et al.* 1999) or from OB reservoirs in the soil (Murillo et al., 2007). These observations led us to examine the hypothesis that different genotypes may be specialized in different routes of transmission.

The aim of this study was to identify SeMNPV genotypes potentially involved in vertical transmission by breeding the progeny obtained from adults reared under both field and laboratory conditions. We also assessed the biological activity of different isolates of SeMNPV in terms of pathogenicity (mean lethal concentration, LC_{50}) and virulence (mean time to death, MTD) to determine whether certain genotypes were more prone to adopt vertical or horizontal routes of transmission.

Material and methods

Field-sampling and laboratory rearing of insects

Field populations of *S. exigua* were sampled in July and September of 2006 and 2007 in the greenhouses of Almería (southern Spain). Adults were attracted to UV light traps and caught after being drawn to a white blanket at sunset. Insects were individually placed in plastic containers. Females were allowed to lay eggs for 1-2 days. Twenty neonate larvae from each female were collected, individualized and reared through to the adult stage. Larvae were monitored daily for symptoms of viral disease. NPV-killed larvae were stored at -20ºC for restriction endonuclease analysis (REN) of viral DNA. Each of the NPV-killed offspring was considered to have succumbed to a vertically transmitted infection. As controls, a *S. exigua* population was continuously reared in the insectary facilities of the Public University of Navarre at 25°C, 16:8 light:dark photoperiod, and 60–70% relative humidity. Fifteen pairs of adults were selected from this population and their progeny reared through to the adult stage and monitored as mentioned above.

DNA extraction and REN analysis

To determine the genotypic diversity present in SeMNPV isolates, viral DNA was purified from OBs as described by Muñoz *et al.* (1999), digested with *Bgl*II, loaded in 0.7% agarose gels with TAE buffer (40mM tris-acetate; 1mM EDTA) and electrophoresed at 16 V overnight. Ethidium bromide stained gels were then visualized in a UV transiluminator, photographed and examined using the Chemi-doc software (Bio-Rad, Alcobendas, Spain).

Determination of dose-mortality response and speed of kill

The pathogenicity of Se-AL1, Se-AL2, Se-OX4, Se-G24, Se-G25 and Se-G26 was determined by inoculating *S. exigua* second instar larvae (L_2) with their respective OBs using the droplet-feeding method. Six concentrations containing between 3×10^3 and 2.5×10^5 OBs/ml of each viral inoculum were prepared to produce mortalities between 5 and 95%. The different suspensions and an OB-free solution were fed to seven batches of larvae, each containing thirty individuals, for each virus treatment. Larvae were individually transferred to 24-well tissue-culture plates containing diet and reared at 25 ± 2ºC. Mortality was recorded after seven days. Bioassays were performed three times. Dose-mortality data were subjected to probit analysis using the POLO-PLUS (Le Ora Software, 1987). To determine time-mortality responses, second instars were inoculated with an LC_{90} dose of OBs as described above. Mortality was recorded every 8 h for 7 days. Time-mortality data were subjected to Weibull survival analysis using the GLIM program.

Results and discussion

Molecular identification of vertically transmitted SeMNPV isolates

OBs extracted from larvae that developed an NPV infection either from the progeny of field-collected adults or from laboratory-reared adults were considered as vertically transmitted isolates. The *Bgl*II REN profiles of these isolates could be assigned to one of seven different genotypic variants (Se-OX1 to Se-OX7) from the *S. exigua* laboratory culture progeny (Fig. 1, lanes 1-7) and two (Se-AL1, and Se-AL2) from the *S. exigua* field progeny (Fig. 1, lanes 1 and 3). The Se-AL1 and Se-OX3 profiles were identical to those previously reported for a soil isolate, Se-G26 (Murillo et al., 2007) and a genotype from a field isolate (SeSP2-C) collected during an epizootic from an infected larva (Muñoz et al., 1999). Se-AL2 had the same *Bgl*II

profile as Se-OX1, which was the predominant genotype found among the progeny of laboratory-reared adults.

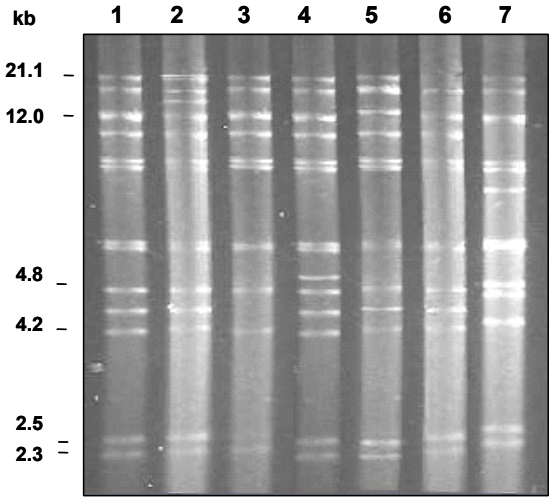

Figure 1. *Bgl*II profiles of genomic DNA extracted from OBs purified from the progeny of laboratory-reared and field-collected adults. Variants were isolated from NPV-killed larvae collected from laboratory-reared adults (Se-OX1 – Se-OX7, lanes 1-7). Genotypes identified in progeny of field-collected adults (Se-AL1 and Se-AL2) are shown in lanes 3 and 1, respectively. The molecular sizes of some of the fragments are shown to the left of the panel in kb.

Biological activity of vertically and horizontally transmitted variants

The biological activity of vertically transmitted isolates Se-AL1, Se-AL2, and Se-OX4, was compared to that of horizontally transmitted isolates previously reported (Se-G24, Se-G25 and Se-G26) in terms of pathogenicity and virulence. Se-AL1, Se-G25 and Se-G26 were the most pathogenic variants tested, with LC_{50} values of 4.4 x 10^4 - 7.5 x 10^4 OB/ml whereas the LC_{50} values of the other variants ranged from 1.6 x 10^5 to 3.4 x 10^5 OBs/ml. With a MTD value of 89.5 h, the Se-OX4 variant killed larvae significantly faster than the other five isolates, while most pathogenic isolate, Se-AL1, had the slowest speed of kill (110 h).

Overall, vertically and horizontally transmitted variants did show consistent differences in their insecticidal properties, in terms of pathogenicity (LC_{50} value) or speed of kill. There was also no consistent relationship between pathogenicity and speed of kill for all the variants taken as a whole, suggesting that genetic factors that do not influence transmission route are likely involved in determining the insecticidal characteristics of this virus.

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The selection of defective and complete co-occluded genotype mixtures in a nucleopolyhedrovirus occurs during entry into midgut epithelial cells

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Abstract: An isolate of the *Spodoptera frugiperda* multiple nucleopolyhedrovirus comprises a stable proportion of deletion genotypes (such as SfNIC-C) that lack *pif1* and *pif2* rendering them non-infectious *per os*. Deletion genotypes survive by complimentation with a complete genotype (SfNIC-B) in co-infected cells. To determine whether selection for particular ratios of complete and deletion genotypes occurs mainly during the establishment of the primary infection in insect midgut cells or during subsequent systemic infection, we examined genotype frequencies in insects inoculated with OBs comprising different co-occluded mixtures of genotypes. Dramatic changes in genotype frequencies were observed between the OB inoculum and budded virus samples taken from larvae inoculated with OBs comprising 10% SfNIC-B + 90% SfNIC-C, indicating that a marked reduction of SfNIC-C genotype had occurred in the insect midgut due to the immediate elimination of all non-infectious OBs that originated from cells that had been infected only by SfNIC-C. In contrast, immediate changes were not observed in OBs comprising mixtures of 50% SfNIC-B + 50% SfNIC-C or those comprising 10% SfNIC-B + 90% SfNIC-C. Subsequent changes in genotypic frequencies during the systemic infection were fairly small in magnitude for all genotypic mixtures. We conclude that the prevalence of defective genotypes in the SfNIC population likely depends on a balance between the frequency of OBs produced in cells infected by SfNIC-C alone and selection for fast-replicating deletion genotypes. Selection is probably modulated by changes in the average number of genomes that infect cells during the infection period.

Key words: Nucleopolyhedrovirus, defective genotypes, infection, selection.

Introduction

In a Nicaraguan isolate of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfNIC), nine plaque purified variants have been identified (named from SfNIC-A to SfNIC-I). The only complete genotype was named SfNIC-B (Simón et al. 2004). SfNIC-C posses a large deletion (16.4 kb) that affects a number of genes including *pif1* and *pif2* and it is therefore unable to infect larvae *per os*. However, this genotype can survive by complementation in cells co-infected with the complete genotype (SfNIC-B). Remarkable cooperative behavior has been demonstrated to occur in co-occluded mixtures comprising SfNIC-B and SfNIC-C. The potency of OB mixtures comprising 75% of SfNIC-B and 25% SfNIC-C is ~2.5-fold higher than that of SfNIC-B OBs alone (López-Ferber et al., 2003). Recent studies with *pif1*/*pif2* deletion recombinants have revealed that it is the deletion of this region that is responsible for the observed potentiation of the genotypes and that the proportions of complete and deletion

genotypes present in mixtures subjected to serial passage *per os* rapidly converge to a genotype composition that results in the highest virus transmissibility (Clavijo et al., in press).

Whether this selection for specific ratios of genotypes occurs mainly during the establishment of the primary infection process in insect midgut epithelial cells or during the later development of the systemic infection of the host by budded virus (BV) is unknown. In an attempt to elucidate this question, we analyzed the frequency of SfNIC-B and SfNIC-C genotypes at different time points throughout the infection process in larvae inoculated with different co-occluded mixtures of these two genotypes.

Material and methods

Insects and viruses

Larvae of *Spodoptera frugiperda* were obtained from a laboratory colony and maintained on semisynthetic diet (Greene et al., 1976). Two distinctive genotypes, SfNIC-B and SfNIC-C, purified *in vitro* from the wild-type Nicaraguan isolate (SfNIC) were obtained from a previous study (López-Ferber et al., 2003). OBs of SfNIC, SfNIC-B and SfNIC-C were amplified in fourth instars, collected and quantified by counting. Viral DNA extraction was performed and restriction endonuclease analysis was used to verify the DNA profiles of both purified genotypes and the wild-type isolate (Simón et al., 2008).

Experimental virus populations

Purified OB suspensions of the SfNIC-B and SfNIC-C genotypes were dissolved in alkaline buffer to release ODVs. Each ODV-containing supernatant was then mixed in one of three different proportions: 90:10, 50:50 and 10:90. These mixtures were named (INJ), as they were injected into groups of 50 *S. frugiperda* fourth instars (~8 µl/larva). The OBs obtained from these larvae were named (D_0) and were used as inoculum to infect second instar larva *per os*. Groups of 50 larvae were inoculated with one of the three co-occluded mixtures at a concentration of $1.2x10^6$ OBs/ml (~90% mortality). Groups of eight larvae were sampled every 24 h during a five day infection period to obtain the hemolymph.

Quantification of the relative proportions of genotype mixtures

The relative proportions of SfNIC-B and SfNIC-C present in: i) the ODV mixtures that constituted the co-injected samples (INJ), ii) the D_0 samples of OB inocula and, iii) the BVs from hemolymph samples taken at 1 to 5 days post-infection were analyzed by semi-quantitative PCR using primers designed by Simón et al. (2004) that differentially amplify the two genotypes. PCR reactions were stopped at 19 cycles. The relative proportions were estimated by densitometric analysis using an image analysis program.

Results and discussion

Genotype frequencies differed in each experimental population. In larvae inoculated with OBs comprising (10% SfNIC-B+ 90% SfNIC-C), a dramatic change in genotype frequencies occurred between inoculation (D_0) and the day 1 sample in which the relative prevalance of SfNIC-B increased over five-fold, whereas that of SfNIC-C decreased by two-fold. We assume that insects infected with a high proportion of SfNIC-C will result in a high proportion of cells infected by SfNIC-C genotype alone, generating inoculum OBs (D_0) that will not be able to infect the midgut. These OBs would be cleared in the following *per os* passage. Only OBs that were produced in cells infected by at least one SfNIC-B genome will be capable of infecting the midgut cells in the following peroral passage. As a result, the frequency of SfNIC-C in the

population will be quickly reduced. Mathematical modeling predicts that SfNIC-C would disappear unless an advantage exists for this genotype, such as a faster rate of replication (Simón et al., 2006).

In the period from 2 to 5 days post-infection, the frequencies of SfNIC-B and SfNIC-C varied less significantly, with SfNIC-B gradually increasing its relative abundance to 65% at 5 days post-infection. Although smaller, this variation could indicate that competition efficiency for the host resources between both genotypes is taking place and different selection pressures may occur on both genotypes during the infection. In larvae fed with 50% SfNIC-B + 50% SfNIC-C, no major changes were observed during the period of infection, and the final genotype ratios were 57% SfNIC-B + 43% SfNIC-C at 5 days post-infection. In this situation it is likely that a high proportion of the inoculum (D_0) OBs were produced in cells coinfected by both genotypes. Finally, in the experimental population consisting (90% SfNIC-B+ 10% SfNIC-C) frequencies of genotypes changed quite significantly by day 3, when the relative proportion of SfNIC-C increased to 20% and stabilized at this level during days 4 and 5 post-infection.

In conclusion, the equilibrium frequency of deletion genotypes in the population appears to represents a balance between the elimination of PIF1/PIF2 deficient ODVs in the insect gut and positive selection for fast-replicating deletion variants that may be favored during the systemic infection phase in each insect. The magnitude of within-host selection is likely modulated by changes in the average number of genomes that infected each cell during the infection period. Testing these hypotheses requires additional research.

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Effects of the *Spodoptera littoralis* granulovirus on the development and reproduction of cotton leafworm (Lepidoptera: Noctuidae)

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Abstract: Laboratory studies were performed on insecticide activity of *Granulovirus* on cotton leafworm *Spodoptera littoralis* (Boisduval) larvae. The *S. littoralis* granulovirus (SpliGV) was tested against first, third and fifth instars larvae fed on alfalfa leaf disks treated with different OB concentrations, resulting in LC_{50} values of $3.74x10^4$ OB/larvae, $6.86x10^5$ OB/larvae and $1.1x10^7$ OB/larvae, respectively. Lethal times decreased with increasing doses and the fifty lethal times (LT_{50}s) to similar mortality percentage (60-70%) were 27.98, 21.90 and 15.93 days for first, third and fifth instars, respectively. Sublethal effects were studied by treatment of third instar larvae at the LC_{50} equivalent dose. Larval development time and pupation period were not affected by SpliGV treatment. However, significant differences were found in the oviposition and viable eggs per days when both sexes came from treated larvae. Spermatophore number, fecundity and egg viability were not affected, but female longevity increased in combinations in which female came from treated larvae. In addition, the effects of SpliGV on male and female internal reproductive system were studied. In 9 days old pupae coming from larvae treated with SpliGV smaller amount of primary and secondary spermatocytes were observed, and 3 days old adults showed a little amount of spermatids and sperm bundles. No effects were found on the oocyte production in both virgin and mated females.

Key words: *Granulovirus*, *Spodoptera littoralis*, sublethal effects, reproduction, development, gametogenesis

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Biological activity and compatibility with chemical pesticides of a Colombian granulovirus isolated from *Tecia solanivora*

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Abstract: The Guatemalan potato moth *Tecia solanivora* is the most limiting potato pests in Venezuela, Colombia and Ecuador. In a previous work, a native granulovirus isolated from *T. solanivora*, appeared to be one promising tool for managing this insect and the other potato moth *Phthorimaea operculella*. The compatibility with chemical pesticides used for potato crops protection was determined. Biological activity was not affected after 24 hour of contact, suggesting that native granulovirus could be used combined with these agrochemicals in an Integrated Potato Crop Management Programme.

Key words: baculovirus, granulovirus, potato, Guatemalan potato moth

Introduction

Potatoes are central to the culture and diet of Andean people and occupy the fourth rank among the world's principle food crops (FAO, 2008). However, Andean potato production is threatened by two moths *Tecia solanivora* and *Phthorimaea operculella* (Lepidoptera: Gelechiidae), whose larvae destroy potato tubers. Because distribution of both moths often overlap in Colombia, it is necessary to develop tools to manage them simultaneously.

Only a few chemical insecticides are registered for controlling these moths, and their efficacy is not well determined (Notz and Gualdrón 2000). Currently, a registered biopesticide based on a Peruvian granulovirus isolated from *P. operculella* (PhopGV) has been successfully used in Colombia for managing the Guatemalan moth in stored potato. This virus isolate appears to be efficient for controlling *T. solanivora*. However, an isolate isolated from the same insect host could be more virulent to this insect than Peruvian granulovirus isolated from a different potato moth.

In a previous work a native isolate of granulovirus VG003 isolated from *T. solanivora* was selected due to its high biocontrol activity towards *T. solanivora*, and its yield on viral particles. The scope of this study was to investigate its compatibility with chemicals.

Materials and methods

Lethal concentrations over P. operculella and T. solanivora larvae under laboratory conditions

Bioassay was performed by using suspensions of purified virus (Caballero *et al.*, 2001) of native VG003 and foreign Peruvian isolates. Eight different virus dilutions adjusted from 1.5

x 10^4 to 5 x 10^9 occlusion bodies (OBs)/mL by OD 450 nm measurement were evaluated by spraying two ml of each suspension on three potato tubers with the nebulizer described by Carrera *et al.*, (2008). Inoculated tubers were placed individually in plastic flasks and infested with 15 neonatal larvae of *T. solanivora* or *P. operculella*. Treatments were maintained at 25ºC ± 2°C for 25 days, when tubers were analyzed for determining the larvae mortality. Experiment was repeated five times and dose response data were analyzed by using Probit analysis (Finney, 1971) to determine the LC_{50} and LC_{90} concentrations.

Compatibility with chemical pesticides

Four insecticides (Carbofuran, Profenofos, Chlorpyrifos and Dimetomorph) and four fungicides (Mancozeb, Metalaxyl, Cypermethrin and Cymoxanil) frequently used for managing potato insect pest and diseases, were reconstituted in water by using two times the field application recommended dosage and 1 ml of each pesticide dilution was mixed with 1 ml of purified virus VG003. Mixtures were maintained at 20°C for 24 hours in a temperature regulated bath and then, OBs were purified by differential centrifugations and using a sucrose gradient (45%, 65% and 80%). Purified virus suspensions were used to prepare new suspensions adjusted to 3 x 10^6 OBs/ml, which were used to perform a bioassay following the previously described methodology. The bioassay included a positive control treatment corresponding to a non-exposed virus and an absolute control in which potato tubers were not inoculated. Mortality percentages were used to determine the efficacy by the Schneider-Orelli equation (Zar, 1999), which was analyzed by ANOVA test (95%).

Results and discussion

Lethal concentrations over P. operculella and T. solanivora larvae under laboratory conditions LC_{50} and LC_{90} of Colombian and Peruvian granulovirus are presented in Table 1. The Colombian isolate VG003 was significantly more virulent for *T. solanivora* than for *P. operculella*, VG003 lethal concentrations were statistically lower over its original host (*T. solanivora*), in comparison with the obtained over the alternative host (*P. operculella*).

In the other hand, the Peruvian isolate PhopGV, was significantly more virulent for its original host *P. operculella* than for *T. solanivora*. For both isolates, the lethal concentrations were lower when bioassay was carried out over the insect from which granulovirus strains were originally isolated, which could be related with a specific adaptation of each viral isolate to different hosts (Léry *et al.*, 1998).

Table 1. Lethal concentrations (OBs/ml) of VG003 and Peruvian PhopGV isolates over *P. operculella* and *T. solanivora* larvae under laboratory conditions

Insect	Viral isolate	LC_{50} (OBs/ml)	Lower limit	Upper limit	LC_{90} (OBs/ml)	Lower limit	Upper limit
Phthorimaea operculella	**VG003**	1.3 x 10^7 a	9.0 x 10^6	2.0 x 10^7	1.3 x 10^9 a	6.8 x 10^8	3.0 x 10^9
	PhopGV	3.4 x 10^6 b	2.3 x 10^6	4.9 x 10^6	1.3 x 10^8 b	8.0 x 10^7	2.5 x 10^8
Tecia solanivora	**VG003**	3.6 x 10^6 b	2.5 x 10^6	5.1 x 10^6	2.2 x 10^8 b	1.2 x 10^8	4.6 x 10^8
	PhopGV	1.5 x 10^7 a	9.6 x 10^6	2.6 x 10^7	7.2 x 10^9 a	2.5 x 10^9	2.8 x 10^{10}

The LC_{50} of VG003 isolate obtained in the present work is similar to the one previously obtained by Chaparro *et al.*, (2008) for VG003 isolate over *T. solanivora* larvae, when used a

different potato tubers inoculation method. In the mentioned work, inoculation was made with a brush, which was utilized to apply 2mL of viral suspension on the surface of each potato, obtaining a LC_{50} of 1.6 x 10^6OBs/ml.

Compatibility with chemical pesticides

Virus only treatment at 3 x 10^6 OBs/ml resulted in a 66.8% mortality, while in treatments with both virus and pesticides, mortality ranged from 64.5% to 80.1%. The differences are not statistically significant, suggesting that OBs were not inactivated by the chemical pesticides evaluated. To verify if larvae mortality in the treatments was caused by the virus infection or by a residual content of chemical pesticides in the samples, the percentage of collected larvae with typical granulovirus infection symptoms was calculated. Virus-only treatment presented the highest symptomatic larvae percentage (40%). Mixtures of virus and agrochemicals, gave lower values (between 22.2% and 37.8%). ANOVA tests did not detect statistical differences between treatments. These results could suggest that viral pathogenicity was not affected for evaluated chemical products.

Virus tolerance to pesticides could be attributed to a protective effect of OBs (Figure 1), which protect the virions exposed to adverse environmental conditions. Tamez (2002) demonstrated when this protective action of the granule when testing n-propanol in a nucleopolyhedrovirus spray-dried formulation, which did not affect the insecticidal activity. In other work, Villamizar and Martínez, (2008) evaluated the compatibility of one isolate of Spodoptera frugiperda nucleopolyhedrovirus with 16 organic solvents, concluding that all solvents evaluated were compatible with the virus, as they did not affect viral activity.

Although in our experimental conditions virus particles per se are unaffected by the chemical compounds tested, baculovirus efficacy, can be altered in many ways by the effects of chemical pesticides on the host insect, that should be carefully analysed.

The Colombian isolate VG003 demonstrated high potential to control both potato moths under an integrated pest management programme due to its biocontrol activity and its compatibility with chemical pesticides used in potato crops.

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Presence of nuclear polyhedrosis viruses in populations of pine looper *Bupalus piniarius* L (Lepidoptera: Geometridae)

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Abstract: Baculoviruses are potential agents for the control of different forest pests. Research on baculoviruses of forest pests were carried out in the University of Latvia, Institute of Biology. The aim of our studies was to obtain new isolates of nuclear polyhedrosis viruses (NPVs) and to investigate occurrence of persistent infections in *Bupalus piniarius* populations. Monitoring of *B. piniarius* populations was done in different regions of Latvia. The insects collected from natural habitats were checked for presence of NPV. In 2008 we observed very low NPV infection level (mortality <5%). NPVs were isolated from *B. piniarius* by subjecting asymptomatic larvae to stress-factors: extreme temperatures, NsNPV, 0.5% $ZnSO_4$ or 1.0% H_3BO_3. An optimised method of virus DNA determination by PCR was used for *in–situ* determination NPVs in different populations of *B. piniarius* (Lepidoptera: Geometridae). PCR confirmed that endemic insect populations had NPV infection.

Key words: nuclear polyhedrosis viruses, pine looper, persistent infection

Introduction

In nature, nuclear polyhedrosis viruses (Baculoviridae), which are frequently associated with outbreak and declining populations of Lepidoptera, cause diseases of insects and can control the population size of their hosts. NPVs are considered to be safe biological insecticides and have a great potential in pest control (Entwistle, 1997). Virus may persist within the host insect population in an occult or latent state (Bilimoria, 1991). Persistent baculovirus infections have been identified in a number of host species and are proposed as a mechanism for vertical transmission of the virus (Hunter-Fujita et al., 1998). There is little information on persistence and transmission of viruses in pest populations with low density. Knowledge on interaction between baculoviruses and host insect helps to develop an effective strategy of biological control. The development of new laboratory techniques allowing viruses to be identified at low concentrations and in individuals that are asymptotic is altering our understanding of occurrence of viruses in populations.

Researches on baculoviruses of forest pests have been carried out in the Institute of Biology since 1965. Local virus strains and isolates have high activity in the climatical conditions of Latvia (Zariņš, Eglīte, 1993). The pine looper *B. piniarius* is often present in the pine stands in Latvia (Ozols, 1985). In 1992, NPVs were isolated from pine looper's larvae (Zariņš, 2001). Recently, the Laboratory of Experimental Entomology of Institute of Biology, University of Latvia, has been studying the NPVs occurrence and persistence. The aim of our studies was to obtain new isolates of NPVs and to investigate occurrence of persistent infections in *B. piniarius* populations.

Material and methods

Monitoring of pine looper population density

Since 1990 Latvian State Forest Research Institute "Silava" has monitored *B. piniarius* population density. In 2008 *B. piniarius* larvae and pupae were collected from natural habitats – pine forests. Collection of insect material was done in 4 trapping stations located in Kurzeme: Ance, Engure, Padure and Venta. In one station, situated in Vidzeme's region Valka, the *B. piniarius* population density (number of pupae in 1 m^2 of litter) and the weight of pupae were recorded (Fig 1.).

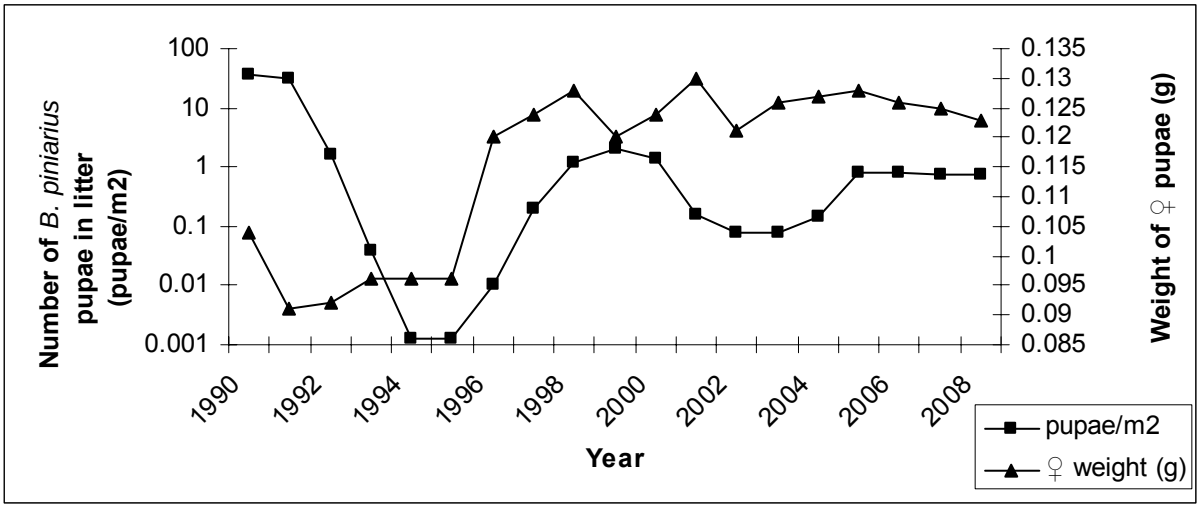

Figure 1. *Bupalus piniarius* population density (number of pupae in litter) and average weight of pupae (female) since 1990 (average means from observed plots).

Insects' collection and rearing

To examine virus infections in natural insect populations, asymptomatic living insects were collected from natural habitats using standard methods. Collected living insects were reared in the laboratory - in isolators on fresh natural foods (pine needles). Rearing of larvae until pupation or death was followed by examination of cadavers to confirm presence of pathogen.

Direct microscopic examination

The presence of NPV in larval tissues was detected by direct examination of larval tissue smears, using phase contrast or after staining (Adams & Bonami, 1991; Lacey, 1997). For ultra structural examination, polyhedrae were dissolved in 1% NaOH for 5 min and negatively stained with 2% phosphotungstic acid. The preparations were observed in an electron microscope (JEM-100C, JEOL, JAPAN).

Activation of persistent infection

Virus infection was activated by subjecting field collected asymptomatic larvae to following stress-factors: NPV isolated from other insects (Ns NPV 2 x 10^7 polyhedrae/ml), extreme temperatures (24 hours at +4 ±2 ^oC, then 48 h at + 32 ±2 ^oC); the food treated with 0.5% $ZnSO_4$ and 1.0% H_3BO_3, inadequate food – semidried pine needles. Tests were performed in 4 repetitions. Dead larvae were frozen with liquid nitrogen and homogenized in distilled water

with 0.1 % SDS and used for DNA tests. Individuals were catalogued and kept frozen at – 18 ^{o}C until DNA analysis was made.

Determination and identification of viral DNA

Extraction of viral and host genomic DNA from individual insects (larvae and pupae) was done using Genomic DNA Purification Kit (MBI Fermentas). We used nested PCR and primers that correspond to the polyhedrin gene (Jankevica, 1999). The PCR reaction mixture (MBI Fermentas) was used. The PCR was performed as described by Jankevica (1999). At the end of the reaction the resulting amplification products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized under short wave UV light, the size of DNA fragment was detected (Saville et al. 1997).

Results and discussion

Monitoring of *B. piniarius* population showed that all populations now are in pre-phase of pest's mass multiplication and larvae are healthy and in good physiological condition. We did not observed mortality higher than 17.5 % in studied regions. In Padure all collected larvae pupated. In locality Venta larvae had high vitality, only 5 % of them died and microscopical examination did not show the presence of pathogens. Larval mortality in Engure, Valka, Ance caused by NPV was 5.0 ± 2.2 %, 2.5 ± 2.0 % and 2.5 ± 2.0 %, respectively.

It is known that virus infections in either the latent or persistent state provide the pathogen with the option of remaining associated with host for its lifetime without causing serious disease (Posse et al., 2008). We observed significantly higher mortality after subjecting healthy *B. piniarius* larvae collected in Padure and Venta, to stress-factors. Obtained data showed that populations of pine looper had a persistent NPV infection that was influenced by stress-factors and caused illness of insects. In variants with extreme temperatures larval mortality was very high 96.9 ± 6.0% ($P \leq 0.05$), microscopical examination and use of PCR based method confirmed that 89.7 ± 3.3% of dead larvae carry NPV (Table 1). Ten days after subjecting to stress-factors highest amount of polyhedra was produced in variants with extreme temperatures.

Table 1. Activation of virus infection of *Bupalus piniarius* using different stress-factors.

Stress-factor	**Instar of larvae**	**$LT_{50} \pm sLT_{50}$, days**	**Corrected mortality ± SE, %**	**Presence of NPV in dead larvae, %**
Neodiprion sertifer NPV	III - IV	25.2 ± 3.9 a	56.9 ± 6.2 b	100 ± 1.6 a
Extreme temperatures	III - IV	15.4 ± 1.8 b	96.9 ± 6.0 a	89.7 ± 3.3 b
1.0 % H_3BO_3	III - IV	*	31.0 ± 4.2 c	51.7 ± 3.0 d
0.5 % Cu SO_4	III - IV	26.5 ± 1.7 a	60.9 ± 3.9 b	48.6 ± 2.9 d
Inadequate food	III - IV	27.2 ± 1.9 a	54.9 ± 2.9 b	75.7 ± 1.7 c
^ Means with different letters in each column differed significantly $P \leq 0.05$, - * LT_{50} were not calculated, corrected mortality did not achieve 50%				

Stress-factors: the food treated with 0.5% $ZnSO_4$, NPV isolated from *Neodiprion sertifer* and inadequate food – caused mortality of larvae 60.9 ± 3.9%, 56.9 ± 6.2% and 54.9 ± 2.9%, respectively. In variants with Ns NPV we determined 56.9 ± 6.2% larval mortality and confirmed that 100 % of dead larvae showed presence of NPV. These results concur with data obtained by Huges et al. (1997), that use of other virus can challenge progression of virus infection. Further, the virus may be transmitted vertically to the next generation.

We concluded that persistent NPVs infections are detectable in endemic *Bupalus piniarius* populations. Next step of investigations will be vertical transmission of NPVs to offspring larvae.

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Application of juvenile hormone analogue and optical brightener technologies to the production of *Spodoptera frugiperda* multiple nucleopolyhedrovirus

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Abstract: Final instar larvae of *Spodoptera frugiperda* grew to a maximum weight of 622 ± 13 mg on diet treated with 1% piriproxyfen, a juvenile hormone analogue (JHA), or 695 ± 17 mg on diet treated with 1% fenoxycarb, both of which were significantly greater weights than observed in larvae that developed on untreated diet (512 ± 9 mg). Virus mortality in insects inoculated with SfMNPV occlusion bodies (OBs) was approximately 50% in all treatments, reflecting the resistance of late instar *S. frugiperda* larvae to infection. However, JHA treatment did not result in a significant increases in the total OB yield, or OBs per mg larval weight, compared to untreated infected insects. We determined whether resistance to infection could be overcome by inoculation with mixtures of OBs and optical brighteners. Potentiation of OB activity was >2500-fold in mixtures with 1% Leukophor AP or Blankophor BA, or >15-fold in mixtures with 0.1% of either brightener, compared to SfMNPV OBs alone. We conclude that SfMNPV production was not increased in JHA treated larvae because the JHA did not result in a supernumerary instar in *S. frugiperda*. However, optical brighteners were highly effective in overcoming developmental resistance to infection in this species.

Key words: baculovirus production, piriproxyfen, fenoxycarb, leukophor AP, blankophor BA

Introduction

Baculovirus-based insecticides have to be produced in living insects. This represents a constraint in the commercial development of baculovirus-based products because mass rearing of insects is costly, and because insect cultures often show changes in vigor that result in temporal variation in the quality and quantity of insects that can be infected during the virus production process. Recently, we showed that treatment of late instar *Spodoptera exigua* larvae with juvenile hormone analogues (JHA), such as fenoxycarb or methoprene resulted in the production of a supernumerary instar that was larger and weighed more than untreated final instar insects (Lasa et al., 2007). Infection of the JHA-treated larvae with high doses of virus occlusion bodies (OBs) resulted in 2.7 to 2.9-fold increases in total OB yields per larva compared to yields from untreated infected insects. In the present study we examined whether JHA technology could be effectively applied to increase the production of a multiple nucleopolyhedrovirus (SfMNPV) of the fall armyworm, *Spodoptera frugiperda*, that is a serious pest of maize and sorghum in the Americas. We also examined the potential of two stilbene optical brighteners to reduce the concentration of OBs required for lethal infection of this pest.

Material and methods

Insects, virus, JHA and optical brighteners

Larvae of *S. frugiperda* were obtained from a laboratory colony maintained in the insectary of the Universidad Pública de Navarra, Pamplona, Spain. The insects were subjected to the following conditions during rearing and during the experiments described below: 26 ± 2ºC, 60 ± 5% R.H., 16 h: 8 h L:D photoperiod. Larvae were reared using a wheatgerm-based semisynthetic diet described previously (Muñoz et al., 2001). OBs of a Nicaraguan isolate of SfMNPV were fed to fourth instars that were subsequently reared on uncontaminated semisynthetic diet. Patently diseased larvae were collected, triturated in 0.1% SDS and OBs were collected by filtering through muslin and centrifugation at 90 *g* for 5 min to eliminate insect debris. Two JHAs were obtained in the form of commercial insecticide products. Fenoxycarb was obtained as a water dispersible granule formulation (Zambu, 25% WG, Agro Artes, Castellón, Spain), whereas pyriproxifen was obtained as a liquid formulation (Juvinal, 10% EC, Kenogard, Barcelona, Spain). The stilbene optical brighteners used in this study were Blankophor BA (Bayer, Barcelona, Spain) and Leukophor AP (Croma, Gipuzcoa, Spain).

Effects of JHAs on insect growth and OB production

Recently-moulted fifth instars were placed individually in 25 ml plastic pots that had been filled to a depth of 2 mm with semisynthetic diet. The surface area of the exposed diet was 625 mm^2. Groups of 30 larvae were randomly assigned to one of the following treatments involving applications to the surface of the diet: (i) water (control) (ii) 1% piriproxyfen, (iii) 1% fenoxycarb, (iv) SfMNPV OBs alone (v) SfMNPV OBs + 1% piriproxyfen, (vi) SfMNPV OBs + 1% fenoxycarb. In all cases virus treatments were based on a concentration of 1000 OBs/mm^2 diet surface (625,000 OBs/pot). All treatments were applied in a volume of 55 µl of water. Larvae were weighed individually 12 days after starting the experiment. To determine OB production, ten infected larvae from each treatment were randomly selected, individually homogenized in 600 µl sterile distilled water, washed twice, suspended in a total volume of 2 ml and subjected to counting in triplicate in a Neubauer haemocytomer. The experiment was performed three times.

Effects of optical brighteners on the lethal concentration of SfMNPV OBs

To determine the effect of two optical brighteners on 50% lethal concentration values of SfMNPV, trays of semisynthetic diet, 2 mm in depth, were subjected to surface contamination treatments involving water alone (control), one of five concentrations of SfMNPV OBs estimated to result in between 10 and 90% mortality, and mixtures of SfMNPV OBs and either Leukophor AP or Blankophor BA at concentrations of 0.1 or 1.0% (wt./vol.). Each treatment was applied to the surface of the diet, spread gently over the diet surface, and allowed to dry for 45 mins, after which time the diet was cut into 400 mm^2 squares and placed into 25 compartment plastic dishes. Groups of 25 recently moulted forth instars were individually placed in each of the compartments of each dish and incubated for 7-8 days until pupation or death. The experiment was performed three times. LC_{50} values were calculated by logit regression in GLIM.

Results and discussion

For insects that were not infected by virus, treatment with either JHA resulted in a significant increase in the maximum weight attained by larvae compared to the controls ($\chi^2 = 83.1$; d.f. =

2; $P < 0.05$). The average maximum weight of untreated insects was 512 ± 9 mg at 5 days after the start of the experiment, after which the insects began to pupate. This compares to maximum a weight of 695 ± 17 mg in fenoxycarb-treated insects, that were significantly heavier than piriproxyfen-treated insects (622 ± 13 mg) ($Z = 3.70$; $P < 0.05$), both at 9 days post-treatment. Unlike the situation in *S. exigua*, JHA treatment did not induce a supernumerary moult, but instead extended the duration of the final instar, which continued to grow until death, an effect that has also been observed in *S. littoralis* (Gelbič & Neměc, 2001) and *Bombyx mori* (Miranda et al., 2002).

Virus mortality in insects inoculated with SfMNPV OBs was approximately 50% in all treatments, reflecting the resistance of late instar *S. frugiperda* larvae to NPV infection (Table 1). Infected larvae that were treated with piriproxyfen were significantly heavier immediately prior to death than untreated or fenoxycarb-treated insects (Kruskal-Wallis $\chi^2 = 6.45$; d.f. = 2; $P = 0.04$). However, treatment with piriproxyfen or fenoxicarb did not result in a significant increase in the total OB yield per insect compared to untreated insects (Kruskal-Wallis $\chi^2 = 0.97$; d.f = 2; $P = 0.62$), and no differences were observed in the production of OBs per mg larval weight in any of the treatments (Table 1).

Table 1. Effect of JHA treatments on larval weight, total OB yield and OB yield per mg larval weight in *Spodoptera frugiperda* larvae inoculated with SfMNPV.

Treatment	Weight of larvae (mg)	Total OB yield per larva ($x10^9$)	OBs per mg larval weight ($x10^6$)
SfMNPV alone	328.3 ± 12.9 a	2.73 ± 0.19 a	8.30 ± 0.53 a
SfMNPV + 1% piriproxyfen	393.4 ± 18.7 b	2.61 ± 0.22 a	6.94 ± 0.55 a
SfMNPV + 1% fenoxicarb	357.9 ± 25.4 a	2.90 ± 0.24 a	8.47 ± 0.69 a

Figures followed by similar letters did not differ significantly for comparisons within columns (Mann-Whitney U-test, $P > 0.05$).

To determine whether resistance to SfMNPV infection could be overcome by inoculating insects with mixtures of OBs and optical brighteners, the concentration-mortality response was determined in fourth instars inoculated with SfMNPV and Leukophor AP or Blankophor BA. The estimated LC_{50} value of SfMNPV OBs alone was 384 OBs/mm^2 of diet surface (Table 2). OBs in mixtures with optical brighteners were significantly more pathogenic than SfMNPV OBs alone ($\chi^2 = 111.9$; d.f. = 4; $P < 0.05$). Potentiation of OB activity was >2500-fold in mixtures with 1% of either brightener, or >15-fold in mixtures with 0.1% of either brightener, compared to SfMNPV OBs alone. The brighteners were equally effective synergists; no significant differences were detected in the potentiation activity of the optical brighteners when applied at a concentration of 1% ($t = 0.076$; d.f. = 1; $P > 0.05$) or 0.1% ($t = 1.28$; d.f. = 1; $P > 0.05$).

These results showed a clear effect of optical brightener concentration on the degree of potentiation in mixtures of optical brighteners and OBs, in line with previous observations on SfMNPV (Martínez et al., 2003). The cost of these products has also been highlighted as an issue of concern (Martínez et al., 2000), but at 3.16 €/kg for Blankophor BA and 3.10 €/kg for Leukophor AP, these compounds can be considered economical. It seems likely that increased pest control and/or reduced quantities of OBs necessary in each application mean that the use of optical brightener formulations of SfMNPV may represent an attractive option for control of this important pest.

Table 2. Logit analysis of the concentration-mortality response of *Spodoptera frugiperda* fourth instars fed SfMNPV OBs alone or in mixtures with one of two optical brighteners.

Treatment	Regression[a]	LC_{50} (OBs/mm^2)	Range of 95% C.I.		Relative potency
			upper	lower	
SfMNPV	y = 0.7175 x + 8.57	384.8	1105.2	134.0	1
SfMNPV + 1% Blankophor BA	y = 0.7175 x + 2.89	0.14	0.24	0.08	2738
SfMNPV + 0.1% Blankophor BA	y = 0.7175 x + 6.30	16.2	37.1	7.1	23.8
SfMNPV + 1% Leukophor AP	y = 0.7175 x + 2.92	0.14	0.25	0.08	2640
SfMNPV + 0.1% Leukophor AP	y = 0.7175 x + 6.58	24.1	59.7	9.7	16.0

[a]A test for non-parallelism was not significant ($\chi^2 = 9.30$; d.f. = 4; $P > 0.05$).

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Detecting positively selected genes in geographically distinct *Spodoptera frugiperda* nucleopolyhedrovirus genotypes: Potential applications in the development of biological insecticides

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Abstract: The complete genomic sequences of three *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV) genotypes purified from North, Central and South American wild-type isolates were compared in an effort to identify genes potentially involved in virulence or in determining host species barriers. As a first step, the sequencing of the complete dominant genotype within the SfMNPV Nicaraguan isolate, named SfNIC-B, was performed, analyzed, and compared to the complete genome sequences of two other genotypes from the US (SfMNPV-3AP2) and Brazil (SfMNPV-19). The nucleotide sequences of these three genomes were highly conserved and collinear. The genome size of SfNIC-B was the largest and comprised 143 ORFs. Selection pressure analysis over the SfNIC-B ORFs resulted in pairwise ω value combinations greater than one (positive selection) for ten ORFs. These genes are currently under analysis to identify potential positively-selected sites (amino acids) in each single ORF. Positively selected genes will be functionally examined in future studies.

Key words: *Spodoptera frugiperda*, nucleopolyhedrovirus, genomic analysis, positive selection analysis.

Introduction

Spodoptera frugiperda is a severe pest of maize, rice and sorghum throughout most of the American continent. Several SfMNPV isolates from Nicaragua, the US, and Brazil, with potential for the control of *S. frugiperda*, have been assayed against insects under laboratory conditions but their pathogenicity is lower than required for the development of bioinsecticidal products. The identification of factors determining insecticidal performance would facilitate the development of recombinant viruses with a greater likelihood of commercial acceptance but, so far, identification of such genes has not been an easy task. Analysis of gene content between different genotypes of the same virus population has allowed identification of genes determining, for example, the number of virions per OB (Simón et al., 2008), but those directly involved in pathogenicity and virulence remain unidentified. The advent of new bioinformatic tools such as selection pressure analysis (Yang, 2007) may be of great assistance in this process. With that aim, the complete genome sequences of three SfMNPV genotypic variants, SfMNPV-3AP2 (Harrison et al., 2008), SfMNPV-19 (Caldas-Wolff et al., 2008), and SfNIC-B (this paper) were compared and their ORFs subjected to pairwise selection pressure analysis.

Material and methods

Viruses

The SfMNPV isolate was initially collected from diseased larvae in Nicaragua (SfNIC) and then propagated in *S. frugiperda* larvae. Nine SfNIC genotypic variants were isolated by plaque purification (SfNIC-A to SfNIC-B) (Simon *et al.*, 2004), of which SfNIC-B was the most abundant in the wild-type population. A SfNIC-B bacmid DNA was constructed (Simón et al., 2008) and purified by alkaline lysis and CsCl gradient and used for sequencing (Sistemas Genómicos S.L., Paterna, Spain).

DNA sequence analysis

Sequence alignment and gene-parity plots were performed using NCBI BLAST alignment tools (Altschul *et al.*, 1990) to examine genome organization and the order of homologous ORFs. PAML software (Phylogenetic Analysis by Maximum Likelihood) (Yang, 2007), was used to analyze evidence for selection acting on ORFs of SfNIC-B, SfMNPV-19 and SfMNPV-3AP2. Predicted amino acid sequences were aligned using ClustalW (Thompson *et. al*, 1994). PAML uses a maximum-likelihood approach to determine the numbers of non-synonymous (amino acid changing) substitutions per non-synonymous site (δ_N) and of synonymous (silent) substitution per synonymous site (δ_S). The δ_N to δ_S ratio, ω, is a measure of natural selection acting on the protein. Genes with ω=1 are classified as undergoing neutral evolution, genes with ω<1 are classified as undergoing negative (purifying) selection, and genes with ω>1 are classified as undergoing positive (diversifying) selection. The analysis was performed using a pairwise comparison method that assumes a single ω value for all codon sites in an entire ORF.

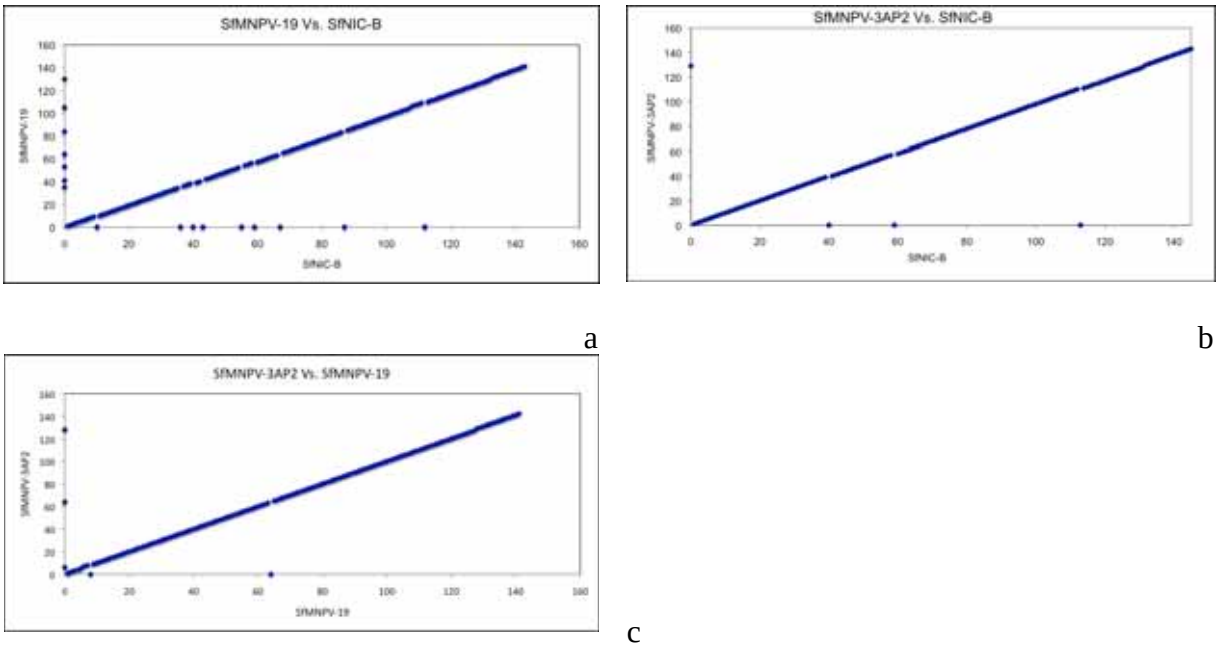

Figure 1: Gene-parity plots comparing ORF content of SfMNPV-19 vs. SfNIC-B (a); SfMNPV-3AP2 vs. SfNIC-B (b) and SfMNPV-3AP2 vs. SfMNPV-19 (c). ORFs present in only one of the compared genomes appear on the axis corresponding to the virus in which they are present.

Results and discussion

Differences in gene content between SfNIC-B, SfMNPV-19 & SfMNPV-3AP2

The genome size of the plaque isolate SfNIC-B was 132,954 bp. The nucleotide sequences of these three genomes were strongly conserved (over 99% identity), indicating that they belong to the same baculovirus species. Gene arrangement was almost completely collinear between all isolates, whereas minimal differences were observed in gene content between isolates (Fig. 1). Isolate SfNIC-B presents nine (10, 36, 40, 43, 55, 59, 67, 87, 112) and three (40, 59, 113) ORFs that were absent in SfMNPV-19 and SfMNPV-3AP2, respectively, while SfMNPV-19 ORFs 35, 41, 53, 64, 84, 105, and 130 and SfMNPV-3AP2 ORF113, were absent in SfNIC-B. In contrast, SfMNPV-3AP2 ORFs 6, 64 and 128 were absent in isolate SfMNPV-19 while SfMNPV-19 lacked SfMNPV-3AP2 ORFs 8 and 64 (Fig. 1). These results suggest a lower level of gene conservation between SfMNPV-19 and SfNIC-B. Other small differences between the three genomic sequences were also found (data not shown) and motivated further studies on the microcolinearity of genomic regions with tandem duplications, gene loss, translocations and inversions.

Identification of positively selected genes in SfNIC-B, SfMNPV-19 & SfMNPV-3AP2

Ten genes of unknown and putative predicted functions were identified as being positively selected (Table 1). Of these, four had positive ω values for all three pairwise comparisons. This number of positively selected genes was greater than that found by Harrison *et al.* (2003) between the *Rachiplusia ou* and *Autographa californica* MNPVs. Nevertheless, the large number of amino acids with invariant ω values (ω=0) made an accurate detection of positive selected genes difficult using this approach and must be complemented by models that allow detection of positively selected sites within each protein coding sequence.

Table 1: Selection pressure values (ω) for positively selected SfMNPV ORFs.

NIC-B ORF	BLAST homology	ω (NIC-B/19)	ω (NIC-B/3AP2)	ω (19/3AP2)
19	Hypothetical protein	1.1	1.3	0.0
23	Hypothetical protein	5.8	1.7	0.3
58	ODV-E66	1.2	1.3	0.4
68	38 KDa protein	99.0	99.0	NC*
80	CQ30	2.0	99.0	2.1
92	LEF3	1.2	1.3	0.0
101	P94	2.1	1.7	0.3
125	Hypothetical protein	99.0	2.0	99.0
132	P26B	1.2	1.2	99.0
135	Hypothetical protein	99.0	99.0	2.0

*NC: not calculated due to the absence of mutations

In evolutionary terms, negative or purifying selection events ensure that deleterious mutations cannot invade a population by the elimination of non-synonymous mutations (amino acid-changing) at a faster rate than synonymous ones. In contrast, positive selection allows non-synonymous nucleotide substitutions, which may cause alterations in the activity of the encoded protein, facilitating the adaptation to a new host species and/or overcoming host defences. Studies on positive selection may thus contribute to the discovery of novel insecticidal gene activities.

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Bacteria

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Efficacy of *Bacillus thuringiensis* ssp. *tenebrionis* against different European populations of *Tribolium confusum* in combination with spinosad

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Abstract: The potential of using *Bacillus thuringiensis* Berliner ssp. *tenebrionis* (Bacteria: Bacillaceae) and spinosad against several European populations of *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) larvae was examined. The insecticide formulations used were Novodor that contains 3% of *B. thuringiensis* ssp. *tenebrionis* (AI) and NAF-313 that contains 0.12% of spinosad (AI). At the lowest dose rate of *B. thuringiensis* ssp. *tenebrionis*, there were significant differences among the mortality of the populations tested. With the increase of the *B. thuringiensis* ssp. *tenebrionis* dose, these differences were reduced and all populations had similar morality levels. The addition of spinosad also increased mortality of the exposed *T. confusum* larvae. All larvae died on wheat treated with spinosad and the highest dose rate of *B. thuringiensis* ssp. *tenebrionis*.

Key words: *Bacillus thuringiensis* ssp. *tenebrionis*, spinosad, *Tribolium confusum*, populations

Introduction

The continuous use of contact insecticides, also known as grain protectants, in stored-products is basically among the most commonly used methods for the control of pests at the post-harvest stage. However, many of these substances are very toxic to mammals and harm the environment. At the same time, several insect species have developed a considerable level of resistance. All these characteristics, in conjunction with the consumers' demand for residue-free food, makes essential the evaluation of new, reduced-risk substances, against these pests. Among the alternatives proposed, insect pathogens, such as entomopathogenic fungi, bacteria, viruses and nematodes, are likely to have a good potential for this purpose. The use of *Bacillus thuringiensis* Berliner ssp. *tenebrionis* (Bacteria: Bacillaceae) has been evaluated against several stored-product pests, such as Coleoptera and Lepidoptera, with mixed results, given that different species or different populations of the same species respond differently to different bacterial isolates. *B. thuringiensis* is naturally-occurring in storage facilities (e.g., Moore et al., 2000). Nevertheless, resistance to *B. thuringiensis* is an issue of major importance, and it is expected that this characteristic may be a limiting factor to a wider *B. thuringienis* use (Tabashnik, 1994). Hence, it is essential to investigate potential combinations of *B. thuringiensis* with other, low mammalian toxicity, compounds, in order to moderate any potential loss in efficacy. One of the most promising newer bacterial formulations is the bacterial-based insecticide spinosad. Spinosad is based on metabolites of the actinomycete *Saccharopolyspora spinosa* Mertz and Yao and it has been evaluated with success against several stored-grain insect species (e.g., Subramanyam et al., 2007; Athanassiou et al., 2008a, b, c; Chintzoglou et al. 2008). Since 2005, spinosad has been registered as a grain protectant

in the USA (Subramanyam, 2006) and it is expected that in the near future it will be one of the dominant grain protectants in the globe. Still, the combinations of spinosad with *B. thuringiensis* ssp. *tenebrionis* has not been evaluated so far. In the present work, we examined the potential of using the above substances against several European populations of *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) larvae.

Materials and methods

The insecticide formulations used were Novodor (Hellafarm S. A., Amaroussion, Greece), that contains 3% of *B. thuringiensis* ssp. *tenebrionis* (AI), and NAF-313 (DOW AgroSciences, LLC, Indianapolis, USA), that contains 0.12% of spinosad (AI). The European populations of *T. confusum* were from Greece, Portugal, UK, Germany and France [for more details on the populations used see Vayias et al. (2006)]. All populations were reared in wheat flour plus 5% brewer's yeast at 25 ^{o}C 70±5% r.h. and continuous darkness. Larvae, 3rd-5th instar, were used in the bioassays. The commodity used was wheat (var. Mexa). Six lots, of 1 kg wheat, were prepared. Four were sprayed with *B. thuringiensis* ssp. *tenebrionis*, at 1500 and 3000 ppm. From these four lots, one for each dose was sprayed with spinosad, at 1 ppm. Finally, from the remaining two lots, one lot was sprayed with spinosad only, and the other one with sterile distilled water and served as a control. From each of these lots, three samples, of 50 g each, were taken for each population and placed in cylindrical glass vials. On each vial, 20 larvae were placed and all vials were then placed in incubators at the conditions mentioned above. Larval mortality was assessed 7 d later. The entire experimental procedure was repeated three times, by preparing new lots each time. All data were submitted to a two-way ANOVA, for treatment and insect population. Means were separated by the Tukey-Kramer (HSD) test, at a probability level of 5% (Sokal & Rohlf, 1995).

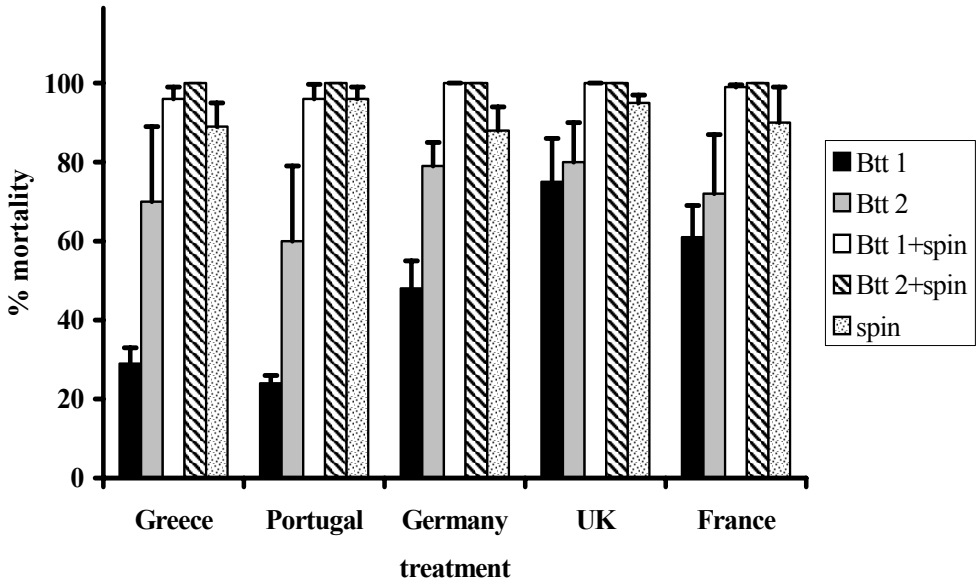

Figure 1. Mean larval mortality (%+SE) of five European populations of *T. confusum* larvae after exposure to *B. thuringiensis* ssp. *tenebrionis* and spinosad (Btt 1: 1500 ppm, Btt 2: 3000 ppm, spin: spinosad).

Results

Control mortality was low, and usually did not exceed 5%; hence, mortality in the untreated wheat was not included in the analysis. Both main effects were significant, but the interaction was not (treatment df=4, 50; F=38.1; P<0.01; population df=4, 50; F=2.7; P=0.03; treatment x population df=16, 50; F=1.3; P=0.19). At the lowest dose rate of *B. thuringiensis* ssp. *tenebrionis*, there were significant differences among the mortality of the populations tested (Fig. 1). Generally, at this dose, the Greek and Portuguese populations were less susceptible than the population from UK. However, with the increase of the *B. thuringiensis* ssp. *tenebrionis* dose, these differences were notably reduced and all populations had rather similar mortality levels, ranging between 60-80%. The addition of spinosad also increased mortality of the exposed *T. confusum* larvae, and more than 95% of the larvae were dead at the lowest *B. thuringiensis* ssp. *tenebrionis* dose. Moreover, all larvae died on wheat treated with spinosad and the highest dose rate of *B. thuringiensis* ssp. *tenebrionis*. Finally, on wheat treated with spinosad only, mortality ranged between 89 and 96%.

Discussion

The populations tested here varied on their susceptibility to spinosad dust (Athanassiou et al., 2008a) and also to diatomaceous earth formulations (Vayias et al., 2006). In light of the present findings, the same populations have different susceptibility level to *B. thuringiensis* ssp. *tenebrionis*, when it is applied at the low dose rate. However, the increase of dose increased mortality of all populations; however, larval survival was high, and reached 40% in the case of the Portuguese population. *B. thuringiensis* ssp. *tenebrionis* has proved effective against *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae), but the effect varied according to the spore/crystal formulation (Kaelin et al., 1999). Moreover, Beegle et al. (1996) found differences among isolates of *B. thuringiensis* subspecies against larvae and adults of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae). On the other hand, *T. confusum* is considered among the less susceptible stored-grain insect species to spinosad and dose rates higher than 1 ppm are required for 100% mortality. Generally, spinosad is slower acting in comparison with many conventional insecticides used as grain protectants and mortality might had been higher at longer intervals. Still, longer intervals are related with pupation and adult emergence, while generally, it is well established that adults are less susceptible than larvae. However, based on the findings of the present study, the simultaneous use of *B. thuringiensis* ssp. *tenebrionis* and spinosad increased the efficacy, regardless of the population and the *B. thuringiensis* ssp. *tenebrionis* dose. Similarly, Saleem et al. (1995) found that there is a synergistic effect of pyrethroids with *B. thuringiensis*, against larvae of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). In that study, the authors noted that this effect was expressed more vigorously in the combination of *B. thuringiensis* with cypermethrin than with permethrin. Our results indicate that the combined use of *B. thuringiensis* ssp. *tenebrionis* with spinosad should be further evaluated, under a wider range of cases (species, commodities, doses etc.). Spinosad is likely to serve as a mean to control resistant population, since one of the basic drawbacks on the use of *B. thuringiensis* ssp. *tenebrionis* is the development of resistance. Moreover, given that, for a satisfactory level of control, *B. thuringiensis* ssp. *tenebrionis* should be applied at relatively high dose rates, the simultaneous presence of spinosad is likely to reduce the required *B. thuringiensis* ssp. *tenebrionis* dose in storage facilities.

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Translocation of *Bacillus thuringiensis* in plants and insecticidal activity

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Abstract: *Bacillus thuringiensis* was isolated from within the tissues of cotton plants from fields that had never been treated with commercial formulations of this bacterium. Bt *kurstaki* marked by transformation with a plasmid encoding GFP could be introduced to cotton plants by application of spores to the roots. Such bacteria appear to migrate through the xylem and could be recovered from all parts of the plant. Leaves from inoculated plants were able to cause toxicity when fed to *Spodoptera frugiperda.*These results give further insight into the natural ecology of Bt and may provide a novel method for insect control.

Key words: *Bacillus thuringiensis*; endophytes

Introduction

Despite its extensive use in insect control for decades, the natural ecology of *Bacillus thuringiensis* (Bt) remains poorly understood. Bt can be successfully isolated from the soil, from dead insects, and from the phylloplane (Schnepf et al., 1997; Damgaard, 2000). Although Bt can be found on the phylloplane and is most typically used in the control of insects that live there, it is unclear how the bacteria become available at the leaves.

It has recently been observed that Bt inoculated into soil sown with clover seeds could end up in the leaves after the seeds germinated and grew (Bizzarri and Bishop, 2007). However, how the Bt might have made its way from the soil to the leaves was not explained (e.g., it could have been brought up on sprouts). We have discovered that Bt can naturally be found inside plants. Furthermore, Bt introduced into the soil can be taken up by plants and translocated throughout the plant. Such translocated Bt living inside plants have insecticidal activity. These results open up new horizons for understanding the natural ecology and evolution of Bt and use of Bt in insect control.

Materials and methods

Isolation of Bt from field plants

Cotton plants were collected and extensively washed before surface sterilisation i 5% hypochlorite, 5 min; 90% ethanol, 5 min. Transverse sections were cut using a sterile scalpel and the newly exposed surface was pressed onto NYSM agar. Colony morphology, crystal formation, crystal protein profile analysis by SDS PAGE, PCR amplification of toxin genes and toxicity assays were used to identify the resulting colonies.

Inoculation of plants with Bt

Spores of Bt *kurstaki* strain HD1 expressing GFP (Btk::GFP) was inoculated at the roots of 28 day old cotton seedlings at a does of 10^8 cells in 5 ml. Over time, plants were selected and surface sterilised for sectioning, plating and microscopic analysis.

Insect assays

Single leaves (approximately 12 cm^2) from treated or control plants were collected into sterile Petri dishes prior to the addition of 10 second-instar *S. frugiperda* larvae from a Bt *kurstaki* HD1-sensitive colony. Each treatment was carried out in triplicate and larval mortality was assessed after 7 days.

Results and discussion

Bt from plants in the field

Seven colonies were isolated from throughout the plants and showed Bt-like morphology. One strain showed production of crystals containing 130- and 70-kDa proteins and PCR amplicons indicating the presence of *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry1B* and *cry2A* genes: a pattern similar to Bt *kurstaki* HD1. This strain was also toxic to *S. frugiperda*, *Anticarsia gemmatalis* and *Plutella xylostella*).

Introduction of Bt

Btk::GFP introduced into the soil was taken up by the plant and could make its way into any part of the plant. The bacterium was able to persist in plant tissues for at least 7 weeks following an inoculation (Table 1, column "S"). Fluorescent bacteria were never recovered from control plants that had not been inoculated, thereby excluding contamination as a source of fluorescent Bt in the experimental plants.

Table 1: Presence of Btk:GFP in plants after root inoculation.

	Soil		Root		Stem		Petiole		Leaf	
Weeks	Control	Bt	Control	Bt	Control	Bt	Control	Bt	Control	Bt
0	-	+	-	+	-	+	-	+	-	+
1	-	+	-	+	-	+	-	+	-	+
2	-	+	-	+	-	+	-	+	-	+
3	-	+	-	+	-	+	-	+	-	+
4	-	+	-	+	-	+	-	+	-	+
5	-	+	-	+	-	+	-	+	-	+
6	-	+	-	+	-	+	-	+	-	+
7	-	+	-	+	-	+	-	+	-	+
8	-	+	-	+	-	-	-	+	-	+

Fluorescence microscopy was employed to visualize bacteria in plant sections. Individual bacteria, fluorescent with GFP and motile, could be seen inside the plant in such areas as the cotton petiole (representative image shown in Figure 1). The association of the bacteria with the lumen of the xylem, suggests that they are being translocated through the plant in the ascending water column.

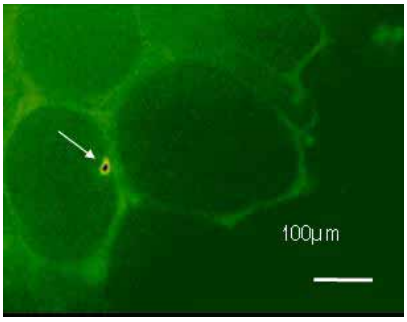

Figure 1: Btk::GFP within a cotton petiole

Toxicity of leaves

Although there was significant variation among experimental treatments, mortality was always observed with both the single and weekly-inoculated plants, typically at a level of 10-20%, and never observed with non-treated plants.

Implications

There are two dramatic implications of this work. First is for understanding the evolution of Bt. Bt is an insect pathogen and tends to target insects that live in the phylloplane. Yet an important reservoir of this organism resides in the soil. How can the bacteria move between these localities? Our results here provide a rational explanation. Bt in the soil can be taken up into the plant and deposited into the leaves, where it can intoxicate insects. Either via insect droppings (Bizzarri and Bishop, 2007) or via occasional dead insects falling into the soil, the bacterium can recycle back to the soil reservoir.

The second area that this work impacts is biological control. Although high levels of lethality were not seen here, our experiments suggest that Bt applied directly into the soil can serve as a source for biological control from pests. Since such Bt would be shielded from UV in sunlight (which damages the crystals), it could serve as a protected source of biological control over periods of time. The levels of insect toxicity reported herein were achieved without optimisation and it is likely that an understanding of the interactions between Bt and host plants will allow specific adaptation of methodologies to suit both the Bt strain deployed and the particular plant host, to attain viable levels of control for plant protection in the field.

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Does *Diabrotica*-resistant Bt-maize promote pests like fruit flies and aphids? - Indications from biosafety research on effects of Cry3Bb1-Bt-maize on Diptera

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Abstract: Within a research cluster of the German Ministry for Education and Research different maize cultivars (Bt-maize MON 88017, isogenic partner DKC 5143, conventional cultivars Benicia and DK315) were grown in a field experiment and the species assemblage of Diptera was analysed. Additionally feeding trials with plant residues were conducted in the laboratory with saprophagous Diptera larvae.

The hatching rates of flies and midges with saprophagous larvae ranged between app. 800 to 1,000 ind/m² and season, but were not significantly different between the several treatments. Also, feeding trials with decomposing larvae of *Lycoriella castanescens* (Diptera: Sciaridae), which were fed with Bt- or non-Bt-maize litter, roots or pollen did not show any significant differences between the four maize cultivars regarding mortality of the larvae, rates and duration of pupation and hatching rates. Nevertheless, all larvae which had fed on Bt-maize contained Cry3Bb1-Bt-toxin up to 263 ng/g larva. However, out of the Diptera-families with phytophagous larvae the hatching rates of Chloropidae (frit flies) from Bt-maize were significantly higher than in all other treatments. Even Diptera families with predacious larvae were significantly more abundant in Bt-maize plots in comparison to plots with non-Bt-cultivars. These effects were mainly caused by a high abundance of *Aphidoletes aphidimyza*-larvae (Diptera: Cecidomyiidae) in Bt-maize plots. The larvae of this species are predators of aphids. Results of another working group within the research cluster found that aphid numbers were significantly higher in Bt-maize in comparison to the other cultivars (Rauschen, pers.comm., RWTH-Aachen). The results indicate that MON 88017 Bt-maize obviously favours pest organisms, which are non-targets of the Bt-toxin transferred to the crop plant by genetic modification.

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Bacillus thuringiensis susceptibility variation among *Ostrinia nubilalis* populations

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Abstract: *Ostrinia nubilalis,* Hübner, (Lepidoptera: Crambidae) has been considered one of the most important corn pests and nowadays is widely controlled with transgenic plants expressing one activated *Bacillus thuringiensis* (Bt) toxin (Cry1Ab). However, this insect species is a secondary pest of other organic farming crops where it is kept under control with Bt formulations. We have tested the susceptibility of an *O. nubilalis* European laboratory strain to a model formulation (Standard Bt HD-1-S-2005) and to their protoxin components; Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa. The principal components of the formulation, Cry1Ab and Cry1Ac, were as toxic as the whole model formulation. Lower toxicity was found for Cry1Aa and Cry2Aa (about 10 and 100 times, respectively). Reported results from North American pointed to a different susceptibility pattern of *O. nubilalis* strains showing a similar toxicity of the tested protoxins and a higher activity of the formulated product. Though the bioassay methodology makes difficult the comparison of results obtained in different laboratories, intrapopulation susceptibility pattern variation has been reported in other lepidopteran species. The present study shows this also happens in *O. nubilalis* and suggests that a susceptibility screening could help to increase the effectiveness of the Bt-based bioinsecticide by choosing the most efficient product for the target population treatment.

Key words: *Ostrinia nubilalis*, Bt toxins, susceptibility, population variability.

Introduction

Some *Bacillus thuringiensis* (Bt) strains produce during sporulation, several protein types in a parasporal aggregate (the "crystal"). These proteins are denominated Cry proteins or Bt-toxins, and they have insecticidal properties. Commercial formulations based on Bt strains are sprayed as an environmental friendly tool in organic farming to control lepidopteran pests. To exert its toxic action, Cry proteins must be activated by solubilization and tripsinization in the insect midgut.

Genetically modified corn expressing one activated Bt protein (Cry1Ab) have been developed to provide an effective protection of the crops to its most relevant pest; the lepidoptera species *Ostrinia nubilalis* Hübner, the European corn borer. However, this insect species is a polyphagous pest damaging several crops such as pepper, sugar beet or carrot which are treated with commercial Bt formulated products successfully. Different commercial insecticide formulations from different Bt strains containing different types of Cry proteins or different amounts of the same toxins have been developed. These formulations are mainly based on strains of the serovar *kurstaki* and resembles to the pioneer strain HD1. Indeed, the proposed standard to Bt product potency comparison is based in such strain. The last standard Bt (Standard Bt HD-1-S-2005) has been manufactured by Valent Biosciences (Libertyville, IL), and is also used for quality control of the well-known product Dipel ES (composed by spores and crystals of a *B. thuringiensis* var. *kurstaki* strain).

Several toxicity analyses have been realized with the objective to determine the susceptibility of different *O. nubilalis* strains to Cry1Ab, but few analyses have been performed with other type of Cry proteins. In the present study, a laboratory strain of *O. nubilalis*, not exposed to Bt for more than 10 years was used to determine the susceptibility pattern to single crystal proteins present in the HD1 strain (Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa) and to the Standard Bt HD-1-S-2005 as example of Bt formulate.

Materials and methods

Insect rearing

O. nubilalis individuals were obtained from a colony maintained in the laboratory of INRA (Le Magneraud, France). Larvae were reared on artificial maize diet (Lozzia & Manachini, 2003). Insects were grown at 25°C, with a photoperiod of 16:8 (L:D) hours and 60% of humidity.

Bt toxins and Standard Bt preparation

All protoxins were produced and purified following reported methods. Cry1Aa and Cry2Aa were produced from recombinant *B. thuringiensis* EG1273 (Ecogen Inc., Langhorn, PA) and EG7543 (Monsanto Co., Chesterfield, MO) strains according to the protocol described by Li *et al.* (2004). Cry1Ab was produced as inclusion body from a recombinant *Escherichia coli* PDB140 strain (Herrero *et al.,* 2004) supplied by R.A. de Maagd (Wageningen, NL). CryAc was obtained from recombinant *E. coli* ECE53 strain provided by Bacillus Stock Centre (Ohio State University, OH), following the protocol described by Ge *et al.* (1991). Protoxins were solubilised in carbonate buffer (pH 10.5) and quantified by densitometry after a SDS-PAGE, using BSA as standard. The formulation Standard Bt HD-1-S-2005 was obtained as a powder from Valent Biosciences and dissolved in carbonate buffer (pH 10.5).

Bioassays

Bt proteins were supplemented to the surface of the maize diet at seven different doses with protein concentrations ranging from 6-4374 ng/cm^2 for Cry1Aa, 0.13-97 ng/cm^2 for Cry1Ab, 1-729 ng/cm^2 for Cry1Ac, and 4-2700 ng/cm^2 for Cry2Aa. The formulate concentration Standard Bt ranged from 0,11-81 ng/cm^2. Two ml of diet were placed in each cell of a 128-cell bioassay tray (2 cm^2/cell) and 50 µl of the product solution were added to the surface, including a carbonate buffer control without toxin. Single neonate larva (0 to 24 h old) was placed in each well and 16 larvae were used in each dose, performing the experiments, at least, per duplicated. Bioassay trays were placed in a controlled chamber at 25°C, with 60% of humidity at constant darkness. Mortality was scored after seven days and analyzed with Probit analysis (Finney, 1971) using the POLO-PC program (LeOra Software, Berkeley, CA) which provide LC_{50} and slope parameters. Absence of significance in the variation of the slopes between bioassays tested by Probit analysis allowed the potency calculations.

Results and discussion

The present work was focused on the control of *O. nubilalis* with commercial Bt products which mainly contain CryAb and other 3 additional protoxins (Cry1Aa, Cry1Ac and Cry2A). The slopes obtained from the bioassays of the different protoxins and the Standard Bt sample were not significantly different amongst them indicating a similar larval response. The analyses indicate that Cry1Ab and Cry1Ac were the most effective protoxins, with similar potencies that were also similar to the Standard Bt potency value. Cry1Aa and Cry2Aa were

also toxic but with significantly lower potencies (0.4 and 0.02, respectively) than the Standard Bt. Assuming that the protein crystal composition of the Standard Bt is similar to the reported for the *B. thuringiensis* var. *kurstaki* HD1 strain (Masson *et al.* 1990), Cry1Ab and Cry1Ac would account for most of the toxicity observed in the product.

A similar analysis (Li *et al.*, 2005) was performed with an *O. nubilalis* strain from Kansas (USA). In this case the experimental results differed qualitatively respect to ours since the LC_{50} values were no significantly different among the four protoxins tested, and the Dipel ES formulate (Valent Biosciences) showed higher toxicity than the individual protoxins (fig 1). Also, the susceptibility of *O. nubilalis* to Cry1Ab toxin (present in 80% of commercial available Bt corn), has been determined in several studies (Farinos *et al*., 2004, Saeglitz *et al.*, 2006) showing differences of up to 11 times among the populations.

The different toxin susceptibility values obtained by the distinct laboratories could be due to several factors (Crespo *et al.*, 2008) such as differences in methodology of the bioassays (surface treatment or diet incorporation, days of exposure) as well differences in toxins preparations (protoxins or activated proteins, differences in the activation protocol) or in toxin sources. However, the bioassays performed in the same laboratory can show up a consistent comparative pattern of toxicity.

Intraspecific variation due to geographic variation (as the European strain analyzed in this study and the Kansas strain from USA) is one of the most reliable explanations to the different susceptibility patterns. Similar variation results have been reported for other lepidopteran species such as *Plutella xylostella* (Gonzalez-Cabrera *et al.*, 2001), *Spodoptera frugiperda* (Monnerat *et al.* 2006) or *Spodoptera exigua* (Hernández-Martínez *et al.* 2008). The present study demonstrate that this toxin susceptibility variation also happens in *O. nubilalis* and suggests that the commercial formulates may have different effectiveness depending on the *O. nubilalis* population.

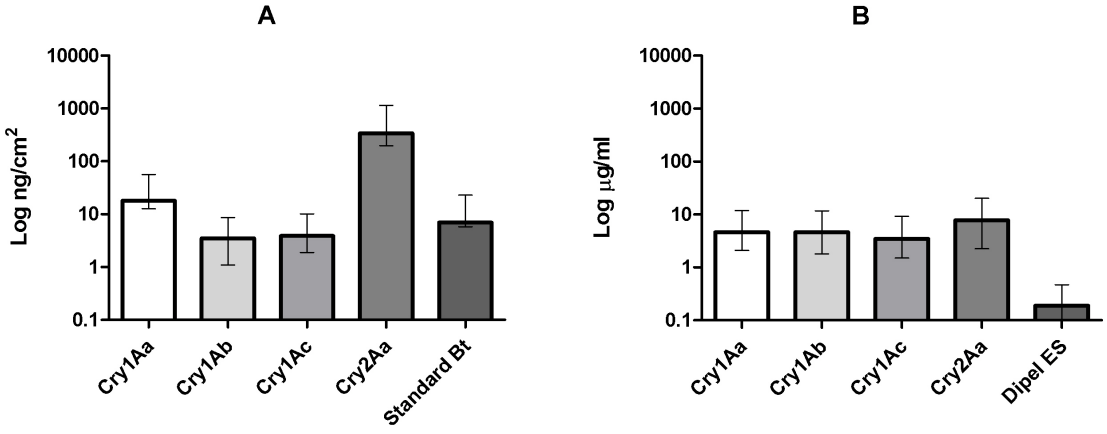

Figure 1. Susceptibility (LC_{50} values) of an European (A) and an American (B) *O. nubilalis* colonies to 4 Bt protoxins and a Bt-based formulate. A) Bioassay data obtained in the present work by using diet surface technique. B) Bioassay data obtained by Li *et al.* (2005) by using diet incorporation technique and Dipel ES as Bt-formulate.

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Consumption of *Bacillus thuringiensis kurstaki* causes an immune response in *Bt-r*esistant and *Bt*- susceptible *Trichoplusia ni* colonies

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Abstract: Increasing evidence implicates a role for the innate immune system of *Trichoplusia ni* in mitigating the effects of spore-crystal formulations of *Bacillus thuringiensis kustaki* (*Btk*). We studied the immune response to *B. thuringiensis kurstaki* (*Btk*) in susceptible and resistant *T. ni* after consumption of low doses of *Btk*, and after an injection challenged with a cocktail of bacteria. We measured the expression of genes encoding antimicrobial peptides (AMPs) in the fat body, and the differential number of circulating hemocytes in resistant and susceptible individuals.

Key words: Insect immunity, *Btk*-resistance, antimicrobial peptide, hemocyte, phenoloxidase

Introduction

Bacillus thuringiensis continues to be the most abundantly applied biological pesticide. Its insecticidal activity is due to a proteinaceous inclusion body that is produced during sporulation, and comprises several endotoxins. Historically, *Bt* has been delivered as a spore-crystal formulation that contains several strain-specific toxins along with viable endospores, however more recent technology has enabled the cloning of these bacterial toxins, the genetic transformation of valuable crop plants, and ultimately the systemic expression of these toxins in several crop plants (Vaeck, 1987).

A high level of resistance to *Btk* spore crystal formulations has evolved in cabbage loopers (*Trichoplusia ni*) in commercial greenhouses in British Columbia, Canada (Janmaat and Myers, 2003). We can induce tolerance to *Bt* in reverted susceptible lines with prior exposure to sublethal levels of *Btk.* High levels of resistance can be maintained by regular exposure to *Btk* (Ericsson et al., 2009). We also found differences in the baseline and inducible immune response to a standardized immune challenge with non pathogenic bacteria or *Btk.* Here we highlight these results and report on our more recent findings.

Materials and methods

Rearing of Bt-resistant and Bt-susceptible Trichoplusia ni lines

Two lines were established on a wheat-germ based diet and reared at 26^{o}C, 16:8 (L:D) and uncontrolled humidity. The BTR line has been maintained for >75 generations through periodic selection experiments with *Bt kurstaki* HD-1 strain (DiPel WP, Valent Biosciences) during laboratory culture.

Cabbage looper immune challenge

Third instar larvae from each colony were challenged with injections of bacteria (*Escherichia coli* and *Staphylococcus epidermidus)* or *Bt* doses. Controls received no immunological

challenge. At various time intervals midgut, fat body, and or hemolymph tissues were collected.

RNA isolation, cDNA synthesis, quantitative real-time PCR
Total RNA from fat body and midgut tissues was extracted and used to synthesize cDNAs. Target-specific primer sets were designed to amplify known inducible AMPs. Quantitative real-time PCR was used to compare the transcriptional activity from each tissue, each treatment group and each colony. A 239bp fragment of the *Trichoplusia ni* Elongation Initiation Factor 4A (EIF4A) gene was used to normalize cDNA samples.

Differential hemocyte counts
Fourth instar larvae were weighed to the nearest mg, and a 3ml aliquot of hemolymph was added to 10ml of Schneider's insect medium supplemented with sodium bicarbonate and calcium carbonate (pH: 6.8), and viewed on an Improved Neubauer hemocytometer.

Results and discussion

Antimicrobial gene expression
The induction of humoral components was assessed for each treatment by comparing the transcript levels of several putative AMP genes from fat body tissues. Differences in transcript levels were found after injections with bacteria and after consumption of *Btk.*

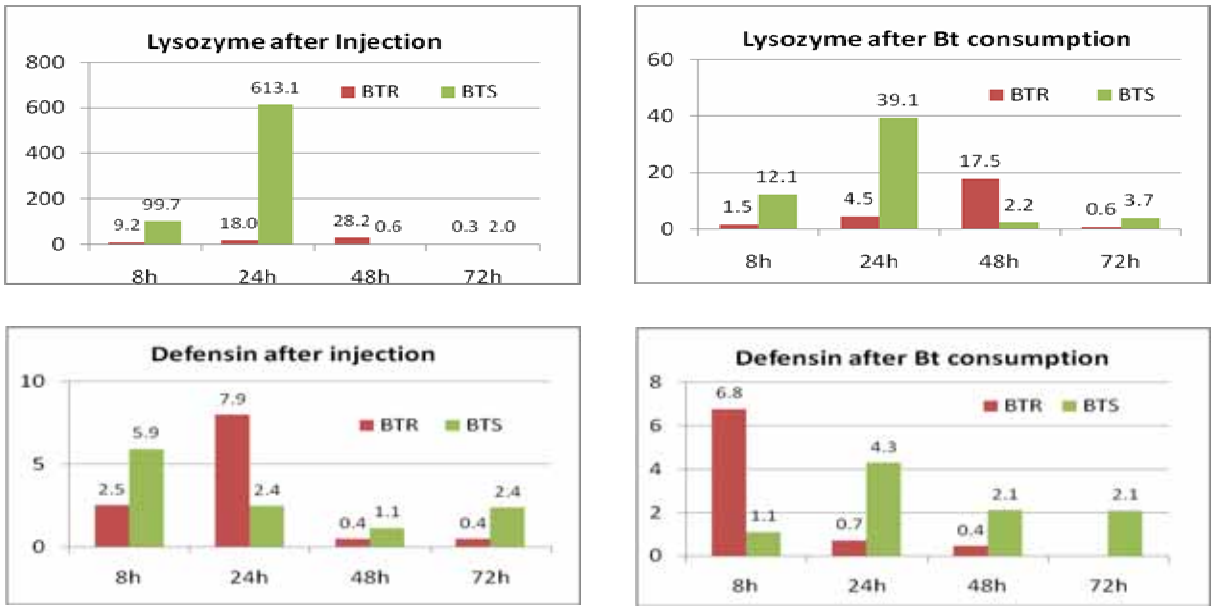

Figure 1. Transcription levels of lysozyme and defensin in larvae of *Trichoplusia ni* after injection with bacteria triggering the immune response or after consumption of Bt.

Differential hemocyte counts
Exposure to *Btk* or microbial injections caused significant changes in the total number of circulating hemocytes in Bt-RS but not in Bt-R larvae, whereas the baseline levels in untreated controls also differed. In Bt-RS, bacterial injections caused increases in the number of hemocytes, whereas consumption of *Btk* caused reductions in total numbers.

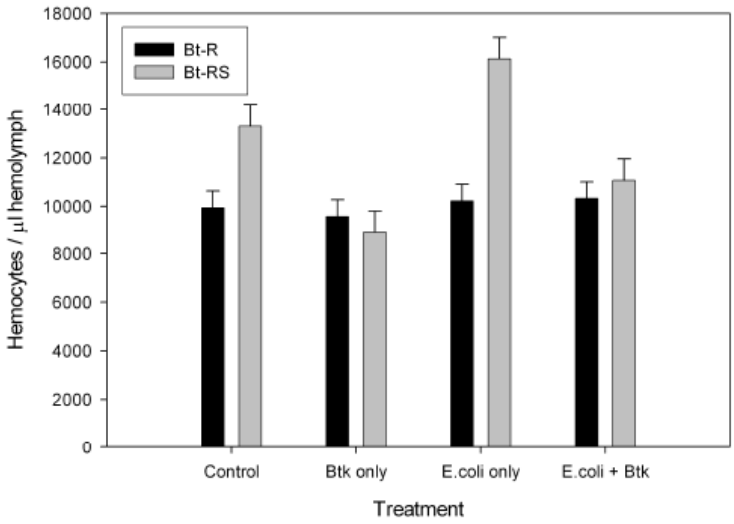

Fig 4. Changes in total hemocyte count per µl hemolymph 24 hours after treatment. Shown are the mean values (+/- SE). (From Ericsson et al., 2009)

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Cloning and expression of modified *Bacillus thuringiensis cry1A.105*, *cry2Ab2* and *cry3Bb1* genes from Bt-maize event MON89034 x MON88017

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Abstract: Numerous studies on potential effects of the transgenic plant with multiple insect control Cry proteins have been performed in the last years. These studies often require a large amounts of Cry protein to asses the biosafety of these plants. Cry proteins expressed in *Escherichia coli*, which are considered as equivalent to the plant produced proteins, are used as surrogates. In our work, the modified genes *cry1A.105*, *cry2Ab2*, and *cry3Bb1 of Bacillus thuringiesnsis*, encoding δ-endotoxins against lepidopteran and coleopteran larvae were isolated from Bt maize hybrid MON89034 x MON88017 by PCR. The Cry1A.105 is a modified Cry1A protein with amino acids sequence identity to Cry1Ab, Cry1Ac and Cry1F proteins of 90.0%, 93.6% and 76.7%, respectively. The Cry2Ab2 and Cry3Bb1 are variants of the wild-type proteins isolated from *Bacillus thuringiensis* subsp. *kurstaki* and *kumatomosiensis*, respectively. The ORFs of these genes were cloned separately in to pET-28b+ vector and expressed in *E. coli* strain BL21-DE3 under the control of T7-promoter. When expressed in *E. coli* the proteins accumulated as inclusion bodies. As expected, the Cry1A.105 and Cry3Bb1 were expressed as 133 kDa and 77 kDa, respectively, while Cry2Ab2 had a size of 65 kDa, which is slightly smaller than predicted. After purification, their purity was estimated in SDS gel electrophoresis. Bioassays using neonate larvae of *Ostrinia nubilalis* were conducted to examine the toxicity of Cry1A.105 and Cry2Ab2 proteins. The synergy effect of these both proteins against *O. nubilalis* will be discussed. The toxicity of Cry3Bb1 protein was examined against larvae of the Colorado potato beetle.

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Occupational exposure to airborne *B. thuringiensis* in environments treated with Dipel

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Abstract: Knowledge of the natural occurring bioaerosols in horticulture environments can aid in the risk assessment of introducing new organisms into the working environment through biocontrol agents. Few studies have quantified vegetable growers' exposure to *Bacillus thuringiensis* (*Bt*) during working hours. In this study we have collected air samples from greenhouses and open fields obtained from workers breathing zone and studied the presence of *Bt* and mesophilic bacteria. The vegetable growers worked in 3 greenhouses or open fields where Dipel had been applied and in 5 greenhouses or fields where Dipel was not applied. Preliminary results showed that vegetable growers to some extent were exposed to *Bt*, although the level did not seem to resemble any hazard.

Key words: Occupational exposure, Dipel, *Bacillus thuringiensis*, mesophilic bacteria, greenhouse, field vegetables.

Introduction

Dipel, a biocontrol agent with the active ingredient *Bacillus thuringiensis* (*Bt*) subspecies *kurstaki* has been utilised for the improvement of crop production for more the than 50 years. *Bt* has only been reported to have caused adverse health effects in a few human cases (Jensen *et al.* 2002). However, an immunological response can still be detected in individuals that have been exposed to the bacterium (Bernstein *et al.* 1999, Doekes *et al.* 2004). The spraying of Dipel onto plants may cause the agent to become aerosolized and hereby inhalable by humans. Measuring 1.4 µm in aerodynamic diameter (Seaver *et al.*, 1999), *Bt* can potentially be deposited in the nose, in the throat and in the lungs down to the bronchi. Particles which have settled on surfaces after the spraying, can be reintroduced into the air from the leaves by mechanical influence such as the activities performed by workers. The air quality in greenhouse and in open field has only sparsely been studied. Thus we know very little about the microflora vegetable growers are inhaling during a working day. Furthermore, greenhouse workers airway exposure to *Bt* in vegetable productions has been studied to an even lesser extent. The aim of this study was to determine vegetable growers' exposure to *Bt*. Simultaneously the natural flora of mesophilic bacteria was quantified, which can aid in the interpretation of Dipels significance in the environment.

Materials and methods

Air samplings were performed in 2007-2008 in tomato and cucumber greenhouses and in open fields producing cabbage. The vegetable growers included both organic and conventional growers. In three out of eight productions, Dipel was applied to control lepidopteran pests. In one greenhouse production of tomatoes, Dipel was repeatedly used to spot treat infested plants during the summer. Air sampling was performed on a day were

treatment of plants occurred and again in the fall, when old plants were cleaned out of the greenhouse. In open fields, Dipel was used by one cabbage grower and one celery grower. Air sampling was performed days to months after the treatments.

To measure the exposure of the workers airways to airborne microorganisms, employees were carrying GSP (CIS, inhalable sampler, by BGI, Waltham, MA, USA) air samplers throughout the work day (6-7 hours). Samplers were mounted with polycarbonate filters. After air sampling, filters were washed in an extraction solution before plating onto agar plates using a modified CAMNEA method (Palmgren *et al.* 1986). Cultivable mesophilic bacteria were estimated on nutrient agar at 25°C and cultivable *Bacillus* sp. of the cereus-group were estimated on *B. cereus* selective media (BCSM) at 30°C. Microorganisms were identified by morphology studies and quantified as cfu (colony forming units) per m^3 air. Further characterization of *Bacillus* sp. stains was performed by PCR analysis.

Preliminary results and conclusions

In the tomato greenhouse 89 % of the workers were exposed to the applied Dipel on the day of the spray. Again in November 29 % of the workers cleaning the greenhouse were exposed to Dipel. In the open fields workers were exposed to Dipel up to 70 days after the application of Dipel. The average concentration of airborne mesophilic bacteria in greenhouse was $5.6x10^4$ cfu/m^3 and in open fields it was $8.1x10^3$ cfu/m^3. In the tomato greenhouse, workers exposure to Dipel was a factor 40 and a factor 50 lower then the exposure level for mesophilic bacteria, respectively, for the summer and the fall measurement. In open fields the exposure to Dipel was a factor 20 lower than the mesophilic bacteria. *Bacillus* sp. strains of natural origin were only infrequently detected in the air samples. *Bt* subspecies *kurstaki* resembling the insecticidal strain in Dipel was not recovered from environments were no Dipel had been applied. Even though *Bacillus* sp. is a common dweller in soil and can be found on plants, our study shows that members of the *B. cereus*-group are not frequent constituents of the airborne microflora in greenhouses and open fields. The introduction of Dipel to the environments was therefore an introduction of a new factor, which needs to be assessed in regard to health risks. However, looking at the natural occurrence of mesophilic bacteria in the environments, as a baseline for the environment, it indicates that the application of Dipel does not increase the workers exposure to airborne bacteria significantly in the studied cases.

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Insecticidal activity in root-associated, plant-beneficial pseudomonads

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Abstract: Root diseases and pests are a serious problem in agricultural crops, causing each year important yield losses. Biological disease and pest control with microbial agents applied to soil or plant material has evolved as a promising alternative strategy. Plant-beneficial fluorescent pseudomonads that operate in the rhizosphere are well-characterized for their antifungal activity that helps them protect plants against root diseases caused by pathogenic fungi. Recently, we have made the exciting discovery that some disease-suppressive *Pseudomonas fluorescens* strains exhibit also potent insecticidal activity. Anti-insect action is linked to a genomic locus encoding a novel large protein toxin that we have termed Fit for *P. fluorescens* insecticidal toxin. The Fit toxin is related to potent insect toxins of *Photorhabdus luminescens*, a mutualistic bacterium of insect-invading nematodes. A first survey of the occurrence and molecular diversity of the insect toxin locus in root-associated pseudomonads indicates that the *fit* genes may be present in specific subgroups of these bacteria that include the well-characterized biocontrol agents CHA0 and Pf-5. In our current work, we investigate the molecular characteristics of the Fit toxin locus, focusing on regulatory elements and signals that control toxin production and transport. To get insight into the (agro)ecological function of the *Pseudomonas* insect toxin, we monitor the biological activity of Fit in bioassays involving insects and rhizosphere microanimals and try to identify accessory *Pseudomonas* products that sustain anti-insect action of the toxin. The occurrence of antifungal and anti-insect activities in root-colonizing pseudomonads highlights not only the impressive arsenal of features that these bacteria possess to manipulate their rhizosphere habitat, but points also to new possibilities to protect the health of agricultural crops.

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Development of a new microbial insecticide based on C*hromobacterium subtsugae*

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Abstract: Marrone Organic Innovations has licensed a technology based on a novel species of *Chromobacterium* from USDA and is developing it into a microbial bioinsecticide. The development work includes media optimization to maximize the yield of secondary metabolites responsible for insecticidal activity as well as formulation development for increased efficacy and storage stability. Bioactive compounds are extracted from fermentation broths and the resulting crude extracts are fractionated for compound isolation and identification. Our studies confirm the previous data from USDA; the insecticidal activity of fermentation broths develops over time and coincides with the cell death during the stationary growth phase. Cell-free extracts have good activity against insect pests. The active compounds in the whole-cell broth seem to be heat-stable but some activity is lost during freeze drying. Work is in progress for media optimization, formulation development and active compound identification. Spectrum testing against various insect pests is continuing through bioassays as well as greenhouse and field studies.

Key words: Biopesticide, insecticide, *Chromobacterium*

Introduction

Natural product insecticides have a potential to play an important role in controlling pests in both conventional and organic farms. Secondary metabolites produced by microbes (bacteria, actinomycetes and fungi) provide novel chemical compounds which can be used either alone or in combination with known compounds to effectively control insect pests and to reduce the risk for resistance development.

The development of a microbial pesticide starts with the isolation of a microbe in a pure culture. It then proceeds with efficacy and spectrum screening using *in vitro*, *in vivo* or pilot scale trials in a greenhouse and in the field. At the same time, active compounds produced by the microbe are isolated and identified. For the commercialization of a microbial pesticide, the microbe has to be economically produced by fermentation at an industrial scale and formulated with biocompatible and approved additives to increase efficacy and to maximize the ease of application as well as storage stability under field conditions.

In 2000, Dr. Martin and her coworkers at USDA isolated a purple-pigmented bacteria (PRAA4-1) from a forest soil in Maryland (Martin et al., 2007a). In the initial screening, they found this bacteria to be toxic to Colorado potato beetle and other insect pests. This motile, Gram-negative, bacteria was identified as a new species of Chromobacteria, *Chromobacterium subtsugae* sp. nov (Martin et al., 2007b). It is a facultatively aerobic, motile, Gram-negative betaproteobacterium with polar flagella. Colonies formed at 2-3 days on an L-agar at 25°C are initially cream colored, gradually turning light to dark violet during the following 24 hours. Colonies of PRAA4-1 grow well on peptone based media with an optimum at 25°C, pH 6.5-8.0, and with 0-1.5 % (w/v) NaCl (Martin et al., 2007a). Marrone Organic Innovations

(MOI) has licensed this technology from USDA, and is now developing it into a commercial biopesticide.

Materials and methods

Extraction of active compounds

C. subtsugae was grown at MOI in L-broth at 25 ^{o}C for 8 days and the growth was monitored by OD_{600nm} measurements (Figure 1). The broth turned purple after 2 days of incubation and starting at day 5, a 25-mL aliquot was extracted each day with ethyl acetate. The crude extracts were analyzed with HPLC and LCQDeca XP Plus LC-MS using positive ion ES ionization.

Bioassay

For optimization of down-stream processing and formulation development, the effect of freeze drying on the product efficacy was tested in a bioassay with beet armyworm (*Spodoptera exigua*). *C. subtsugae* liquid culture was harvested after 8 days of incubation and freeze dried. The freeze dried material was dissolved in water in a series corresponding to 10-fold, 5-fold, 1-fold and 0.1-fold concentrations relative to the starting material (whole cell broth). Leaf disks of green bell pepper (*Capsicum annuum*) were treated with each test material and placed in small plastic containers. Three first instar larvae of beet armyworm were placed in each container, and closed containers were incubated in a growth chamber at 27°C. All treatments were run in five replicates.Bt at a commercial rate (BT Worm Killer; 15% a.i. at 256x dilution) and water were used as positive and negative controls, respectively. After 72 hours of incubation, the treatments were evaluated for % mortality and growth inhibition.

Results and discussion

Growth curve

In a flask culture at 25 ^{o}C, the stationary growth phase of *C. substsugae* was reached after 3 days.

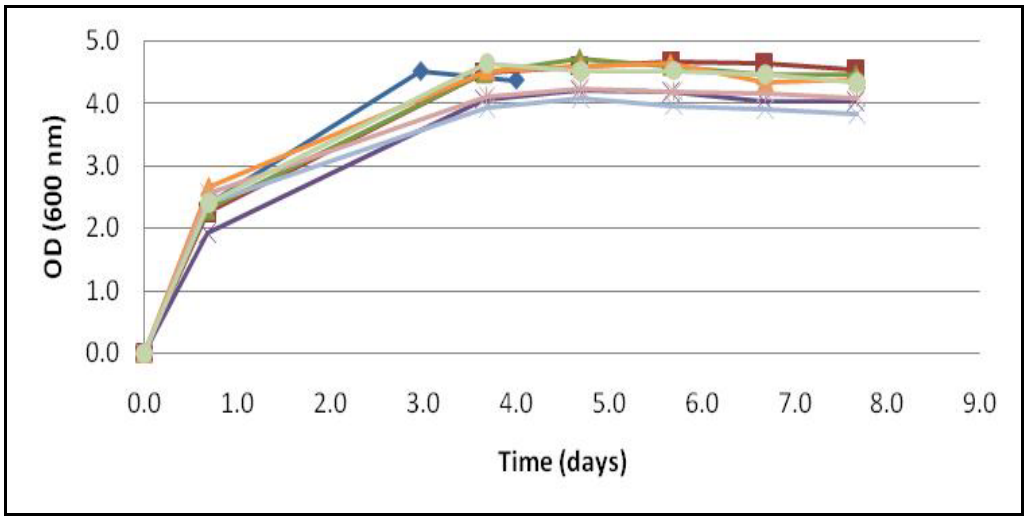

Figure 1. Growth of *Chromobacterium subtsugae* in L-broth at 25°C

Extraction of active compounds

The amount and efficacy of crude extracts obtained from each sample are presented in Table 1. The results showing increased efficacy after 7 days of fermentation indicate that the bioactive compound is produced in the stationary growth phase, which confirms the results obtained by Martin et al. (2007a). Isolation and identification of active compounds are on the way using bioassay-guided fractionation.

Table 1. Amount of crude extract obtained from 25-mL of broth at each sampling time and the efficacy of each extract in a beet armyworm bioassay.

Sample	mg of crude extract	Mortality %
5d	10.2	0
6d	10.8	0
7d	11.2	75
8d	18.4	100

Bioassay

Mortality results from the leaf disk bioassay with third-instar beet armyworm larvae are presented in Table 2. The active compounds in the whole-cell broth seem to be heat-stable but some activity is lost during freeze drying.

Table 2. Effect of freeze drying and sample concentration of the freeze dried material on first-instar beet armyworm larvae mortality in a leaf disk bioassay.

Treatment (concentration of freeze dried material)	Mortality (%)
10x	80.0ab
5x	100.0a
1x	50.0b
0.1x	46.7b
Fresh whole cell broth (1x)	80.0ab
Water	36.7b
Bt (0.06% a.i.)	100.0a
LSD ($p < 0.05$)	47.4

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We would like to thank Dr. Phyllis Martin and her coworkers in the Insect Biocontrol Laboratory at USDA, ARS, Beltsville, MD for the background data.

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Preliminary studies for the attract-and-kill strategy against the mosquito *Culex pipiens* employing oviposition pheromone and *Bacillus thuringiensis* subsp. *israelensis*

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Abstract: The attract-and-kill strategy requires an intelligent and an efficient combination of an attractant and a killing agent such as a pheromone and an insecticide respectively. The production of the synthetic oviposition pheromone of the mosquito species *Culex quinquefasciatus* (Diptera: Culicidae) was already achieved and its combination with a microbial insecticide was tested. Furthermore two microbial larvicides based on *Bacillus thuringiensis* subsp. *israelensis*, commonly used in Greece, were tested in the laboratory against *Culex pipiens* biotype *molestus* (Diptera: Culicidae) as agents that can keep water free from mosquito larvae. Larvicidal activity, over a 50-day period, revealed no good results. The results from oviposition bioassays revealed that all the tested larvicidals repel gravid females of laying eggs for the first two days. However, when synthetic pheromone is combined with one of the microbial agent, oviposition bioassays revealed the same attractant pattern as synthetic pheromone independently.

Key words: *Culex pipiens* biotype *molestus*, synthetic oviposition pheromone, *Bacillus thuringiensis* subsp. *israelensis*.

Introduction

Control agent such as oviposition pheromones (Laurence and Pickett 1985), plant extracts (Isoe et al. 1995) and skatole waters (Olagbemiro et al. 2004), which act as oviposition attractants, could be valuable tools in mosquito control programs. Semiochemicals are biomolecules that spread information between individuals and their finding has introduced the attract-and-kill strategy with many advantages (Stetter and Folker 2000). The oviposition pheromone of *Culex quinquefasciatus* was recently synthesized and tested for its bioactivity on *Cx. pipiens* with very promising results (Michaelakis et al. 2005). The objective of this study was to evaluate the attractiveness of a synthetic pheromone (SP) combined with a microbial killing agent, *Bacillus thuringiensis* subsp. *israelensis*-Bti. Additionally, the effect of two commercially available microbial larvicides was tested over a 50-day period.

Material and methods

Insect rearing

A *Cx. pipiens* biotype *molestus* colony was maintained in the Benaki Institute, (Kifissia, Greece) for more than two decades. (Michaelakis et al. 2005, Michaelakis et al. 2007).

Synthetic pheromone
The oviposition pheromone (6-Acetoxy-5-hexadecanolide, SP) was obtained according to a method recently described (Michaelakis et al. 2005).

Control agents
Two commercial microbial products (commonly used in Greece) of Bti were tested. For positive control the agents pyriproxyfen and temephos were also tested at the doses of 2 mg/L and 0.15 mL/L, respectively. The dosages were equivalent to the lowest recommended label rates for each active substance except for Bti, where the highest recommended label dose was chosen.

Larvicidal bioassays
The bioassay method was based on the standard test for determining the susceptibility of mosquito larvae to insecticides (WHO 1981). However, in the present study, instead of larvae of 3rd and early 4th instars, one-day egg rafts were used (70 ± 5 eggs per egg raft). Bioassays were performed on day 5, 20, 35 and 50 after preparation of dilutions (day 0).

Oviposition bioassays
Two-choice oviposition experiments for SP were set according to already described bioassay method (Michaelakis et al. 2007). The similar procedure was followed in order to assess the effect of the presence of the microbial larvicide in the oviposition site. Product A was chosen in this set of experiment and the beaker with the water and SP had been replaced by the larvicide. Each oviposition bioassay was lasted five days.

Results and discussion

All insecticides were evaluated against newly hatched larvae of *Cx. p. molestus* at a specific concentration. The tested bioassay solutions were stored for 50 days under constant conditions before use (post treatment days). The efficacy of all larvicides is shown in Table 1 where adult emergence is presented for the control and each insecticide and for every post treatment day.

Table 1. Adult emergence (number) for each treatment: Bti (product A and B), control and positive control (pyriproxyfen and temephos). Mean number of 5 replicates (±SE) for every post treatment day (see text).

	Time (days)			
	5	**20**	**35**	**50**
Bti (product A)	60 ± 3,3	65 ± 2,4	61 ± 5,4	69 ± 2,2
Bti (product B)	62 ± 2,1	64 ± 3,1	68 ± 4,8	67 ± 3,3
Control (H_2O)	67 ± 2,1	65 ± 3,7	68 ± 2,1	67 ± 5,1
Positive Control (Pyriproxyfen)	1 ± 0,9	22 ± 4,7	17 ± 1,9	38 ± 3,9
Positive Control (Temephos)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

The results indicated that temephos (positive control) killed all the hatched larvae (100% mortality). Although pyriproxyfen (positive control) significantly differed from the control

(water free from larvicide), the evaluation showed excellent mortality level only for the first five days. As far as Bti is concerned, it had no bioactivity against newly hatched larvae of *Cx. pipiens* (Table 1). Efficacy of Bti was not significant different from the control. The bioactivity of the Bti derives from a variety of factors (Becker et al. 2003). The most important of these factors are: a) Species and instar sensitivity (Larvae lose their sensitivity to bacterial toxins as they develop). b) Temperature, size of the water body, state of nutrition and sunlight also play significant role in Bti bioactivity and c) The protein crystal (inactive protoxin) must be ingested by the target insect, and this depends on its feeding habits.

As concern the oviposition bioassays, the results of the SP with the use of filtered paper, during a period of 5 days, are shown in Figure 1. The first three days the attraction was significant different from the control (~60-72%) and after the fourth day the attraction was almost the same with the control.

In Figure 1 except the SP attraction, the oviposition effect by Bti (product A and B) is also shown. For the first two days Bti product A repelled gravid females from laying eggs. For the rest four days the attraction level reached almost control levels. For the product B, the repellent pattern was constant for 5 days. These findings are in accordance with the negative effect on oviposition activity by larvicides (Maw 1970, Ritchie and Long 2003, Beehler and Mulla 1993).

Even though Bti product A showed repellent activity; its combination with SP rendered water attractive (from 16% attraction to 57% for the first day, Figure 1 and 2). The attraction reached higher levels during second and third day where attraction was 68% and 69 % respectively **(**Figure 2). After the third day the attraction of SP with Bti followed the same pattern with SP by itself.

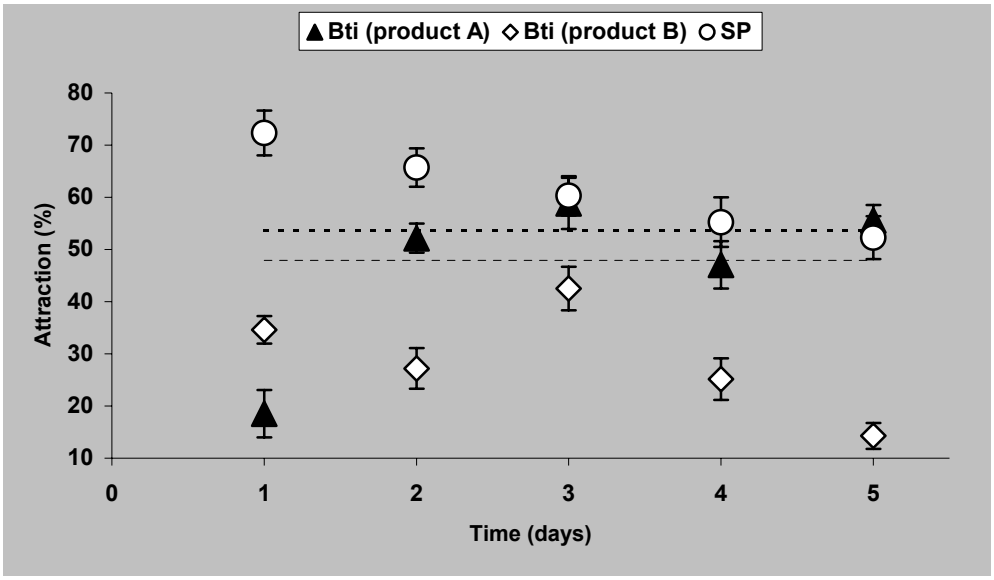

Figure 1. Response to dose of 1 μg of SP (○) and how Bti product A (▲) and Bti product B (◊) affect oviposition. Broken lines represent the upper and lower values of the control mean ± SE (50.1 ± 2.1%, n=10).

Overall, SP after the third day stopped to attract gravid females independently the larvicidal agent. This happens due to the fact that pheromones are volatile and thus they do not remain stable for a long period. These preliminary studies are the basic step for the

implementation of attract-and-kill strategy. The following step will be the use of microencapsulated oviposition pheromone (Michaelakis et al. 2007) combined with Bti products that can provide also slow release activity.

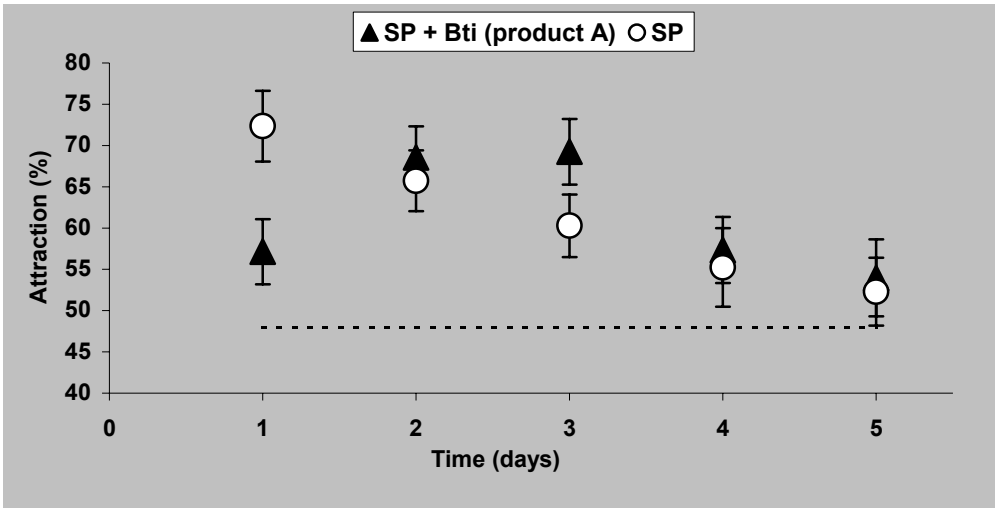

Figure 2. Response to dose of 1 μg of SP combined in water with Bti product A (▲). Broken lines represent the upper and lower values of the control mean ± SE (50.1 ± 2.1%, n=10).

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High activity of a *Bacillus pumilus* strain against *Ceratitis capitata*

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Abstract: *Ceratitis capitata* is one of the world's most destructive and damaging fruit pests. Chemical insecticides are the most used control method, but they are unspecific, environmentally harmful, and in most cases inefficient. Given all these disadvantages and the fact that no efficient field-applicable product is commercially available at the moment, searching for new alternative methods is essential. The entomopathogenic bacteria from *Bacillus* genus are natural agents for biological control of invertebrate pests. Here we report the isolation of a novel strain of *B. pumilus* 15.1 highly toxic against *C. capitata* larvae. We demonstrate that toxicity is revealed only when sporulated cultures are exposed to low temperatures. Isolation of this pathogenic bacterium to *C. capitata* could signify an important finding for the future development of new control strategies.

Key words: Medfly, *Bacillus pumilus*, pathogenic, microbial control.

Introduction

The Mediterranean fruit fly, *Ceratitis capitata*, is considered a highly invasive agricultural and economically important pest throughout the world. In less than 200 years this species has expanded from its native habitat in the sub-saharan Africa to become a cosmopolitan species. The Medfly causes serious economic losses, frequently between 25-50% of the crop, although heavy infestations may cause the complete loss of crops. Populations of *C. capitata* have been shown to be extremely difficult to reduce, using very different approaches. Despite the previous studies no efficient field-applicable product is commercially available at the moment; therefore, searching for new strategies is of paramount importance.

Entomopathogenic bacteria from the *Bacillus* genus are natural agents for biological control of invertebrate pests and are the basis of many commercial insecticides. Three species of *Bacillus* have been mass-produced and commercialized: *B. sphaericus, B. thuringiensis*, and *Penibacillus popilliae* (formerly described as *B. popilliae*).

B. pumilus is a ubiquitous bacterium with diverse described activities. Some *B. pumilus* strains show interesting characteristics like fungicidal activity, and are used as biological control agents for phytopathogenic fungi. *B. pumilus* is not considered as a classical pathogen insect like *B. thuringiensis* or *B. sphaericus*. In fact, there is only one patent report of a *B. pumilus* strain active against insects, such as the corn rootworm (*Diabrotica undecim-punctata*) and the armyworm. The finding of this novel entomopathogenic strain could be useful to develop new control methods to reduce *C. capitata* populations and crop damage.

Materials and methods

Insect rearing
Laboratory conditions for rearing purposes and bioassays were 25 ± 2°C and 65 ± 5% relative humidity, with a photoperiod of 16:8 h (L:D). Both larvae, and adult flies were fed on an artificial diet based on proteins and sugar.

Bacterial isolation
B. pumilus 15.1 was isolated from vegetables wastes collected from an agricultural area in Almuñecar, a locality in the province of Granada (south coast of Spain). Bacterial isolation was performed, with minor modifications, according to the method described by Travers *et al*. (1987). Bacteria were routinely grown in LB, and T3 when sporulation was required.

First instar* C. capitata *larvae bioassays
Sporulated cultures of *B. pumilus* 15.1 (or culture fractions) and control strains were lyophilized and concentrated 10 times before used in bioassays. One hundred microlitres of the bacterial suspension was mixed with 500 µl of larvae diet and homogenized with a sterile toothpick. Bioassays plates were incubated 96 h at 4ºC before a first instar larvae of *C. capitata* was placed in each well. Each bioassay was performed with 48 larvae and replicated at least twice. Culture fractions were obtained by centrifugation (20 min 35.000 x g). Toxicity of vegetative cell was evaluated using a 10 h culture in LB. To test the relation toxicity versus temperature and time bacterial cultures were kept at 4°C and -20°C for 0, 24, 96 and 168 hours before each culture was used in a bioassay.

B. pumilus *15.1 growth curve and TEM observation*
A growth curve in T3 medium (sporulating medium)(Travers et al. 1987) at 30ºC and 240 rpm was performed recording O.D. $_{600\ nm}$ every hour. Each sample was stained with osmium tetroxide and observed under transmision electron microscopy.

***Toxicity tests of* B.pumilus *15.1 on other stages of* C. capitata.**
Adults bioassays were performed in 10x10x10 cm pastic cages. Lyophiliced 15.1 culture was mixed with the adult diet at 1:5 proportion. Eggs bioassays were performed as first instar larvae bioassays using 10 eggs per well. Pupating larvae (third instar larvae) bioassays were performed in a 10x10x10 cm plastic cages with 5 cm of sterile soil mixed with a sporulated 15.1 culture. Pupa bioassays were performed mixing pupae with soil contaminated with a 15.1 culture.

Results and discussion

Toxicity of* B. pumilus *15.1 strain toward* C. capitata *larvae
The activity of *B. pumillus* 15.1 against *C. capitata* is specific of our strain and not a common feature of others *B. pumilus*. This toxicity is not revealed under standard bioassay conditions (Fig. 1, right) but only when the bacterial culture is exposed at 4ºC for at least 96 h (Fig. 1, left). According to our results the potential toxin/s or virulence factor/s are synthesized during the sporulation phase given that vegetative cells are not toxic to the larvae. The number of spores of *B. pumilus* 15.1 was constant along the bioassay, meaning no germination occurred when spores were inside the larvae diet. We hypothesized that the potential virulence factor/s responsible for the toxicity is synthesized during the sporulation, and further transformed or activated when incubation at 4ºC takes place. Activation occurred at -20ºC as well (table 1).

The conditions required for the activation of the virulence/s factor/s against *C. capitata* could explain why this bacterium is not considered a traditional entomopathogen such as *B. thuringiensis* and *B. sphaericus*, given that the incubation of the culture at low temperatures does not form part of any standard bioassay. The effect of low temperature on toxicity is a surprising phenomenon and as far as we know, has only been previously observed once in a *B. thuringiensis* Ormilia strain isolated from Greece (Karamanlidou et al. 1991).

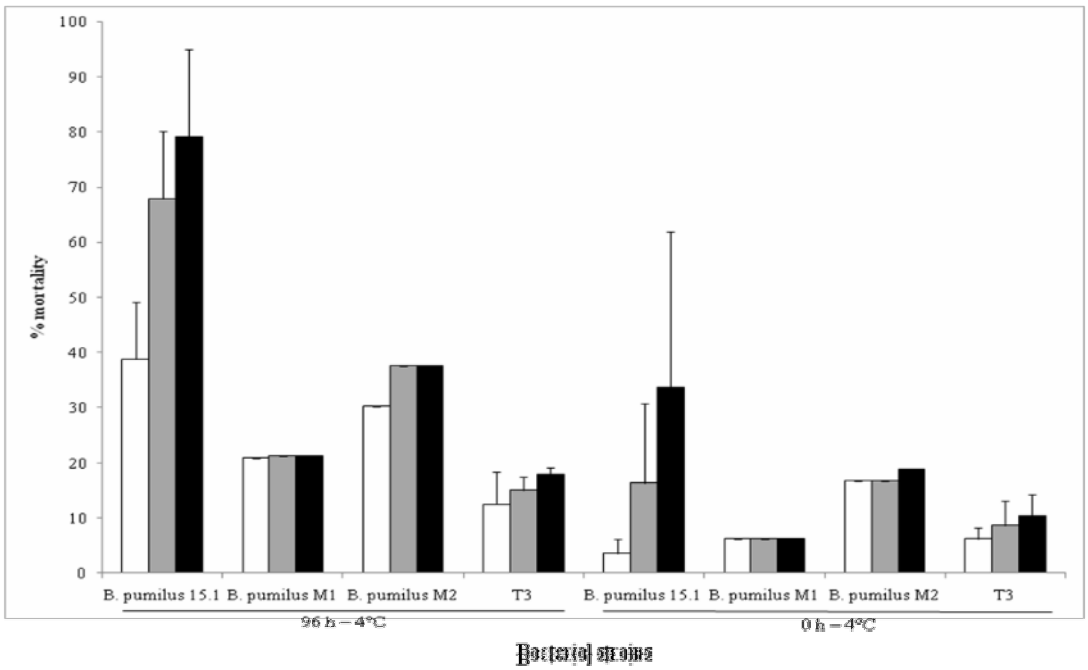

Figure 1. Toxicity result on *C. capitata* larvae of different *B. pumilus* strains.

Table 1. Mortality of *C. capitata* larvae (mean ± SD) 10 days after the beginning of the bioassay. Bacterial cultures were kept at low temperatures (4 or -20ºC) at different times mixed or not with the larvae diet.

Bacterial strain	Exposure time (hours)	Mortality (%)		
		Culture		Culture + diet
		4°C	-20°C	4°C
	0	22.92 ± 0.00	–	16.37 ± 14.45
B. pumilus 15.1	96	93.61 ± 1.94	84.10 ± 2.85	67.92 ± 12.19
	168	66.67 ± 10.42	60.42 ± 18.75	–
	0	16.67 ± 2.08	–	6.25 ± 0.00
B. pumilus M1	96	37.50 ± 4.17	30.21 ± 3.12	21.28 ± 0.00
	168	17.71 ± 1.04	16.67 ± 6.25	–
T3	--	11.11 ± 0.00	16.57 ± 6.16	15.09 ± 2.35

***Toxicity tests of* B.pumilus *15.1 on other stages of* C. capitata.**

In order to determine if the new bacterial strain shows entomopathogenic activity against other life stages of *C. capitata*, we tested toxicity of *B. pumilus* 15.1 against eggs, pupating larvae (third-stage larvae), pupae and adults of Medfly. None of these life stages were susceptible to *B. pumilus* 15.1, so the activity of the strain is specific for the larva stage.

Sporulated culture of* B. pumilus *15.1

Doubling time of *B. pumilus* 15.1 strain in T3 was calculated from a growth curve as 1 hour and 45 min. Observation under transmission electron microscopy of the samples taken through the curve revealed geometric structures lightly stained (form sample 54 h) resembling to those observed in a sporulated *B. thuringiensis* culture and composed by Cry and Cyt toxins. Such structures were observed not only inside the cells but also liberated into the medium (Figure 2). These crystalline structures could be linked to toxicity and further investigation is required.

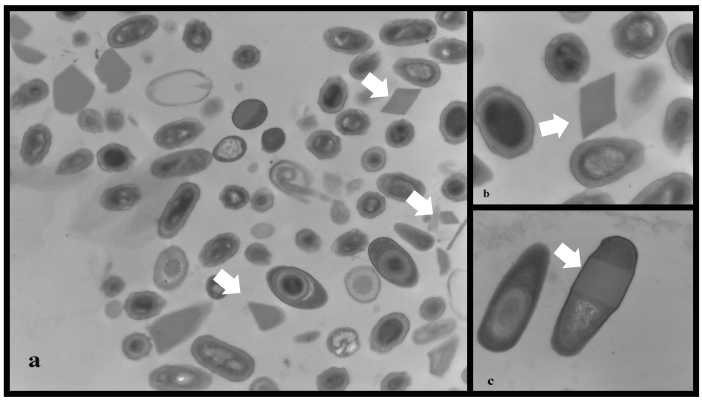

Figure 2. Sporulated culture of *B. pumilus* 15.1 under transmission electron microscopy (TEM). *a*: culture showing spores and crystalline structures, *b*: detail of a crystal, and *c*: cell with a crystalline structure inside.

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Molecular detection of the entomopathogenic bacterium *Pseudomonas entomophila* using Polymerase Chain Reaction (PCR)

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Key words PCR, *Pseudomonas entomophila*, biocontrol

Introduction

The bacterial genus *Pseudomonas* comprises several hundred species of Gram-negative γ-proteobacteria, characterized by high metabolic versatility and production of numerous secondary metabolites. Diverse bacterial isolates from fruit flies (*Drosophila melanogaster*) were tested for their ability to induce systemic immune response in *D. melanogaster* larvae. One isolate was capable of triggering in a reproducible way, *Diptericin* (antimicrobial peptide) expression in both larvae and adults and it was highly pathogenic towards adults and larvae of *D. melanogaster* by oral ingestion. Comparison of its 16S rRNA to existing 16S rRNA sequences clearly demonstrated that this bacterial isolate belongs to pseudomonads and it was designated *Pseudomonas entomophila* (Vodovar et al. 2005). Complete sequencing of the 5.9-Mb *P. entomophila* genome revealed putative virulence factors but experimental evidence for most of them is still lacking (Vodovar et al. 2006). In this study we developed a specific, fast and simple PCR-based detection method useful to detect *P. entomophila* in environmental samples (bacterial isolates or insects)

Materials and methods

The following *Pseudomonas* species were used: *P. entomophila* L48, *P. taetrolens*, *P. veronii*, *P. montelii*, *P. aeruginosa*. The following non-pseudomonads were used: *Flavobacterium saccharophilum*, *Moraxella osloensis*, *Staphylococcus pasteuri*, *Staphylococcus aureus*. *Flexibacter canadiensis*. Bacterial strains were grown on LB agar or TSA at 30°C, for 24-48h, except *P. aeruginosa* and *S. aureus* grew at 37°C. Cultures were stored at 4°C. Bacterial DNA used for PCR was extracted (Spilker et al.2004) and stored at -20° C. In this study three sets of primers (A,B,C) were used: A) PA-GS-F (5'-GACGGGTGAGTAATGCCT A-3') and PA-GS-R (5'-CACTGGTGTTCCTTCCTATA-3'). B) PSEEN-1497F (5'-TTGGTCGAG-CATTGACTCAG-3') and PSEEN-1497R (5'-GAACAAAACGATCCGCATAC-3'). Primers amplify specifically a 570 bp fragment of a putative polysaccharide biosynthesis gene in *P. entomophila* genome showing no homologues in other pseudomonad genomes. C) PSEEN-5525F (GATAAAGCGGTAGCACAATG-3') and PSEEN-5525R (5'-GCTGCTGACTGC-AAGAATCA-3'). These primers amplify a 657 bp fragment of a gene encoding a hypothetical protein in *P. entomophila* genome, showing no homologues in other pseudomonades.

Amplification of target DNA was carried out in 25 μl reaction volume, each containing 2 mM $MgCl_2$, 250 μM (each) deoxynucleoside triphosphates, 0,4 μM (each) primer, 1 U Taq polymerase (HyTest, UK) and 2 μl of whole-cell bacterial lysate, and adjusted to 25 μl by the addition of sterile dH_2O. After initial denaturation for 2 min at 95°C, 25 cycles were completed, each consisting of 20 sec at 94° C, 20 sec at 54° C, 40 sec at 72° C and a final

extension of 1 min at 72°C (Spilker et al. 2004). In the case of duplex PCR we increased the time from 20 sec to 40 sec, for the first two steps of each cycle (denaturation and annealing). Duplex PCR with DNA template of infected insects was performed by increasing the DNA template to 8 μl keeping the same reaction volume, (25 μl). To determine the PCR detection limit, *P. entomophila* was grown in liquid culture, cells were centirfuged (13,000 X g, 2 min), washed with saline (0.9 % w/v NaCl) and then diluted to 10^2 to 10^6 cells per ml. Each suspension was examined by the extraction and amplification procedures outlined above.

Two groups of adult *Drosophila* (25 insects per group) were infected by *P. entomophila*. The two groups of insects were placed in plastic vials, each containing suitable *Drosophila* food. Infection of the first group was by oral ingestion, mixing Drosophila food with 200 μl concentrated bacterial pellet of an overnight *P. entomophila* culture (approx. OD_{600}=200) while the other group was used as a negative control. DNA extraction of *Drosophila melanogaste*r was performed as previously described (Malloch et al. 2006). Supernatant was transferred into a new eppendorf tube and it was directly used or stored at -80°C.

Results and discussion

An already published (Spiker et al. 2004) pair of primers (PA-GS-F and PA-GS-R primers) designed to amplify 16S rDNA in *Pseudomonas* species was used in this study. A 618 bp fragment was amplified only using DNA template originated from *Pseudomonas* species, indicating 100% specificity of these primers to detect Pseudomonads (Figure 1).

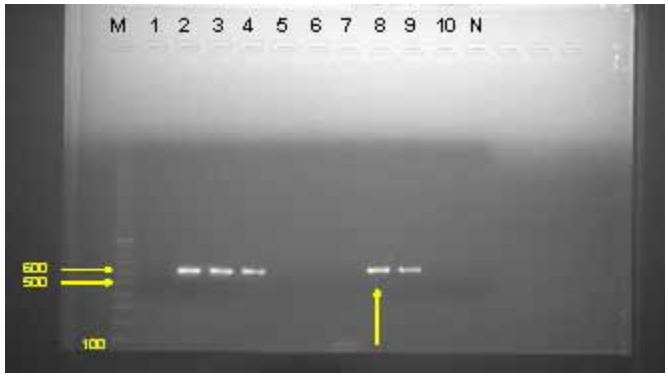

Figure 1. 1) *Flexibacter canadiensis*, 2) *P. taetrolens*, 3) *P. vero*, 4) *P.montelli* 5) *Flavobacterium saccharophilum*, 6) *Moraxella osloensis*, 7) *Staphylococcus pasteuri*, 8) *P . entomophila*, 9) *P .aeruginosa*, 10) *Staphylococcus aureus.* N=Negative Control, M= DNA marker (Biorad). Vertical arrow indicates *P. entomophila*

Primers designed to amplify gene PSEEN 1497 (PSEEN 1497F and PSEEN 1497R) amplified specifically a 570 bp fragment only when DNA template from *P. entomophila* was used (Figure 2). Repetitive PCR using primer pair C failed to amplify the expected product.

PCR detection limit using both primer sets (A and B) was found to be 10^4 cells (Figure 3). We developed a duplex PCR, combining 16S rDNA and PSEEN 1497 primers, which allowed us to detect *P. entomophila* thus differentiating it from other pseudomonads. (Figure 4). Furthermore, duplex PCR was applied to insects experimentally infected with *P.entomophila* confirming the accuracy and the specificity of our method. *P. entomophila* was detected (570 bp fragment) in all infected samples which were tested, (6/6), but not in negative control group (Figure 5). Moreover there is an amplicon (618 bp fragment) present

in the negative control group (non-infected *Drosophila*), indicating the presence of *Pseudomonas* strains, other than *P. entomophila*.

P. entomophila is the first known pseudomonad naturally infecting *D. melanogaster*. Genome analysis revealed putative virulence factors produced by *P. entomophila* but experimental evidence, for most of them, is still lacking. Therefore intensive research is required to elucidate mechanisms involved in entomopathogenicity as well as insect host range of this bacterium.

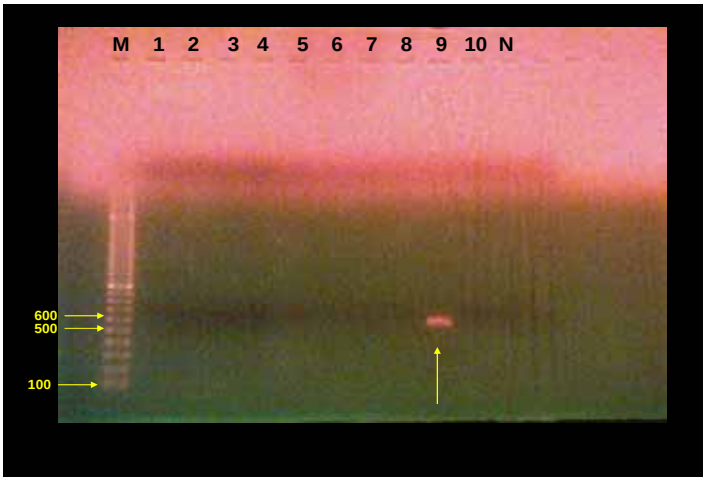

Figure 2. 1) *Flexibacter canadiensis*, 2) *P. taetrolens*, 3) *P. veronii*, 4) *P.montelli*, 5) *Flavobacterium saccharophilum*, 6) *Moraxella osloensis*, 7) *Staphylococcus pasteuri*, 8) *P . aeruginosa*, 9) *P. entomophila*, 10) *Staphylococcus aureus.* N=Negative Control, M= DNA marker (Biorad). Vertical arrow indicates *P. entomophila*

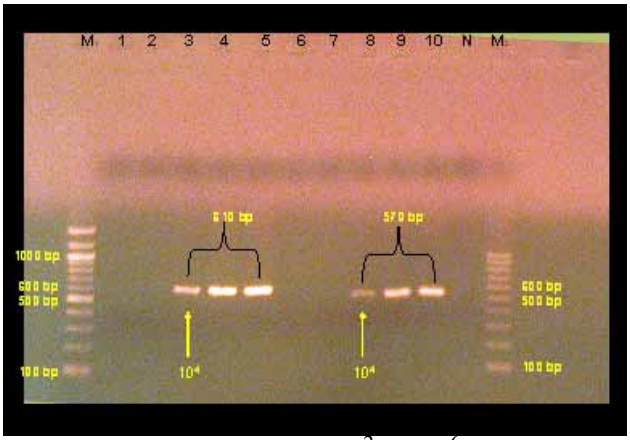

Figure 3. *P. entomphila* DNA corresponding to 10^2 to 10^6 cells per ml was used to determine PCR sensitivity. Vertical arrows indicate the lowest detection limit obtained using primer pairs A and B.

Combining two sets of primers, we developed a specific, fast and simple duplex PCR useful for *P. entomophila* detection. Our method is sensitive enough to detect at least 10^4 cells. Experimental infection of *Drosophila* adults by *P. entomophila* and its subsequent

detection, demonstrates that our method is specific and can be used without prior bacterial isolation from infected insects. To our best knowledge this is the first molecular method aiming to detect *P. entomophila* in environmental samples. The use of our method will facilitate studies related to ecology and insect host range of this entomopathogenic bacterium.

A B

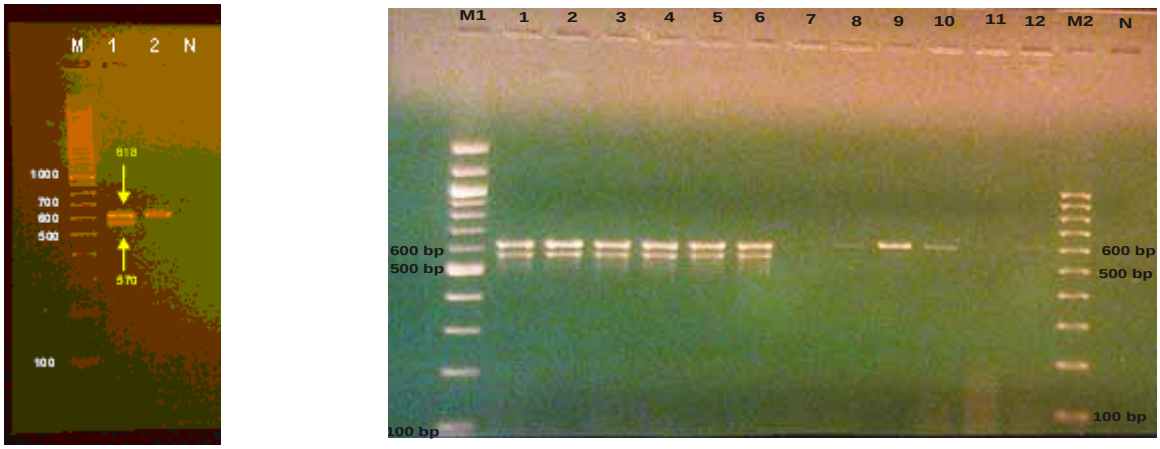

Figure 4. A: Duplex PCR 1) *P. entomophila*, 2) *P. aeruginosa*, N= Negative control. Vertical arrows indicate two amplicons obtained for *P. entomophila.* B: Duplex PCR from *P. entomophila* infected *Drosophila*, (1-6) non-infected (7-12) M1, M2 DNA markers.

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Cloning and expression of modified *Bacillus thuringiensis cry3Bb1* gene from *Bt* maize event MON88017

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Abstract: The gene *cry3Bb1* of transgenic *Bt* maize MON88017 is a modified variant of the wild-type gene of *B. thuringiensis* subsp. *kumatomosiensis* encoding δ-endotoxin against coleopteran larvae. Its open reading frame was amplified from transgenic maize leaves by PCR, cloned into pET-28b(+) vector and expressed in *E. coli* strain BL21-DE3 under the control of T7-promoter. When expressed in *E. coli* the protein accumulated as inclusion bodies. As expected, the Cry3Bb1 was expressed as a 77 kDa protein. Cry3Bb1 produced in *E. coli* and in transgenic plants were compared in SDS gel electrophoresis and Western blot. Bioassays using first instar larvae of *Leptinotarsa decemlineata* will be conducted to examine the toxicity of Cry3Bb1 protein.

Key words: Cry3Bb1, transgenic maize, PCR, expression

Introduction

With the development of genetically modified crops, numerous studies on potential effects of transgenic plants with Cry insect control proteins have been performed. Studies often require large amounts of Cry protein. Cry proteins expressed in *Escherichia coli*, which are considered as equivalent to the plant produced proteins, are used as surrogates. In 2003, *Bt* maize conferring resistance to the western corn borer *Diabrotica* was commercialized. It expresses the Cry3Bb1 protein derived from *Bacillus thuringienis* subsp. *kumatomoensis.* The codon usage of *cry3Bb1* in the construct has been modified from that in the bacterial gene to achieve high expression in maize plant. However, the *cry3Bb1* variant produced in *Bt* maize event MON88017 differs from the wild-type protein in total by six amino acids. In this study we describe the molecular cloning of the modified *cry3Bb1* gene from the *Bt* maize in *E. coli* in order to obtain a large amount of Cry3Bb1 protein equivalent to plant expressed Cry3Bb1 protein. The complete nucleotide sequence of the gene was determined. Western blot analysis was conducted to confirm the identity of the *E. coli* and plant produced Cry3Bb1 protein.

Materials and methods

Bt maize event MON88017 and the near isogenic maize DKc5142 (Monsanto, USA) were grown in greenhouse. Young leaves were collected three weeks after germination and used for extraction of genomic DNA. The *E. coli* strain DH5α was used as cloning host and provided a recombination background. *E. coli* strain BL21-DE3 was used as an expression host. Plasmid pGEM-TEasy (Promega, Germany) were used as cloning vector. Plasmid pET28-NTH derived from pET-28b(+) (Novagen, Germany) and contained the open reading frame (ORF) of *cry3Bb1* as a 1.9-kb *Nco*I/*Eco*RI fragment. pET28-NTH was transformed by electro-poration into *E. coli* DH5α. Plasmid DNA purified from DH5α was digested with *Nco*I and *Eco*RI to check size of vector and insert and was then transformed into *E. coli* BL21-DE3.

Genomic DNA was extracted from Bt maize leaf sample using a modified CTAB method. 100 mg of *Bt* maize leaf were homogenized using liquid nitrogen and incubated with 500 µl CTAB buffer (2% CTAB, 20 mM EDTA, 100 mM Tris-HCl, pH 8, 1.4 M NaCl, 0.2% β-mercaptoethanol) for 1 h at 60°C, then extracted with 500 µl chloroform. The nucleic acids in the aqueous phase were recovered by precipitation with 1 volume of isopropanol, washed with 70% ethanol and dissolved in 100 µl TE buffer. Extracted DNA was used as template.

To amplify *cry3Bb1*, two specific primers Ract1A-upper and Tahsp-lower were designed using the known sequence of first intron of the rice actin (Ract) and the 3′ translated region of wheat heat schock protein gene 17.3 (tahsp) (McElroy et al., 1990; McElwain and Spiker, 1989). Table 1 shows the sequences of the synthesized primers used for amplification and sequencing of *cry3Bb1*. The amplification was carried out for 35 cycles in 50 µl; 2 µl of template DNA (600 ng) was mixed with reaction buffer, 3µM of each primer, 0.3 U of *Taq* DNA polymerase (Axon). Template DNA was denatured (3 min at 94°C) and annealed to primers (45 sec at 50°C), and extensions of PCR products were achieved at 72°C for 3 min. PCR reaction without DNA template was used as negative control.

Table 1. Primers used for amplification and sequencing of *cry3Bb1* from MON88017.

Experiment	**Primer**	**Sequence of primer**
PCR	Ract1A-upper	5′-TCT TTG GCC TTG GTA GTT T- 3′
	Tahsp-lower	5′-GCG ATT AGC CGA TTA CAC A- 3′
Sequencing	T7	5′-ATT ATG CTG AGT GAT ATC CC- 3′
	Sp6	5′-ATTTAGGTGACACTATAGAA- 3′
	1. walking primer	5′- TGC TCT CAC CTC CTT CTA CCA- 3
	2. walking primer	5′-AGA AGG CCT ACT CCC ACC AG- 3′
	3. walking primer	5′-CAG GTG AAG AAG GGG ATG GT- 3′
	4. walking primer	5′-CTT CTC CAG GGG CTC GTC A- 3′

To express the Cry3Bb1 protein, *E. coli* BL21-DE3 containing the pET28-NTH was grown at 37°C in 2-liter flasks containing 500 ml of Luria-Bertani medium. When the cell suspension reached an OD_{600nm} = 0.6, 0.1 mM of IPTG was added to induce the expression of Cry3Bb1. The cells were harvested 4 h after induction by centrifugation. The cells were lysed and the inclusion bodies were washed in 1 M NaCl containing 20 mM Tris HCl (pH 8) and 1% Triton X-100, then in Phosphate-buffered Saline (PBS). The washed inclusion bodies were dissolved in 2 M urea and 100 mM Tris-HCl, pH 12.5, for 1 h at 37°C. The protein solution was then dialysed against 100 mM Tris-HCl, pH 9.0 at 4°C.

Cry3Bb1 proteins expressed in *E. coli* and in Bt maize MON88017 resolved on 10% SDS-polyacrylamide gels were transfered to PVDF-membrane (Hybond^{TM}-P transfer-membrane, Amersham Pharmacia) by electrophoresis in 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS and 20% (v/v) methanol. The membrane was blocked by incubation in 5% (w/v) non-fat dry milk-PBS Tween buffer for 1 h at room temperature. After 5 min rinse in PBST buffer, the membrane was incubated with a Cry3Bb1 protein specific monoclonal antibody at a 1:1000 dilution for 90 min. After washing with PBST, the membrane was incubated for 90 min with a second anti-mouse IgG antibody linked to horse radish peroxidase (Amersham) at a 1:2500 dilution in PBST. After thorough washing with PBST, the binding of the primary antibody to Cry3Bb1 was detected by using chemiluminescent

substrates (1 ml solution A (0.1 M Tris-HCl, pH 8.6 and 0.025% Luminol), 0.5 µl H_2O_2 (30%) and 100 µl solution B (0.11% para-hydroxycoumaric acid in dimethyl sulfoxide).

Results and discussion

Genomic DNA from leaves of Mon88017 was extracted used as template to amplify the *cry3Bb1* gene. Different template concentrations (300 and 600 ng) were applied to PCR. The PCR amplification was successful when 600 ng of template DNA was applied (Fig. 1). The size of the PCR product was 2.5 kb, covering the 1.96 kb ORF of *cry3Bb1* and 0.41 kb of the *Ract* intron and 0.14 kb of *Tahsp*. This PCR product was inserted into pGEM-TEasy vector sequenced using the vector specific T7 and SP6 primer binding sites. Further, four oligos were designed for primer walking to obtain the complete sequence of *cry3Bb1* (Tab. 1).

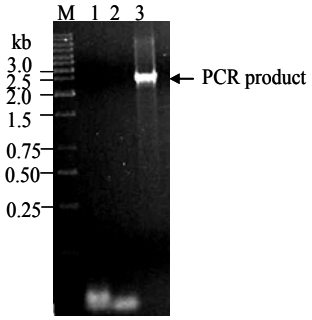

Figure 1. PCR products obtained with specific primers for *cry3Bb1* genes. M: 1 kb Marker; lane 1: negative control; lane 2: 300 ng template, lane 3: 600 ng template

The total length of the cloned *cry3Bb1* gene is 1962 nucleotides, coding for a protein of 653 amino acids with a predicted M_r of 77 000. It shared approximately 59.4% nucleotide and 98.4% amino acid sequence identity with the *cry3Bb1* of *B. thuringiensis* strain EG4961 (Donovan et al., 1992). In addition, the Cry3Bb1 produced in pET28-NTH construct differs from the Cry3Bb1 produced in MON88017 by two amino acids at the position 19 and 222, which could be caused by a mistake in nucleotide incorporation during the PCR amplification.

The Cry3Bb1 protein expressed in *E. coli*.had the expected molecular mass of 77 kDa (Fig. 2). High level expression of protein in *E. coli* resulted in the formation of insoluble aggregates 2 and 3 h after induction with low concentration of IPTG (0.1 mM). In order to analyse the expressed Cry3Bb1 proteins in *E. coli* and in transgenic maize, the inclusion bodies from *E. coli* and total proteins from transgenic maize samples were extracted and loaded on a SDS-Gel. Because the Cry3Bb1 protein was expressed in transgenic maize in low concentration, its band was not visible (Fig. 3A) but on an immunoblotting assay. Cry3Bb1 produced in *E. coli* and in Bt maize were detected in Western blot at the same molecular mass at 77 kDa (Fig. 3B). The strong smearing bands below the 77 kDa band in lanes 2 and 3 could be due to unspecific binding of the antibody to other proteins present in the protein extraction from *E. coli*. No band at 77 kDa was observed in the isogenic maize extraction.

A modified *Bacillus thuringiensis cry3Bb1* gene from Bt-maize event MON88017 was successfully cloned and expressed in *E. coli*. The expression of Cry3Bb1 protein in *E. coli* could provide large amounts of protein used for biosafety research. However, bioassays need to be performed on first-instar larvae of *Leptinotarsa decemlineata* to examine the biological activity of the *E. coli* expressed Cry3Bb1 protein.

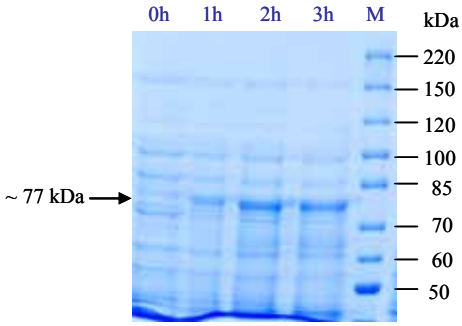

Figure 2. Sodium dodecylsulphate polyacrylamide (SDS) gel eclectrophoresis of total protein isolated from *E. coli* before and after induction with IPTG 0.1 mM

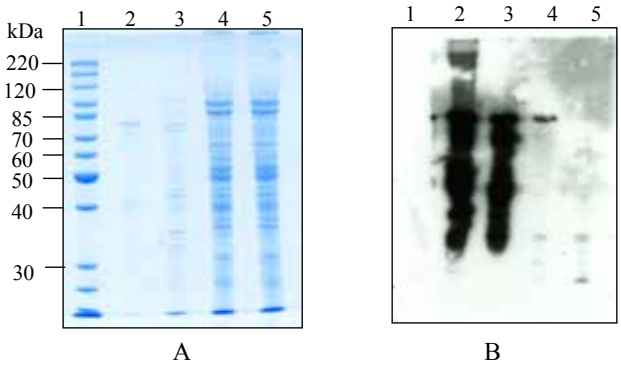

Figure 3. 10% SDS gel electrophoresis (A) and Immunobloting (B) of Cry3Bb1 protein samples expressed from *E. coli* and Bt-maize event MON88017. Lane 1: Marker PageRuler^{TM}, lane 2, 3: Cry3Bb1 expressed in *E. coli* sample 1 and 2, lane 4: proteins extracted from Bt maize, lane 5: proteins extracted from isogenic maize (negative control).

Acknowledgements

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Phylogeny and pathogenicity of *Xenorhabdus* strains for *Spodoptera littoralis*

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Abstract: We used an approach based on a multilocus sequence analysis (MLSA) to address the genealogy within the genus *Xenorhabdus*. The concatenation of four gene sequences – *rec*A, *gyr*B, *dna*N and *glt*X – yields a phylogenetic tree with remarkable robustness that was used to analyse the distribution of the pathogenicity for *Spodoptera litoralis* within the *Xenorhabdus* genus. The results show that the majority of the strains distributed along the phylogenetic tree are not highly pathogenic for *S. littoralis* except *X. nematophila* and *X. indica*.

Key words: *Xenorhabdus*, *Spodoptera littoralis*, phylogeny, pathogenicity

Introduction

Phylogenetic analysis based on 16S rRNA gene sequences failed to resolve with confidence, almost all the phylogenetic relationships at the level of the deepest nodes of the tree within the genus *Xenorhabdus* (Tailliez *et al*., 2006). To address the genealogy of these entomo-pathogenic bacteria, we used an approach based on a multilocus sequence analysis (MLSA) as proposed by Gevers *et al*. (2005). The molecular phylogeny inferred for the concatenated sequences of four gene fragments – *rec*A, *gyr*B, *dna*N and *glt*X – was used to analyse the distribution of the pathogenicity for *Spodoptera littoralis* within the *Xenorhabdus* genus.

Material and methods

Bacterial strains and sequence analysis

We used representative *Xenorhabdus* strains studied previously and their total bacterial genomic DNA was obtained as described previously (Tailliez *et al*., 2006). At the beginning of the study, available sequences of *rec*A, *gyr*B, *dna*N and *glt*X for *Xenorhabdus* (GenBank, http://www.ncbi.nlm.nih.gov/; Donald Danforth Plant Science Center, http://www.xenorhabdus.org/) were aligned using the multiple sequence alignment ClustalW included in the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). PCR and sequencing primers were designed using the conserved regions of the aligned sequences. Sequences obtained were concatenated and then aligned. Phylogenetic trees were calculated using Kimura two-parameters distances (Kimura, 1980) and the neighbor-joining method (Saitou & Nei, 1987) included in the PAUP software (Swofford, 2003).

Assessment of bacterial pathogenicity

Infections of *S. littoralis* fourth-instar larvae with *Xenorhabdus* bacterial cells (10^2-10^3 cells / 20 µl of the appropriate dilution in phosphate-buffered saline of late logarithmic phase cultures in LB broth) were as described elsewhere (Givaudan & Lanois, 2000).

Results and discussion

Phylogeny of Xenorhabdus based on the concatenation of four gene fragments

The neighbor-joining distance tree based on the concatenation of the four gene fragments *rec*A, *gyr*B, *dna*N and *glt*X is presented in Fig. 1. Four clades noted X_I to X_{IV} are delineated. Clade X_I includes seven *Xenorhabdus* species. The bootstrap values (Felsenstein, 1988) are higher than 90 % for the majority of the nodes suggesting a reliable genealogy for this clade. Clade X_{II} includes also seven species. However, the genealogy within this clade failed to resolve with confidence the relationships between the species except between *X. miraniensis* and *X. beddingii* (bootstrap value = 94%), and *X. nematophila* and *X. koppenhoeferi* (bootstrap value = 100%). Clade X_{III} is composed of one species, *X. bovienii*. At least, clade X_{IV} includes five species with high bootstrap values (100 % for the majority of the phylogenetic nodes).

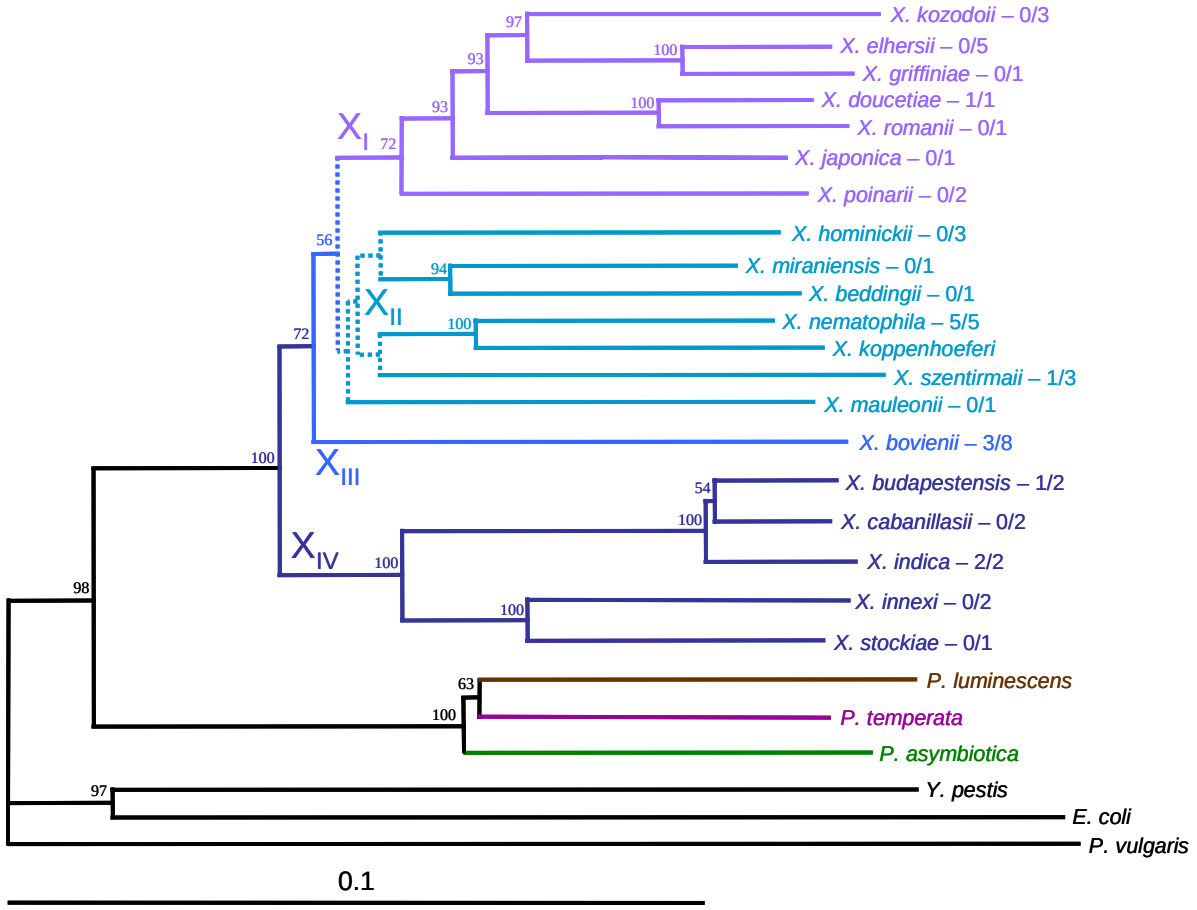

Figure 1. Phylogenetic tree derived from distance analysis of four concatenated protein-coding sequences (*rec*A, *gyr*B, *dna*N, *glt*X). Kimura two-parameters distances were used to construct the neighbor-joining tree. The concatenated sequences of *Photorhabdus* species, *Proteus vulgaris*, *Escherichia coli* and *Yersinia pestis* are used as outgroups. Bootstrap values (percentages of 1000 replicates) of more than 50% are shown at the nodes. Dashed lines indicate unreliable links between groups. Clades X_I to X_{IV} are shown. The bar indicates 10% sequence divergence. The number of virulent *Xenorhabdus* strains for *S. littoralis* on the number of strains tested, are noted next to the species name.

This clade is situated at the basal position of the tree suggesting an early emergence of this lineage. Compare to the 16S rRNA gene phylogeny (Tailliez *et al*., 2006), the concatenation of the four sequences yields a phylogenetic tree with remarkable robustness, likely by increasing the number of phylogenetically informative characters.

Pathogenicity of Xenorhabdus strains for Spodoptera littoralis

Most *Xenorhabdus* strains are highly pathogenic for *Galleria mellonella* with LD_{50} < 100 cells (Akhurst & Boemare, 1990; Akhurst & Dunphy, 1993). However, *X. poinarii* has little pathogenicity for *G. mellonella* when injected alone (LD_{50} > 5000 cells), although it is highly pathogenic when co-injected with axenic *Steinernema glaseri*, its natural nematode host (Akhurst, 1986). Axenic *Steinernema scapterisci* and its *X. innexi* symbiont alone are also less pathogenic for *G. mellonella*. The combination of both partners re-establishes the pathogenicity of the complex towards *G. mellonella* (Bonifassi *et al*., 1999). *X. nematophila* is highly pathogenic for *G. mellonella* (Akhurst & Dunphy, 1993) and *S. littoralis* (Givaudan & Lanois, 2000) with a LD_{50} of less than 20 cells but should be much less pathogenic for other lepidopteran and dipteran (Akhurst & Boemare, 1990).

In our study, the five *X. nematophila* strains, originated from Virginia, France, North Carolina, China and Poland, are all pathogenic for *S. littoralis*. In contrast, only three *X. bovienii* strains (South Carolina, China, Czech Republic) out of the eight tested are pathogenic for *S. littoralis*. For the other *Xenorhabdus* species included in this study, the number of strains tested for each species is low and should be increased to allow drawing general conclusions on the adaptation of *Xenorhabdus* species to insect species. However the results show clearly that the majority of the strains tested are not highly pathogenic for *S. littoralis* (except for *X. nematophila* and *X. indica* strains).

An extensive analysis of the pathogenicity of *Xenorhabdus* strains for various insect species and various doses injected, should allow discovering strains that are much less pathogenic, thus opening new perspectives for the study of the virulence within this genus.

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Field persistence of *Bacillus thuringensis* subsp. *kurstaki*

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Abstract: After an aerial application, the possible persistence of *Bacillus thuringensis* subsp. *kurstaki* strain HD1 (Btk-HD1) in the soil of an oak forest near Florence (Italy), was assessed by means of morphotyping followed by a high throughput staining methodology and PCR analysis. The results show that although the numbers were relatively high on the phylloplane 24h after the insecticidal treatment, individuals belonging to the subsp. *kurstaki* were never detected among the members of the resident edaphic community of *Bacillus thuringiensis*.

Key words: accumulation in soil, microbial pest control.

Introduction

Practical use of microbial pest control is frequent in Italy and the results achieved are very satisfactory. Preparations of *Bacillus thuringiensis* subsp. *kurstaki* (Btk) are commonly applied, in forest and agricultural environments, against lepidopterous insects, especially defoliators. Before the year 1980 only 3% of plant protection products were of biological origin, from then the use of microbial preparations increased. The highest increase was that of *B. thuringiensis* (Bt) preparations, because of their selective mode of action, their limited persistence on plants and their proved safety towards natural enemies of phytophagous insects and towards warm-blooded animals. The only use restrictions of Bt preparations are related to their practical application (aerial application must be authorized by the Ministry of Health), to their potential effects against non-pest lepidopterans and to their possible persistence in soil (Vettori *et al*., 2003) (with risk of accumulation in environments where Bt preparations are frequently used). Therefore studies to verify the hypothesis about the risk of the Bt accumulation in soil were carried out. In fact, although part of the microbial preparation sprayed on plant crowns can reach soil, it is possible that the bacterium may disappear because of well-known phenomena of competition with indigenous microbial community and unsuitable environmental conditions (West *et al.,* 1984).

Materials and methods

Environmental sampling

The present study was carried out in 2007 in an oak forest of Fontesanta Park located near Florence (Italy) at the same time of a treatment (applied by the Regional Government of Tuscany) to control Oak processionary moth (*Thaumetopoea processionea* L.). A commercial preparation (Foray 48 B, 10600 UI/mg) of Btk strain HD1 (Btk-HD1) was distributed by aircraft (2.5 l/ha) against first instar larvae of the moth.

Three different locations, distant approximately 100 mt from each other, were selected and two sampling areas (each one of 1sq mt) were defined in every location. Prior to the treatment one area per location was covered with a nylon sheet that was removed 48h after the Bt application. The treatment was carried out on May 07th, 2007. Soil samples were collected both 24 hours before and after the application. In addition, on both occasions, leaves and litter samples were collected to estimate the initial size of the Btk-HD1 population in the study areas. Again, soil samples were also collected 36 and 84 days after the Bt application. For each area and each assessment date, the soil sample was obtained by sampling at a depth of 2 cm in five different positions of the area, then it was stored at -20°C.

Bacteria belonging to the genus *Bacillus* were isolated from soil and litter samples according to the methodology of De Respinis *et al*. (2006), with the only difference that the serial dilutions of soil suspensions were plated on medium B of King *et al.* (KB,1954). Oak leaves were placed in sterile Erlenmeyer flasks containing 10 ml of phosphate buffer 10mM pH 6.8 and 0.1% (w/v) sodium dodecylsulphate. After 30 min agitation, 0.1 ml aliquots of the bacterial suspensions were serially diluted and plated on KB modified by the addition of cycloheximide (70 mg/l).

Morphotyping and colony staining

After 48h of growth at 30°C, colonies that were beige, with rough, irregular edges, and a "ice crystal" appearance were ascribed to the *Bacillus cereus* morphology group (Bc morphology group includes *B. thuringiensis*, *B. cereus*, *B. anthracis*, *B. mycoides* and *B. weihenstephanensis*). All other colonies were considered to be *Bacillus* spp. Individual colonies that conformed to the Bc morphology group were spotted onto a microscope slide, fixed, stained with Coomassie Brilliant Blue (Ammons and Rampersad, 2002) and viewed under a light microscopy using a 100X immersion oil objective. Parasporal inclusion bodies (crystals) were sought and their shape examined. Colonies containing crystals were classified as Bt.

Molecular characterization by cry1A PCR analysis

Colonies ascribed to the Bc morphology group were subjected to PCR amplification to determine if the insecticidal toxin genes *cry1Aa*, *cry1Ab*, and *cry1Ac*, which are known to be characteristic although not unique of *B. thuringiensis* subsp. *kurstaki* HD1, were present (Valadares *et al*., 2001).

Results and discussion

The presence of Btk isolates, within the Bc morphology group, was not detected on leaves, litter and soil samples collected prior to the Btk-HD1 application. That suggests that the chosen site was suitable for this type of study. Shortly after the application of the biopesticide (24h), Btk-HD1 was re-isolated (Figure 1) both from leaves ($8.1*10^4 \pm 9.5*10^4$ cfu/g) and litter ($7.2*10^4 \pm 1.5*10^4$ cfu/g) however, as indicated by both type of analysis, it was never possible to re-isolate it from soil up to 86 days after the application when surveys were interrupted. The data suggest that the spore load that had reached the soil with the only aerial application carried out was not sufficiently high to allow detection of Btk-HD1 among the members of the indigenous population of Bt (Figure 2) and that the edaphic environment was not favourable to the biocontrol agent proliferation.

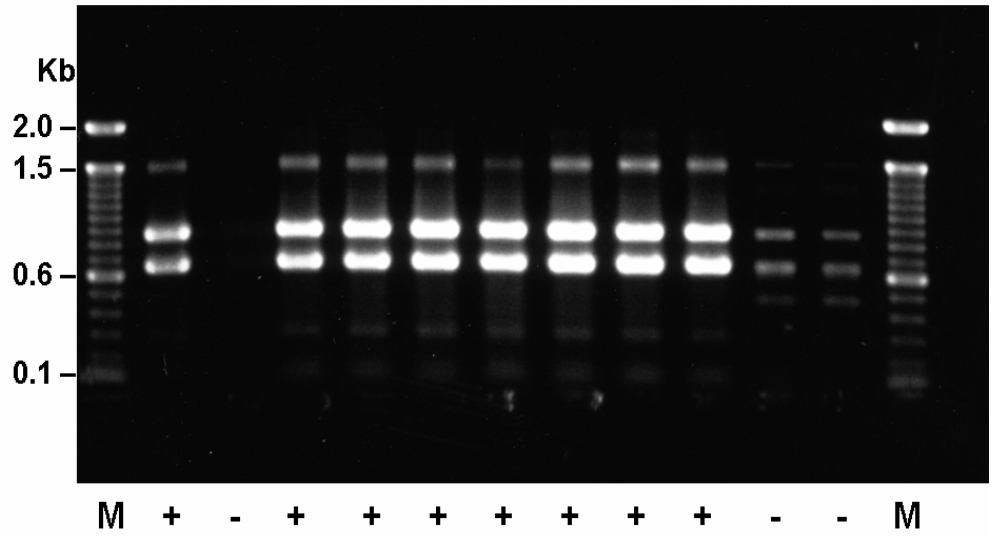

Figure 1. Molecular characterization by cry1A PCR analysis of the Bt morphotypes isolated from a sample of oak leaves 24 h after the aerial application.

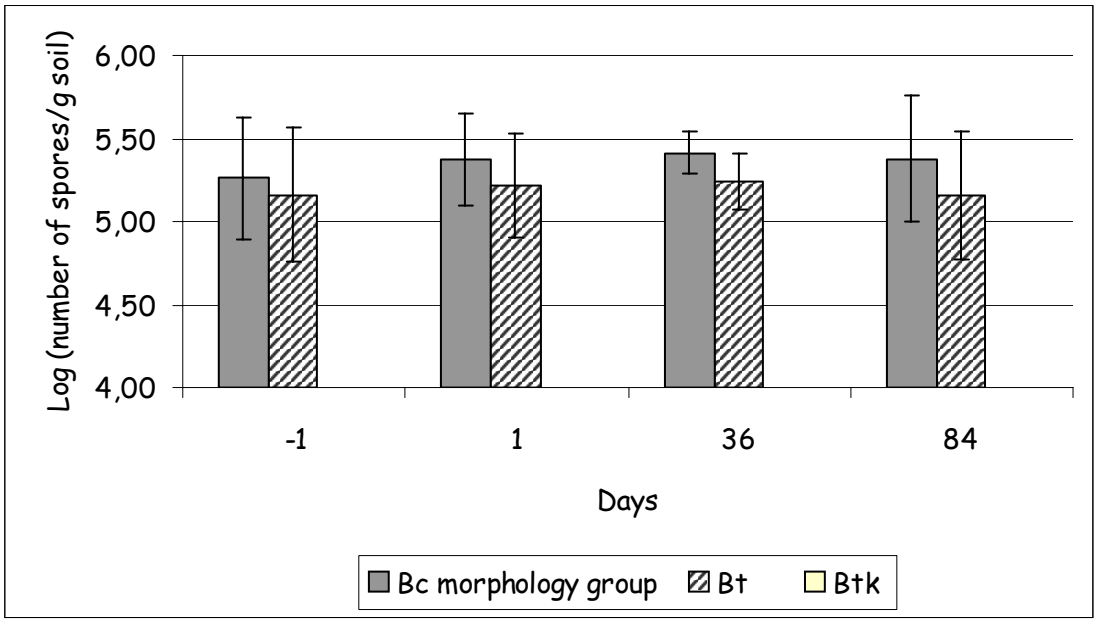

Figure 2. Counts in soil samples of viable spores of *Bacillus cereus* morphology group, of *B. thuringiensis* and of *B. thuringiensis* subsp. *kurstaki*, 1 day before *B. thuringiensis* subsp. *kurstaki*-HD1 application and 1, 36 and 84 days after that.

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How do toxin producing *Bacillus thuringiensis* strains persist in the field? An evolutionary-ecology perspective

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Abstract: The ecology of *Bacillus thuringiensis* (*Bt*) and selective processes maintaining the highly costly cry toxins are still unresolved. Here, we examined whether *Bt* toxins represent cooperative virulence traits and explored the evolutionary ecology of competition between toxin-producing (cooperators) and toxin-null (cheat) strains in the laboratory and the field. We found that *Bt* toxin have the essential properties of cooperative public goods: within-hosts toxin null cheats can out-compete toxin-producing cooperators but cheats cannot infect hosts in the absence of cooperators. In the field, the toxin-producing phenotype shows strong negative frequency dependence at the level of the host plant: patches dominated by toxin-producers are rapidly invaded by cheats and vice versa. Evidence of competition between cheats and cooperators can also be seen in the strong bimodal distribution of cooperator frequency. We argue that the cooperative nature of toxin production can explain several aspects of *Bt* ecology.

Key words *Bacillus thuringiensis*, competition, cooperation, ecology, toxin production

Introduction

The ecology of *Bacillus thuringiensis* (*Bt*) has long been a matter of intense debate. Most fundamentally, there is still disagreement on whether *Bt* is a real pathogen and on how the extremely costly toxin-producing trait is maintained in field populations.

Pioneering work with other bacterial species has shown that bacterial virulence factors can behave as cooperative traits, in other words their production imposes costs upon individuals but conveys benefits to groups or local populations (Griffin et al. 2004; West & Buckling 2003). Here, we examined whether *Bt* toxins represent cooperative traits and explored the evolutionary ecology of competition between toxin-producing (cooperators) and toxin-null (cheat) strains in the laboratory and the field.

Materials and methods

Bacterial strains

A strain of *Bacillus thuringiensi kurstaki* (sequence type 8) was isolated from the leaves of native *Brassica oleracea* on the south coast of the UK. A spontaneous toxin-null mutant of this strain was produced by growing the original isolate in LB broth at 37°C with 0.004% SDS and screening colonies for toxin production.

Field experiment

Greenhouse grown common cabbage *Brassica oleracea* var. capitata were planted out at Wytham field station in a randomized block design, with 12 independent blocks. Within two weeks of planting out 35 third instar *Plutella xylostella* larvae were bagged onto all cabbage plants, except insect free controls. Within 48 hours of releasing insects we applied *Bacillus thuringiensis* with handsprayers. We applied a range of frequencies of cheats and cooperators

(from 0 to 100% cheats) at three different doses (10, 50, and 250 spores μl^{-1}) and a spray free and host free controls. Plants were sampled for *Bt* strains immediately after spraying and 14, 28 and 56 days after spraying.

Screening of toxin-producing strains

Small leaf samples were agitated in sterile sand and saline in order to recover the leaf micro-flora. These samples were pasteurized and plated onto LB agar plates containing polymyxin (Oxoid) in order to select for *Bacillus cereus/B. thuringiensis*. Colonies were transferred to new LB plates, if required, and grown for 7 days at 30°C to induce sporulation. Isolates were stained with Coomassie Blue and examined with oil-immersion microscopy for the presence of proteineceous square or bi-pyrimidal crystals.

Results and discussion

Competition between toxin-producers and cheats in hosts in the laboratory confirmed that *Bt* toxins behave as cooperative public goods. Within hosts cheats grew at least twice as fast as toxin-producers but the ability of cheats to infect hosts declined rapidly as the frequency of toxin producers declined. In the field experiment, we analysed the changes in frequency of toxin-producting strains in the first two time points with a mixed model ANOVA, using plant as a random factor. We found strong negative frequency dependence on toxin production (time*frequency interaction $p < 0.001$; Figure 1) but no significant effect of density.

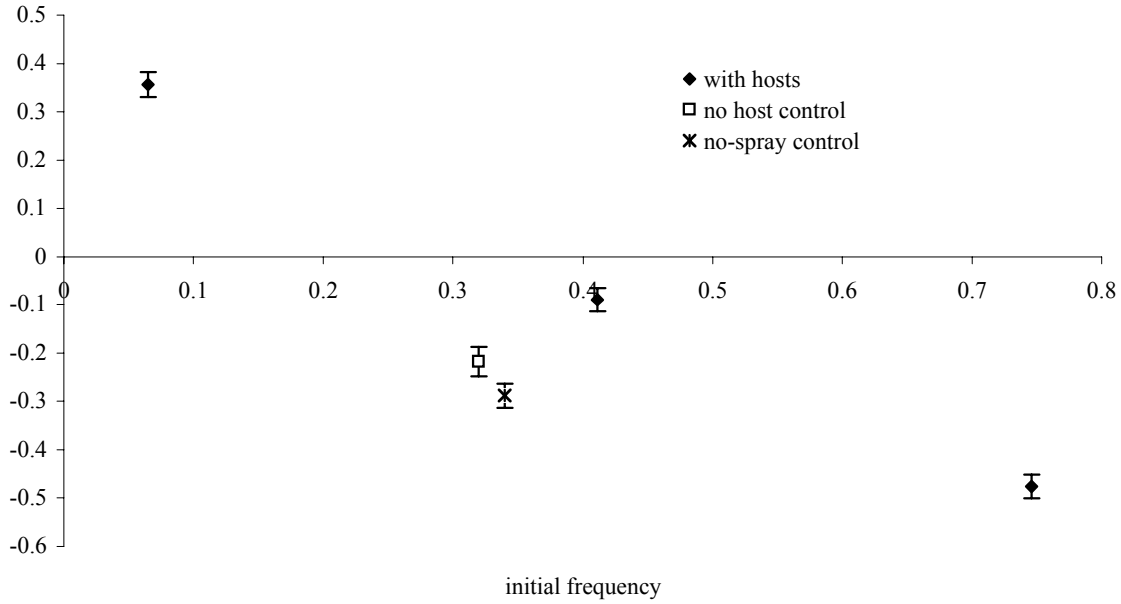

Figure 1. Change in frequency of toxin-producing strains over the first two time points in a field experiment with *Plutella xylostella*.

In other word plants dominated by toxin-producers were quickly invaded by toxin-null cheat strains, and plants dominated by cheats were quickly invaded by toxin-producers. This negative frequency dependence illustrates how toxin production can persist in natural populations of *Bt*. Despite the ability of cheats to invade local patches, cheats cannot persist on the phylloplane in the absence of toxin-producers. As we predicted we also found that the

distibution of frequencies of toxin-producers in patches within plants was strongly bi-modal. The majority of strains were found in patches dominated either by cheats or by cooperators (Figure 2).

It seems therefore that social evolution theory, the theory developed to explain the persistence of cooperation (Hamilton 1964), can explain many of the most problematic features of *Bt* ecology. Toxin-producing strains seem eminently well-adapted to colonize plant surfaces where target hosts are feeding, even in the face of competition from toxin-null strains. It seems likely that toxin production, despite its high cost, can persist in the field because of competition between patches of *Bt* that contain varying frequencies of toxin-producers. However, the rarity of epizootics caused by *Bt* may in part be explained by the vulnerability of patches of pathogenic strains to invasion by avirulent genotypes.

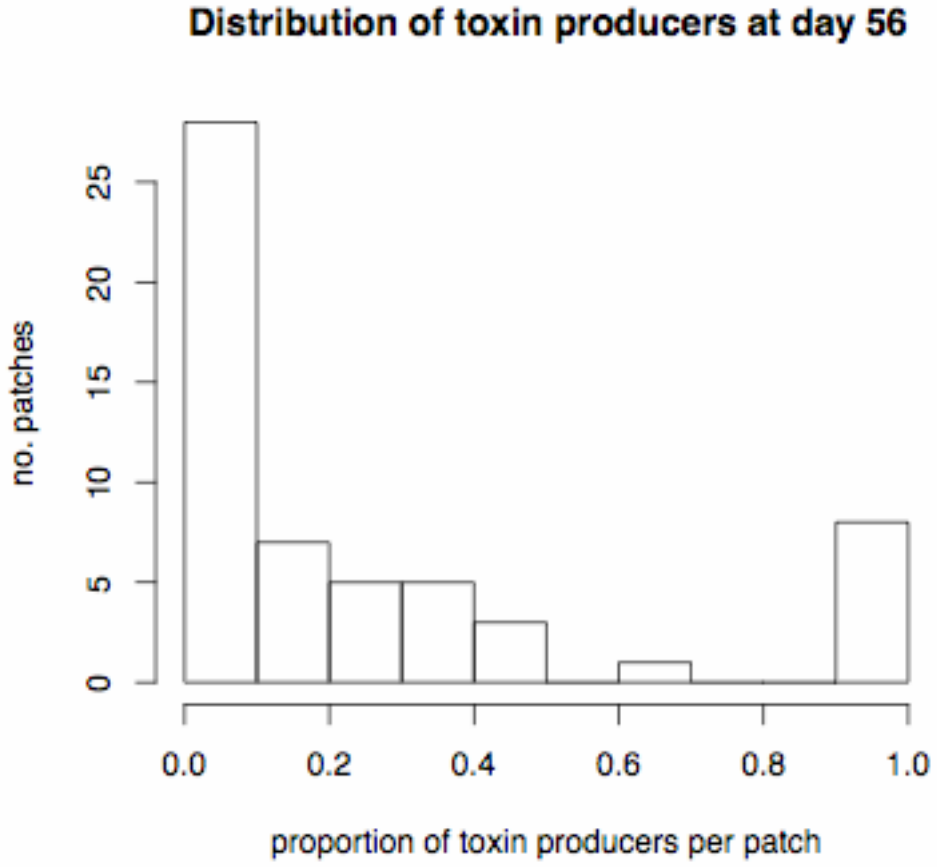

Figure 2. Bimodal distribution of toxin producing strains in the final time point of the field experiment.

Acknowledgements

We thank NERC, BBSRC and the Royal Society for funding.

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Identification, biosynthesis and synthesis of selected natural products from *Xenorhabdus* and *Photorhabdus*

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Abstract: Together with their symbiotic nematodes of the genera *Steinernema* and *Heterorhabditis*, *Xenorhabdus* and *Photorhabdus* are useful biocontrol agents against various insect pests. Moreover, these bacteria are potent producers of new and interesting natural products that might play a role in the complex life cycle of bacteria, nematodes and insects but that might also be useful as antibiotics or other pharmaceuticals. Therefore, we have investigated the biosynthesis and synthesis of several new natural products including new amides, the blue pigment indigoidine, the depsipeptide xenematide, and the cytotoxic xenortides.

Key words: *Photorhabdus*, *Xenorhabdus*, natural products, bioactivity, xenortides, xenematide, indigoidine

Introduction

Photorhabdus and *Xenorhabdus* bacteria live in symbiosis with *Heterorhabditis* and *Steinernema* nematodes, respectively (Goodrich-Blair and Clarke, 2007). The nematode-bacteria complex is highly entomopathogenic and is used commercially against various insect pests. There has been evidence that small molecules (natural products or secondary metabolites) are involved in order to maintain the bacteria-nematode symbiosis as well as to kill the insect and to protect the dead insect from food competitors like other soil bacteria or fungi. During the last two years *Phorhabdus* and *Xenorhabdus* have also been identified as a novel and powerful source of new natural products as several new natural products have been described from these bacteria (Bode, 2009). Moreover, genome sequencing projects have revealed that these bacteria have the potential to produce up to 23 diferent secondary metabolites and up to 7.5% of their genome is dedicated to secondary metabolism. This is more than in *Streptomyces* strains which are a well-established source of secondary metabolites. New natural products are needed as lead structures in the pharmaceutical industry to overcome increasing resistance of human pathogens (e.g. *S. aureus, P. aeruginosa*) and in order to deal with the emerging threat of e.g. tropical diseases allover the world.

Therefore we have initiated an extensive screening programm to identify and isolate new natural products which might be useful in agriculture and medicine. However, as several of the analyzed *Photorhabdus* and *Xenorhabdus* strains produce a mixture o several compounds we have started to identify the compounds produced and than started their chemical synthesis instead of isolating them from the bacterial extracts. Additionally, we have also started to identify the biosynthesis geneclusters involved in the biosynthesis of these natural products in order to learn more about the biochemistry involved and in order to enable future optimization of interesting compounds. Here we present the identification, synthesis, and biological activity of new amides from *Xenorhabdus*, the biosynthesis and synthesis of the insecticidal

xenematide and the cytotoxic xenortides and the biosynthesis of the blue pigment indigoidine from *Photorhabdus*.

New amides from *Xenorhabdus*

2-Phenyl-*N*-(2-phenylethyl)acetamide from *Xenorhabdus* has been described as an apoptosis inducer (Hwang *et al.*, 2003) and within our screening programm, we could identify several producers of this simple compound. A detailed analysis of the producing *Xenorhabdus* strains reavealed the production of several new derivatives that differ in the acyl moiety. However, the *N*-[2-1*H*-indol-3-yl)ethyl] derivatives have also been identified by mass spectrometric analysis. Over all more than 30 new derivatives of this simple amides have been found and several of them have been synthesized in order to test their biological activity. Interestingly, we could show that the new indole derivatives are more active than the known phenylethyl derivatives and that the activity increases in both series of compounds with the chain length of the acyl moiety (Neitzel *et al*., submitted).

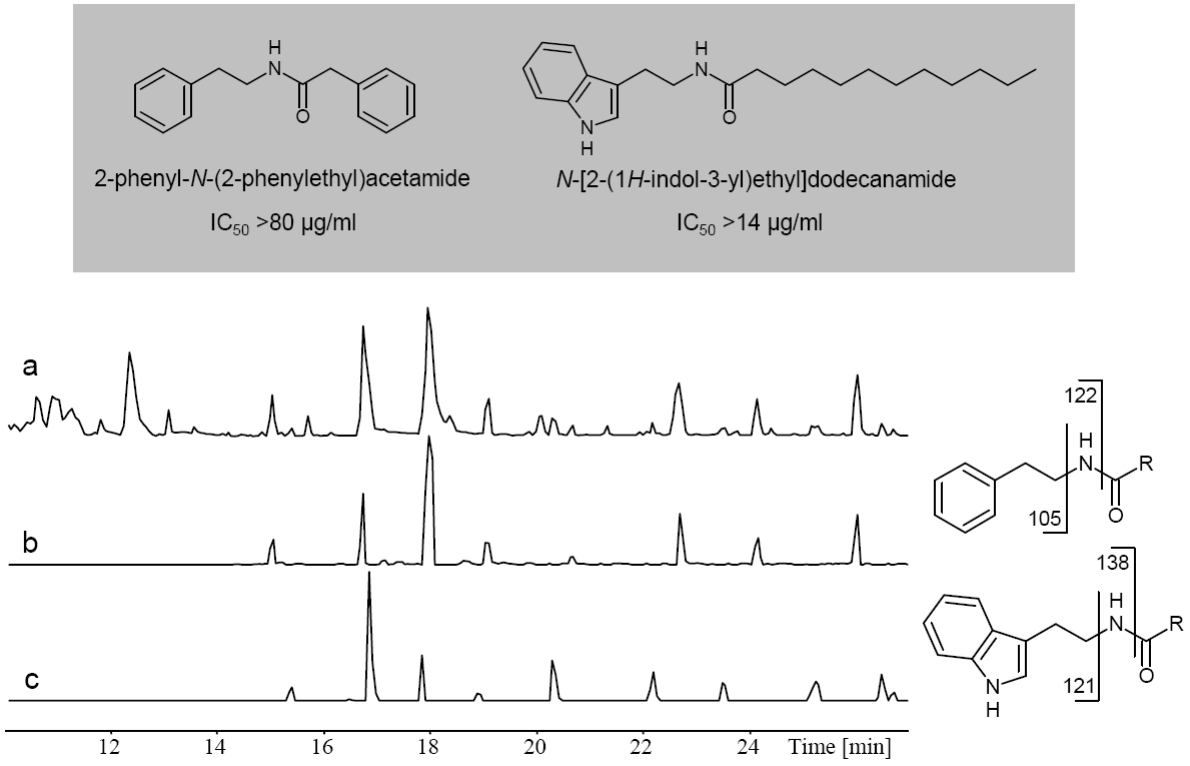

Figure 1. Top: Structure and bioactivity against mouse fibroblasts (L929) of the known compound 2-Phenyl-*N*-(2-phenylethyl)acetamide and a new indole derivative for comparison. Bottom: Typical HPLC/MS analysis of a 2-Phenyl-*N*-(2-phenylethyl)acetamide producing *Xenorhabdus* strains. Full basepeak chromatogramm (a), identified phenylethyl derivatives (b), identified indole derivatives (c). The typical fragmentation and general structure for the phenylethyl and indole derivatives is shown.

Synthesis and biosynthesis of xenematide and xenortides from *Xenorhabdus*

The two peptides xenematide and xenortide A and B have recently been identified from *Xenorhabdus nematophila* (Lang *et al.*, 2008). During our work we could also identify both compounds and could also identify the corresponding biosynthesis gene clusters which allow the identification of the stereochemical assignments of xenematide as shown in Figure 2. Moreover, as the amount of both compoud classes is rather low in our strains, we have chemically synthesized them in order to test their biological activity (Neitzel *et al.*, submitted).

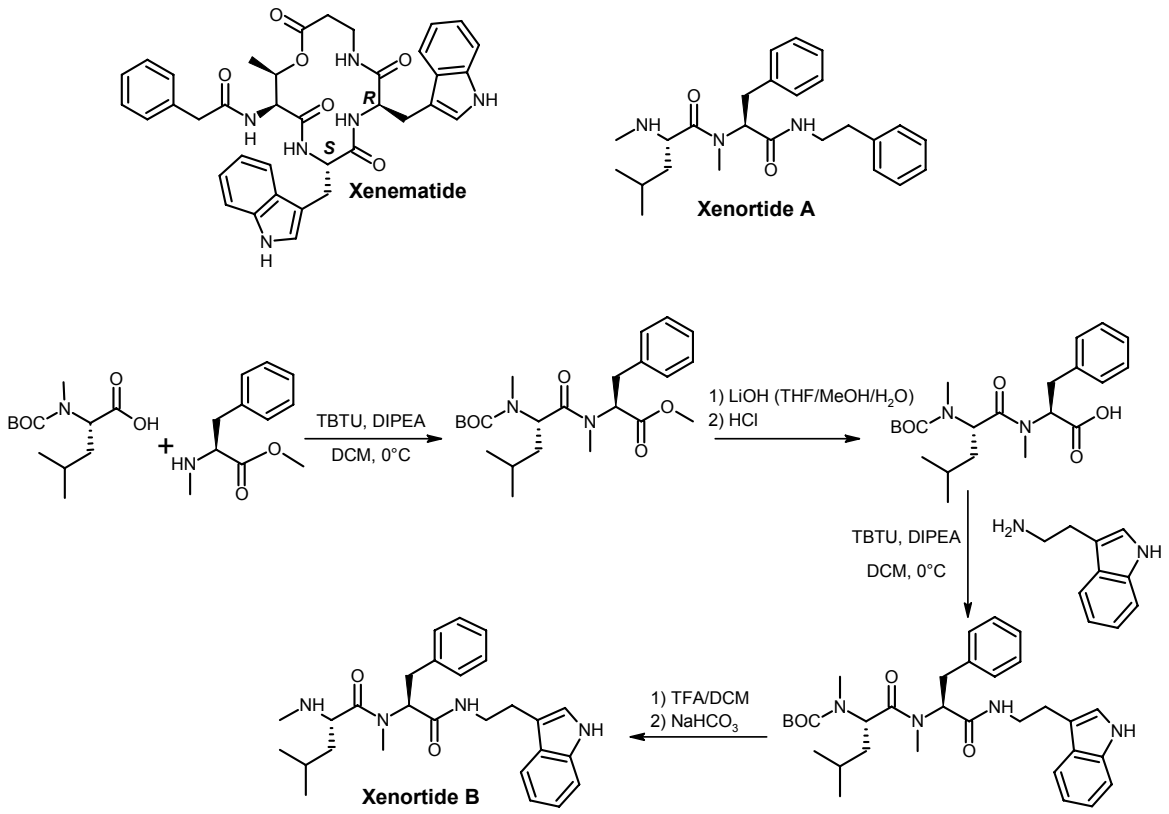

Figure 2. Structures of xenematide (predicted stereochemistry shown), xenortide A and synthesis route to xenortide B.

Biosynthesis of the blue pigment indigoidine in *Photorhabdus*

We could identify a putative indigoidine biosynthesis gene cluster in *P. luminescens* strain TT01. Indigoidine is a blue pigment which was known already from several bacteria like *Erwinia* and *Streptomyces* (Reverchon *et al.*, 2002; Takahashi *et al.*, 2007). We were interested if strain TT01 is actually capable of producing this compound as we have never seen the production of a blue pigment in this strain despite extensive variation of the culture conditions. Therefore we cloned and expressed the putative indigoidine biosynthesis gene cluster in *E. coli*. When a broad-specific phosphopantetheinyl transferase was coexpressed in the resulting strain we could indeed obtain the formation of a deep-blue pigment which was identified as indigoidine my high-resolution mass spectrometry. Additional work led to the

identifation of a new type of regulator in strain TT01 which negatively regulates the production of indigoidine. Moreover, we could show that this regulator is mutated or is completely missing in *Photorhabdus* strains that are green or grey and can still produce indigoidine (Brachmann & Bode, submitted).

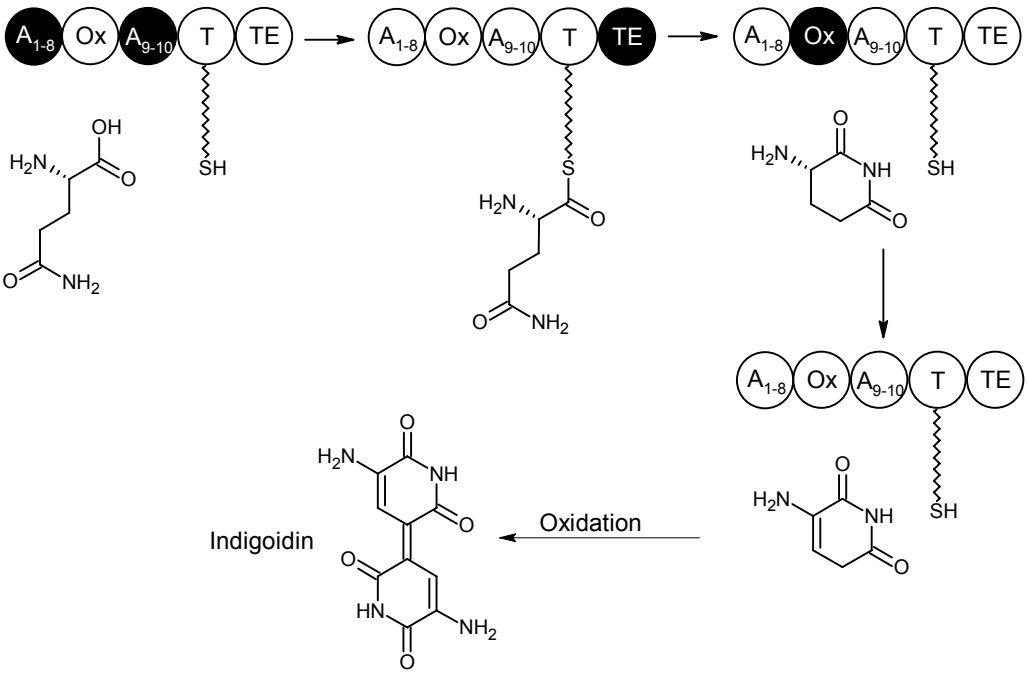

Figure 3. Postulated biosynthesis of indigoidine by the non-ribosomal peptide synthetase IndC. Active domains are shown in black.

Acknowledgements

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Brevibacillus laterosporus preliminary testing in diary farms for the house fly management

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Abstract: A biopesticidal formulation containing spores of a *Brevibacillus laterosporus* strain was employed to test its potential for the management of immature house flies. In outdoor cage experiments, simulating a natural house fly environment, a 58% immature flies inhibition was achieved. In diary farms trials, a reduction in immature fly development was obtained with repeated treatments of the cow pens. In 2003, with a formulation at a concentration of $1x10^8$ spores/ml applied at a dosage of 2 l/m^2, reduction was around 30%. In 2008, with a concentration of $2x10^8$ spores/ml and a dosage of 3 l/m^2, the formulation was able to cause an average depression of immature fly development of 46 %. Therefore, the introduction of this bacterial preparation in Integrated Pest Management programmes in diary farms is promising.

Key words: House fly, *Brevibacillus laterosporus*, field trials

Introduction

The management of many insect pests with high reproductive potential, such as the house fly (*Musca domestica* L.), is always necessary to protect production and health of animals and humans (Cohen et al. 1991). However, human health and environment preservation require the use of insect pest control strategies alternative to the intense application of chemical pesticides. This implies the availability of selective, safe and non-polluting control methods such as the employment of biopesticides. Among these, microbial agents represent an important market segment (Glare and O'Callaghan 2000). Due to their specific mechanism of action, microbial agents are effective only against their target, so that the control of major pests, such as the house fly has necessarily to rely mainly on the conventional management methods available (Moon, 2002). These involve the application of various chemical insecticidal formulations against adults. Safer methods of control for this pest include the use of traps, sugar- and pheromone-based insecticidal baits, the management of breeding sites (fresh manure removal) and the protection and release of natural pupal parasitoids (Hogsette, 1999). More recently, a new discovered strain of the entomopathogenic bacterium, *Brevibacillus laterosporus*, has been proposed as a candidate to be introduced in Integrated Pest Management programs (Ruiu *et al*., 2008a and b). A summary of the main results of recent semi-field and field trials in diary farms with a new formulation based on *B. laterosporus* spores is reported.

Materials and methods

Formulation

The formulation employed in these trials was based on spores of a *Brevibacillus laterosporus* strain active against the house fly (Ruiu *et al*., 2006, Ruiu *et al.*, 2007a). The formulation was

applied at a concentration of $1x10^8$ spores/ml and a dosage corresponding to 2 l/m^2 in both outdoor-cage and field experiments conducted in 2003. In the diary farm trials carried out in 2008, a formulation at a concentration of $2x10^8$ spores/ml and an application dosage of 3 l/m^2 was employed. The controls were always treated with an analogous quantity of water.

Outdoor cage experiment

These semi-field trials were conducted outdoors inside cages (50x30x60 cm) with polystyrene containers (50x30x10 cm) at the bottom. After filling the container with 10 kg of fresh cattle manure, 100 one-week-old lab-reared flies (50 males and 50 females), were released into each cage. In these conditions, flies were allowed to feed and lay eggs on the manure, simulating a natural environment. Every three days, treatments were repeated and flies replaced. After 2 weeks each container was incubated and covered by a polystyrene cap with openings to allow emerging flies to be collected and counted in plastic transparent cylinders (Ruiu *et al*., 2008b).

Diary farm experiments

Field experiments were conducted in Arborea the main diary farm area of Sardinia (Italy). The diary farms selected for experiments, containing on average 100 head, where characterized by house fly infestation, and the cow pen and the dunghill were identified as the main developmental sites for immature stages.

Immature house fly density on the farm was monitored during summer, using modified sampling methods of Loomis *et al*. (1968) and Skoda *et al*. (1996), involving a manufactured 0.5-litre standard metal core sampler. Samples (4 liter each), replicated three times, were collected every week. Sampled manure was incubated and the number of emerging adults recorded (Ruiu *et al*., 2008b).

A total of five (2003) or four (2008) *B. laterosporus* applications were conducted on the surface of the cow pen by a sprinkling can. Treatments were repeated every 3 days over a 2 week period, and immature abundance in treated and control areas was compared.

Results and discussion

Repeated superficial treatments using the *B. laterosporus* formulation caused a significant reduction in immature development ability. In the outdoor cage experiments, the reduction in number of adults that emerged from manure was around 58%, compared to the control (Ruiu *et al*., 2008b).

Similarly, the *B. laterosporus* formulation was able to produce a significant reduction in immature fly development when repeated treatments were carried out on the cow pen of diary farms. In these cases, *B. laterosporus* treatments caused a significant decrease in immature house fly density of about 30% in 2003 (Ruiu *et al*., 2008b) and 46% in 2008 (unpublished data).

These results are in line with those reported in the past by Harvey and Brethour (1960) who mixed formulations containing spores of *B. thuringiensis* var. *thuringiensis*, with cattle faeces. Complete inhibition of larval development was obtained with *B. thuringiensis*, however, in those cases, this effects were likely to be related to the production of β-exotoxin by living bacteria reproducing in manure (Glare and O'Callagha 2000, Jespersen and Keiding 1990).

According to our results, Loomis et al. (1968) obtained a limited reduction in fly emergence (maximum 43%) when manure was treated with other active ingredient (Coumaphos, Co-Ral).

The new *B. laterosporus* spore formulation was more effective in outdoor cage experiments than in field conditions. In fact, in the latter case various environmental factors might be responsible of a wider variability of expected results. Improvements in the formulation insuring a more uniform mixing of spores and manure might enhance the treatment effects.

Everything considered, the efficacy of the treatments on the manure against the house fly is encouraging and provides an important contribution to the prospects of house fly control in natural breeding places (Thomsen and Hammer 1936). In addition, the sub-lethal effects of this microbial agent on immature (i.e. development time) and adult (i.e. fecundity, longevity) house flies, should be taken into account to better evaluate its potential (Ruiu et al. 2006).

Finally, in relation to its compatibility with naturally occurring biological control agents, such as hymenopteran parasitoids (Ruiu et al. 2007b), the new *B. laterosporus* formulation tested in this study has a potential application in biological control and integrated pest management programs of this important pest.

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Concentration of mycotoxins in maize grains and its relation to the European corn borer damage

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Abstract: Maize grains were collected at three localities at the end of two growing seasons. In each variant there were selected the cobs without visible damage and the cobs damaged by the *O. nubilalis* larvae and with the symptoms of Fusarium ear rot. From both variants there were created representative samples and these were analysed for mycotoxin content. Mycotoxin analysis was carried out using appropriate ELISA methods for each toxin. Mycotoxins concentration varied among localities, maize hybrids, years and it was affected by the damage caused by *O. nubilalis* larvae. Mycotoxins level in the grains of Bt maize hybrids was similar to that which was found in the samples from non-damaged cobs of non-Bt maize hybrids.

Key words: maize, *Fusarium*, mycotoxins, *Ostrinia nubilalis*, Bt-maize

Introduction

Mycotoxins are toxic to humans and animals. Their occurrence in food or feed is a significant problem throughout the world. *Fusarium* species are common fungal contaminant of maize grains, which can produce mycotoxins. European corn borer (*Ostrinia nubilalis*) larvae are reported as an important vector of these fungi. The objective of this study was to determine the concentration of deoxynivalenol, fumonisin and zearalenon in grains of various non-Bt maize hybrids and in grains of Bt maize hybrids, to evaluate the damage caused by *O. nubilalis* larvae and define the association between the level of the pest damage and the concentration of mycotoxins.

Materials and methods

Maize grains were collected at three localities at the end of two growing seasons. In each variant there were selected the cobs without visible damage and the cobs damaged by the *O. nubilalis* larvae and with the symptoms of Fusarium ear rot. From both variants there were created representative samples and these were analysed for mycotoxin content. Mycotoxin analysis was carried out using appropriate ELISA methods for each toxin.

Results and discussion

There was high variability in fumonisin content over the years. Generally, the occurrence of this mycotoxin in 2006 was significantly higher than in 2007 ($P <= 0.05$). Contamination of maize grains by the other mycotoxins (DON and zearalenon) was no significantly higher in 2006. In the samples prepared from damaged grains was the concentration of all analysed mycotoxins usually higher compared to the samples created from non damaged cobs. The results are shown in Table 1.

Level of deoxynivalenol from various maize hybrids ranged from 0.28 μg/kg to 4.6 μg/kg in 2006 and from 0.43 μg/kg to 5.15 μg/kg in 2007. Concentration of fumonisin in maize grains was from 0.15 mg/kg to 58.87 mg/kg in 2006 and from 0.02 mg/kg to 16.22 mg/kg in 2007. Zearalenon concentration in maize grains was between 0 μg/kg – 624.7 μg/kg in 2006 and between 0 μg/kg – 462.9 μg/kg in 2007. Mycotoxins level in the grains of Bt maize hybrids was similar to that which was found in the samples from non-damaged cobs of non-Bt maize hybrids. Mycotoxins concentration varied among localities, maize hybrids, years and it was affected by the damage caused by *O. nubilalis* larvae.

Table 1. Mycotoxin concentration in maize grains collected from various maize hybrids. Numbers show average content of deoxynivalenol (DON), fumonisin and zearalenon in grain samples created from undamaged maize cobs and from cobs damaged by the European corn borer larvae with Fusarium ear rot symptoms. ECB – percentage of plants damaged by the European corn borer larvae

Locality/ Year	Maize cobs	ECB	Content of DON (μg/kg)	Content of fumonisin (mg/kg)	Content of zearalenon (μg/kg)
Zavar 2006	Non damaged	85.3	0.53	2.38	21.98
	Damaged	85.3	0.97	14.98	5.57
Solčany 2006	Non damaged	56.3	0.50	1.04	13.22
	Damaged	56.3	0.76	7.94	32.13
Sokolce 2006	Non damaged	43.8	1.03	1.33	14.45
	Damaged	43.8	3.04	20.37	198.02
Sokolce 2007	Non damaged	39.5	1.66	1.16	21.95
	Damaged	39.5	2.70	5.28	191.28
Nitrianska Streda 2007	Non damaged Damaged	32.5 32.5	0.77 0.69	0.06 0.10	12.80 11.28
Nové Zámky 2007	Non damaged Damaged	19.8 19.8	0.99 1.77	0.11 1.20	3.02 71.47

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RVA (Rapid Virulence Annotation) as a functional genomics tool to compare two *Photorhabdus* species with different host ranges

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Abstract: We describe an assumption-free approach, Rapid Virulence Annotation (RVA), for the high-throughput parallel screening of genomic libraries against four different taxa: insects, nematodes, amoeba and mammalian macrophages. These hosts represent different aspects of both the vertebrate and invertebrate immune systems. We have applied RVA to the emerging human pathogen *Photorhabdus asymbiotica* using "gain of toxicity" assays of recombinant *Escherichia coli* clones. We describe a wealth of potential novel virulence loci and attribute biological function to several putative genomic islands, which may then be further characterized using conventional molecular techniques.

Introduction

The growing speed with which the genomes of bacteria can be sequenced is producing an ever expanding knowledge gap between sequence data and its functional annotation. In the case of bacterial pathogens, the identification of virulence factors has typically relied on genetic knock-out and demonstration that virulence is attenuated in a suitable animal model. This approach is not only time consuming and expensive but in vertebrate models ethically debatable. We demonstrate the power of using parallel screens for the identification of virulence genes using three invertebrate infection models and mammalian macrophages in tissue culture, collectively termed them Rapid Virulence Annotation (RVA). RVA relies upon similarities between the immune responses of vertebrates and invertebrates. The innate immune systems of insects and mammals share many common features, both mechanistically and genetically (Khush and Lemaitre, 2000). In addition, much of the basic cellular machinery and pathways that bacterial pathogens can subvert during infection is also well conserved among eukaryotes, presenting common targets. This suggests that virulence mechanisms employed by pathogens of mammals, may work against invertebrates, and vice versa. Indeed, we suggest many virulence strategies evolved initially to combat invertebrates, have subsequently been re-deployed against vertebrates (Waterfield et al., 2004). To test these predictions we conducted an RVA-based screen on a recently emerging pathogen, *Photorhabdus asymbiotica*, (Waterfield et al., 2008), which is a pathogen of both insects and man (Gerrard et al., 2004) The *Photorhabdus* are members of the *Enterobacteriaceae* that live in association with soil dwelling entomopathogenic heterorhabditid nematodes which invade and kill insects (ffrench-Constant et al., 2003). The requirement to overcome the insect immune system and to keep the insect cadaver free of saprophytic organisms in the soil means that all *Photorhabdus* produce a range of bioactive molecules including immune inhibitors, toxins and powerful antimicrobials. The fully annotated genome sequence of the insect-only pathogen *P. luminescens strain* TT01 is available (Duchaud et al., 2008) and the genome sequence of the clinical isolate *P. asymbiotica* ATCC43949 is now completed (http://www.sanger.ac.uk/Projects/P_asymbiotica/). The availability of the two genomes

allows us to correlate the RVA data with regions unique to the human *pathogenic P. asymbiotica.*

Materials and methods

For RVA analysis we sheared the *P. asymbiotica* ATCC43949 genome and cloned it as a cosmid library into recombinant *E. coli*. This library was screened for gain of toxicity (GOT assays) against: the nematode *Caenorhabditis elegans* (nGOT), serving as an oral route model, the single-cell protozoa *Acanthamoeba polyphaga* (aGOT) used as a phagocytosis model, and two caterpillar models (iGOT), *Manduca sexta* and *Galleria mellonella*, both of which represent the more complex insect immune systems. Finally, we used the mouse BALB/c macrophage cell line J774-2 (mGOT) to represent the phagocytic component of the vertebrate immune system. The use of GOT studies in *E. coli*, in which the model host is challenged with individual clones, has the advantage over chromosomal mutagenesis of "unmasking" any virulence factors that would otherwise be hidden due to toxin redundancy or the presence of potent dominant toxins. In order to define the virulence-related regions (RVA regions), the end sequences of cosmids showing an effect were assembled onto a genomic scaffold and regions of minimum genetic overlap within these clusters were identified, often being possible to identify specific open reading frames or operons as gene candidates. Whole genome *in silico* comparison with the strict insect pathogen *Photorhabdus luminescens* TT01 allows the identification of acquired genes potentially involved in human pathogenicity. In parallel to the *in silico* analysis, we have applied RVA to *P. luminescens* TT01. The comparison of both RVA datasets will reveal the commonalities of functional virulence factors for invertebrate pathogenicity in the two *Photorhabdus* species, adding value to the whole genome sequence comparison. Furthermore, the RVA analysis highlights virulence factors likely to be involved in mammalian interactions providing an insight into the mechanisms for cross-species pathogenicity.

Results and discussion

The application of RVA to *P. asymbiotica* gave a high detection rate of candidate gene clusters, encoding both novel and previously described virulence factors, which is a reflection of both the sensitivity of RVA method and also the high level of redundancy in encoded pathogenesis factors in *Photorhabdus* bacteria (ffrench-Constant et al., 2003; Duchaud et al., 2003)).The RVA regions were aligned against the current assembly of the *P. asymbiotica* genome (Fig. 1), each region representing a cluster of cosmids identified either in a single screen (with a minimum of 2 overlapping cosmids) or via a number of parallel screens in combination (Fig. 2). We have now published the complete RVA dataset with detailed diagrammatic summaries of the 21 RVA regions identified (see Waterfield et al., 2008). Several classes of virulence factors were identified by RVA illustrating the robust nature of RVA-based screening including (i) Re-identification of known virulence factors; (ii) dentification of virulence factor homologues from other bacteria; (iii) the functional annotation of secondary metabolite gene clusters and finally (fig 3) (iv) the identification of totally novel virulence gene clusters. A comparison between the genome sequences and the RVA datasets of ATCC43949 and TT01 should prove very revealling.

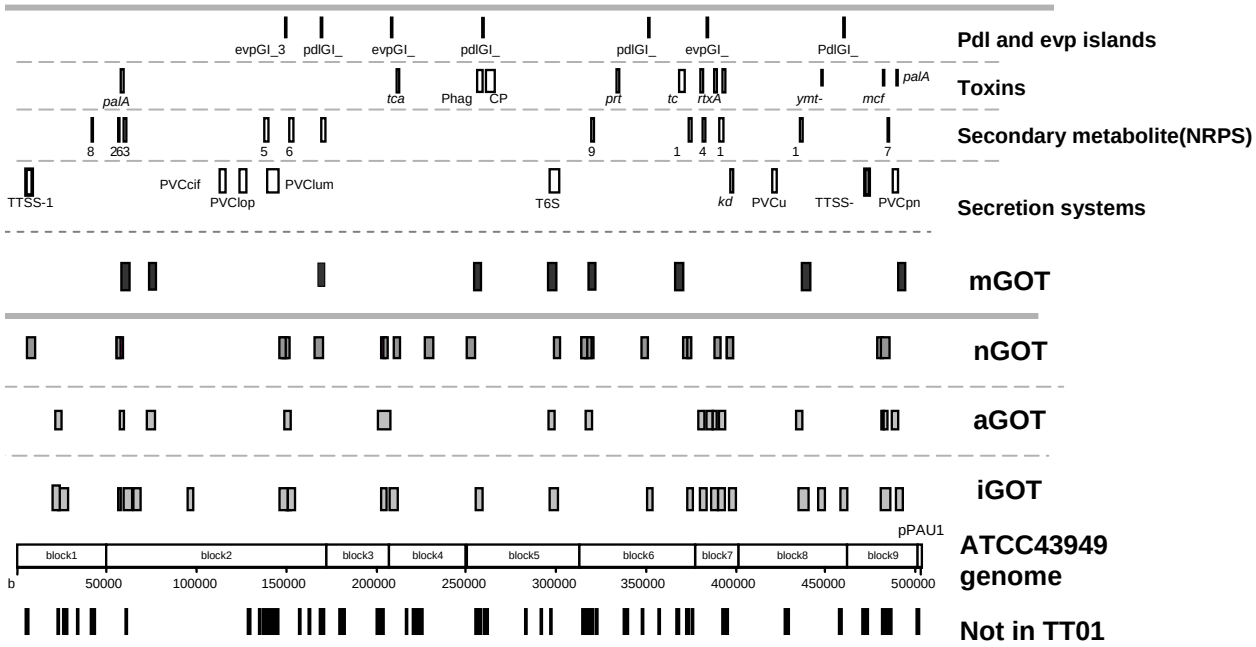

Figure 1. The RVA map of the *P. asymbiotica* whole genome

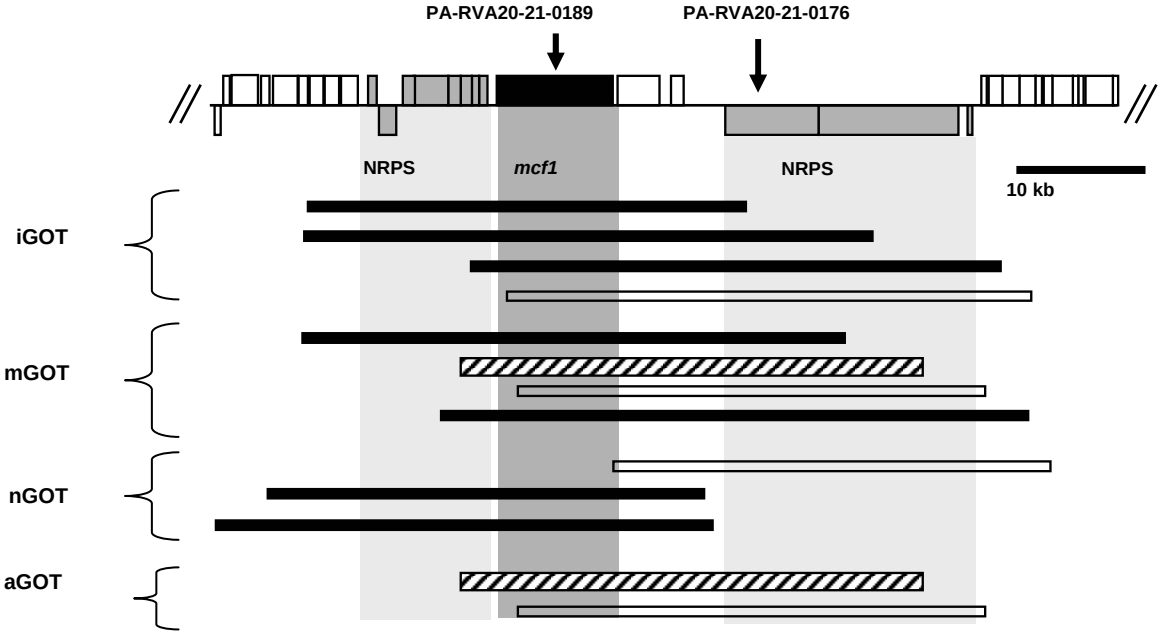

Figure 2. Zoom in of a typical RVA region of the genome showing cosmids toxic to different taxa. This illustrates how GOT-positive cosmids may overlap and define RVA regions. Mcf1 is a known potent lethal toxin. Two NRPS gene clusters also show toxicity.

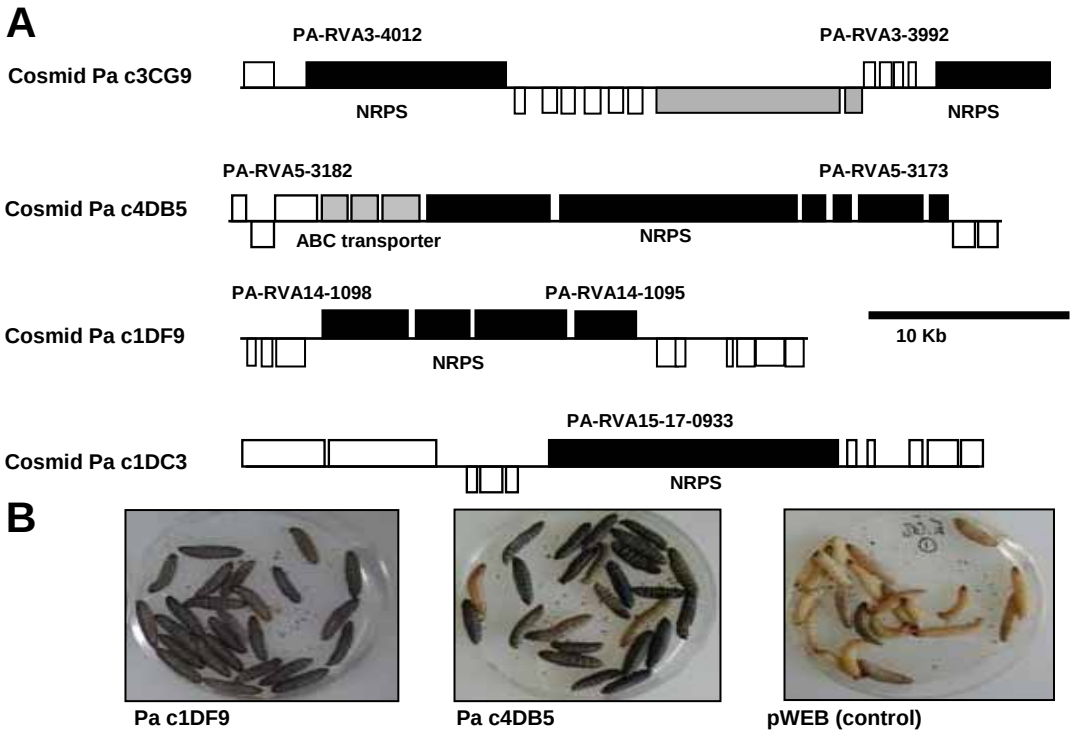

Figure 3. RVA can map biological functions for hard to characterise secondary metabolite gene clusters (A) four positive insecticidal cosmids. Open boxes are ORFs. Above and below the line are the different strands. (B) effect by injection to *Galleria mellonella*.

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Preliminary investigations on bacterioses in cambiophagous beetles with special reference to *Scolytus ratzeburgi*

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Abstract: Bacterial diseases of bark beetles and other cambiophagous insects have been rather poorly recognized (Doane 1960, Wegensteiner 2004). Faced with increasing dieback of both coniferous and deciduous trees in many regions of Europe, this group of insects become more and more significant in forest pathology. During the last decade the growing numbers of cerambycid (longhorn beetles) and scolytid larvae showing typical signs of bacterial diseases were observed in horn beam-oak and Scots pine-birch stands in Wielkopolska and Pomerania regions of Poland. The bacillate bacteria were many times isolated, and in some cases the mortality of *S. ratzeburgi* larvae died in over 90 per cent. In autumn 2008 more precise research was undertaken in order to identify pathogens and to determine areas and strength of the diseases in stands of the National Parks of Wielkopolska and Bory Tucholskie, but materials from other managed forests were also estimated.

Key words: Bacterioses, cambiophagous insects, *Scolytus ratzeburgi*, *Cerambycidae*

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Characterization of Cry1Ac binding in Cry1Ac-resistant *Helicoverpa zea* (Boddie)

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Abstract: The increase in the adoption of *Bacillus thuringiensis* (Bt) transgenic crops, increases the threat the target pest developing resistance. Therefore it is of pivotal interest to contrast the appearance of resistance by establishing in laboratory new resistant strains and characterize the mechanism of resistance. Previous work with a colony of laboratory-selected Cry1Ac resistant *Helicoverpa zea* (AR) showed that resistance was not linked to a reduction in binding. Subsequently, however, AR was outcrossed with another laboratory colony that had been infused with insects collected from corn fields, and reselected for resistance to Cry1Ac toxin. The resulted colony (AR1) is more than 200-fold resistant to Cry1Ac toxin, in contrast to the previous one (AR), which was about 100-fold resistant to Cry1Ac toxin. The characterization of the resistance in the present work demonstrate that there are alterations in total and reversible binding in AR1 compared to the control. These results suggest that alterations in the binding properties of Cry1Ac to brush border membranes may account for at least part of the resistance of the new Cry1Ac-selected strain of cotton bollworm.

Key words: Bt toxins, mode of action, Bt resistance

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Susceptibility of *Tortrix viridiana* L. to *Bacillus thuringiensis* var. *kurstaki* at different level of larval development

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Abstract: Recently out-breaks of the green oak leaf roller *Tortrix viridana* L. (Lepidoptera, Tortricidae) were recorded in Natural Reserve in Sicily (Italy) where treatments are generally forbidden. The commercial, social and environmental value of the wood in the forest needs to be preserved and biological control could be applied in particular case. To optimise the permitted biological treatment, not only in terms of product amount but also for the time of application baseline susceptibility of *T. viridiana* from the Natural Reserve of Ficuzza (Palermo, Italy) to a commercial formulation of *Bacillus thuringiensis* was determined for neonates and 2, 5, 10 and 15 day old larvae. The differences in susceptibility of the different larval development were recorded. For neonates the calculated DL50 was 0.63 µg/ml, while after five days it was 10.01 µg/ml. The same doses had little effect on the older larvae, showing a clear decrease in susceptibility with age and larval growth. A relationship was also found between susceptibility and body length. The implications of these data in controlling this pest in the natural reserve are discussed**.**.

Key words : Green oak leaf roller , oak, natural reserve

Introduction

Various investigations have supplied evidence that defoliation by the larvae of lepidopteran species plays a predominant role in the outbreak of oak decline. The most important defoliating insects are *Operophthera brumata* L., *Tortrix viridana* L. and, in warmer regions, *Lymantria dispar* L.. The green oak leaf roller *T. viridana* L. ranks amongst the most important pests of *Quercus spp.* particularly in Italy and other European countries (Serra et al. 2002; Schroder and Scholz 2005). Recently, high *T. viridiana* infestation was recorded on *Q. pubescens* Willd., in the Natural Reserve of Ficuzza, 3.600 hectares of Mediterranean forest dominated by evergreen oak (*Q. ilex*) and downy oak (*Q. pubescens*), where severity of its attack varies from year to year, depending on climatic conditions. With regard to the control, both chemical and biological, of *T. viridana,* clear evidence of successful prevention of increased oak mortality is still lacking (Thomas et al. 2002). Since, in any case, spraying is critical due to side-effects and hardly calculable factors in planning and application the elaboration of reliable criteria for the recognition of endangered stands and increased risk of defoliation is indispensable (Thomas et al. 2002). Italian law forbids chemical treatment, but biological products can be applied with special permission (L.R. 14/88//DA.970 10/06/91//DA.365/44 26/07/2000). Listed among the micro-organisms applied for biological control of Lepidopteran defoliators is *Bacillus thuringiensis* (Bt), which is actually one of the most used and this employment is being increasingly intensified. Although Bt biosafety is quite well documented, Bt application in the Natural Reserve in Italy requires a special permission and the optimisation of the treatment to achieve the maximum result with the minimum amount of xenobiotic. Therefore it is important to know the pest's susceptibility to Bt and the moment of application. Commercial products based on Bt have been specifically

used against Lepidopteran pests including *T. viridiana* on *Quercus spp*., and quite good results have been claimed in Germany and in Bulgaria (Vankova and Svestka 1976, Bochev 1989; Mikulskaya et al. 2004). Luciano and Lentini (1999) applied Bt to control other oak defoliators on *Q. suber* and reported no negative effect on the woodland ecosystem. In Sardinia (Italy), where *Q. suber* is of great economic importance, forests infested by *Lymantria dispar* L. were sprayed directly from the air, using aircraft. However a specific study on the susceptibility of *T. viridiana* to Bt is not available. Moreover it is important to remember that in the case of Bt, its activity bioassay is based on a reference standard microbial strain and *Ephestia kuehniella* Zeller, or on *Trichoplusia ni* Hb. as the insect test (Gelernter & Navon 1996). Therefore, knowing the baseline susceptibility of the *T. viridiana* population in a particular area is really important to forecast the development of resistance and to optimise Bt use. Moreover it is important to recognise easily and practically, in the natural reserve, the level of the development of the larvae and to correlate them with the better amount of product that should be used. Costa and Gomez-Filho (2002) demonstrated that body length measurements can be used as an accurate index for lepidopteran larval growth. Moreover the authors found a relationship between body length and larval weight. The present research was aimed at detecting the differences in susceptibility for the development period from *T. viridiana* larva to *B. thuringensis* var. *kurstaki*, and setting up a baseline as a data base for further investigations.

Materials and methods

Insect collection

T. viridiana larvae and eggs were collected directly from branches of heavily infested *Q. pubescens* in the Ficuzza forest (Palermo Italy). The material was put into a climate chamber (25 ± 0.5°C, 18:6 h (L:D), 75 ± 5 % RH), and taken alive to the laboratory. The larvae were classified on the basis of body length and the days from hatching.

Bioassay and data analysis

The bioassay was done by treating the larvae, neonates and the 2, 5, 10 and 15 day-old larvae, with different 6 doses (1 = 0.625 µg/ml ; 2 = 1.25 µg/ml; 3 = 2.5 µg/ml; 4 = 5.0 µg/ml; 5 = 10.0 µg/ml; 6 = 25.0 µg/ml plus the non treated control), of a commercial product based on *B. thuringiensis* var. *kurstaki*. The bioassay was performed according to McGuire et al. (1997) on oak leaves. One the bioassay was read, the LC50 was determined through Probit analysis. Data of susceptibility, day of age and body length were submitted to a regression analysis calculated with SPSS Version 9.0.

Results and discussion

Baseline larval susceptibility to commercial Bt, of *T. viridiana* from a Natural Reserve in Sicily, was evaluated. The baseline susceptibility of *T. viridiana* to Bt evaluated for the neonates larvae was 0.63 µg/ml and the DL 95 was 15.9 µg/ml. After 2 days, susceptibility almost halved, the value being 1.74 µg/ml. The larvae submitted to the bioassay 5 days from birth had values of CL 50 and CL 95, a tremendous increment (CL50 = 10.01 µg/ml and DL95= 52.8 µg/ml), demonstrating that susceptibility had decreased 15-fold. The Probit analysis results are not shown for the 10 and 15 day larva, the mortality of these older larvae was really low with the doses used, and only the 25.0 µg/ml dose gave more than 10% mortality for the 10 day larvae. Our findings concord with results reported for two other Lepidopterans: susceptibility of *Ostrinia nubilalis* Hb. to a commercial product of Bt (Lozzia

and Manachini 2004) and *Chilo suppresalis* (Walker) to the toxin CryIAb derived from Bt (Alina et al. 2000). Moreover, Prota et al. (1996) claimed that Bt concentrations used directly in the field need to be doubled in the case of the *L. dispar* larvae are at stage III. Bochev (1989) reported that the Bt treatment against *T. viridiana*, should be made against instars 1-3 and must be completed at least 10 days before pupation. Similar results were found also in the *Heliothis virescens* in 4, 6 and 8-d-old age-classes survived longer than 2-d-old larvae on Bt cotton (Bommireddy & Leonard, 2008) The results show the importance of age for Bt effectiveness against Lepidopterous. In field conditions it could be useful to know how susceptibility changes in relation to parameters such as body length, rather than having to recognise the different instars, so we also tried to investigate whether any relationship exists between susceptibility and larval parametres.

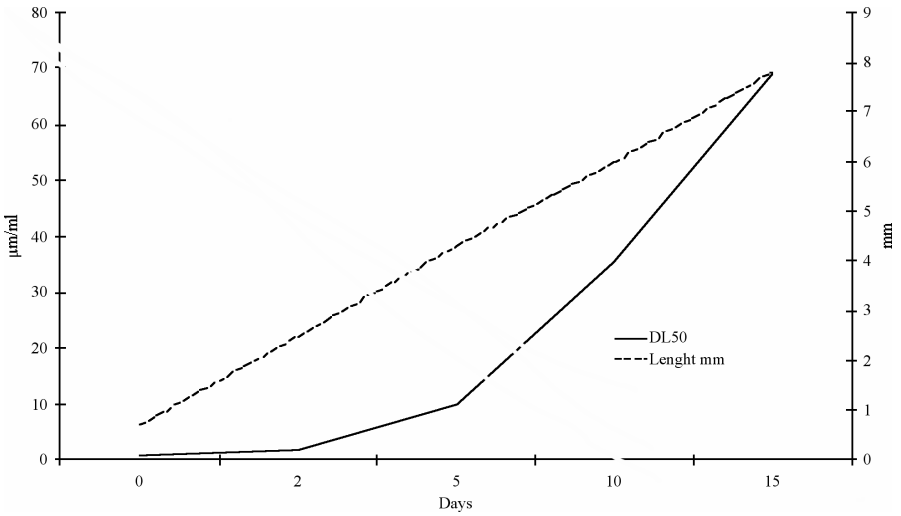

Figure 1. Relationship of susceptibility to *Bacillus thuringiensis*, body length and days of life of *Tortrix viridiana* larvae .

Table 1. Mean of body length (mm) of *Tortrix viridiana* larvae of different age

Larvae	Body length	Standard error
Neonates	0.71	0.03
2 days old	2.50	0.07
5 days old	4.30	0.07
10 days old	6.10	0.05
15 days old	7.81	0.09

Note the strong correlation (r=0.977; Fig. 1) between susceptibility and days of larval life; there is also correlation between body length and susceptibility (0.923) as well for body length and larval age (0.982). Table 1 shows the average body length of the larvae at different days from birth. It is interesting to notice that Huang et al. (1999) claim that the decrease in susceptibility to commercial Bt according to Lepidoptera larval age can be expressed as a cubic function, and explained on the basis of body mass. Costa and Gomez-Filho (2002) found that the use of body length measurements to evaluate the growth performance can be

particularly useful in studies of immature insects under field conditions, when more accurate measures are not possible or even desirable to obtain. Moreover they claim that it urges other workers to undertake similar studies as a means of exploring this phase of insect life cycle in a moreecological context. Thus we have try to find a practical method based also on this finding to optimize the control of phytophagous in particular conditions.

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Studies on the proteolytic processing and binding of *Bt* toxins Cry3Bb1 and Cry34Ab1/Cry35Ab1 in the midgut of Western corn rootworm (*Diabrotica virgifera virgifera* LeConte)

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Abstract: The Western corn rootworm (WCR) is one of the most economically important corn pests worldwide. One possibility for controlling this pest is the cultivation of *Bt*-corn. However, widespread cultivation of *Bt*-corn may increase the probability of the development of pest populations resistant to the respective *Bt* toxins. To establish test systems for identifying resistance mechanisms in the case of resistance development, different parameters involved in the processing of *Bt* toxins in the midgut of third instars larvae of WCR (European strain) were studied. The proteolytic processing of *Bt* toxins Cry3Bb1 and Cry34Ab1/Cry35Ab1 by WCR midgut juice was examined, but no degradation of any of these toxins was observed. Ligand-blot binding analyses with Cry3Bb1 as well as Cry34Ab1 and Cry35Ab1 revealed specific receptors in the WCR midgut epithelium. The molecular weights for Cry3Bb1, Cry34Ab1, and Cry35Ab1 receptors were characterized as having molecular weights of approximately 30 kDa, 110 kDa, and 50 kDa, respectively.

Key words: Cry3Bb1, Cry34Ab1/Cry35Ab1, resistance mechanisms, midgut, gut juice, gut epithetlium, proteolytic processing, binding analyses

Introduction

The Western corn rootworm (WCR) is one of the most economically important corn pests worldwide. In Europe, the WCR was observed for the first time at the beginning of the 90s, spreading since that time and emanating further from South East Europe. One possibility to control this pest is the cultivation of *Bt*-corn. For *Bt*-corn with activity against the WCR, different genes encoding *Bt* toxins were introduced into corn, such as Cry3A by Syngenta, the binary Cry34Ab1/Cry35Ab1 by DOW AgroSciences and Pioneer, and Cry3Bb1 by Monsanto.

However, widespread cultivation of the resulting *Bt*-corn may increase the probability of the development of pest populations resistant to the respective *Bt* toxins. The resistance can occur at any step of the toxic pathway, which includes as its main steps the enzymatic digestion of *Bt* toxins with proteases present in the midgut juice and the binding of *Bt* toxins to specific receptors of the midgut epithelium.

With this background, parameters of the processing of the *Bt* toxin Cry3Bb1 and the binary *Bt* toxin Cry34Ab1/Cry35Ab1 in the midgut of third instars larvae of the WCR (European strain) were examined. These examinations are of basic interest but also lead to test systems for the identification of resistance mechanisms in potentially resistant individuals. In addition, the binding sites of Cry3Bb1 as well as Cry34Ab1 and Cry35Ab1 were characterized.

Materials and methods

Midguts
In Germany, the WCR is under quarantine. Thus, the rearing of the required insects (third instars larvae of a WCR European strain) and the midgut preparation was done in the quarantine ward of the company BTL Bio-Test Labor GmbH in Sagerheide. From their laboratory, the midguts were sent to the Institute for Biological Control in Darmstadt. For studies on the proteolytic processing of *Bt* toxins, pure midgut juice was extracted by centrifugation (Eppendorf centrifuge 5417R) at 20,000 g for 30 minutes at 4°C. To prepare the midgut epithelia for binding studies, the midguts were cut lengthwise and the gut contents together with the peritrophic membrane were removed by rotating the epithelia three times in washing buffer (200 mM Tris, 20 mM $CaCl_2$, pH 5.75).

Bt toxins
The *Bt* toxin Cry3Bb1 was provided by Monsanto. The binary *Bt* toxin Cry34Ab1/Cry35Ab1 was produced according to the method described by Baum *et al.* (2004) using the *Bt* strain from which DOW AgroSciences transferred the toxin genes to the respective *Bt*-corn. To confirm the toxic effect of the 14 kDa (Cry34Ab1) and 44 kDa (Cry35Ab1) proteins, additional bioassays with a *Diabrotica*-related organism, the Green dock leaf beetle (*Gastrophysa viridula*), revealed a mortality of 80%.

Proteolytic processing
Within the studies on the proteolytic processing, it was determined if the *Bt* toxins Cry3Bb1, Cry34Ab1, and Cry35Ab1 were digested by the WCR midgut juice. Proteolytic processing of the toxins was simulated *in vitro* by incubating the toxins with midgut juice. For two samples, 6 µl of the toxin solution (28 µg Cry3Bb1, 21 µg Cry34Ab1, or 29 µg Cry35Ab1) were diluted in 15.6 µl buffer (200 mM Tris, 20 mM $CaCl_2$, pH 5.75), and 2.4 µl midgut juice diluted 1:10, 1:25, or 1:100 was added. After incubation at room temperature, the reaction was stopped for 5 minutes at 95°C. For sample preparation, a Lämmli denaturation buffer (Roti-Load1 no. K929.1) was used in a ratio of 4:1 (12 µl of each sample and 4 µl buffer). Because *Bt* toxins are proteins, they and the products of their break down were described with the aid of SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) using 12% gels (BioRad). The sample input was 11 µl.

Binding analyses
To study the binding of *Bt* toxins to specific receptors in the midgut epithelium, ligand-blot binding analyses were carried out. First, intact brush border membrane vesicles (BBMVs) were produced from midgut epithelia (Wolfersberger *et al.* 1987) and the *Bt* toxins were labelled with biotin (Dendolf *et al.* 1993). BBMV proteins - separated with SDS-PAGE and transferred to a PVDF membrane - were incubated with biotin-labelled toxins. Binding of biotin-labelled toxins was evidenced with a streptavidin horseradish peroxidase conjugate coupled for ECL detection.

Results and discussion

Proteolytic processing
The *Bt* toxin Cry3Bb1 showed a 77 kDa band. The toxin was not processed after a 60-minute incubation with 1:25 diluted WCR midgut juice (Fig. 1 a) or after a 60-minute incubation with 1:100 diluted WCR midgut juice (Fig. 1 b).

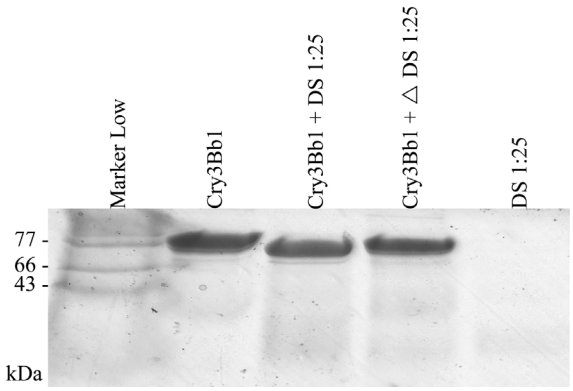

Figure 1a. Processing of *Bt* toxin Cry3Bb1 by 1:25 diluted midgut juice from *Diabrotica* (60 min incubation) [DS = midgut juice; △ = heated]

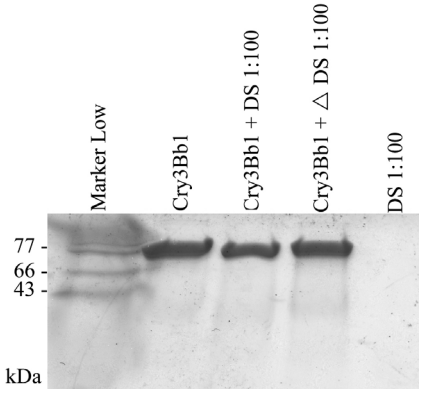

Figure 1b. Processing of *Bt* toxin Cry3Bb1 by 1:100 diluted midgut juice from *Diabrotica* (60 min incubation) [DS = midgut juice; △ = heated]

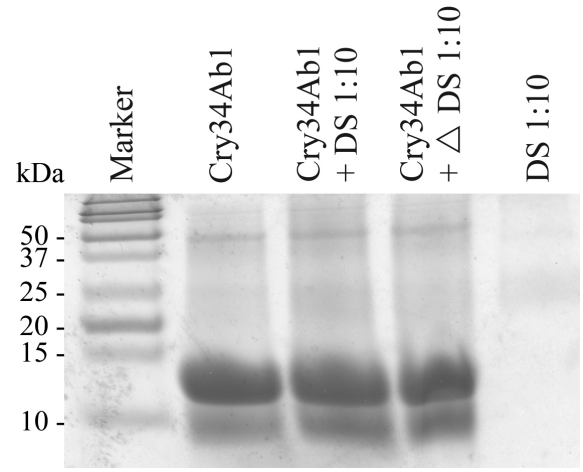

Figure 2a. Processing of *Bt* toxin Cry34Ab1 by 1:10 diluted midgut juice from *Diabrotica* (30 min incubation) [DS = midgut juice; △ = heated]

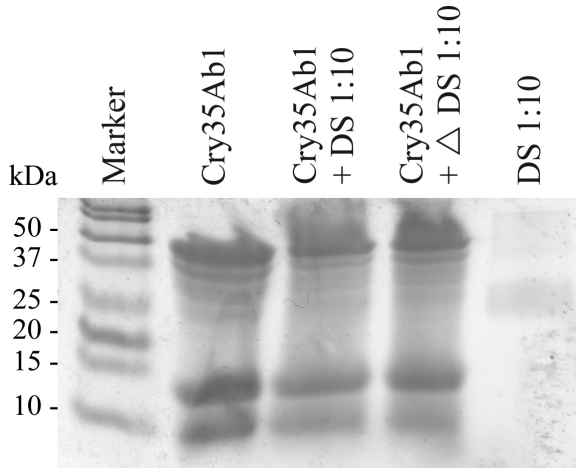

Figure 2b. Processing of *Bt* toxin Cry35Ab1 by 1:10 diluted midgut juice from *Diabrotica* (30 min incubation) [DS = midgut juice; △ = heated]

The toxin Cry34Ab1 showed an approximate 14 kDa band and Cry35Ab1 an approximate 44 kDa band (Ellis *et al.* 2002). After a 30-minute incubation with 1:10 diluted midgut juice, neither toxin was processed and they remained unchanged (Fig. 2 a, Fig. 2 b).

Thus, in the studies on the proteolytic processing of Monsanto´s transgenic corn toxin Cry3Bb1 and DOW AgroSciences binary toxin Cry34Ab1/Cry35Ab1 by midgut juice from the Western corn rootworm, no degradation of the toxins was observed. However, it is conceivable that in the case of genetically conditioned protease changes, the digestion of the toxins into ineffective breakdown products could occur.

Binding analyses

Because *Bt* toxins bind to specific receptors of the midgut epithelium, ligand-blot binding analyses with the toxins Cry3Bb1 and Cry34Ab1/Cry35Ab1 were carried out. The toxin Cry34Ab1 showed binding to an approximate 110 kDa WCR BBMV protein (Fig. 3 a) while the toxin Cry35Ab1 bound to an approximate 50 kDa protein (Fig. 3 b). After the addition of

increasing amounts of biotin-labelled Cry3Bb1, the bands for the biotin-labelled Cry34Ab1 and Cry35Ab1 receptors became weaker, whereas the approximate 30 kDa bands corresponding to the Cry3Bb1 receptor became stronger (Fig. 3 a, Fig. 3 b). Binding competition experiments have to be performed to establish whether both binary toxins Cry34Ab1 and Cry35Ab1 compete for Cry3Bb1 toxin binding sites.

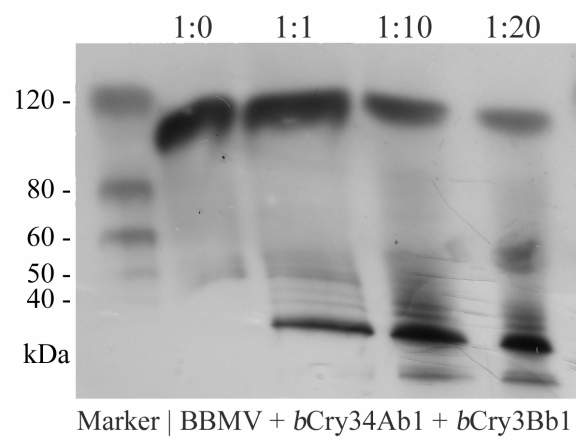

Figure 3a. Binding analysis with biotin-labelled toxins Cry34Ab1 and Cry3Bb1

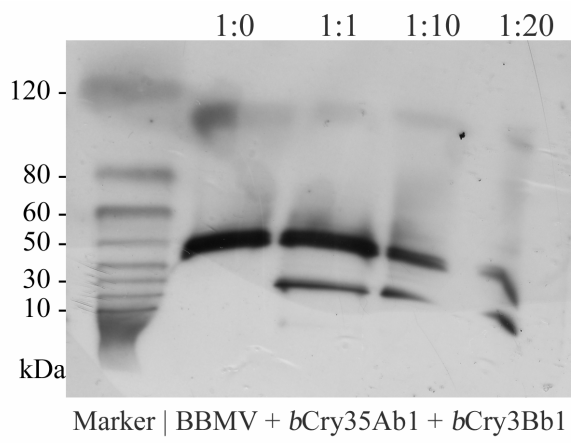

Figure 3b. Binding analysis with biotin-labelled toxins Cry35Ab1 and Cry3Bb1

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Potential effects of *Bacillus thuringiensis* against adults and older larvae of *Rhynchophorous ferrugineus*

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Abstract The Red Palm Weevil, *Rhynchophorous ferrugineus* (Oliv.) (Coleoptera, Curculionidae) is the most important pest of the date trees in many countries. It reached Italy in 2004. One of the alternatives for its control could be the utilization of *Bacillus thuringiensis* (Bt), an entomopathogenic bacterium characterized by its production of insecticidal crystal proteins. Larvae and adults of *R. ferrugineus* were assayed on a meridic diet containing a commercial product of Bt active against Coleoptera. The LC50 for adults was very high superior to 2.0 mg/ml. Larval mortality, body weight, and number of surviving larvae that did not gain significant weight (<0.5 mg per larva) were also recorded after 21 days. Actual and practical mortality were calculated for RPW larvae. The calculated LC50 value of Bt based on practical mortality was 0.45 mg/ml. Negative effect of Bt treated diet on the weight of RPW larvae was recorded.

Key words: Red Palm Weevil, larval weight, biological control, practical mortality

Introduction

Originally from tropical Asia, the Red Palm Weevil (*Rhynchophorus ferrugineus* (Olivier)) is the most dangerous and deadly pest of many palm trees. It reached the Mediterranean in the 1980s, and was first recorded in Sicily (Italy) in 2005 (Longo et al., 2008). Pests introduced by other regions have often remained above damage thresholds because there are no natural enemies to control them, which allowed them to express, to the maximum, their biotic potential. We know few natural pathogens for *R. ferrugineus*, and its control, even by chemical means, is far from satisfactory in the urban areas and especially in the case of RPW attack to *Phoenix canariensis*. Pathogens belonging to the *Bacillus* genus (*B. sphaericus, B. megaterium* and *B. laterosporus*) and *Pseudomonas aeruginosa* have been reported in Egypt, but none of them showed good pathogenicity (Bunerjee et al., 1995; Salama et al., 2004). The potential use of microbial pathogens is still under-investigated. Entomopathogenic bacteria, show notable action specificity and can offer valid alternatives to the indiscriminate use of synthetic products. The products that are available for biological control of other pests on the market are based mainly on *Bacillus thuringiensis* Berliner (Bt) (Sanchis & Bourguet 2008). Several different strains of Bt are isolated and often used against Coleoptera (Krieget al., 1983; Ferro & Gelernter, 1989; Wang et al., 2008).

Information has come to light with regard to the potential pathogenicity of different strain of *B. thuringiensis* versus pest belong to Curculionidae (Weathersbee et al., 2006, Martins et al., 2007 a b). Since entomopathogenic nematodes and *Bauveria bassiana* (Patent 2007) are the only biological agents currently available for *R. ferrugineus* suppression and acceptable control is not always achieved, *B. thuringiensis* would be a welcome addition to RPW management programs in urban areas.

Due to RPW recent introduction and little knowledge regarding its pathogens this research aim to investigate the potential of Bt commercially as biological control.

Materials and methods

Insect collection and rearing

Larvae and adults were collected from infested palms mainly *Phoenix canariensis*, cut down following phytosanitary measures for the control and eradication of *Rhyncophorus ferrugineus* Regional Decree 6 March 2007) with the collaboration of the Regional Department of Phytosanitary Structural Intervention for Regional Service Unit No. 53 Region of Sicily, Department of Agriculture and Forestry Department and the Sicilian Regional Forestry Company, Region of Sicily. For the bioassay older larvae were used as the RPW rearing protocol and set up is still in progress. The samples will then be transported to the laboratories of the University of Palermo where they were kept and raised in air-conditioned cabinets for subsequent testing.

Bioassay

The biological activity of commercial, *Bacillus thuringiensis* subsp. *kurstaki pathotype H- 3A, 3b* registered against Coleopetera (*Leptionotarsa decemlineata*) was evaluated initially against adult and older larvae exposed to treated insect diet. For the adults two types of diet were used: 1. mix of banana and agar, 2. dish of palm trees while for the larvae a diet based on apple fruits. The mortality was observed after 7 days and after 21 days. The bioassay was carried out with six different concentrations (0.1 mg/ml; 0.3 mg/ml; 0.5 mg/ml; 1.0 mg/ml; 1.5 mg/ml; 2.0 mg/ml). One bacteria free control was included. Larval mortality, larval weight, and the number of surviving larvae that did not gain significant weight (<0.5 mg per larva) were recorded on the 20/21th days after inoculation. The bioassay for larvae was carried out with treated diet at the same concentrations used for adults. Three replicates (10 larvae each) for each concentrations plus the control were performed. Larval weight was estimated on 10 larvae randomly collected from the replicates for each concentrations.

Data analysis

One the bioassay was read, the LC50 was determined through probit analysis. Due to the low mortality and the high weight of the RPW larvae a modification of the method described in Huang et al.(2007) was performed. Larval mortality was analyzed with two criteria: actual larval mortality and 'practical 'mortality (Wu et al., 2009). Actual larval mortality was calculated using the number of dead larvae divided by the total number of larvae assayed. The practical mortality was obtained using the equation: Practical mortality (%)=100 x (number of dead larvae +number of surviving larvae that had a body weight of <0.5 mg per larva)/total number of insects assayed.

Data of the larval weight were analyzed by the General Linear Models Procedure, and differences among treatment means were determined by Tukey's studentized range test (SAS Institute 1990), calculated with SPSS Version 9.0. Differences among means were considered significant at a probability level of 5 percent ($P \leq 0.05$).

Results and discussion

Adults

Bioassays with diet contaminated by spores and δ-endotoxin of the bacterium indicated that adults were susceptible to infection, while a lower mortality was observed in the case of palm trees dishes treated with Bt. In both of the cases the concentration of the product to be efficacy was rather high.

Actual larval mortality

The mortality of larvae exposed to treated diet was negligible after 7 days and we recorded it after 20/21 days. The CL 50 was 1.27 mg/ml (Slope SE= 1.7 ± 0.3) and the CL90= 88.8 mg/ml. These values are rather high if compared with the value recorded for neonates of Lepidoptera and for the susceptibility of *L. decemlineata* (Ferro & Gelernter 1989, Weathers-bee *et al.* 2006; Sanchis & Bourguet, 2008) however it is comparable with the results obtained for other Bt strain used against other (Martins *et al.* 2006; Porcara *et al*. 2008; Wang *et al*. 2008).

Moreover even if the mortality of older larvae exposed to treated diet was negligible there were some the effects on larval weight (Table 1) and behaviour (reduction in feeding and movement) were observed.

Practical mortality and effect on weight

Practical mortality of the RPW larvae on non-Bt control diet ranged from 0 – 8 % across all the bioassays. The larval practical mortality of RPW treated diet was high (>50%) even at the lowest concentration (0.5 mg/ml) tested, and it reached 85% at 2.0 mg/ml. The calculated LC50 value of Bt based on practical mortality was 0.45 mg/ml (with a 95% Confident level of 0.38 – 1.58 mg/ml).

In conclusion the potential use of *Bt* in controlling RPW should be more investigated as, at least with this strain, the degree of control achieved might be inadequate.

Table 1. Mean of the weight of older larvae of *R. ferrugineus* exposed to diet containing *Bt* and to the control recorded 3 weeks after the inoculation

Treatment (mg/ml)	Weight (g) ±SE, n=10)
Control	4.20 (0.31) a
0.1	4.18 (0.29) a
0.3	3.90 (0.35) a
0.5	3.71 (0.41) a,b
1.0	3.55 (0.51) b
1.5	2.55 (3.02) b
2.0	2.49 (0.28) b

Means within a column sharing the same letter were not significantly different ($P > 0.05$, Tukey's studentized range test (SAS Institute 1990).

Activity for *B. thuringiensis* against older larval stages of insects is not normally expected; nevertheless, the dose-dependent mortality we observed for larvae exposed were reproducible even if rather low. However the weight of older instar larvae were reduced compared to those of the control, indicating that midgut damage or feeding inhibition may have occurred among larvae that survived to the treatments.

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Regulation of the expression of Fit insect toxin locus genes in the root-associated biocontrol pseudomonad CHA0

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Abstract: The root-colonizing *Pseudomonas fluorescens* strain CHA0 is a biocontrol agent of soil-borne plant diseases caused by fungal and oomycete pathogens. Remarkably, this plant-beneficial pseudomonad is also endowed with potent insecticidal activity that depends on the production of a large protein toxin termed Fit (for *P. fluorescens* insecticidal toxin). In our present work, the genomic locus encoding the *P. fluorescens* insect toxin is subjected to a detailed molecular analysis. The Fit toxin gene *fitD* is flanked upstream by the *fitABC* genes and downstream by the *fitE* gene that encode the ABC transporter, membrane fusion, and outer membrane efflux components of a type I protein secretion system predicted to function in toxin export. The *fitF*, *fitG*, and *fitH* genes located downstream of *fitE* code for regulatory proteins having domain structures typical of signal transduction histidine kinases, LysR-type transcriptional regulators, and response regulators, respectively. The role of these insect toxin locus-associated control elements is being investigated with mutants defective for the regulatory genes and with GFP-based reporter fusions to putative promoter regions upstream of the transporter genes *fitA* and *fitE*, the toxin gene *fitD*, and the regulatory genes *fitF* and *fitH*. Our preliminary findings suggest that the three regulators interact with known global regulators of biocontrol factor expression to control Fit toxin expression and secretion.

Key words: *Pseudomonas*, biocontrol, insecticidal, GFP-based reporters, regulators, root colonization

Introduction

Microbial control of insects that cause damage to agricultural crops as pests and vectors of diseases is a promising alternative to classical chemical control. To date, the commercially most successful microbial agent is *Bacillus thuringiensis* which produces the crystal (Cry) proteins, a family of potent insecticidal toxins (Bravo et al., 2007). There is ample interest in the discovery of alternative, non-Bt related bacterial toxins with anti-insect activity. In recent years, *Photorhabdus* bacteria that live as mutualists in the intestines of entomophagous nematodes have emerge as a potent source of novel insecticidal proteins for crop protection (ffrench-Constant et al., 2007).

Recently, we have identified a chromosomal region in the plant-beneficial bacterium *Pseudomonas fluorescens* CHA0 that encodes a large novel protein toxin (Péchy-Tarr et al., 2008). The toxin is related to the Mcf (Makes caterpillars floppy) insect toxins of the entomopathogen *Photorhabdus luminescens* (Daborn et al., 2002). This is remarkable as strain CHA0 is a plant colonizer with no known insect association, but is characterized for its capacity to protect crop plants against soil-borne diseases caused by fungal pathogens (Haas & Keel, 2003; Haas & Défago, 2005). We termed the novel toxin Fit for *P. fluorescens* insecticidal toxin (Péchy-Tarr et al., 2008). The genomic locus encoding the Fit toxin is also present in the closely related *P. fluorescens* biocontrol agent Pf-5. When injected into the hemocoel of larvae of the tobacco hornworm *Manduca sexta* and the greater wax moth *Galleria mellonella*, even low doses of the two Fit toxin-producing *P. fluorescens* strains

killed the insects (Péchy-Tarr et al., 2008). Mutants with deletions in the Fit toxin gene were significantly less virulent to the larvae. When expressed from an inducible promoter in a non-toxic *Escherichia coli* host, the Fit toxin gene was sufficient to render the bacterium lethal to both insect hosts, confirming the anti-insect potency of the Fit toxin (Péchy-Tarr et al., 2008).

The Fit toxin gene is part of a conserved locus containing eight genes predicted to contribute to toxin transport and regulation of toxinogenesis (Fig. 1). The *fitD* gene, encoding the toxin, is flanked upstream by the *fitABC* genes and downstream by the *fitE* gene that encode components of a type I protein secretion system showing remarkable similarities to RTX toxin transporters (Péchy-Tarr et al., 2008). The *fitF*, *fitG*, and *fitH* genes situated downstream *fitE* encode regulatory functions. The deduced product of *fitF* is predicted to be a membrane-bound, signal-accepting hybrid histidine kinase-response regulator. The protein encoded by *fitG* is related to members of the family of LysR-type transcriptional regulators and *fitH* encodes a response regulator.

We have begun to develop a set of GFP-based reporter constructs to study regulatory mechanisms and signals controlling *fit* gene expression. Our approach also implies the creation of specific mutations in Fit locus genes that encode presumable regulation and secretion functions.

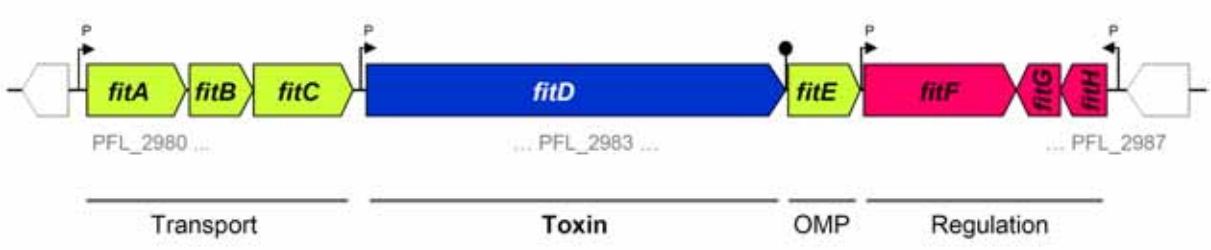

Figure 1. Organisation of the Fit insect toxin locus in *P. fluorescens* CHA0. Putative functions encoded by the locus are indicated below the arrows representing the *fit* genes. The corresponding locus tags in the annotated sequence of *P. fluorescens* Pf-5 are PFL_2980 through PFL_2987. P, putative promoter. ↾, rho-independent transcription terminator.

Materials and methods

P. fluorescens *strains*

P. fluorescens wild type CHA0 is a Fit toxin-positive biocontrol agent of soilborne diseases that produces multiple exoproducts with antifungal activity (Haas & Keel, 2003; Péchy-Tarr et al., 2008). The mutant derivative CHA89 has the *gacA* gene disrupted by a kanamycin resistance determinant (*gacA*::Ω-Km^r) and is defective for exoproducts controlled by the GacS/GacA global regulatory system (Laville et al., 1992; Haas & Défago, 2005). A gene replacement technique (Baehler et al., 2006; Péchy-Tarr et al., 2008) was used to introduce a Δ*fitH* in-frame deletion mutation into the chromosome of *P. fluorescens* CHA0, generating the *fitH* mutant CHA1158.

GFP-based reporter fusions to fit *genes*

Fragments of 0.7 kb, 0.3 kb and 0.5 kb containing the *fitA*, *fitF* and *fitH* promoter regions (Fig. 1) were amplified from CHA0 DNA by PCR using the PrimeSTAR® HS high fidelity DNA polymerase kit (Takara Bio Inc.). The fragments were cloned into the eGFP expression vector pPROBE-TT (Miller et al., 2000) and checked by sequencing. The resulting reporter

plasmids pME8203, pME8207 and pME8205 carrying the *fitA-gfp*, *fitF-gfp*, and *fitH-gfp* fusions, respectively, were electroporated into *P. fluorescens* CHA0 and its mutant derivatives.

Reporter expression assays

Aliquots of 10 µl of overnight cultures of bacteria carrying the GFP-based reporters (adjusted to an OD_{600} of 1.0) were used to inoculate 10 ml of tryptic soy broth (TSB) contained in 50-ml flasks. Cultures were incubated at 30°C with shaking at 180 rpm. Green fluorescence was measured with a Fluostar fluorescence microplate reader (BMG Labtechnologies) throughout the exponential and stationary growth phases (Baehler et al., 2005). For each measurement, the green fluorescence value was divided by the corresponding OD_{600} value giving the specific fluorescence of the cells expressed as relative fluorescence units (Baehler et al., 2005). The green fluorescence emitted by CHA0 cells carrying an empty pPROBE-TT vector was determined for background correction.

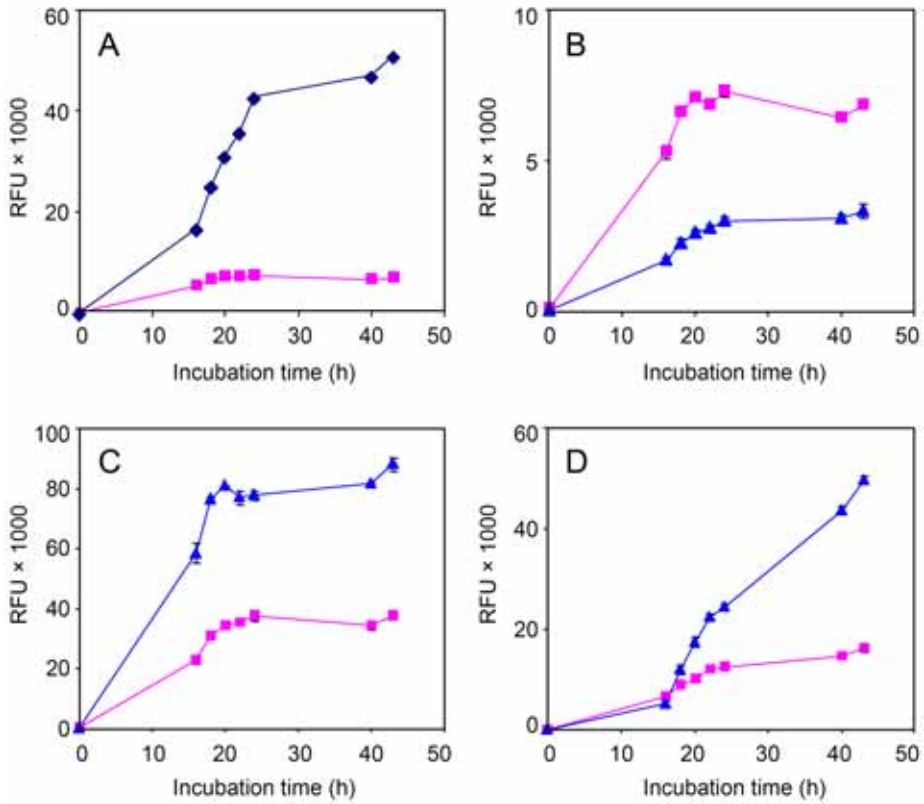

Figure 2. Effect of the FitH and GacA regulators on the expression of **(A, B)** *fitA-gfp* (pME8203), **(C)** *fitF-gfp* (pME8207), and **(D)** *fitH-gfp* (pME8205) reporter fusions in *P. fluorescens* wild type CHA0 (squares, ■), the *fitH* mutant CHA1158 (diamonds, ◆), and the *gacA* mutant CHA89 (triangles, ▲). Relative GFP fluorescence intensities (RFU) were determined for bacteria cultured in TSB at 30°C. Means and standard deviations from three replicate cultures are shown. The experiment was repeated twice with similar results.

Results and discussion

A preliminary transcript analysis predicts five major transcriptional units for the Fit locus, comprising the individually transcribed *fitD*, *fitE* and *fitF* genes, and the polycistronically

transcribed *fitABC* and *fitGH* genes (our unpublished findings). To get an insight into the regulation of these units, we have begun to monitor the expression of GFP-based reporter fusions to the *fitA*, *fitD*, *fitF* and *fitH* genes in *P. fluorescens* CHA0 and its mutant derivatives. Here, we present first results on the role of the Fit locus-associated regulator FitH and its interplay with the GacS/GacA regulatory system in *fit* gene regulation.

The expression of the *fitA-gfp* reporter was strongly enhanced the *fitH* mutant (Fig. 2A), indicating that FitH acts as a repressor of transport gene expression. Expression of the reporters for the other *fit* genes was not affected by the *fitH* deletion (data not shown). In the GacA-defective mutant CHA89, the expression of the *fitA-gfp* reporter was significantly lower than in the parental strain CHA0 (Fig. 2B), pointing to positive control of transport gene expression via the GacS/GacA two-component system. By contrast, the GacS/GacA system appears to exert a marked negative effect on the expression of the genes encoding the FitF and FitH regulators, as the expression of the corresponding reporters was strongly enhanced in the *gacA* mutant (Fig. 2C and 2D). Bacterial growth was checked and was not significantly different among all strains tested (data not shown).

Together, these results highlight FitH and GacS/GacA as important regulators of the *P. fluorescens* Fit locus, affecting in particular expression of the *fitA* and *fitF* genes. FitH negatively regulates *fitA* expression, whereas GacS/GacA act as positive regulators of *fitA* expression, possibly by an indirect effect via downregulation of *fitH* expression. Our findings add the Fit locus genes to the growing list of genes encoding biocontrol, defense and competitivity traits controlled by the GacS/GacA system in *P. fluorescens* CHA0 (Laville et al., 1992; Haas & Keel, 2003; Haas & Défago, 2005). Further experiments are underway to investigate the role of the hybrid regulator FitF and the control of Fit toxin gene expression.

Acknowledgements

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Toxicity of *Bacillus thuringiensis* Cry proteins against the olive moth *Prays oleae*

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Abstract: *Prays oleae* (Bernard) is one of the most important pests of olive orchards in countries from the Mediterranean basin. *Bacillus thuringiensis* is being included in Integrate Pest Management programs as an effective biological control agent. For the first time, in this work, activity agains third-instar *P. oleae* larvae of nine Cry proteins have been analysed. All Cry proteins tested were toxic agains *P. oleae* showing Cry2Aa, Cry1Ca and Cry1Aa the highest larval mortality percentages. Toxicity of the most active toxin as mean lethal concentration (LC_{50}) value was also calculated.

Key words: *Prays oleae*, *Bacillus thuringiensis*, Cry δ-endotoxins

Introduction

The microlepidopteran *Prays oleae* (Bernard) (Lepidoptera: Yponomeutidae) is one of the major insect pests that cause significant damages in mediterranean olives orchards. The life cicle of this species is synchronized with olive tree cicle having three generations per year: filophagous (feeding on leaves), anthophagous (feeding on flowers) and carpophagous (feeding on fruits) (Arambourg, 1986). The most important damages ocurred during carpophagous generation when larvae of *P. oleae* cause a high percentaje of fruit fall (De Andrés, 1991).

Chemical insecticides are being commonly used for the control of this pest. In addition, *Bacillus thuringiensis* is being included in Integrated Pest Management programs as an effective biological control agent. *B. thuringiensis* is a gram-postive bacterium that produces paraesporal crystal composed of proteins with specific insecticidal properties (Cry δ-endotoxines) agains several species of insects (Schnepf *et al.*, 1998).

Since this species shows difficulty in rearing and laboratory maintenance data of individual Cry proteins toxicity are very scarce (Hernández-Rodríguez *et al.*, 2009). In this work, toxical activity of nine Cry proteins against *P. oleae* were analysed for the first time. This study provides significant data on the contribution to better use of *B. thuringiensis* and optimized biological control of this pest insect.

Materials and methods

A total of nine Cry proteins were used in this study: Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, Cry1Da, Cry1Fa, Cry1Ja and Cry2Aa. They were supplied by Prof. Juan Ferré from Departament de Genètica (Universitat de València, Spain) and were prepared from recombinant *B. thuringiensis* strains expressing a single toxin from Ecogen (Langhorne, PA). Cry proteins were solubilized and trypsin-activated for toxicity assays (Hernández-Rodríguez *et al*., 2008 and 2009). Since *P. oleae* can not be reared under laboratory conditions third-instar larvae were utilized for the bioassays. All larvae were collected during filophagous and anthophagous generations from olive orchards in Cabra, province of Córdoba (Analusia, Spain). Biossays were carried out using a semiartificial diet composed by 800 ml of water, 80 g of fresh olive leaf flour,

34 g of brewer's yeast, 32 g of wheat germ, 18 g of agar, 14 g of casein, 4.5 g of ascorbic acid, 1.3 g of benzoic acid, and 1.1 g of nipagin. Diet were deposited in a tube and a 15 µg drop of Cry proteins solution were spread on the surface of the diet. Ufter let dry, one larvae was transfered to each tube. Two kind of byossays were performed. First, a single concentration of 16 µg/ml of Cry proteins solution and two replicates of 30 larvae (plus a control treatment) were used per toxin. In adittion, four concentration in geometrical progretion (two replicates of 30 larvae per concentration, plus a control treatment) were used to calculate activity of more toxic protein. Larvae were maintained under laboratory conditions(25 ± 2 ºC, 65 % relative humidity, and 14:10 h light/dark photoperiod) and mortality was recorded after seven days. Abott-corrected mortality were used to compare larval mortality percentages. Concentration–mortality data were subjected to Probit regresión analysis (Finney, 1971) using the POLO-PC program (LeOra Software, 1987) to calculate the mean lethal concentration (LC50) of more toxic protein.

Results and discussion

All Cry proteins tested were toxics against *P. oleae*. Average of mortality caused by this toxins are showed in Figure 1. Proteins that caused a higher percentaje of mortality were Cry2Aa and Cry1Ca above 80% of dead larvae (88.97 ± 0.2 and 86.21 ± 2.54 respectively). On the other hand, Cry1Ba caused lower percentaje of mortality (below 15%). Used *B. thuringiensis* comercial strains are mainly composed by Cry1Ac (De Liñán, 2007). In the present work, Cry1Ac produced around 67% of larval mortality being the third toxin in order of toxic activity. In addition, mean lethal concentration (LC_{50}) value for most toxic protein (Cry2Aa) was calculate being 1.20 µg/ml.

This work provides, for the first time, significant data of most common lepidopteran-specific Cry proteins toxicity, indicating that several of these toxins (in addition of Cry1Ac) can be used for a best control of *P. oleae*.

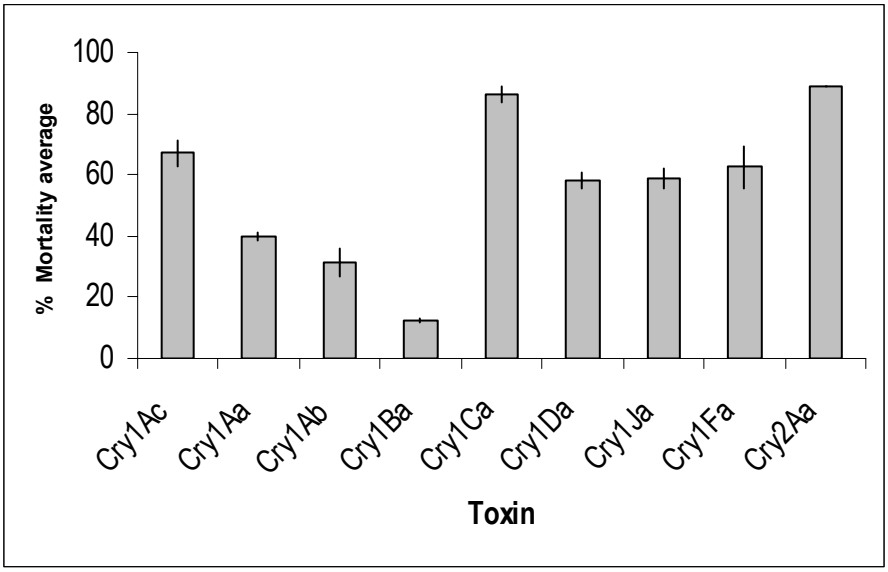

Figure 1. Average of mortality percentaje caused by Cry proteins against third-instar larvae of *Prays oleae*. Vertical lines show estandar error.

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Occurrence and molecular diversity of the Fit insect toxin locus in plant-beneficial pseudomonads

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Abstract: The application of plant-beneficial pseudomonads provides a promising alternative to chemical pest management in agriculture. The fact that *Pseudomonas fluorescens* CHA0 and Pf-5, both well-known biocontrol agents of fungal root diseases, exhibit also potent insecticidal activity is of particular interest, as these plant-beneficial bacteria naturally colonize the rhizosphere of important crop plants. Insecticidal activity in these strains depends on a novel locus encoding the production of a protein toxin termed Fit (for *P. fluorescens* insecticidal toxin). To gain a better understanding of the ecological relevance of the *Pseudomonas* anti-insect activity, we have begun to investigate the occurrence and molecular diversity of the Fit toxin genes among root-associated pseudomonads. To this end, we have screened a large world-wide collection of fluorescent *Pseudomonas* sp. isolated from the roots of different plant species using molecular fingerprinting techniques. The strains are already well characterized for exoproduct patterns and disease-suppressive ability and are currently being tested for insecticidal activity in a greater wax moth larvae assay system.

Key words: *Pseudomonas*, biocontrol, insecticidal, diversity, evolution

Introduction

The ecologically important group of the root-colonizing, plant-beneficial pseudomonads is well known for the ability to suppress plant diseases, stimulate plant growth and enhance nutrient availability to the plant and therefore is widely recognized as a crucial component of soil ecosystem productivity (Haas & Keel, 2003; Haas & Défago, 2005). Recently, we discovered that some fluorescent pseudomonads capable of controlling soilborne diseases exhibit also insecticidal activity, which depends on a novel locus encoding a protein toxin termed Fit (for *P. fluorescens* insecticidal toxin). *Pseudomonas fluorescens* strains CHA0 and Pf-5 kill larvae of the greater wax moth *Galleria mellonella* within less than two days when injected into the haemocoel already at low cell numbers. By contrast, mutants with deletions in the *fitD* gene encoding the insect toxin are significantly less toxic (Péchy-Tarr et al., 2008).

Remarkably, the novel *Pseudomonas* toxin is related to the insect toxin Mcf (Makes caterpillars floppy) of *Photorhabdus luminescens*, an entomopathogen that lives in an intimate association with insect-invading nematodes (Daborn et al., 2002). We have begun to investigate the occurrence and molecular diversity of the Fit toxin gene among root-associated pseudomonads to better understand the ecological relevance of insecticidal activity in these plant-beneficial bacteria.

Materials and methods

Bacterial strains

We rely on a large world-wide strain collection of fluorescent *Pseudomonas* spp. isolated from the roots of different plant species (Keel et al., 1996; Wang et al., 2001; Frapolli et al.,

2007). *Photorhabdus* strains isolated from entomopathogenic *Heterorhabditis* and *Steinernema* nematodes were kindly provided by Jürg Grunder (ZHAW Wädenswil).

***Screening for presence and diversity of* fitD**
A set of specific primers was designed based on the public available genome sequences of *P. fluorescens* Pf-5 and *P. luminescens* TT01. PCR products were separated on agarose gels, purified and sequenced using standard protocols following the instructions of the manufacturers. A phylogenetic framework was reconstructed using the Neighbor-joining and Minimum evolution methods implemented in MEGA4 (Tamura et al., 2007).

Insect toxicity assays
Washed bacterial cells from overnight cultures in Luria broth were suspended in 0.9% sterile NaCl solution and diluted to an OD_{600} = 0.1. Aliquots of 5 µl, corresponding to an injection dose of 3 x 10^4 cells, were injected into the haemolymph of individual ultimate-instar *G. mellonella* larvae (Péchy-Tarr et al., 2008). Larvae injected with sterile NaCl solution served as control. Treated larvae were incubated in Greiner six-well plates at room temperature and scored as live or dead to evaluate the toxicity of the bacterial strains.

Results and discussion

Frequency of* fitD *among root-colonizing pseudomonads and toxicity of* fitD*-positive strains
Twenty *fitD*-positive strains were identified and their *fitD* gene was partially sequenced to ensure the reliability of the method. Sequencing of the 16S rDNA revealed that all *fitD*-carrying strains identified belong to a specific subgroup of fluorescent pseudomonads producing the two antifungal compounds 2,4-diacetylphloroglucinol and pyoluteorin (data not shown) described previously (Keel et al., 1996; Wang et al., 2001; Frapolli et al., 2007).

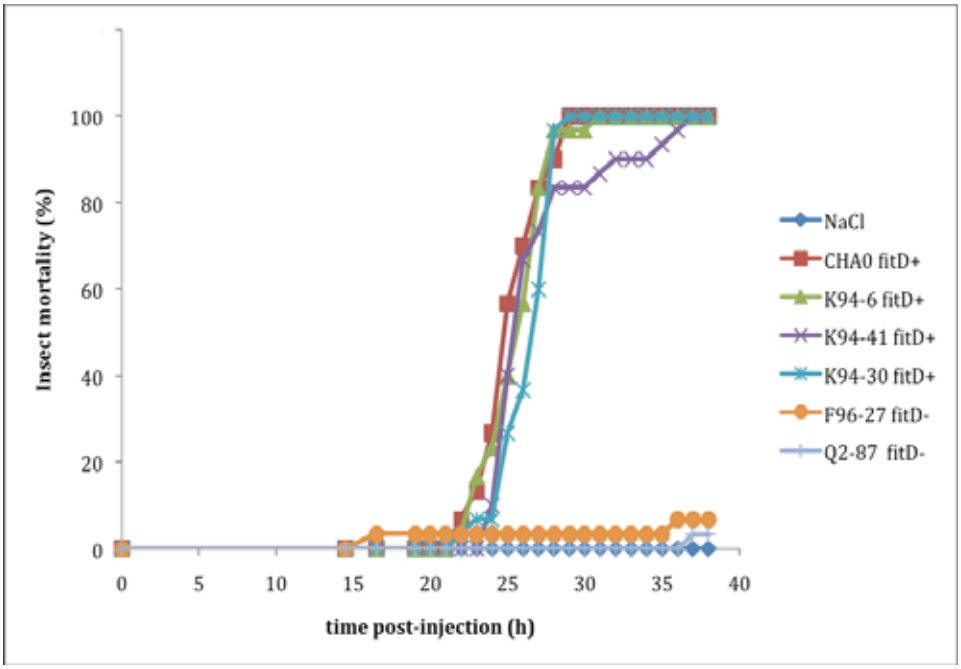

Figure 1. Relative toxicity of select root-colonizing *P. fluorescens* strains to larvae of *G. mellonella*. Bacteria were injected at 3 x 10^4 cells per larva. Controls received sterile NaCl solution. Thirty larvae were tested in each treatment.

A selection of three *fitD*-harboring strains was tested for insecticidal activity in greater wax moth larvae and compared with *fitD* non-containing strains (Fig. 1). In accordance with previous toxicity assays using strains CHA0 and Pf-5 (Péchy-Tarr et al., 2008), all *fitD*-harboring strains killed the larvae within 36 h. By contrast, closely related, but *fitD*-negative *P. fluorescens* strains exhibited virtually no toxicity. At this stage, the presence of the Fit toxin appears to correlate strongly with anti-insect activity in the tested strains (Fig. 1).

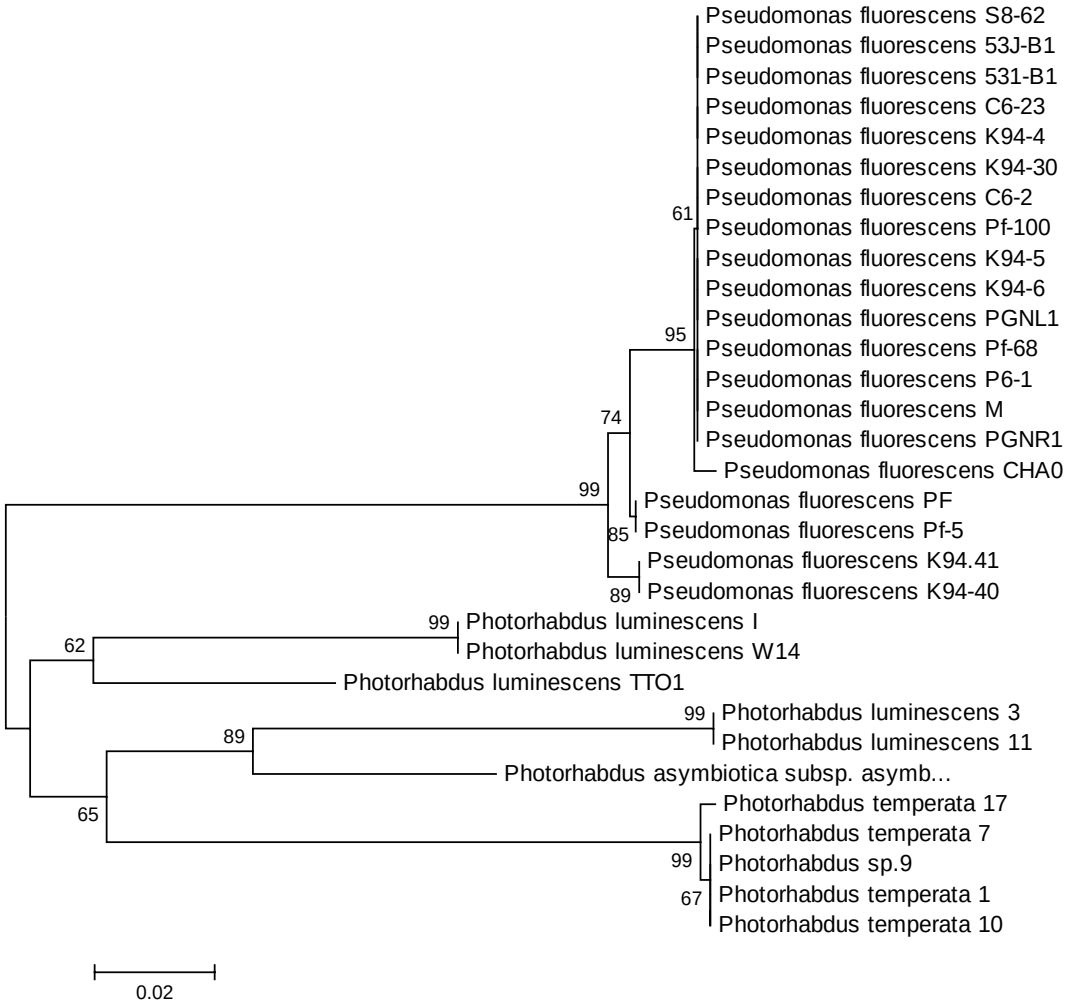

Figure 2. Phylogenetic relationship among 20 *P. fluorescens* and 11 *Photorhabdus* strains based on partial amino acid sequences of the *fitD* gene. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.49 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. There were a total of 247 positions in the final dataset.

Diversity of the Fit toxin gene

To investigate the molecular diversity and to characterize the evolutionary history of the Fit toxin gene, a 0.74-kb fragment of *fitD* as well as of the *mcf1* gene of *Photorhabdus* strains was amplified and sequenced. Based on these sequence data, a pairwise comparison revealed a rather low molecular diversity of the *fitD* fragment analysed, reflecting the close relatedness

of the identified strains. This is most likely a consequence of the fact that the locus is present only in a specific subgroup of the 2,4-diacetylphloroglucinol and pyoluteorin-producing pseudomonads. The results are in accordance with a Southern hybridization experiment in which a *fitD* probe of CHA0 was tested on a strain selection (data not shown).

Acknowledgements

We thank Jürg Grunder (ZHAW) for access to the *Photorhabdus* strain collection and Sarah Hofmann for help with some experiments. We gratefully acknowledge support from the Swiss National Science Foundation (project 3100A0-120121/1) and the Swiss State Secretariat for Education and Research (project SER C07.0026, COST action 862).

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Interaction of loop 1 of *Bacillus thuringiensis* Cry3Aa domain II with Colorado Potato Beetle midgut epithelium cells generates cytoxicity

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Abstract: We have previously proposed a brush border membrane vesicle (BBMV) associated ADAM metalloprotease as a *Bacillus thuringiensis* Cry3Aa toxin receptor in Colorado Potato Beetle (CPB). A peptide corresponding to loop 1 of Cry3Aa domain II (pep-rec peptide) was shown to be involved in the toxin recognition by the ADAM metalloprotease. In this study, we have found that pep-rec peptide displays a cytotoxic effect against CPB dissociated midgut epithelium cells (MECs). MECs treated with pep-rec peptide exhibited a mortatlity of 15.7 %. FITC-labelled pep-rec peptide bound to a portion of intact CPB midgut epithelium, mainly in the apical surfaces of columnar cells and in basolateral contact regions between cells, as well as to immobilized CPB BBMV proteins. By performing confocal laser microscopy we found that both, pep-rec peptide and Cry3Aa, were internalized into MECs exhibiting a characteristic punctate pattern on the plasma membrane and in patched areas concentrated in a region of the cytoplasm near the apical cell surface. MECs treatment with Methyl-β-Cyclodextrin (MCD), an effective cholesterol membrane extracting compound, caused the loss of pep-rec peptide intracellular punctate pattern, although had no effect on pep-rec peptide toxicity. In contrast, around 2.4-fold reduction of Cry3Aa toxin mortality in MECs or larvae exposed to Cry3Aa in the presence of MCD was observed. Pretreatment of CPB midgut BBMV with MCD showed a dose-dependent effect and a concentration of 5 mM MCD resulted in complete inhibition of Cry3Aa toxin membrane associated proteolytic processing. Results indicate that the plasma membrane cholesterol plays a key role in Cry3Aa mode of action. Taking together all these data we propose a two step mechanism of Cry3Aa toxicity in CPB. As a first step, Cry3Aa would partition in lipid rafts (since cholesterol depletion prevents Cry3Aa cleavage and reduces its toxicity). In the second step, the interaction of loop 1 of Cry3Aa domain II with a CPB ADAM metalloprotease would take place, generating toxicity

Fungi

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Potential of *Lecanicillium* spp. for use against the melon aphid and cucumber powdery mildew

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Abstract: The melon aphid *Aphis gossypii* and the cucumber powdery mildew fungus *Erysiphe cichoracearum* D.C. f. *cucurbitacearum* Poteb. are the major pest organisms of cucumbers in both open and closed environments in Georgia. Key elements of our present investigations are the establishment of pathogenicity of introduced *Lecanicillium* spp. to the above mentioned pest organisms. Results of our laboratory experiments demonstrated activity against both aphid and powdery fungus justification for further investigations under field conditions.

Key words: *Melon aphid,* cucumber powdery mildew, strains *Lecanicillium lecani, Erysiphe cichoracearum.*

Introduction

Vegetable-growing is considered one of the more important productive sectors in the economy of Georgia. The melon aphid *Aphis gossypii* is causing great damage to vegetable cultures in both open field and closed environments. In Georgia, only the mycoinsecticide *BotaniGard ES* based on *Beauveria bassiana* (Bals.) Vuill. is used against aphids and greenhouse whitefly (Chkhubianishvili *et al*., 2006 a,b; 2007; 2008; Kakhadze *et al.,* 2008). At present, other mycopesticides have been developed and used in different countries, among them *Lecanicillium* spp. against aphids (Faria & Wraight, 2007). It is also known that *Lecanicillium* spp. possess the ability for simultaneous control of aphids and the cucumber powdery mildew fungus, *Sphaerotheca fuliginea* (Kim et al., 2007). The aim of our study was to determine the susceptibility to *Lecanicillium* spp. to the melon aphid and the cucumber powdery fungus *Erysiphe cichoracearum* D.C. f. *cucurbitacearum* Poteb. in Georgia.

Material and methods

The melon aphid, *Aphis gossypii* was collected from cucumber plants in private greenhouse farms (East Georgia) and propagated on the cucumber plants cultivar "Zazulia" (Baltic) in pots under laboratory conditions (25-26ºC, 75-80% RH). The isolates of *Lecanicillium* spp. were obtained from Canada in the network project STCU - GNSF - # 4326. Two different isolates were selected for testing. One was initially isolated from the commercial product Vertalec and was identified as *Lecanicillium longisporum* and the second LRC 216 (=DAOM 19849) could not be designated to any presently described species based on rDNA sequences (J. Bissett, pers. comm.). The isolates were cultured on potato dextrose agar (PDA) for 14-20 days at 23-24ºC. The conidia suspensions were filtered through a sterilized cheese-cloth and

enumerated using a haemocytometer. A $2x10^7$ conidia per ml suspension was used for treatment of *A. gossypii* and cucumber powder mildew fungus, applied with a hand-held sprayer. Dead aphids were collected, placed in Petri dishes with damp filter paper and placed in humidity chamber (100% RH). Only aphids with fungal symptoms were considered as individuals killed by the fungi (Goettel & Inglis, 1997). Mortality of *A. gossypii* was corrected for control mortality using Abbott's formula (Abbott, 1925). Statistical analysis of each bioassay was conducted according to two-way ANOVA (GrafPadPrism5).

The effect of *Lecanicillium* spp. against powdery mildew, *E. cichoracearum was* determined on diseased leaves of potted cucumber. The same conidia suspensions as described above at $2x10^7$ conidia/ml were used to treat cucumber plants at the 2-3 leaf stage. All plants exhibited a similar degree of powdery mildew infection. Nine replicate plants were used for each treatment with the control being treated with sterile water. One, eight and eleven days following treatment, diameters of powdery mildew spots were determined and compared with the controls.

Results and discussion

The susceptibility of the melon aphid and the cucumber powdery fungus to two isolates of *Lecanicillium* spp. were determined (Figs 1 & 2). The Vertalec isolate exhibited high pathogenicity against *A.gossypii* when compared with the LRC isolate. The standard error for Vertalec was –0.09, for LRC –1.08, for the control 3.05. *Lecanicillium* spp. in the three different days (3, 5 and 7 days after conidia treatment), $P<0.001$ (Fig. 3).

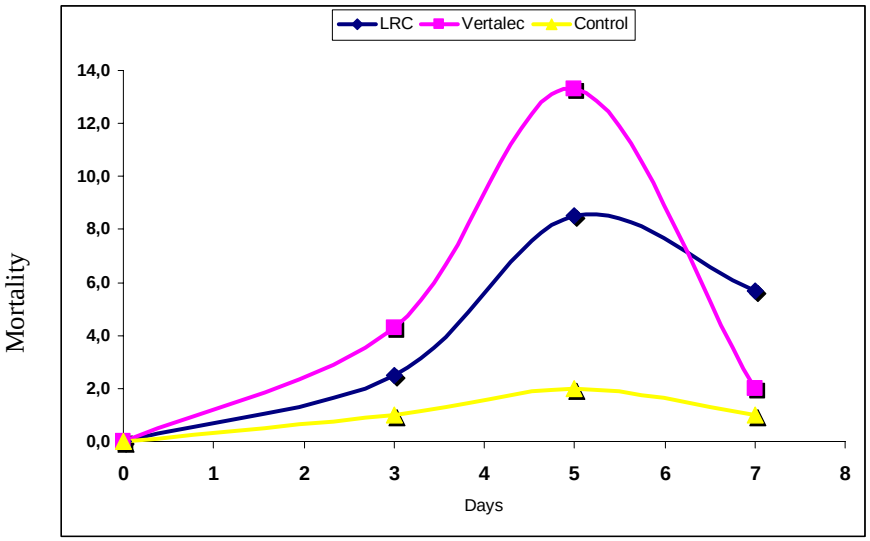

Figure 1. Average mortality of the greenhouse aphids with *Vertalec* and *LRC* strains

The range of spot diameters of powdery mildew after treatment with Vertalec were from 0.63 to 0.82 cm, from 0.60 to 3.33 cm when treated with LRC and from 0.58 to 7.87 cm in the controls (Fig. 4). Treatment with Vertalec significantly reduced spot size of *E. cichoracearum* as compared to the controls. The results are presented in Fig.4

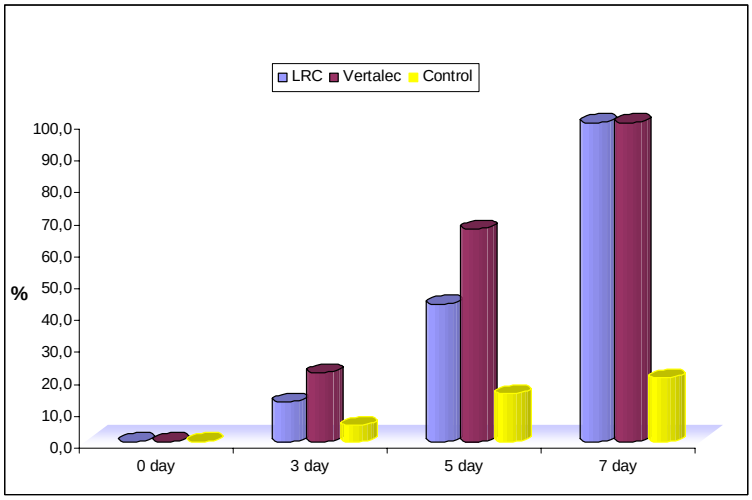

Figure 2. The greenhouse aphid percent cumulative mortality corrected for control mortality

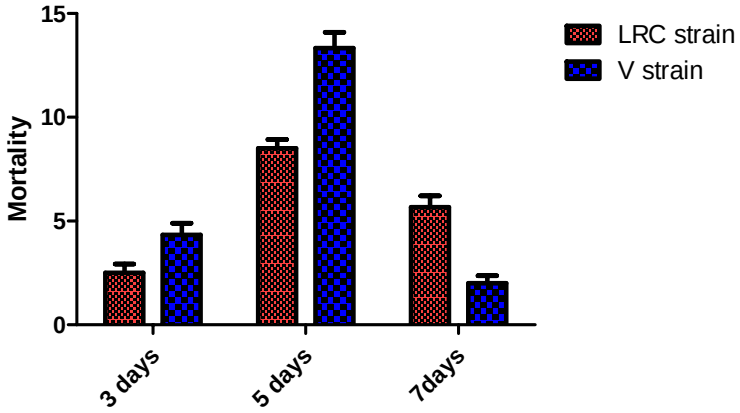

Figure 3. Mortality of aphids treated with Vertalec or the LRC strain of *Lecanicillium* spp. over time

Criteria for mycosis assessment

The cornerstone of the presented investigations is to consider that this was the first attempt to study fungal pathology of aphids in Georgia. The preliminary information provides possibility to continue the investigations under field conditions. It was established for the first time that *Lecanicillium* species are antagonistic to *E. cichoracearum*, a pathogen species responsible for cucumber powdery mildew.

Acknowledgements

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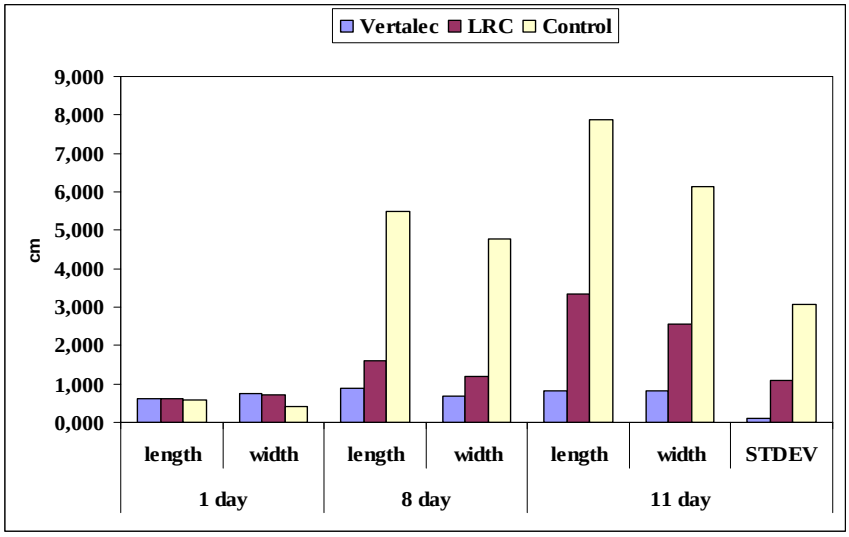

Fig. 4 The strains' effects on the powdery mildew spot diameters (in average)

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Biocontrol of whiteflies based on *Lecanicillium lecanii*

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Abstract: A native isolate of the fungus *Lecanicillium lecanii* Vl026 originally isolated from an adult of *Trialeurodes vaporariorum* demonstrated biocontrol activity against *T.vaporariorum* and *Bemisia tabaci*. In order to develop a formulated biopesticide, initially, a massive production medium was standardized by evaluating two solid substrates supplemented with two nutrient solutions. No significant differences were observed among treatments as compared with substrates supplemented only with water obtaining a yield of 2.1 x 10^9 conidia per cm^2 in S1 substrate after 8 days of incubation at 25°C, which was selected to produce this fungus. Produced conidia were separated and used as active ingredient for biopesticide. The product was designed as a wettable powder for foliar application, in which, a sunscreen and a drying protectant was included. This formulation presented concentration of 10^{10} conidia.g^{-1}, germination of 96%, moisture content of 4.2% and a protection against ultraviolet radiation of 100% and demonstrated efficacy.

Key words: Whiteflies, biological control, biopesticides

Introduction

Whiteflies are considered one of the world's major agricultural pest groups, attacking a wide range of crop hosts and causing considerable crop loss, as direct feeding pests and virus vectors (Bellotti et al., 1999). These insects are of great concern because of their ability to transmit viral diseases and develop tolerance and resistance against pesticides (Faria and Wraight 2001, Cardona et al., 2001). For this reason, the objective of the present work was to develop a formulation based on a native isolate of *Lecanicillium lecanii* easy to apply and with adequate technological characteristics and high biocontrol activity.

Material and methods

Entomopathogenic fungus

The isolate of *Lecanicillium lecanii* VL026 previously selected for its high biocontrol activity against *T. vaporariorum* and *B. tabaci* was supplied by the Microorganisms Collection of the Biological Control Laboratory (Center of Biotechnology and Bioindustry, Corpoica).

Mass production and formulation

A biphasic fermentation process for *L. lecanii* was using 4 days liquid inoculum production in an airlift fermentor and subsequent inoculation of a solid matrix. For solid fermentation, substrates based on cereals supplemented with 2% yeast extract and 2% malt extract were incubated at 25°C for 8 days. Once an adequate culture process for mass production was selected, the separation process of the biomass was standardized by centrifugation. From the obtained biomass, pre-formulation surveys were made. The active ingredient of the biocontrol agent (conidia) and the possible excipients of the prototypes were characterized, determining

their particle size, humidity, volume, fluidity and pH. Simultaneously, the effect of the excipients on the viability of the microorganism was determined through a three-month compatibility study, consequently selecting the most innocuous. Afterwards, the formulation prototype was further improved evaluating different processes of coating and drying as well as carrying out physical and microbiological characterization of the prototype and determining their stability under storage conditions. The efficacy of the final formulation was then determined under field conditions.

Efficacy studies

The efficacy of the formulation based on *L. lecanii* at a concentration of $1x10^7$ conidia.mL^{-1} was determined against *T. vaporariorum* and *B. tabaci* on commercial tomato crops (var. Chonto) in the greenhouse. It was also evaluated against *B. tabaci* in melon. IPM plots were compared with a conventional treatment (farmer's) consisting on the application of chemical insecticides. Presence of adults and immature stages was determined weekly and total yield at harvest was recorded. Treatments consisted of: 1) conventional chemical management (farmer), 2) application of the *L. lecanii* and Acetamiprid according to population levels, 3) application of biopesticide every 15 days starting from the first record of eggs or adults and 4) integrated pest management (IPM), including yellow traps, Thiamethoxam, *L. lecanii* and Acetamiprid application according to population levels. The trial was arranged in a randomized complete block design with four treatments and four replicates of each treatment. Each plot consisted of six rows of plants, each 10 m long. *T. vaporariorum* adults captured in a monitoring trap located in the top third of a plant were quantified. Eight days after each biopesticide application, the percentage of *T. vaporariorum* nymph infection was evaluated and after completion of the crop cycle, yields were assessed.

L. lecanii was also evaluated for controlling *B. tabaci* in a cherry tomato and in melon under commercial greenhouse and field conditions, respectively. Treatments consisted of: 1) conventional management (application of Thiamethoxam), 2) application of *L. lecanii* according to population levels with a total of ten applications. *B. tabaci* adults captured in yellow monitoring traps were quantified. After completion of the crop cycle, yields were assessed. For tomato each treatment was evaluated in an area of 816 m^2, divided in two plots containing eight rows of 45 m. Ten applications of Thiamethoxam or *L. lecanii* were performed in treatments 1 and 2, respectively, from the fifth day after transplantation until day 82. For melon each treatment was evaluated in an area of 800 m^2 containing eight rows of 90 m. Eight applications of Thiamethoxam and six applications of *L. lecanii* were performed in treatments 1 and 2, respectively, during the first 40 days after planting.

For the field trials, the data were analysed using ANOVA with significant level ($P<0.05$) and analyzed using Duncan's multiple range test.

Results and discussion

Mass production and formulation

When substrates based on cereals (A1) or cereals supplemented with yeast and malt (S1) were evaluated for mass production of *L. lecanii* VL026, no significant differences were observed ($P>0.0001$) among the evaluated treatments. After 8 days of incubation at 25°C, yields for the substrate Al were $1.6x10^9$ conidia/cm^2 and $1.3x10^9$ conidia/cm^2 when they were supplemented with yeast extract and malt extract. For S1 there were 1.6 $x10^9$ conidia/cm^2 and 1.9 $x10^9$ conidia/cm^2 respectively, while when nutrient solution was replaced by water, $2.1x10^9$ conidia/cm^2 was obtained. Therefore the latter substrate was selected for VL026 production.

Conidia separated from its culture medium were microbiologically and physically characterized and formulated. The product based on *L. lecanii* was designed as a wettable powder for foliar application with a concentration of 10^{10} conidia g^{-1} with a conidial germination of 96%, moisture content of 4.2%, particle size of <45 µm and a protection against UVC radiation of 100% after six hours of exposition.

Efficacy studies

The percentage of *T. vaporariorum* nymph infection was evaluated eight days after application and production was measured at the end of the crop cycle. Adults captures were 118.8; 148.9; 128.5 and 115.8 for the treatments 1) chemical management, 2) *L. lecanii* and Acetamiprid application according to population levels, 3) application of *L. lecanii* each 15 days, and 4) IPM, respectively. The infection produced by *L. lecanii* in sampled nymphs were 1.3%, 36.6%, 68.1% and 25.0%, respectively, whereas the tomato crop yields expressed as t ha^{-1} were 64.44; 67.84; 68.01 and 67.26, respectively (Table 1).

Table 1. Effect of *L. lecanii*, chemical and integrated control on *T. vaporariorum* in tomato (Chonto) under commercial greenhouse conditions

Treatment	# Applications				Nymphs with *L. lecanii*	Yield	Profit
	Bio.	Carb.	Acet.	Thia.	(%)	(t ha^{-1})	($US)
Chemical	--	1	4	--	1.3 d	64.44	4,352
Bio + Chem.	6	--	1	--	36.6 b	67.84	5,559
Biocontrol	11	--	--	--	68.1 a	68.01	5,606
IPM	2	--	1	1	25 c	67.26	5,600

Bio: *L. lecanii*; Carb: Carbofuran; Acet: Acetamiprid; Thia: Thiamethoxam. Applications were performed for *L. lecanii* at 200-500 nymphs/trifolium and chemicals at 500 nymphs/trifolium. The values with the same letter are not significantly different according to Duncan $P<0.05$.

Table 2. Effect of *L. lecanii* and chemical insecticides on *B. tabaci* in cherry tomato and melon under commercial greenhouse conditions

Treatment	Production (t ha^{-1})	Production costs ($US)	Incomes ($US)	Profit ($US)	Benefit/ cost index
Tomato Biocontrol	22.7	2,778	9.93	7.14	3.5
Tomato Chemical	21.9	3,334	9.58	6.24	2.8
Melon Biocontrol	29.4	5,073	9.68	4.61	1.9
Melon Chemical	25	5,023	8.23	3.21	1.6

Benefit/cost index: Income/production cost. 1 kg tomato: $0.43 US, kg melon: $0.43 US

When the biopesticide based on *L. lecanii* was evaluated against *B. tabaci* on a commercial cherry tomato a higher yield was observed with 22.7 t ha^{-1}, whereas in conventional management plots a yield of 21.9 t ha^{-1}were obtained with a benefit cost index of 3.5 and 2.8, respectively. Similar results were obtained in a commercial melon (Table 2).

The present investigation explored the potential of *L. lecanii* as effective tool to control two whiteflies, which often coincides in the same crop and thus need to be controlled in both with the same product. It has been demonstrated that the formulation with *L .lecanii* is an effective tool to control whiteflies *T. vaporariorum* and *B. tabaci*. The use of unformulated

fungal product is generally not advisable, although many biological control products on the market based on fungi fall into this category. The performance of unformulated products may also be adversely affected by unfavourable environmental conditions, such as low relative humidity and high UV radiation. Formulated products, containing such materials as diluents, humectants, adherents and UV-protectants, have great potential to provide better, more consistent results. Formulated conidia of *L. lecanii* developed in the present work were more resistant to UV radiation than unformulated conidia . They are also more efficient against both whiteflies under field conditions, as demonstrated also by Alves et al. (1998).

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Side-effect of the entomopathogenic fungus *Lecanicillium muscarium* on the predatory mite *Phytoseiulus persimilis*

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Abstract: In biological control, different beneficial organisms have to be combined for an effective management. If entomopathogenic fungi will be integrated, than the effect on non-target organisms like beneficial arthropods has to be considered as well. Because of the high importance of the predatory mite *Phytoseiulus persimilis* in biological control, side effects of *Lecanicillium muscarium* on this species were assessed. In two standardised bioassay in Petri dishes and on plants (*Phaseolus vulgaris)* individual mites were dipped in suspension or put on leafs after spraying with *L. muscarium* at different spore density. Results indicate pathogenicity of the fungus towards the predatory mite, however, the risk of infection decrease, the closer the conditions are to application density used under practical conditions. At concentration of 10^6 and 10^7 spores ml^{-1} no risk for predatory mite is expected on the plant.

Key words: *Lecanicillium muscarium*, syn. *Verticillium lecanii*, *Phytoseiulus persimilis*, biological control, side-effects, predatory mite, entomopathogenic fungus

Introduction

A high efficacy of entomopathogenic fungus *Lecanicillium muscarium* (syn. *Verticillium lecanii*) was indicated in many experiments against sucking insects. The same was recorded for our strain V 24, which has a high virulence against the white fly and different species of thrips (Sermann and Büchner, 1998; Alavo et al., 2001; Dimitrov, 2005; Meyer, 2007). To combine different beneficial organisms in biological control systems it is necessary to examine their compatibility. In case of the entomopathogenic fungus *L. muscarium* we have a great interest to assess the level of risk for non-target important animal antagonists. For this reason, investigations were carried out on the side effect of strain V 24 of *L. muscarium* (Zare and Gams, 2001) and trials were started at first with the predatory mite *Phytoseiulus persimilis.* We looked at adhesion of spores, mortality of mites and development of population of *P. persimilis* after different conditions of contact.

Material and methods

In standardised Petri dish trials the efficacy of the fungus after direct (dipping of *P. persimilis* into fungal spore solution) and indirect exposure (spraying of fungal suspension on leaves with *Tetranychus urticae*) of predatory mites was determined. In a second standardised biotest *Phaseolus vulgaris* were colonized with *T. urticae*. Twelve plants were sprayed with *L. muscarium* V 24 at different spore density from $2x10^5$ up to $2x10^8$. After application 2- 3 predatory mites were put on each leaf. Pots were transferred into special cages (40 x 40 cm) in a growth chamber. Counting of alive, dead or mouldy dead individuals was 4, 7, 9 and 11 days after inoculation.

Table 1. Treatments for application of the entomopathogenic fungus *Lecanicillium muscarium* on predatory mite *Phytoseiulus persimilis*

Test method	**Application form**	**Spore density Spores ml^{-1}**
Fluorescence marked conidia	Dipping and spraying	2x10^5 2x10^6 2x10^7
Petri dish	Dipping	2x 10^5
	Dipping or spraying resp.	2x10^6
	Dipping or spraying resp.	2x10^7
	Dipping or spraying resp.	2x10^8
On plant	Dipping or spraying resp.	2x10^6
	Dipping or spraying resp.	2x10^7

Results and discussion

The results differ according to spore density and exposure. Spores can adhered on the body of predatory mite, but up to 85% of spores were lost within 24 h. On the other hand, it was demonstrated that predatory mites may also pick up spores from the substrate. No danger is related to exposure of a spore density of 2 x 10^5 spores ml^{-1}. At higher density of 10^6 and 10^7 spores ml^{-1} the number of dead individuals much depends on humidity conditions. Only few predatory mites were dead on plants with lower humidity (4.2 % mortality at 2 x 10^6 and 12.7 % at 2 x 10^7 spores ml^{-1}).

Table 2. Corrected mortality of *Phytoseiulus persimilis* after application of *Lecanicillium muscarium* V 24 at different spore density and application method

Application Method	**Biotest**	**Spore density (spores ml^{-1})**	**Corrected mortality (%)**
Dipping	Petri dish	2x10^6	12,2
		2x10^7	67,1
	Plant	2x10^6	8,9
		2x10^7	29,8
Spraying	Petri dish	2x10^6	9,8
		2x10^7	38,1
	Plant	2x10^6	4,2
		2x10^7	12,7

Under more humid conditions in Petri dishes the number of living predatory mites was lower than on plants at a spore density of 10^7 spores ml^{-1}. For practical use spore density of

10^6 and 10^7 are more important and results indicate that an infection of predatory mites is possible at these spore concentrations. But this risk will be less under conditions similar to those in greenhouses. In consequence, these results have to be checked under greenhouse conditions as well.

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Entomopathogenic fungi and the codling moth, *Cydia pomonella*: Comparison of different fungal isolates and the effect of CpGV resistance on the infectivity of fungi

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Abstract: Biological control of the codling moth by its granulovirus is a success story. It has never been questioned until the emergence of resistant *Cydia pomonella* populations in Germany. Especially organic farmers, which have not many effective alternatives available, are highly interested in the development of other biocontrol agents against this key pest insect. Entomopathogenic fungi are well known as important, natural mortality factor of diapausing larvae and could be exploited as part of a resistance management strategy, together with the use of pheromones, *Bacillus thuringiensis* and entompathogenic nematodes. In this study, we therefore compared the infectivity of three different fungi (*Beauveria* sp., *Isaria* sp. and *Lecanicillium* sp.), which had been isolated from *C. pomonella* larvae, with three different fungi originating from commercial products (Naturalis L®, PreFeRal® and Mycotal®) in bioassays at 20°C against 5th instar larvae of a Cp-GV-susceptible and a less susceptible population. Primarily, each pair of fungal isolates (e.g. *Beauveria* sp. from codling moth and *B. bassiana* from Naturalis L®) was compared at three different concentrations (10^6, 10^7, 10^8 spores per ml) by a dipping method using 20 larvae per treatment. In a final assay, all isolates were compared at one concentration (10^7 spores/ml), using a susceptible and a completely resistant *C. pomonella* population.

All fungal isolates could infect and kill larvae of the three different *C. pomonella* populations. In the three pairwise comparisons of isolates, usually the larvae of the susceptible population died faster, than the larvae of the less susceptible population, except for Mycotal®, were the average survival time of the susceptible population was longer (7 days, compared to 4 days, respectively). Only with *Beauveria* the wild isolate was more infectious than the commercial isolate. In the other combinations, the commercial isolate was as good as the wild isolate (*Lecanicillium*-pair) or even better (*Isaria*-pair). When all fungi were compared, there were no differences in the susceptibility of the two codling moth populations and the most infectious fungus was the wild *Beauveria* sp. isolate. In conclusion, there is a high potential for the development of new biocontrol preparations from wild isolates, but commercial isolates could also be used effectively. The susceptibility of different *C. pomonella* populations towards different entomopathogenic fungi varies, but the degree of CpGV resistance does not seem to have any influence on the susceptibility to entomopathogenic fungi.

Key words: Entomopathogenic fungi, *Cydia pomonella*, Cp-GV resistance, infectivity

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Metarhizium anisopliae interactions with ticks' eggs

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Abstract: Efficacy of two entomopathogenic hyphomycetes fungi, *Metarhizium anisopliae* var. *acridum (M. an. ac.*) and *M. anisopliae* var. *anisopliae (M. an. an.*) was evaluated against eggs of three tick species – *Hyalomma excavatum*, *Rhipicephalus annulatus* and *Rhipicephalus sanguineus*. Eggs laid by surface-sterilized females were spread on conidia-impregnated filter paper. Although *M. an. an.* differed in their virulence to the tested ticks, they reduced the hatching percentages of eggs of all three tick species to 0–32% compared with 80–90% in the control eggs. The *M. an. ac.* strains were highly virulent to *H. excavatum* and *R. sanguineus* eggs, reducing the hatching percentages to 2–6 %, but had no influence on hatching of *R. annulatus* eggs. The effects of lipid fractions from tick eggs surface on the development of conidia were tested. Both germination of *M. an. an.* conidia and the formation of appressoria were stimulated by extracts from egg cuticles of all three tested tick species. However, the stimulating effect was lower when the conidia were exposed to lipids from relatively less susceptible *R. annulatus* eggs than when exposed to lipids from *H. excavatum* or *R. sanguineus* eggs. Conidia of *M. an. ac.* exposed to such lipid extracts did not germinate or form appressoria.

Key words: Biological control, *Metarhizium anisopliae,* tick eggs, *Hyalomma excavatum, Rhipicephalus (Boophilus) annulatus, Rhipicephalus sanguineus*

Introduction

Entomopathogenic fungi seem to play an important part in preventing the increase of tick populations, but still relatively little information exists on their specific role in killing tick eggs. Entomopathogenic fungi were never isolated from tick eggs and relatively rarely from other tick stages, in nature (Costa et al., 2002, Fernandes et al., 2004, Polar et al., 2005) even though they caused high mortality of tick eggs under laboratory and field conditions (Kaaya et al., 1996, Bittencourt et al., 2000, Gindin et al., 2001). Our previous experiments demonstrated the low virulence of two *Metarhizium anisopliae* var. *acridum* (*M. an. ac*) strains to *Rhipicephalus annulatus* eggs, larvae and engorged females (Gindin et al., 2001), but Onofre et al. (2001) observed that two strains of this fungus reduced the hatching success of *R. microplus* eggs by 70–80%. The epicuticle of both insect and tick eggs is covered with a layer of wax, which is the initial site of the fungus-host interaction. The lipids associated with the insect epicuticle can stimulate or inhibit the germination of entomopathogenic fungal conidia (Szafranek et al., 2001, James et al., 2003). The cuticular lipids of the fungus-resistant adults tick *Amblyomma americanum* were found to inhibit the germination of *Beauveria bassiana* conidia, whereas no inhibition was observed by the cuticular lipids deriving from adults of the susceptible tick *A. maculatum* (Kirkland et al., 2004).

This study evaluates the ovicidal effect of several *M. an. ac*. and *M. an. an*. strains on eggs of three tick species – *Hyalomma excavatum, R. annulatus* and *R. sanguineus*, including the role of egg cuticular lipids on the germination and formation of appressoria of two *M. anisopliae* subspecies.

Material and methods

Fungi and bioassay

Fungal strains were grown on Sabouraud dextrose agar (SDA, Difco) for 2-3 weeks at 25 °C, conidia were suspended in sterile distilled water + 0.01 % Triton X-100 and filtered through Miracloth. The suspensions were vortexed and sonicated for 5 min. Conidia concentrations were determined with a hemacytometer.

R. annulatus ticks were surface sterilized by dipping in 0.5 % (w/v) methyl p-hydroxy-benzoate and 0.1% sodium benzoate (5 min. in each). The females were incubated in sterile Petri dishes with moist filter paper for oviposition at 25 ºC. Only eggs laid during the first 6 – 7 days after drop off were used. Filter paper was impregnated with 0.5 ml of sterile water containing 0.01% Triton X-100 without (control) or with fungal conidia at 10^6 conidia per ml. Eggs (50–100 per dish) were distributed on the filter paper with a camel-hair brush and incubated at 25°C and 100% RH. The numbers of infected eggs (mycelia development) and the numbers of emerged larvae were counted. Each treatment was replicated four or five times for each tick species and for each strain, and the whole bioassay was repeated twice.

Extraction of lipids from egg cuticles

Batches of 360-mg eggs were transferred to glass vials, immersed in 3 ml of 100% analytical grade pentane and agitated by hand for 5 min. Pentane was removed to a clean vial and evaporated down to 120 µl under N_2 gas, and 20 µl aliquots (equivalent to 60 mg of eggs) were immediately placed on cover slips. Immediately after evaporation of the solvent, the cover slips with the lipids spots were used for a germination assay.

Conidia development assay

Petri dishes with 2% LE-Agarose (SeaKem, Cambrex) were inoculated with 0.1 ml of conidia suspension containing 1×10^6 conidia per milliliter. The cover slips with a spot of the lipids from the egg surfaces were placed on inoculated Agarose. The Petri dishes were sealed with Parafilm and incubated at 25ºC for 12 or 42 h. Then, cover slips on were placed on a glass slide with Lacto phenol blue and the numbers of germinated conidia and the numbers that developed appressoria were counted. Each test included four to six repetitions for each solvent and was repeated two or three times. About 100 conidia were counted per repetition. Cover slips treated only with solvents were used as controls.

Results and discussion

In the present study, eggs of three tick species were found to be highly susceptible to three *M. an. an*. strains (7, K and 108) which reduced their hatchability to 0–8.5%, compared with 81–89% in the control groups (Table 1). The isolate *M. an. an.* - 43 was less pathogenic to *R. sanguineus* and *R. annulatus* eggs but, nevertheless, reduced their hatchability to 23.5–30.6%, and no *H. excavatum* eggs hatched. At 7 d PI, *R. annulatus* eggs were more resistant to most *M. anisopliae* strains than those of *R. sanguineus* or *H. excavatum* (Table 1). *M. an. ac.* also exhibited high efficacy in reducing the hatchability of eggs of two tick species: *H. excavatum* and *R. sanguineus*. However, *R. annulatus* eggs were almost totally resistant to the two tested *M. an. ac*. strains, even at very high doses of conidia (Table 1).

The various substances on the surface of host cuticle may determine the arthropod's susceptibility or resistance to fungi. The present study demonstrated that non-polar lipids extracted from egg cuticles of the three tested tick species stimulated both the germination of *M. an. an.* conidia and the formation of appressoria. Nearly 100% of the germinated conidia

that were kept in contact with extracts from *R. sanguineus* or *H. excavatum* eggs, formed appressoria within 42 h of exposure. The stimulating effect of the extracts from *R. annulatus* eggs was found to be less powerful, stimulating only 27–30% of the germinated conidia to form appressoria (Table 2).

Table 1. Mean percentage of infected eggs (7 d PI) and hatchability (21 d PI) of eggs infected with *Metarhizium anisopliae* strains and incubated at 25^0C and 100% RH

Fungus	*Rhipicephalus annulatus*		*Rhipicephalus sanguineus*		*Hyalomma excavatum*	
Fungus	Infected eggs (Mean % ± SD)	Hatchability (Mean % ± SD)	Infected eggs (Mean % ± SD)	Hatchabiliy (Mean % ± SD)	Infected eggs (Mean % ± SD)	Hatchabiliy (Mean % ± SD)
M.an.an						
7	66.7 ± 4.0A	0a	87.7 ± 9.6B	8.2 ± 2.2b	98.0 ± 4.4B	1.8 ± 2.0a
K	53.5 ± 7.4A	0a	69.5 ± 11.6AB	8.5 ± 2b	97.0 ± 3.9B	0a
108	38.0 ± 13.0A	2.0 ± 2.2a	80.6 ± 3.5B	2.2 ± 4.3a	95.1 ± 6.5C	0a
43	11.7 ± 6.0A	30.6 ± 5.6b	42.1 ± 4.7B	23.5 ± 9b	66.8 ± 18.0C	0a
M.an.ac						
5	0A	91.5 ± 3.3b	10.9 ± 2.3B	2.5 ± 2.9a	7.5 ± 2.7B	1.7 ± 2.0a
11	0A	78.4 ± 3.5b	13.1 ± 2.7B	3.0 ± 2.3a	10.0 ± 4.6B	5.8 ± 5.4a
Control	0A	85.0 ± 6.5a	1.0 ± 1.9A	81.3 ± 3.0a	0.8 ± 2.2A	89.0 ± 2.2a

Means within a row followed by the same letters (capitals for infected eggs; lower case for hatchability) are not significantly different ($P < 0.001$).

Table 2. The effect of pentane extract from surface of tick eggs on *M. anisopliae* var. *anisopliae* conidia germination (CG) and mean percentage conidia forming appressorium (CFAp)

	Exposure time of conidia to Pentane lipids extracts	
Tick species	CG (Mean* % ± SD), 12 h PI	CFAp (Mean* % ± SD), 42 h PI
R. annulatus	57.0 ± 12.6B	30.3 ± 5.2B
R. sanguineus	98.5 ± 1.0C	100C
H. excavatum	57.6 ± 10.0B	100C
Non (control)	2.5 ± 2.4A	0A

Means within a column followed by the same letter are not significantly different ($P < 0.001$).

In the case of *M. an. ac*., although eggs of two tick species (*H. excavatum* and *R. sanguineus*) were highly susceptible to its infection, none of the conidia of *M. an. ac*.-5 germinated after contact with any of the lipids extracts within 12 h of contact, and hardly any (1.8–6.5%) after 42 h of contact. No appressoria were observed among the few germinated *M. an. ac.* -5 conidia exposed to either lipid fraction. Thus, it is clear that the substance(s) which induce conidial germination are neither lipids nor lipid-dissolved compounds.

Control of ticks can be applied at two main points: while they feed on their vertebrate host or on the ground while resting or questing. The present results suggest that entomopathogenic fungi can be considered as a potential means for reducing tick populations by

killing egg masses of economically important ticks. However, field experiments should be performed, so as to demonstrate the potential contribution of fungi to tick population control.

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Further progress with *Metarhizium* microsclerotial production

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Abstract: Microsclerotium production by *Metarhizium anisopliae*, previously reported for flask scale, was successfully achieved at a 100-Liter fermenter scale. The resulting granular formulations readily conidiated on water agar or in moist soil to the same extent as reported for flask fermentations. Both Pharmamedia and Hycase M were suitable substitutes for the original casamino acids, with the former being slightly superior in terms of subsequent conidial production in soil. Both were slightly superior to casamino acids in terms of efficacy against the sugarbeet root maggot in non-sterile clay soil. Rates, as low as 20 mg microsclerotial formulation per 100 g non-sterile clay soil yielded 50% larval sugarbeet root maggot mortality within 1 week and > 90% after 2 weeks.

Key words: *Metarhizium anisopliae*; formulation; microsclerotia; root maggot

Introduction

Granular formulations of the entomopathogenic ascomycetes, *Metarhizium anisopliae* and *Beauveria bassiana*, have historically taken several forms, e.g., conidia bound to inert or nutritive carriers, spent medium from solid substrate fermentation, dried mycelium, or mycelial fragments or blastospores embedded in an alginate matrix. All, however, suffer various disadvantages: expense, short room-temperature shelf-life, improper sizing for standard farm implements. Jaronski and Jackson (2008) and Jackson and Jaronski (2009) discovered that under certain liquid culture conditions, namely high glucose (36 g/L) and C:N ratios in excess ≥30:1, *M. anisopliae* produces undifferentiated, melanized, compact, hyphal aggregates that we termed microsclerotia (MS). These bodies strongly resemble the microsclerotia of plant pathogenic fungi and can be easily prepared into granular formations by filtration of liquid cultures and simple air drying. The formulations can then be sized by sieving to match specifications of most agricultural granular applicators. The MS conidiate readily when rehydrated. MS granules were highly efficacious against sugarbeet root maggot, *Tetanops myopaeformis* (SBRM) in soils and superior to conventional granules composed of a nutritive carrier (maize meal) coated with conidia (Jaronski and Jackson, 2008). Our research had several objectives: (1) assessing yields and conidial production by MS produced in 100 L fermentation; (2) examining the effect of substituting more economic nitrogen sources on yield, subsequent conidial production, and efficacy by MS formulations; and (3) identifying minimal use rates for efficacy against larval SBRM in soil bioassay.

Material and methods

The *M. anisopliae* used through all experiments was Novozymes Biologicals Strain F52 (ATCC 90448), passed through *Tetanops myopaeformis* larvae. For the culture experiments, conidia were obtained from 2-3 week-old potato dextrose agar cultures. The medium throughout these experiments consisted of a basal salts and vitamin medium with glucose as

carbon source and casamino acids as nitrogen source, to achieve a carbon concentration of 36 g/l and a C:N ratio of 36:1 (Jackson and Jaronski 2009). For liquid media experiments (100 ml, the casamino acids were substituted with Pharmamedia™ (Traders Protein) or HyCase™ M (Quest Int.) to achieve the same C level and C:N ratio. For scale-up tests, three-day old, 3 l precultures, as above, were used to inoculate 100 Liters of medium (using casamino acids as N source) in a Biostat D100 fermenter (B. Braun) equipped with three rushdin impellors and side baffles. Fermentation conditions were 28°C., 400 RPM and 50 SIPM.

Flask cultures were harvested after 4 days. Diatomaceous earth (HYFLO®, Celite Corp.) was added at a rate of 5% (w/v) and the cultures filtered under vacuum through Whatman No 54 filter paper; the resulting filter cakes were broken up by pulsing in a kitchen food blender, layered in shallow aluminum trays, and air-dried overnight in the air flow to a final moisture of <5% (gravimetric). The dry material was passed through a No. 20 mesh onto a No. 30 mesh sieve to collect granule particles 0.6-1.5 mm diameter. These MS-containing granules (MSg) were stored at 5-8°C in vacuum-sealed polyethylene bags. The 100 l cultures were also mixed with HYFLO (5% w/v) but biomass was concentrated with a rotary drum vacuum filter. The moist filter cake was crumbled into smaller pieces, which were then ground in a commercial grinder (Quadro Engineering Co.) and subsequently air-dried overnight.

Conidial production by MSg was determined by sprinkling 25 mg of unsieved formulation onto the surface of each of 2 water agar plates, which were then incubated at 28° C for 8 days. Each plate was then flooded with 5 ml of sterile water, the conidia mechanically dislodged, available liquid was pipetted from each plate and its volume measured, and conidial concentrations determined hemocytometer count. Conidial production in soil was studied in the non-sterile clay soil used for bioassays, described below, and determined in soil samples by serial soil dilution plating on semi-selective medium (Chase et al., 1986).

For bioassays the MSg was sieved to produce granules 0.7-1 mm diameter. These were then incorporated into an air-dried, clay soil, that was then wetted with deionized water to 15% field saturation (-2.33MPa with an Aqualab™ WP4 meter, Decagon Inc.). The treated or control soil was then dispensed equally into three 60-cc plastic condiment cups. The cups were sealed and placed on a layer of water-moistened paper towel (to maintain humidity) in a large, lidded, plastic container, and incubated at 25° C. After 1 week, the soils were infested with 10 L3 SBRM larvae/cup. Larval mortality was determined weekly for two weeks. MS granule preparations from the nitrogen source substitution experiments were bioassayed at 100 and 200 mg granules per 100 grams dry soil. We also conducted a separate series of bioassays using doses of 20, 50, 75 or 100 mg / 100 grams soil. All bioassays consisted of three replicate cups of 10 SBRM larvae each, and were conducted 2-3 times in their entirety.

Results and discussion

Scale-up to 100-liter fermentation

Microsclerotial production ranged from 1300-2100 g biomass, equivalent to or slightly lower than in <1l flasks (equivalent to 1800 g/100 l, Jackson & Jaronski, 2009). Conidial yield was slightly lower than that from <1 l flask fermentations (~$8x10^8$ conidia/g MS, Jackson & Jaronski, 2009). Yields of formulated MS were on the order of 7 kg per 100 l fermentation.

Substitution of nitrogen sources

Pharmamedia and Hycase M produced similar biomass and microsclerotia/ml culture as casamino acids. Use of Pharmamedia resulted slightly faster conidial production in soil but after 7 days conidia per gram of MS formulation was not significantly different among the three nitrogen sources.

Table 1. *Metarhizium anisopliae* F52 microsclerotial biomass produced on a 100-Liter scale, gross weight of resulting formulation (using 5% w/v HYFLO during harvest), and mean conidial production on water agar

Batch	Biomass (g)	Formulation (Kg/100 L)	Mean Conidia/g MS	S.D.
E071210	2060	7.06	5.0E+08	1.27 E+08
E080112	1308	7.30	5.6 E+08	2.83 E+08
E080119	1468	7.47	4.4 E+08	7.07 E+08
E080208B	1917	6.31	5.8 E+08	7.07 E+08
Mean	1688	7.03	5.2 E+08	
S.D.	357.8	0.512	0.65 E+08	

ANOVA $F_{3,4}$= 1.55; p = 0.33

Table 2. Effect of nitrogen source on conidial production by 100 mg microsclerotial formulation in 100 g non-sterile clay soil at 15% WHC (-2.33 MPa) and 24 C. Means in any column followed by different letters are significantly different (Tukey's HSD test, p = 0.05).

	Day 4		Day 7		Day 14	
Fermentation N Source	CFU/g soil	conidia/g of MS	CFU/g soil	conidia/g of MS	CFU/g soil	conidia/g of MS
Casamino Acids	1.11E+06	1.11E+09 b	1.62E+06	1.62E+09 a	1.91E+06	1.91E+09 a
Pharmamedia	1.98E+06	1.99E+09 a	2.40E+06	2.40E+09 a	2.29E+06	2.29E+09 a
HyCase M	9.76E+05	9.76E+08 b	1.90E+06	1.90E+09 a	1.89E+06	1.89E+09 a

Table 3. Effect of nitrogen source on efficacy of microsclerotial granules in non-sterile clay soil at 15% WHC (-2.33 MPa) and 24°C. Means in any column followed by different letters are significantly different (Tukey's HSD test, p = 0.05).

Fermentation	Day 7		Day 14	
N Source	100 mg/100g soil	200 mg/100g soil	100 mg/100g soil	200 mg/100g soil
Casamino acids	57% c	98% a	100% a	100% a
Pharmamedia	83% b	95% a	100% a	100% a
Hycase M	100% a	100% a	100% a	100% a
Untreated	0% c	0% b	0% b	0% b

Multiple dose bioassay

The lowest dose of 20 mg/100 g soil resulted in 50% SBRM mortality within 1 week and 87% after 2 weeks (Fig. 1). Higher doses, 50+ mg/100 g soil caused 100% larval mortality within 1 week. The 20 mg rate represents 1 granule per 6.25 CC soil (0.16 granules/cc), based on the size and bulk density of these MSg granules. In contrast, at least 4 granules/cc soil were needed with F52 conidia on a maize-meal, nutritive carrier to achieve high SBRM mortality and 3 weeks were necessary for full SBRM mortality to be expressed from exposure to F52 conidia on maize meal (Jaronski et al., 2007). Thus, much lower field rates are feasible with MS granules. Given the 7 kg yield of MSg formulation, a 100 l fermentation would produce enough material for 1.4-3.5 ha if formulation was incorporated in a 12-cm, band-over-row, 3 cm deep, or 28-44 ha, if applied in furrow, in a crop planted on 61 cm row spacing.

Alternatively, 7 kg MSg could be sufficient for direct incorporation into 10-27 m^3 potting medium.

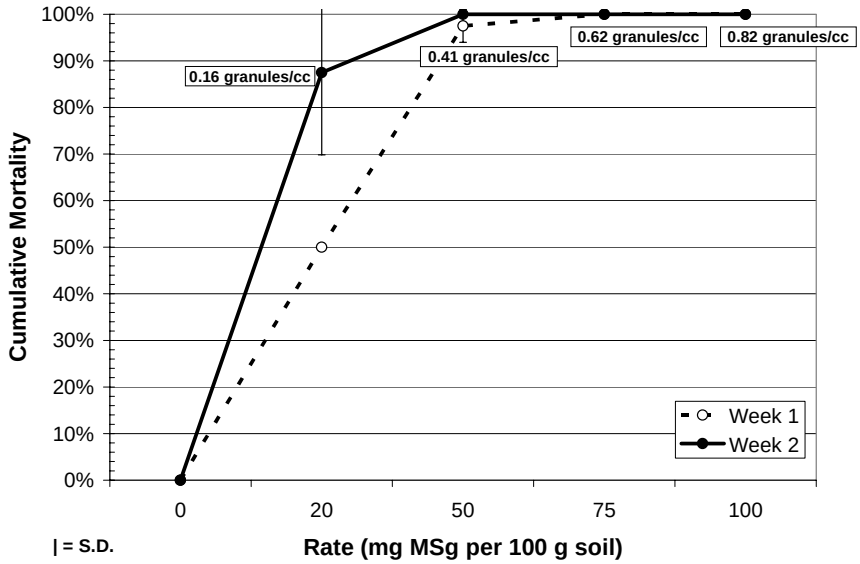

Figure 1. Cumulative mortality of sugarbeet root maggot larvae incubated in clay soil inoculated with different rates of microsclerotial granules (MSg). Data in boxes represents the number of granules per cc of soil, based on size and bulk density of the granules

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DNA polymorphisms in hybrid strains of entomopathogenic fungi *Lecanicillium* spp.

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Abstract: The entomopathogenic fungi *Lecanicillium* spp. are exploited commercially as useful biological control agents all over the world. Three strains, Vertalec and Mycotal with a high specific virulence against aphids and whiteflies, respectively, and B-2 with a high ability to colonize leaves were fused to obtain a new strain combining the beneficial characteristics into one strain. We investigated diagnostic fragments among these parental strains and some hybrid strains in order to detect molecular markers to distinguish hybrid strains. The internal transcribed spacer (ITS) and intergenic spacer (IGS) region of ribosomal DNA (rDNA) were analyzed by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) and subsequently investigated DNA haplotypes by combining primers, which amplified the ITS region and microsatellites. A uniform biased tendency of the DNA banding pattern was observed depending on the combination of parental strains. Finally, we detected unique DNA patterns generated from genome profiling (GP).

Key words: *Lecanicillium* spp., rDNA, PCR-RFLP, genome profiling

Introduction

Lecanicillium spp. (formerly *Verticillium lecanii*) are ubiquitous, entomopathogenic, mitosporic fungi that are pathogenic to various arthropods and have an extremely wide host range (Goettel et al., 2008). They are widely used as commercial microbial control agents, e.g. in the products Vertalec® and Mycotal® used against aphids and whiteflies, respectively. The B-2 strain, isolated from the green peach aphid in Japan by Koike et al. (2004) has a high colonization ability on living cucumber leaf surfaces even under conditions of low relative humidity. Aiuchi et al. (2004 and 2008) carried out protoplast fusion among these three strains to obtain hybrid strains that have a wide host specificity and can persistence on leaves at low humidity.

V. lecanii was re-classified into the genus *Lecanicillium* and divided again into several species based on morphological observation and molecular analyses by Zare and Gams (2001). From the new classification point, the fusion of Vertalec (*Lecanicillium longisporum*) x Mycotal (*L. muscarium*) appears to be an interspecific hybridization. On the other hand, Mycotal x B-2 (*L. muscarium*) is intraspecific.

In a previous study, genomic DNA and mitochondrial DNA (mtDNA) of the hybrid strains were analyzed by molecular method in order to assess the success of the protoplast fusion (Aiuchi et al., 2008). In both analyses, a uniform biased tendency of the banding pattern was observed depending on the combination of the parental strains. These fragments were difficult to utilize as molecular markers. In this study, we analyzed the hybrid strains by

various molecular methods, by restriction fragment length polymorphisms (RFLP), microsatellite analyses and genome profiling (GP) and compared the results with those of the parental strains. A final goal of the study was to detect diagnostic banding patterns which can be used as molecular markers.

Material and methods

Fungal strains and DNA extraction

This study used Vertalec, Mycotal (Koppert, NL), B-2 and hybrid strains, 2aF43 (Mycotal x B-2) and AaF42 (Vertalec x Mycotal). Mycelium DNA was purified using a Nucleon PhytoPure kit (Scotlab Ltd., Strathelyde, UK) according to the manufacture's instruction.

DNA amplification and RFLP analysis

The ITS and IGS region of rDNA was amplified using the primer pairs ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and CNL12 (5'-CTGAACGCCTCTAAGTCAG-3') and CNS1 (5'-GAGACAAGCATAT-GACTACTG-3'), respectively. PCR amplification was carried out by using thermal cycling protocols previously described in Sugimoto et al. (2001). PCR products were individually digested with the following restriction enzymes: *Sau*3AⅠ, *Msp*Ⅰ, *Hae*Ⅲ, and *Rsa*Ⅰ (Takara Bio INC., Japan). The restriction products were separated on 3% agarose gels by electrophoresis and visualized by ethidium bromide staining.

Combined use of primers to amplify the ITS region and microsatellites

Fourteen regions were amplified using the following primer together with primers ITS4 and ITS5:GCC, GTG, TGTC, GACA, $(GAA)_6$, $(GGAT)_4$,$(CA)_8$GT. Amplification used the same thermal cycling protocols as those used in ITS region PCR; however, in this case, annealing temperature was reduced to 45°C. The PCR products were separated and visualized as described above.

Genome profiling

Two methods, random PCR and temperate gradient gel electrophoresis (TGGE) were used. Random PCR was performed using four primers pfM12 (FITC 5'-AGAACGCGCCTG-3'), B83 (FITC 5'-CAGGCCGAAGTC-3'), B85 (FITC 5'-ACGGGTCGTAAC-3'), and B89 (FITC 5'-ACTAACCTGGAC-3') (Hatakeyama et al., 2008). The random PCR reaction mixtures were adjusted according to Hatakeyama et al. (2008). PCR conditions were: 95°C for 2 min followed by 35 cycles of reactions at 94°C for 15 sec, 40°C for 30 sec and 50°C for 30 sec, and a final extension at 72°C for 1min. The electrophoresis conditions for TGGE were acording to Hatakeyama et al. (2008). The gels were stained with ethidium bromide and photographed.

Results and discussion

RFLP analysis and combined amplification of the ITS region and microsatellites

All RFLPs using four enzymes and the fragments amplified by the combined primers resulted in patterns of either parental strain. The hybrid strains AaF42 (Vertalec x Mycotal) exhibited the patterns of Mycotal, while 2aF43 (Mycotal x B-2) was the B-2 type. These results were also reported by Aiuchi et al. (2008). As a consequence of these results a genetic predominance is proposed as follows: Vertalec< Mycotal< B-2. In other words, *L. longisporum*< *L. muscarium*.

Genome profiling

Random PCR was performed using DNA extracted from hybrid strains and parental strains as templates and the B83 primer. It was difficult to distinguish between hybrid and parental strains with one dimension electrophoresis, because random PCR amplified plural numbers of products. When these PCR products were analyzed by TGGE, significant differences were noted (Fig. 1A-E). The profile image of the migration pattern of AaF42 (Fig. 1E) was similar to Mycotal (Fig. 1B), and 2aF43 (Fig. 1D) was similar to B-2 (Fig. 1C), apparently. That of Vertalec (Fig. 1A) was unique. However, several specific bands were observed. The profile images using other primers confirm the uniform biased tendency of the DNA banding pattern obtained in the analysis of the ITS and microsatellite region.

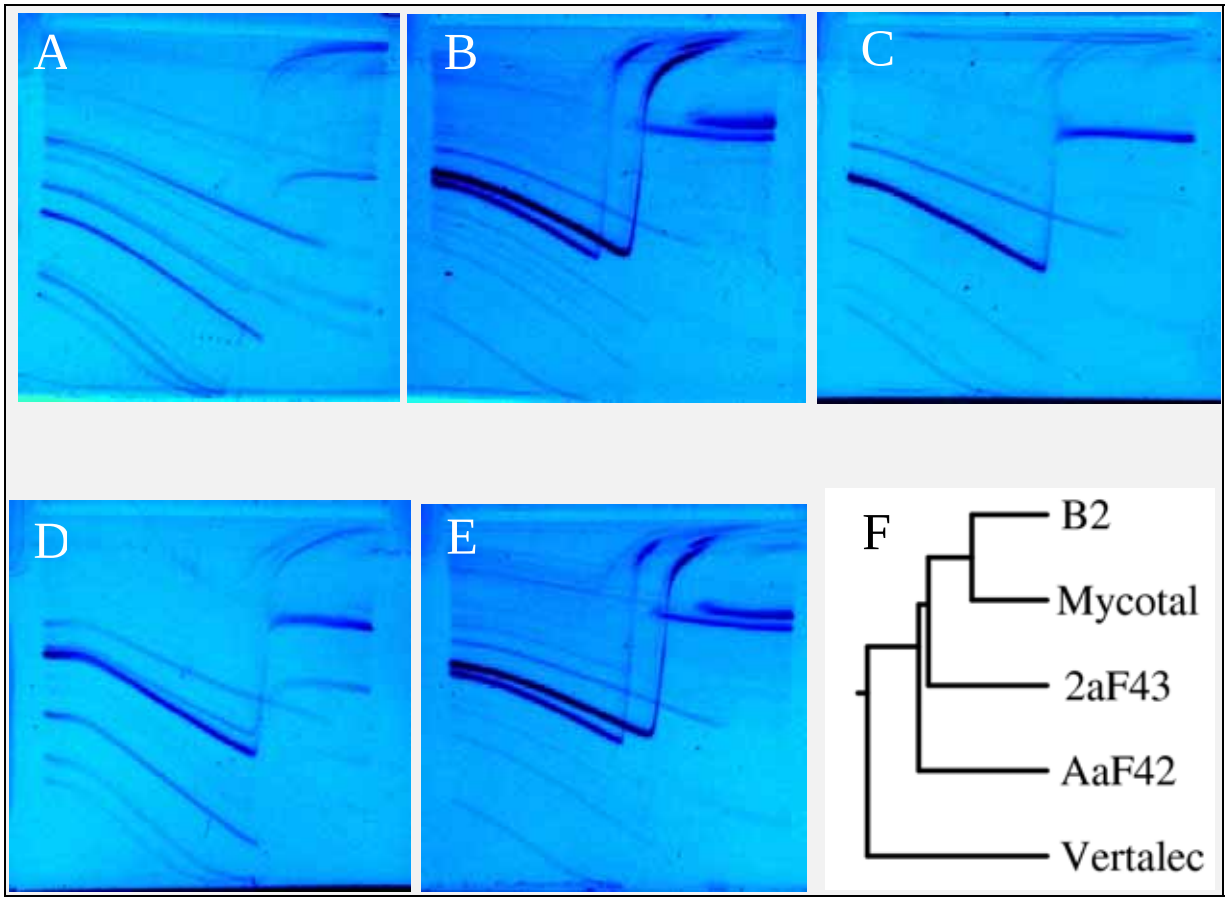

Figure 1. Genome profiling using the B83 primer and dendrogram of parental and hybrid strains. A to E show the profiling images of TGGE. A: Vertalec, B: Mycotal, C: B-2, D: 2aF43, D: AaF42. E: Dendrogram reflecting genetic similarities of strains based on their *PaSS* value. Cluster analysis was performed using the unweighted pair group method analysis (UPGMA).

GP estimates the similarity of genomes by comparing species identification dots (Spiddos) extracted from the profile images as the pattern similarity score (Pass). Pass was shown to be useful for quantitatively measuring the closeness between species (Naimuddin et al., 2000).

Phylogenetic analyses were performed using the unweighted pair group method analysis (UPGMA) based on their Pass value (Fig. 1F). Mycotal and B-2 are closely related because they belonged to the same species (*L. muscarium*). However, 2aF43, result of intraspecific

hybridization, was not so close. This genetic distance suggested that complicated insertions or deletions occurred. Takatsuka (2007) noted the genetic relatedness of isolates was associated with geographical location in *Beauveria bassiana*. Thus, genetic relatedness may differ among the Japanese (B-2) and commercial isolates (Mycotal and Vertalec) despite being from the same species. AaF42, resulting from interspecific hybridization, is less related to Mycotal. Consequently, the GP analysis can be recommended to detect subtle genetic differences.

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Fungal BCAs in viticulture: Compatibility with common fungicides

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Abstract: According to EUROSTAT, approximately 150,000 tons of fungicides were sold in the EU 15 in 2001. Seventy percent of them were applied in viticulture. Thus, the application of fungal BCAs has to be coordinated with the necessity of the conventional control of fungal pathogens. We tested the effect of fourteen common fungicides on the growth of a *Metarhizium anisopliae* isolate which is intended to be used for the control of grape phylloxera. The ASTM standard D 5590-00 was applied to determine the susceptibility of *Metarhizium* to fungicides (Standard Test Method for Determining the Resistance of Paint Films and Related Coatings to Fungal Defacement by Accelerated Four-Week Agar Plate Assay). Polyram (Dithiocarbamates) and CAA-fungicides (Melody Combi, Forum Star) inhibit the growth of *Metarhizium* when half of the recommended concentrations are used. No inhibition of growth could be observed when *M. anisopliae* was exposed to QoI-fungicides (Collis, Flint, Equation Pro), DMI-fungicide (Topas, Systhane), amines (Prosper), quinolines and pyrimidines (Vento), PA-fungicides (Ridomil Gold Combi), phosphonic acid (Phosfik) or wettable sulphur. Also Electis (dithiocarbamates) lead to no inhibition of growth. Most fungicides induced a visible increase of sporulation when used at low concentrations.

Key words: Biocontrol, *Botrytis*, fungicide, Hyphomycetes, *Metarhizium*, *Vitis*

Introduction

Fungal pathogens are a major problem in viticulture as most *Vitis vinifera* varieties have no natural resistance against alien pathogens such as powdery and downy mildews, which were introduced into Europe during the 19th century. Their control is generally achieved by wide-spread application of fungicides. In 2001 approximately 150,000 tons of fungicides were sold in the EU 15, of which seventy percent were applied in viticulture (EUROSTAT). This number is even more remarkable because viticulture covers only 8 % of the total crop aerea in the EU 15. Grapevine receives about 30 kg fungicides per hectare and year (Phytowelt 2003), two-times more than used for fruit trees. Besides fungal pathogens, grapevine suffers from severe infestation with different insect pests, among them grape phylloxera *Daktulosphaira vitifoliae* (Fitch). Former studies demonstrated that grape phylloxera can be controlled by the entompoathogenic fungus *Meta-rhizium anisolpliae* (Kirchmair et al. 2004a,b). But the potential application of the fungal BCA may be restricted by the extensive use of chemical fungicides. In the presented study we tested the *in-vitro* effect of 14 commonly used fungicides on the growth of *M. anisopliae*.

Material and methods

Fungal strains

Metarhizium anisopliae (CBS 123710) was obtained from the culture collection of the Institute of Microbiology, University of Innsbruck, Austria. *Botrytis cinerea* was isolated from grapes at the phylloxera trial sites in Geisenheim, Germany (Kirchmair et al., 2004b).

Fungicides

Tested fungicides (Table 1) were used according to the producers' specifications. Half and double of the recommended concentrations were also used in the experiments.

Table 1.Fungicides tested in agrar plate tests and indications for powdery (*Erysiphe necator*) or downy (*Plasmopara viticola*) mildew

Fungicide	Use	Group	Recommended Concentration
Collis	Powdery mildew	QoI-fungicide (**Q**uinone **o**utside **I**nhibitors)	0.04 %
Flint		QoI-fungicide	0.015 %
Prosper		Amines	0.05 %
Wettable sulphur		Sulphur	0.6 %
Systhane 20 EW		DMI-fungicide (**D**e**M**ethylation **I**nhibitors)	0.015 %
Topas		DMI-fungicide	0.015 %
Vento		Quinolines and pyrimidines	0.025 %
Electis	Dawny mildew	Dithiocarbamate	0.45 %
Equation Pro		Cyanoacetamideoxime, QoI-fungicide	0.04 %
FORUM Star		CAA-fungicide (**C**arboxylic **a**cid **a**mides)	0.12 %
Melody Combi		CAA-fungicide	0.15 %
Polyram WG		Dithiocarbamate	0.2 %
Ridomil		PA-fungicide	0.15 %
Phosfik		Phosphonic acid	0.2 %

Compatibility tests

Compatibility tests were carried out according to the ASTM standard D 5590-00 (2005). Fungi were incubated on potato dextrose-agar (PDA) for ten days at 25 °C. Spore suspensions were prepared in 0.85 % (w/v) NaCl containing 0.5 % (v/v) Tween 80 (final concentration: 1.2 spores ml^{-1}). Filter papers (3.5 × 2.5 cm, Schleicher & Schuell grade 556) were autoclaved and dried and subsequently soaked in the fungicide solutions/suspensions. Samples were air dried for 24 h and placed at the centre of agar plates. Uncoated filter paper was used as control. A sterilized atomizer was used to apply a thin coat of spore suspensions to each specimen. All experiments were performed in triplicates. Plates were incubated at 25° C and 85 % to 90 % relative humidity. Growth was rated weekly.

Results and discussion

The effect on the growth of *M. anisopliae* exposed to the tested fungicides is presented in Table 2. Polyram (dithiocarbamates) and CAA-fungicides (Melody Combi, Forum Star) inhibited the growth of *M. anisopliae* when half of the recommended concentrations were used. No growth inhibition was observed when *M. anisopliae* was exposed to QoI-fungicides (Collis, Flint, Equation Pro), DMI-fungicide (Topas, Systhane), amines (Prosper), quinolines and pyrimidines (Vento), PA-fungicides (Ridomild Gold Combi), phosphonic acid (Phosfik) or wettable sulphur. Most fungicides induced a visible increase of sporulation of *M. anisopliae* when used at low concentrations (Fig. 2). A different effect was determined when fungicide susceptibility of the grey mold *Botrytis cinerea* was tested. This fungus reacted with the formation of sclerotia to low doses of fungicides (Fig. 2).

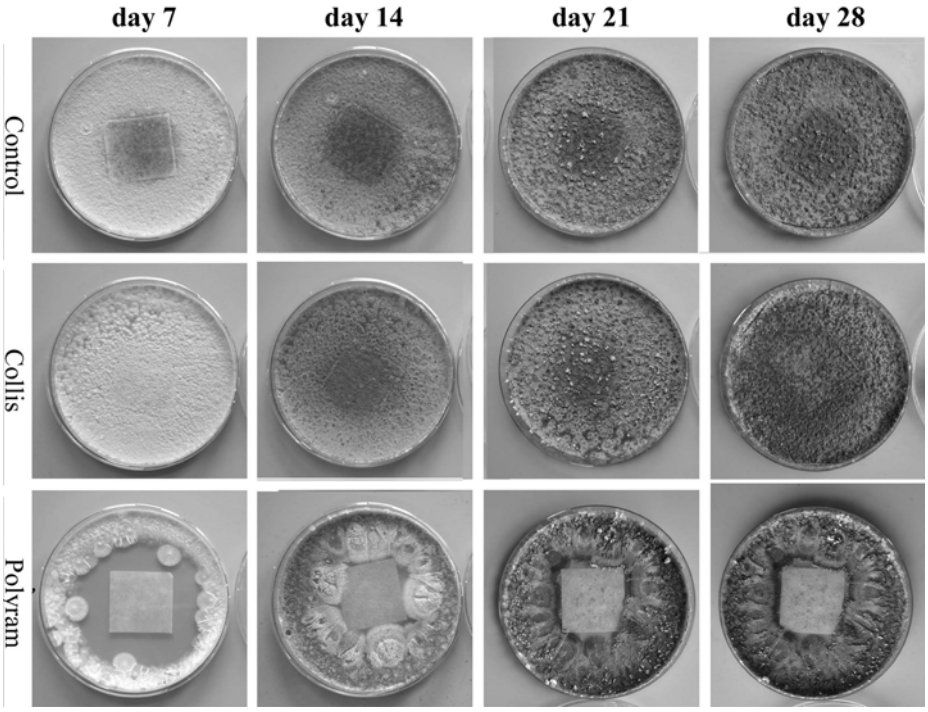

Figure 1. Testing the effect of fungicides on the growth of *Metarhizium anisopliae* according to the ASTM standard 5590-00: Control, fungicide with no effect on the growth rate (Collis), 'effective' fungicide (Polyram).

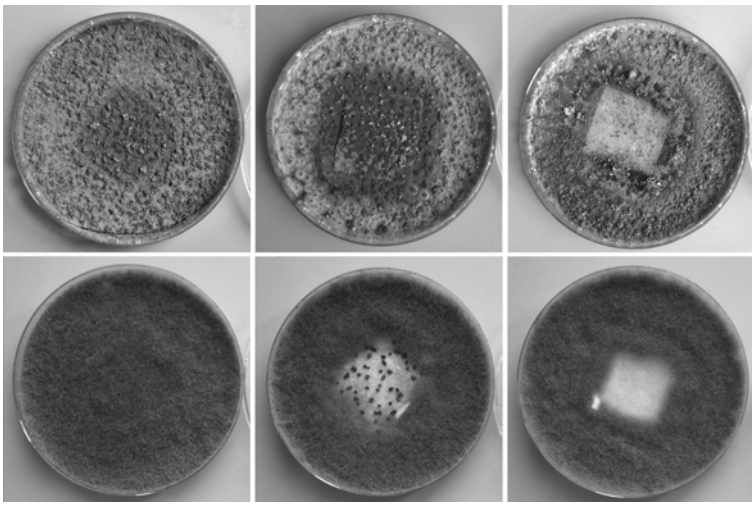

Figure 2. Effect of Ridomil on *M. anisopliae* (above) and *Botrytis cinerea* (below). Left to right: Control, 0,15 % Ridomil, 0,3 % Ridomil: Enhanced sporulation (dark areas) of *M. anisopliae* and formation of sclerotia of *B. cinera* could be observed at a Ridomil concentration of 0.15 %.

Although the active ingredient of Electis is in the same chemical group as Polyram (dithiocarbamates) and has the same mode of action, it did not cause any inhibition of growth of *M. anispliae,* indicating that data on the effect of a fungicide cannot be logically applied to other fungicides of the same chemical class. Luz et al. (2007) reported major differences in susceptibility against the dithiocarbamate fungicide Manzate of *M. anisopliae* var. *acridum*

and var. *lepidiotum*. As resistance or susceptibility against fungicides seems to be strain specific it should be the task of future research to test fungicide susceptibility of other potential fungal biocontrol agents. The combination of an application of fungicides and the fungal BCA *M. anisopliae* is possible although the application of Polyram (dithiocarbamates) and CAA-fungicides should be avoided.

Table 2. Rating of fungal growth on filter papers coated with fungicides according to ASTM standard D 5590-00: 0 = no growth, 1 = traces of growth (<10 %), 2 = light growth (10-30 %), 3 = moderate growth (30 -50 %), 4 = heavy growth (60 % to complete coverage). Fungicides, which inhibited the growth of *Metarhizium* are printed in bold.

Fungicide	Concentration [%]	Rating			
		day 7	day 14	day 21	day 28
Control		4/4/4	4/4/4	4/4/4	4/4/4
Collis	0.02 / 0.04 /0,08	4/4/3	4/4/4	4/4/4	4/4/4
Electis	0.225 / 0.45 / 0.9	4/4/4	4/4/4	4/4/4	4/4/4
Equation Pro	0.02 / 0.04 /0,08	4/4/3	4/4/3	4/4/3	4/4/4
Flint	0.0075 / 0.015 / 0.03	4/4/3	4/4/3	4/4/3	4/4/4
Forum Star	**0.0625 / 0.125 / 0.25**	**3/1/0**	**3/1/0**	**4/2/0**	**4/3/1**
Melody Combi	**0.075 / 0.15 / 0.3**	**2/0/0**	**3/0/0**	**3/1/1**	**4/2/2**
Phosfik	0.1 /0.2 / 0.4	4/4/4	4/4/4	4/4/4	4/4/4
Polyram	**0.1 / 0.2 / 0.4**	**0/0/0**	**0/0/0/**	**1/1/1**	**2/2/2**
Prosper	0.025 /0.05/0.1	4/4/3	4/4/4	4/4/4	4/4/4
Ridomil Gold Combi	0.075 / 0.15 / 0.3	3/2/0	4/4/1	4/4/1	4/4/2
Systhane	0.0075 / 0.015 / 0.03	4/4/4	4/4/4	4/4/4	4/4/4
Topas	0.0075 / 0.015 / 0.03	4/4/3	4/4/4	4/4/4	4/4/4
Vento	0.0125 / 0.025 / 0.05	4/4/4	4/4/4	4/4/4	4/4/4
Wettable sulphur	0.3 / 0.6 / 1.2	4/4/4	4/4/4	4/4/4	4/4/4

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Mechanisms important for the epidemic development of *Neozygites floridana* in *Tetranychus urticae* populations

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Abstract: In this study we aimed at understanding the mechanisms that affect an epidemic development of *Neozygites floridana* in a *Tetranychus urticae* population. This was done by comparing how many spores a cadaver infected with a *N. floridana* isolate could produce and at what distance and in which directions they could be thrown on a coverslip at temperatures relevant to the northern hemisphere (13, 18 and 23°C). The highest number of spores were produced at 13°C at a number of 1,886 per cadaver. Numbers of spores thrown at 18°C and 23°C were 1,733 and 1,302, respectively. Temperature had a significant effect on sporulation. Most of the spores were thrown at a distance of 0–0.6 mm from the cadaver. Cadavers placed on the underside of a coverslip were able to throw spores back up on the coverslip surface. A whole plant bioassay was also conducted to reveal where on a plant *T. urticae* infected with *N. floridana* die and sporulate. Cadavers showed a different vertical distribution on the cucumber plant compared to healthy spider mites. Most of the cadavers were located at the lower to the middle part of the plant, while healthy spider mites were more evenly distributed on the whole plant.

Key words: *Tetranychus urticae*, *Neozygites floridana*, spore discharge, temperature, in-plant distribution

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) has a broad host plant range and is an annual pest of many crops throughout the world (Greco et al., 2005). Use of acaricides is the main strategy to control *T. urticae* in field crops and development of resistance in *T. urticae* populations is common (Graham & Helle, 1985, Devine et al., 2001). Predators are widely used in biological control of spider mites and are effective when applied before the spider mite population is too high. Biological control by the use of predators is, however, unsatisfying when applied at high spider mite populations. The mite-pathogenic fungus *Neozygites floridana* (Weiser and Muma) (Zygomycetes: Neozygitaceae), an important natural enemy of *T. urticae*, is, however, known to be prevalent when spider mite populations are high (Elliot, 1998) and might be a good candidate under these situations (van der Geest et al., 2000).

To be able to succeed in using *N. floridana* for biological control of *T. urticae* we need, however, to understand the mechanisms that affect an epidemic development of *N. floridana* in a *T. urticae* population. The aim of this study was therefore to reveal the effect of temperature on sporulation of *N. floridana* (numbers, distance and direction of spores thrown). Further we aimed at understanding where on a plant *N. floridana* infected *T. urticae* die and sporulate.

Material and methods

Mites and fungi used in the experiment
T. urticae and *N. floridana* (NCRI 271/04) used in the experiment were collected in a commercial strawberry field at Ås in the southeastern Norway. The isolate was kept in an *in vivo* culture on *T. urticae*.

Coverslip bioassay on number and distance of spores
A coverslip bioassay was conducted at temperatures relevant to the northern hemisphere (13, 18 and 23°C) to compare how many spores a cadaver infected with the Norwegian *N. floridana* isolate could produce and at what distance they could be thrown. Cadavers were placed both on top (all three temperatures) and on the underside (only at 23°C) of the coverslip to see whether cadavers placed on the underside of a coverslip were able to throw spores back up on the coverslip surface (leaf surface). This is important for the epidemic development of *N. floridana* since the majority of *T. urticae* are located on the underside of the leaf.

Whole plant assay on location of cadavers
To obtain knowledge about where on a plant *N. floridana* infected *T. urticae* die and sporulate, a growth chamber experiment with cucumber plant was conducted at 18°C.

Results and discussion

Number and distance of spores thrown
The highest number of spores were produced at 13°C at a number of 1,886 per cadaver. Numbers of spores thrown at 18^{o}C and 23°C were 1,733 and 1,302, respectively. Temperature had a significant effect on sporulation (P=0.002). Most of the spores were thrown at a distance of 0–0.6 mm from the cadaver at all three temperatures. A few spores were, however, thrown at a maximum distance of 6.6–7.2 mm at 13°C. Cadavers placed on the underside of the coverslip threw most of the spores up on the underside of the coverslip at a distance of 0–0.6 mm from the cadaver. A surprisingly low rate (33%) of the spores thrown, landed on the surface below the cadaver.

Location of cadavers on whole plants
Cadavers showed a different verical distribution on the cucumber plant compared to healthy spider mites. Most of the cadavers were located at the lower to the middle part of the plant, while healthy spider mites were more evenly distributed on the whole plant.

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Rhizosphere competence of insect pathogenic fungi in the control of *Othiorhynchus sulcatus* in strawberries under cold climatic conditions

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Abstract: The vine weevil, *Othiorynchus sulcatus*, is a serious pest in strawberries in Norway and biological control methods are needed to combat this pest. In this study, the rhizosphere competence of two cold active Norwegian fungal isolates (*Metarhizium anisopliae* isolate NCRI 250/02 and *Beauveria bassiana* NCRI 12/96 and the well known Ma43, originating from Austria) were tested by estimating fungal concentrations counting colony forming units (CFUs) in the bulk and rhizosphere soil surrounding the strawberry plant roots. The highest numbers of *B. bassiana* NCRI 12/96 CFUs were recorded in the rhizosphere at 1.87 x 10^9 per liter soil 3 months after application. The highest numbers of *M. anisopliae* NCRI 250/02 CFUs were recorded in the rhizosphere at 2.41 x 10^9 per liter soil 1 year after application. Numbers of CFUs of *M. ansiopliae* Ma43 CFUs were generally lower than for the Norwegian isolates, but also for this isolate a higher fungal concentration was found in the rihzosphere soil than in the bulk soil.

Key words: *Othiorynchus sulcatus*, *Beauveria bassiana*, *Metarhizium anisopliae*, rhizosphere competence, cold active

Introduction

The vine weevil, *Othiorynchus sulcatus*, is the most important species among 8 root weevil species that can cause damage to strawberries in Norway (Hesjedal, 1982). The vine weevil has long been recognised as a pest in temperate regions worldwide. In recent years it has become an increasingly serious pest, much due to withdrawal of broad-spectrum insecticides and the increased use of polyethylene mulch.

Mycelia formulation of the insect pathogenic fungus *Metarhizium anisopliae* is registered for use against *Otiorhynchus* spp. in several countries but no fungal control agents are avilable for control of *O. sulcatus* in Norway. All developmental stages of *Otiorhynchus* spp. are susceptible, but best control has been achieved against larvae (Moorhouse *et al*., 1992). A number of studies have shown that *M. anisopliae* and *Beauveria bassiana* have good potential against *Otiorhynchus* spp. (Cross *et al*., 2001).

In field grown strawberries, good control with *M. anisopliae* has been reported when environmental conditions for the fungus are favourable (Oakley, 1994). Temperatures in excess of 15°C are required for good control by most fungal isolates. Low temperature is therefore a major restricting factor for use of fungi outdoors (Gillespie *et al*. 1989, Soares *et al*. 1983). Isolates with low temperature optimum could therefore be well suited for field conditions in Northern Europe, where soil temperatures at the time when most larvae are found in the soil in autumn are 10-12°C. Norwegian *M. anisopliae* and *B. bassiana* isolates have shown promising results against *O. sulcatus* larvae at low temperatures in laboratory bioassays (Hjeljord & Klingen 2005). One of the Norwegian *M. anisopliae* isolates has also

shown good competition with other soil fungi in laboratory experiments (Hjeljord & Meadow 2005).

In addition to being cold tolerant, rhizosphere competence is important for fungal control agents that are used to control root feeding pests. "Rhizosphere competence" has been defined when considering biological control agents as "the ability of a microorganism, applied by seed treatment, to colonize the rhizosphere of developing roots" (Baker 1991).

In this study we aimed at testing the rhizosphere competence of two different cold active Norwegian isolates in a semi field experiment in Norway and compare them with the well known Ma43 (also known to have many other names (Eilenberg 2008)).

Material and methods

A semi field ring experiment was conducted in Ås in South Eastern Norway from May 2007 to September 2008. Twenty concrete rings were filled with 280 l of soil. Bare root strawberry plants, cultivar Korona, were planted (15 May 2007) in soil inoculated with barley colonized with fungal isolates at the rate of $1x10^9$ to 1 l of soil. Two Norwegian fungal isolates *(M. anisopliae* isolate NCRI 250/02 and *Beauveria bassiana* NCRI 12/96) and the well known Ma43, originating from *Cydia pomonella* in Austria, were tested. To estimate fungal concentrations in the bulk and rhizosphere soil surrounding the strawberry plant roots, a destructive sampling method was used and soil was collected 3 months (17.08.07), 5 months (15.10.07), 12 months (16.05.08) and 15 months (28.08.08) after application of fungal isolates.

Soil samples of bulk and rhizosphere soil were air dried at room temperature for about 4 days and a subsample was diluted with distilled water and Tween and plated on a selective medium. Inoculated agar plates were incubated at 25^oC in darkness and colony forming units (CFUs) were counted after 6 and 12 days.

Results and discussion

The highest numbers of the Norwegian *B. bassiana* NCRI 12/96 CFUs were seen in the rhizosphere at $1.87x10^9$ per liter soil in August 2007, three months after application. The highest numbers of the Norwegian *M. anisopliae* NCRI 250/02 CFUs were seen in the rhizosphere at $2.41x10^9$ per liter soil in May 2008, one year after application. Numbers of CFUs for the *M. ansiopliae* Ma43 were generally lower than for the Norwegian isolates, but also for this isolate a higher fungal concentration was found in the rihzosphere soil than in the bulk soil (Figure 1).

This study is the first that showed rhizosphere competence of insect pathogenic fungi in strawberry. It is also the first study that show that *B. bassiana* is among the rhizosphere competent fungi. Both Norwegian isolates of *B. bassiana* NCRI 12/96 (ARSEF 5510) and *M. ansiopliae* NCRI 250/02 performed better than the commercially avilable *M. ansiopliae* Ma43 isolate under Norwegian field conditions. The Norwegian isolates *M. ansiopliae* NCRI 250/02 and *B. bassiana* NCRI 12/96 (ARSEF 5510) may be interesting for the field control of soil inhabiting pests in colder climates and deserves further testing. The fungal consentrations in the soil one year after application seem to be great enough to kill *O. sulcatus* in strawberry, but this needs to be confirmed in a field study including the pest insect itself.

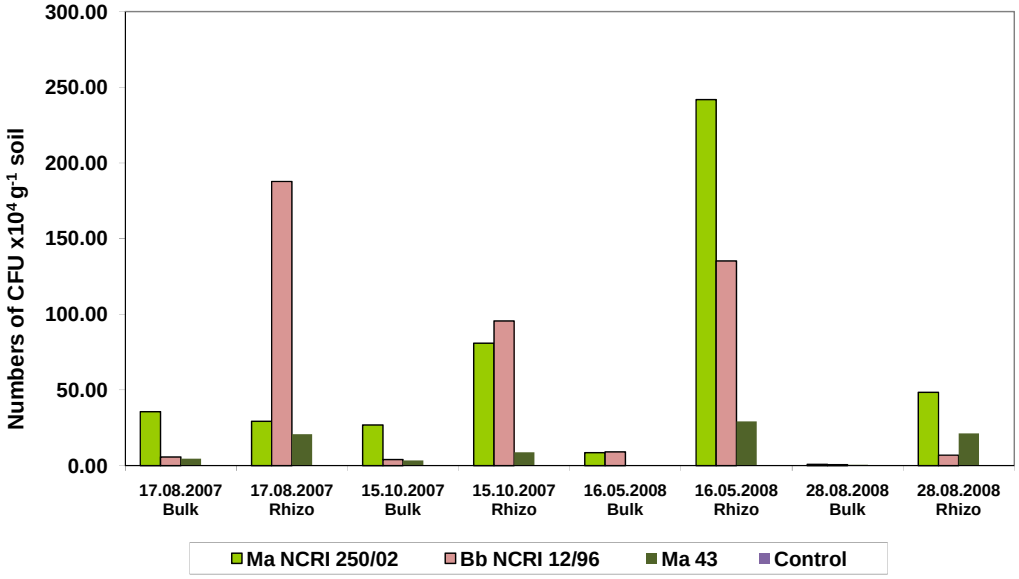

Figure 1. Fungal concentration in the bulk soil and rhizosphere soil around strawberry plants.

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Persistence of the entomopathogenic fungus *Lecanicillium muscarium* under outdoor conditions

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Abstract: Results of laboratory trials, which demonstrated the efficacy of the entomopathogenic fungus *Lecanicillium muscarium* against mining larvae of the horse chestnut leafminer moth *Cameraria ohridella* led to outdoor trials. One aspect of these trials was to determine the persistence of two fungi, the commercial product Mycotal® (Koppert, NL) and strain V24 from our collection. Different spore concentrations and the influence of an oil-containing adjuvant (Koppert, NL) on persistence were tested. The persistence of the fungus was determined through the number of colony forming units (cfu) after impressing the leaves on agar plates. The fungus could be detected until 14 days post application (dpa), with differences between the variants, despite most unfavourable weather conditions, like above-average of temperature and hours with sunshine as well as low humidity and heavy rainfall. In all variants dead and moulding larvae were found within the mines.

Key words: *Lecanicillium muscarium*, entomopathogenic fungus, *Cameraria ohridella*, persistence

Introduction

In laboratory trials, the effectiveness of the entomopathogenic fungus *Lecanicillium muscarium* strain V24 (strain from own collection) against mining larvae of the horse chestnut leafminer moth *Cameraria ohridella* Deschka & Dimic was successfully demonstrated. Larvae were infected and mouldy inside the mines. The mortality was 100% (Kalmus, 2008). Strain V24 can be distinguished from other isolates by its effectiveness within a wide range of temperature as well as tolerance to low humidity (Hetsch et al., 2005; Meyer et al., 2005; Lerche et al., 2008). To study the potential of *L. muscarium* in the field, an outdoor trial followed.The efficacy of V24 was compared with the commercial product Mycotal® (Koppert, NL). The persistence of the fungi on the plant surface were also assessed.

Material and methods

The trial took place on 3 years old horse chestnut seedlings. Test conditions like *L. muscarium* isolates tested, spore concentration and the addition of an oil-containing adjuvant (Addit; Koppert, NL) are summarized in Table 1. Each variant contained 12 seedlings. An initial population of *C. ohridella* was infested in April 2008. The first application took place on the 7th of May with 500 ml suspension per variant and was repeated at intervals of 14 days up to the 15th of September. The determination of the persistence of *L. muscarium* took place between the 21st of June and the 5th of July 2008. One, seven and 14 days post application (dpa) the number of colony forming units (cfu) was counted after impressing leaves (12 leaves per variant and time) on plates with selective-agar for entomopathogenic fungi. To notice the influence of direct and indirect sunlight, upper and lower surfaces of leaves were separately examined. After incubation (5d, 20°C) the cfu were counted and the average per

cm^2 was determined. The average surface of the leaves assessed was 30 cm^2, so the presented results were related to this surface area. The temperature and humidity were measured directly within the trial area. Records on the duration of sunlight and precipitation were received from a weather station in Berlin-Dahlem.

Table 1. Variants of the trial

***L. muscarium* as:**	**Spore concentration per ml**		**Addit**	**Name of the variant**
	$1,5x10^7$	**$1,5x10^8$**		
Mycotal®	X			My
Mycotal®	X		x	MyA
V24	X			L7
V24	X		x	LA7
V24		x		L8
V24		x	x	LA8

Results and discussion

The temperature and duration of sunlight were above average (www.proplanta.de/web/themen.php?Siteid=1140008702&Weiter=99&Mehr=99&Fu1=1214406183&Fu1Ba=1140008702), why the relative humidity usually was below 80%, less than the required minimum for successful germination and development of entomopathogenic fungi. The fungus was also exposed to precipitation on 6 days. *L. muscarium* was detected on the plant surface until 14 dpa. One day post application My and MyA had fewer cfu than treatments L (strain V24). In all variants the cfu decreased rapidly until 7 dpa. Remarkable is the following increase of the inoculum in the variants L7 and L8 (without Addit). An influence of direct or indirect sunlight was visible on the persistence of *L. muscarium*. On 1 dpa cfu from upper and lower surfaces were still similar, whereas on 7 dpa cfu were higher on the lower surfaces. Only in LA8 a delay of the decrease of inoculum was detected. Despite 14 days of direct sunshine, cfu were sporadically detectable in the variants L7, My and MyA, but L8 and LA8 had higher counts. The presence of the fungi was also confirmed by the presence of moulding cadavers spontaneously infected with *L. muscarium* in all variants. Thus development of the fungus under outdoor conditions is possible.

The ability of the fungus *L. muscarium* to persist unfavourable weather conditions over a period of 14 days is remarkable because UV radiation can limit viability and low humidity delays the development of the fungus (Braga et al., 2002; Hetsch, 2005; Lee et al., 2006) and through rain the fungus can be washed from the leaf (Lerche, 2008). The decrease of the inoculum over 14 days might be caused by the shadow on the lower leaf surface providing some kind of protection against adverse environmental conditions. However, the reason for the increase of the cfu 14 dpa need further clarification. It could have resulted from a too small random sample as well as growth of the fungus on the cadaver of the host or saprophytic development of the fungus on the surface of the leaf.

The use of the Addit had no positive impact to the persistence of *L. muscarium*. It contains oil and perhaps the negative influence of UV radiation was even amplified. The use of spore concentrations $>10^{10}$ could not increase the cfu recorded after application. Laboratory examinations show that the spores of strain V24 cluster in higher concentrated suspensions (Wolff 1998). In the case of Mycotal® clusters were already found at concentrations at 1,5 x 10^7. Maybe the amount of inoculum will be decreased by clusters.

That could explain the similarity of the variants of V24 as well as the small amount of cfu in the variants of Mycotal® from one day past application. Even under outdoor and also in case of adverse climatic conditions, *L. muscarium* persists on the leaf and is able to germinate, infect and kill larvae of *C. ohridella* within their mines.

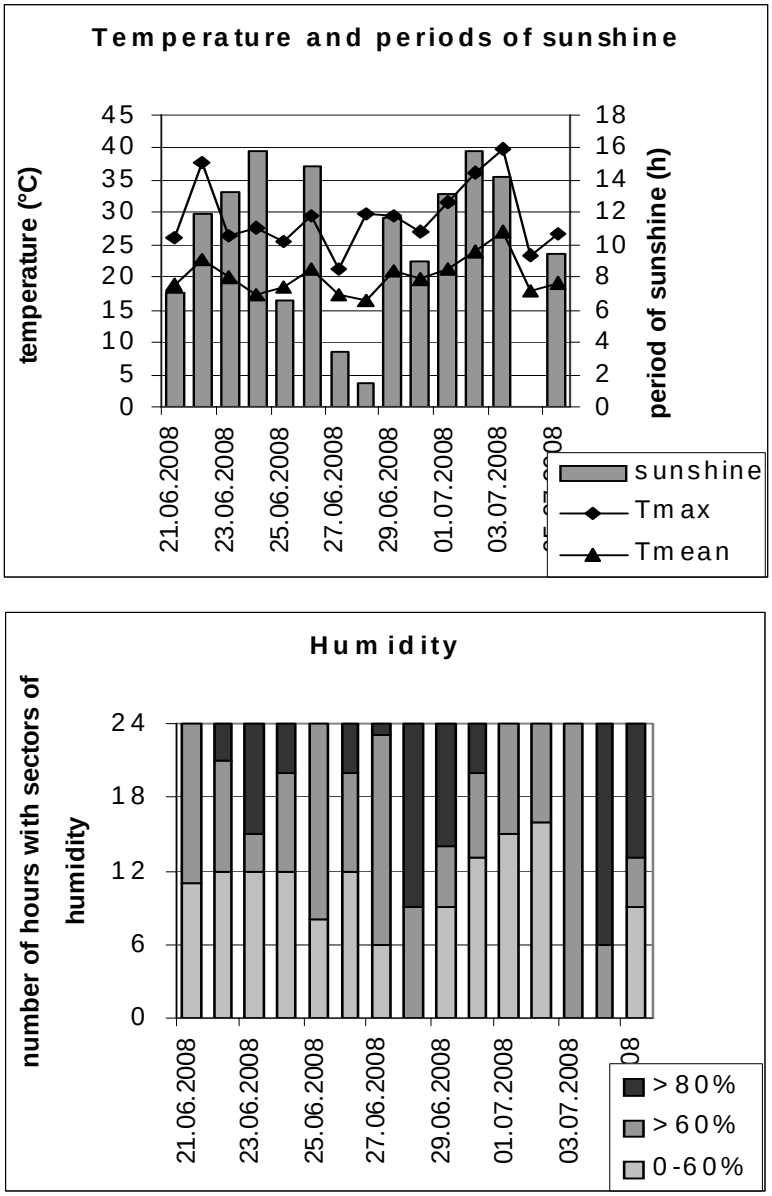

Figure 1. Temperature, period of sunshine and humidity during the carrying out of the test

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The potential of *Beauveria bassiana* and *Metarhizium anisopliae* strains for control of the cattle tick *Rhipicephalus annulatus*

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Abstract: The pathogenicity of 3 *Beauveria bassiana* and of 5 *Metarhizium anisopliae* strains from Israel, Ethiopia and the Palestinian Authority against engorged *Rhipicephalus (Boophilus) annulatus* females and eggs was examined. Two *B. bassiana* and 4 *M. anisopliae* strains were found to be highly virulent toward ticks (>50% mortality) regardless of their geographic or host origin. The infection was evidentially transmitted from the females to the eggs they laid, resulting in a reduction of hatching to 0-12.5% by the virulent strains and 25-50% by the lower virulent strains. Direct inoculation of eggs by all tested strains except one resulted in a complete inhibition of larvae hatching.

Key words: Tick control, *Metarhizium anisopliae*, *Beauveria bassiana*, *Rhipicephalus annulatus*

Introduction

Among the entomopathogenic fungi species tested for their pathogenicity to ticks the most pathogenic were *B. bassiana* and *M. anisopliae*. They cause high mortality of many tick species, of various life stages and reduce the egg laying capability and larval hatchability (e.g. Kaaya et al., 1996; Gindin et al., 2001; Samish et al., 2001; Polar et al., 2005). While the susceptibility of the ticks to several fungal strains varies, the mycoses in most tick stages developes relatively slow. Thus, the major requirement in the selection of pathogens for controlling ticks was always their virulence toward different tick stages. However, this strategy did not include other important characteristics of tick-fungi interaction such as transmission of infection from an infected to a non-infected host both among ticks at the same life stage and also among ticks at different life stages. Transmission of the pathogen can appear both on the vertebrate host surface while the tick feeds as well as on ground while the female tick oviposits. The pathogen produces a new generation of conidia on the tick's cadavers and its survival depends on micro-environment conditions in the transmission sites. In the present study, we evaluated the virulence of fungal strains toward *R. annulatus* females and eggs and the transmission potential of the fungal strains collected in Israel, the Palestinian Territories and Africa. Three of the strains were isolated from ticks.

Materials and methods

Fungi

Metarhizium anisopliae var. *anisopliae* and *Beauveria bassiana* were grown on Sabouraud dextrose agar (SDA, Difco) for 2-3 weeks at 25°C. The conidia were suspended in sterile distilled water containing 0.01 % Triton X-100 and filtered through Miracloth. The

suspensions were vortexed and sonicated for 5 min. Spore concentrations were determined with a hemacytometer.

Ticks

Rhipicephalus (Boophilus) annulatus ticks were fed on calves and kept under laboratory conditions (28°C and 70–80 % RH). Ticks were surface-sterilized by dipping in 0.5 % (w/v) methyl p-hydroxybenzoate and 0.1% sodium benzoate (5 min. in each). The females were incubated in sterile Petri dishes with moist filter paper for oviposition at 25°C. Only eggs laid during the first 6-7 days after drop off were used.

Bioassays with eggs and engorged females

Petri dishes (55 mm) were lined with sterile filter paper and impregnated with 0.5 ml of 0.01 % (v/v) Triton X-100 with or without (control) conidia suspension (1×10^{-6} conidia ml^{-1}). Fifty to hundred eggs laid by surface sterilized females were placed on the filter paper by using a camel hairbrush. Plates were sealed with Parafilm and incubated at 25°C and 100% RH.

Table 1: Pathogenicity of entomopathogenic fungi to engorged *R. annulatus* females 14d PI

Fungal strain	Original host	Geographic origin	Female Mortality (%)	Female Laying (%)	Eggs weight/ female (mg)	Larval hatching* (%)
Beauveria bassiana						
T-2005	*Boophilus annulatus*	Israel	53.3±5.75	40.0±20.0	185.0±2.7	0
PAL-Bb-01	*Ips typographus*	West bank	72.5±21.2	25.0±23.3	145.0±55.0	12.5
PAL-Bb-02	*Agrilus planipennis*	West bank	22.5±22.5	77.5±22.5	296.3±54.8	25
Metarhizium anisopliae						
T-2004	*Rhipicephalus sanguineus*	Israel	55.0±12.5	40.0±42.6	170.0±26.4	0
Ma-7	Unidentified coleopterous	Israel	55.0±9.2	45.0± 9.3	160.0±29.9	0
PAL-Ma-01	*Harpalus caliginosus*	West bank	42.5±22.5	57.5±22.5	187.5±75.9	0
PAL-Ma-02	*Melolontha hippocastani*	West bank	52.5±18.3	47.5±18.3	152.5±51.2	0
Ma-K	*Amblyomma variegatum*	Kenya	65.0±14.0	32.5±14.9	160.0±43.4	0
PPRC-51	*Pachnoda interrupta*	Ethiopia	40.0±21.4	52.5±14.9	156.0±76.6	25
PPRC-21	*Pachnoda interrupta*	Ethiopia	7.5±10.4	80.0±33.8	309.4±45.5	50
PPRC-27	*Pachnoda interrupta*	Ethiopia	20.0±21.4	80.0±21.4	255.5±73.8	50
Control			2.5±7.0	97. ±7.0	330.7±56.8	87.5

* Percent represent the number of dishes observed with larvae out of total replicates

Females were inoculated with conidia by dipping them in conidia suspension (1×10^7spores ml^{-1}) for 5 sec., transferred to dishes lined with moist filter paper (5 females per dish) and incubated at 25 ^{0}C. The humidity in bioassay test was kept at nearly 100 % during the first day after inoculation, and from then on the humidity was reduced to about 90%. Adult mortality and their eggs weight were recorded 14 days PI.

Results and discussion

The virulence of the *M. anisopliae* strains toward the engorged females varied significantly; only four of the strains caused more than 50% mortality (Table 1). Two *B. bassiana* strains out of three were highly virulent. Most of the *M. anisopliae* and *B. bassiana* strains that caused more than 50% mortality resulted in low numbers of females laying eggs. All strains that caused more than 40% mortality of females during the 14 days PI inhibited the females' oviposition, reduced eggs' weight by half. Because the bioassay was conducted at relatively low humidity (about 90%), the emerging of the conidia on the ticks' cadavers took place only 14 d PI. The percent of cadavers on which conidia developed varied from 0 to 33% and was not correlated with strain's virulence. The fungal infection was transmitted from the females' cadaver surface to the eggs they laid. Even though the number of ticks' cadavers with fungal conidia on its surface was low (7.5-33.3%), colonization of the eggs mass with fungi transmitted from infected females was detected in 75-100% of the dishes of the virulent *Metarhizium* strains. No case of eggs colonization with any of the *B. bassiana* strains could be observed. However, apart of faint fungal development on cadaver's and eggs surface 14 days PI, the four most virulent strains completely inhibited any hatching of larvae from eggs laid by infected females. The low virulent strains also caused a reduction of 25-50% of egg hatching in spite of the absent of a visible eggs colonization.

Table 2: Pathogenicity of entomopathogenic fungi to *R. annulatus* eggs

	Fungal colonization on eggs (6 d PI, %)	Larvae hatching (28 d PI, %)	Sporulation on dead larvae (28 d PI, %)
Beauveria bassiana			
PAL-Bb-01	<5	0	100
PAL-Bb-02	89±7	0	100
Metarhizium anisopliae			
M.a-7	23±9	0	100
M.a-K	97±4	0	100
PAL-Ma-01	35±16	0	100
PAL-Ma-02	57±16	0	100
PPRC-51	77±5	0	100
PPRC-27	7±2	24±3	0
Control	0	93±1	0

The direct virulence of the fungi towards tick eggs test demonstrated that all tested fungi, except PPRC-27, infected the *R. annulatus* eggs and completely suppressed larvae hatching (Table 2). The higher efficacy of strains in this test in comparison to the previous one is

probably due to the difference in environmental humidity 100% RH in the later test in comparison to about 90%RH in the previous bioassay.

This study demonstrates that the transmission of *M. anisopliae* and *B. bassiana* from infected females to eggs and then from eggs to emerging larvae take place even when using strains which exhibit low virulence toward *R. annulatus* females. Moreover, fungal transmission and infection to the following tick stages occurred also at lower humidity (about 90%) than necessary for successful female's infection (close to 100%). Such phenomenon can occur in tick oviposition natural sites and increase fungal efficacy.

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Metarhizium anisopliae chlamydospores in tick eggs

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Abstract: *Metarhizium anisopliae* chlamydospores were rarely described previously and the conditions needed for their development were not identified. For the first time, the presence of *M. anisopliae* chlamydospores in the tick eggs and the ambient conditions influencing its formation are reported. The infection of tick eggs by *M. anisopliae* involves the common events such as adhesion, conidia germination, penetration of the egg chorion and development of hyphae in the egg cytoplasm. However, the final fungal developmental stage in egg differs according to the ambient conditions. At high humidity (100%) and moderate temperature (25°C) the fungus emerges out of the dead eggs and forms conidiophores with conidia. Stress conditions, in which the temperature was elevated to 30 °C or the humidity was reduced to 55 – 75 %, induced the production of chlamydospores inside the eggs without emergence of fungi on the eggs surfaces. When eggs with chlamydospores were transferred to favorable conditions (25°C / 100 % RH), conidiogenesis was observed. Observations were done by means of histology and TEM.

Key words: *Metarhizium anisopliae*, *Rhipicephalus annulatus* eggs, chlamydospores, stress conditions

Introduction

The physiological adaptations of entomopathogenic fungi to different climatic conditions increase their potential use as biological control agents against arthropods. Various fungi have different survival strategy under unfavorable conditions, mainly by forming resting structures. Hyphomycetes fungi often form thick-walled hyphae or chlamydospores (Pendland, 1982). *Metarhizium anisopliae* is physiologically adapted to different environments - pathogenic development inside their arthropod hosts and to saprophytic growth (Wang et al., 2005). Such fungi can persist in soil or in insect cadavers for a long time under unfavorable environmental conditions (Arthurs et al., 2001; Milner et al., 2003), however, very seldom morphological resting structures were detected and data on inductive factors for their formation are absent. *M. anisopliae* chlamydospores from hyphal bodies were described within elaterid larvae as well as in haemolymph of the greater wax moth *Galleria mellonella*, 4 - 5 days PI (Zacharuk, 1971; Sewify and Hashem, 2001). According to Hanel (1982), *M. anisopliae* chlamydospores are formed from a dying mycelium, serving as a defence response against colonization of termite cadavers by other saprophytic organisms.

M. anisopliae infecting tick eggs causes dramatic reduction of eggs hatchability and a very high percent of egg mortality. The early tick eggs infection steps of *M. anisopliae* and its final external conidiogenesis on the dead eggs were described for *R. sanguineus* eggs after being inoculated with fungi under favorable conditions (Garcia et al., 2005). However, information on fungus development and survival in tick eggs under less favorable conditions is missing. The aim of the present study was to study the interaction between tick eggs and *M.*

anisopliae under stressing environmental conditions and to demonstrate the possible physiological adaptation, mechanism which guarantees the fungal survival under such environment.

Materials and methods

Fungi

M. anisopliae var. *anisopliae* and *Beauveria bassiana* isolates grown on Sabouraud dextrose agar (SDA, Difco) for 2-3 weeks at 25°C, conidia were suspended in sterile distilled water + 0.01 % Triton X-100 and filtered through Miracloth. The suspensions were vortexed and sonicated for 5 min. Spore concentrations were determined with a hemacytometer.

Ticks rearing

Rhipicephalus (Boophilus) annulatus ticks were fed on calves and kept under laboratory conditions (28°C and 70 – 80 % RH). The female ticks were surface sterilized by dipping in 0.5 % methyl p-hydroxybenzoate and 0.1% sodium benzoate (5 minutes in each). The females were incubated in sterile Petri dishes with moist filter paper for oviposition at 25ºC. Only eggs laid during the first 6 – 7 days after drop off were used.

Eggs

Petri dishes (55 mm) were lined with sterile filter paper and impregnated with 0.5 ml of 0.01 % Triton X-100 with or without (control) conidia suspension (1×10^{-6} conidia ml^{-1}). Fifty to hundred eggs were placed on the impregnated filter paper by a camel hair-brush and incubated at 25 or 30°C in desiccators over an appropriate saturated solution to reach different humidity (sodium chloride for 75 % RH, D (+)-Glucose-Monohydrate for 55 % RH and water for 100 % RH). For each combination, 4 – 5 Petri dishes were prepared and about 50 eggs from each dish were examined by light microscopy on day 14 PI.

Histology

Infected eggs were sampled for fixation at 6, 12, 18, and 24 hours and then daily 2-10 days PI. The eggs were fixed in 3.6 % formalin in 0.1 M sodium cacodylate buffer (pH 7.2) and processed in a Histoprocessor Tissue Tek VIP-5 overnight. The samples were embedded in paraffin wax and sectioned into 3 – 5 µm thick slices. The sections were stained with hematoxylin-eosin (H&E) and with periodic-acid-Schiff (PAS). The sections were examined under a light-microscope.

TEM

Samples of infected eggs were fixed for 2 hours in 3.5 % gluteraldehyde in PBS buffer, pH 7.2, then fixed for 1 hour in buffered (pH 7.2) 1% OsO_4 and dehydrated in a graded ethanol series and acetone series. Then, samples were embedded in Agar 100 resin (Agar Scientific, Cambridge, UK). Sections were cut with a diamond knife on a LKB-ultramicrotome and stained by 5% aqueous uranyl acetate followed by lead citrate. The samples were examined using a Tecnai G2 electron microscope (Fei comp., Philips) and micrographs were taken using Megaview III (SIS).

Results and discussion

The mortality of eggs infected with *M. anisopliae* at 25°C and 100 % RH was close to 100 % and by 8 - 10 days PI conidiogenesis was observed on the surface of all dead eggs (table 1). Elevated temperature (30°C) and/or lower relative humidity (55 or 75 % RH) were found to

induce the development of *M. anisopliae* chlamydospores in tick eggs, a fact confirmed by histological and SEM observations (Fig. 1, left). The conditions during the first 3 – 5 days of incubation were critical for the development of chlamydospores inside the eggs later on (Table 2). Transferring eggs with chlamydospores to 25°C and 100 % RH resulted in fungus conidiogenesis 6 days after transfer (Fig. 1 right).

Table 1: Fungal structures formed on/inside eggs of *Rhipicephalus annulatus* kept under different ambient conditions.

Eggs incubated for 14 days PI at:					
Temperature			25°C		30°C
RH / Fungal stage & location		55 %	75 %	100 %	100 %
Conidia	on egg surface	-	-	+	-
Chlamydospores	inside eggs	+	+	-	+

*At 55 % RH, most of the dead eggs dried before fungus development

Table 2: Influence of different regimes of relative humidity on formation of fungal structures on/inside eggs of *Rhipicephalus annulatus*. All eggs incubated constantly at 25°C and observed for 14 days.

		Days PI when eggs were incubated at 100 % RH before been transferred to 75 % RH			
Fungal structure	Location	0	3	5	7
Conidia	on eggs surface	-	-	-	+
Dividing hyphae	inside eggs	-	+	+	-
Chlamydospores	inside eggs	+	+	-	-

Data were obtained from 4 – 5 egg masses, 50 – 100 eggs in each egg masses.

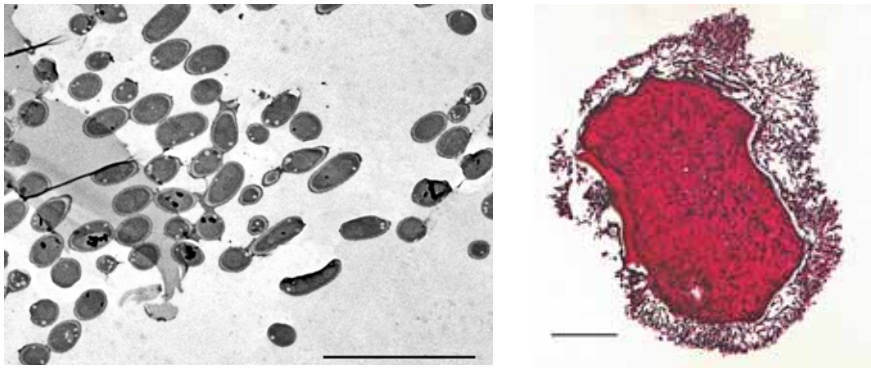

Figure 1. *Metarhizium anisopliae* chlamydospores inside an egg of *Rhipicephalus annulatus* tick. TEM. Scale bar: 10 µm (left) and conidiogenesis of *Metarhizium anisopliae* in a *Rhipicephalus annulatus* egg (right)

The histological and SEM observations shown that chlamydospores have an oval shape 5.3±0.9µm long, 2.5±0.2µm wide and with a 100nm thick wall. Hyphae emerging and consequently conidiogenesis were never observed on the surface of eggs with chlamydospores.

We propose that by analogy with chlamydospores of other fungi (Armengol *et al*., 1999; Urbasch, 1991), the thick-walled *M. anisopliae* chlamydospores are a type of resting structures, which can probably survive under sub-optimal conditions and renew the life cycle under favorable conditions. In this study, *M. anisopliae* exhibited an advantage for controlling tick eggs by its ability to produce chlamydospores under stressing conditions and thus to overcome "difficult" periods and widen the time span of producing infective conidia in ecological niches favorable by ticks.

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The criteria for selecting *Metarhizium anisopliae* thermo-tolerant strains for the control of arthropods on vertebrates

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Abstract: The tolerance of fungi to high temperature is an important criterion for choosing strains for controlling ticks while feeding on the warm skin of vertebrates. The efficacy of *Metarhizium anisopliae* var. *anisopliae* strains with different thermal characteristics was compared in two model experiments: 1) at temperature reflecting the common conduction on animals surface (35°C) and 2) at a relatively short increase of temperature up to the maximum that was detected on cattle skin for limited time (37°C and 40°C). The present study show that isolates suitable for application to cattle must be able not only to germinate and grow at higher temperature but also to fast recover the conidia germination and keep their virulence after temporal rise of temperature above the one in which the fungus develop and infect.

Key words: Tick control, *Metarhizium anisopliae*, temperature, cattle tick, *Rhipicephalus annulatus*

Introduction

The environmental temperature influences the virulence of many entomopathogenic fungi and this can determine the success of pest control under natural conditions (e.g. Thomas et al., 1996; Fargues and Luz, 2000). Applying biopesticide on animal surface must be effective at relatively high temperatures, which in most cases exceed the optimal temperature for germination and growth of the fungi (25-32°C). The temperature on cattle surface in shade fluctuates between 30 and 35°C, reaching on the ears and spine 35–41°C (Polar et al., 2005).

Entomopathogenous fungus, *Metarhizium anisopliae* var. *anisopliae* (*M. an. an.*) is one of the most promising candidates for control of both on- and off-host tick stages (e.g. Kaaya and Hassan, 2000; Samish et al., 2004). However, the various strains of *M. an. an*. differ both in virulence toward ticks and in their thermal characteristics (Polar et al., 2005; Leemon and Jonsson, 2007). The maximal temperature for conidial germination and mycelia growth of *M. an. an*. is 35-37°C, however, thermal death point for their conidia varied from 49 to 60°C (e.g. Fargues et al., 1992; Walstad et al., 1970; Zimmermann, 1982).

For an initial selection of strains that can be effective on animal surface, it must be known which thermal characteristics guarantee fungal survival, development and infection under real environmental conditions. We evaluated two hypothesis based on the assumption that efficacy of *M. an. an.* at similar animals skin temperatures depended 1) on ability to germinate and grow at relatively high constant temperature near fungal maximal temperature; 2) on ability to quickly recover the germination and growth after temporal temperature increasing above the maximal temperature suitable for fungal development.

Materials and methods

Fungi

M. an. an. strains were grown on Sabouraud dextrose agar (SDA) for 2 weeks at 25°C. The conidia were harvested, suspended in sterile distilled water containing 0.01 % Triton X-100 and filtered through Miracloth (Calbiochem, La Jolla, CA). Spore concentration was determined with a haemocytometer.

Ticks

The cattle ticks, *Rhipicephalus (Boophilus) annulatus*, were fed on calves and kept under laboratory conditions (28°C and 70 – 80% RH).

Conidial germination test

Conidia suspension (0.2 ml, 1×10^6 conidia ml-1) was placed in Petri plates with SDA medium and incubated at 25°C, 32°C or 35°C. Germination was observed by mean of light microscope at ×200 magnification.

Colony radial growth test

Sterile filter papers (65 mm) were placed in the middle of Petri dishes amended with SDA. Aliquots of conidia suspension (10 µl, 1×10^6 conidia ml^{-1}) were placed on the filter papers and incubated at 25°C, 32°C and 35°C. For 10 days the colony growth on *x* and *y* axes was recorded.

Tick mortality test

Engorged female ticks were inoculated with fungi by dipping them in conidia suspension (test group) (1×10^7spores ml-1 in sterile distilled water containing 0.01 % Triton X-100) or in sterile distilled water containing 0.01 % Triton X-100 (control group) for 5 seconds. These ticks were transferred to dishes lined with moist filter paper (5 females per dish) and incubated at corresponding temperature regimes and 100 % humidity for 14 days. The following temperature regimes were evaluated: 1) constant temperatures of: 25°C or 35°C during the whole period of the bioassay or 2) temporal temperature of 37°C or 40°C for 6 or 48 hours immediately after dipping following by incubation at 25°C. Tick mortality was recorded daily and eggs were weight 14 d PI. Thirty to forty females were used for each treatment.

Data analysis

The difference in amount of germinated conidia and mortality dates were analyzed by ANOVA (SAS LIFETEST) after arcsine transformation. If differences among means of the treatments were found significant, Tukey's test was used for multiple comparisons among means.

Results and discussion

Development of **M. anisopliae** *strains at constant temperatures or after a high thermal shock*

The percent of conidia, from two strains (Ma-7 and PAL-2) germinating on SDA was 100% at 25°C as well as at 32°C, but was reduced significantly at 35°C (table 1). The radial growth of these strains reduced by half when incubated at 32°C in comparison to 25°C and ceased at 35°C. The third strain, PPRC-51, kept at all tested temperatures a high germination percent and grew at 35°C. The 6-hours increasing of temperature to 37°C did not influence the germination percent and radial growth of 2 strains at their following cultivation at 25°C. Only one strain (PPRC-51) showed a reduction of germination percent after temporal thermal shock.

Table 1. The percent of *Metarhizium anisopliae* conidia, which germinated at different temperature regimes

Strain	Origin	Constant temperature				Kept at 37°C for 6-hours and transferred to 25°C
		25°C	32°C	35°C	37°C	
Ma-7	Israel	100	100	68.0 ±10.4	0	93.3 ± 3.0
PAL-2	West Bank	100	100	55.0 ±2.0	0	98.5 ± 1.0
PPRC-51	Ethiopia	100	100	90.5±5.0	0	52.7 ± 8.8

Based on different thermal characteristics of *M. an. an.*, two contrasting strains were chosen for a comparative bioassay at higher temperature: 1) strain Ma-7 which demonstrated the inhibition of conidial germination and vegetative growth at 35°C, but not at temporal thermal shock and 2) strain PPRC-51 which demonstrated 100% germination and ability to grow at 35°C but 2 times reduction of conidial germination after 6-hours exposure to 37°C thermal shock.

Pathogenicity of Ma-7 and PPRC-51 to* R. annulatus *at 25°C and 35°C

The mortality of ticks treated with Ma -7 or PPRC-51 and incubated at 25°C was significantly higher than in the non-infested control. Elevating the incubation temperature to 35°C increased mortality in the control group by 4 times and significantly reduced the mortality of the ticks inoculated with both fungal strains. At 35°C, the mortality of the ticks treated with PPRC-51 was 2.5 times higher than in the control group unlike the mortality of the ticks treated with Ma-7 which was equal to the degree of mortality in the control group.

Pathogenicity of Ma-7 and PPRC-51 to* R. annulatus *after thermal shock

The short exposure (6 hours) of ticks incubated with Ma-7 to 37°C or 40°C did not influence the tick mortality, which was similar to mortality at 25°C. The longer exposure (48 hours) to both temperatures reduced the ability of the fungus to control the ticks. The other strain, PPRC-51, partially lost its ability to kill the ticks already after the short thermal shock at both temperatures tested. The PPRC-51 exposed for 6h to the elevated temperatures killed 3-4 times less ticks exposed constantly to 25°C.

Female ticks exposed to the higher temperatures laid also fewer eggs. Female ticks inoculated with PPRC-51 or Ma-7 and incubated permanently at 25°C as well as those inoculated with Ma-7 and kept for 6h at the higher temperatures laid half of the amount of eggs laid by uninfected females kept constantly at 25°C. Females incubated with PPRC-51 conidia and exposed for 6h to the higher temperatures laid the same amount of eggs as the uninfected females kept constantly at 25°C.

The present study shows that the ability of fungi strains to keep their virulence at relatively high temperature does not guarantee their efficacy after temporal increasing temperature above those suitable for fungal development. The temperature of mammalian skin varies and is influenced by the environment. During midday, the skin temperature may be relatively high for several hours. Therefore, initial selection of thermotolerant strains for "on host" tick control must be based not only on the ability of the conidia to germinate and grow at constant temperature but also on their ability to germinate fast and to keep their efficacy after a temporal thermal shock at maximal temperatures which may occur on cattle surface (37–41°C). One of the possible ways to overcome the problem is to use on mammals simultaneously two strains with different thermal characteristics.

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Phylogenetic characterization of entomopathogenic fungi from Uzbekistan

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Abstract: As part of a wider study of the biocontrol potential of entomopathogenic fungi from Central Asia, the pathogenicity of a number of insect-derived fungal isolates from Uzbekistan was assessed and its previous morphological assignment to the genera *Beauveria, Metarhizium, Fusarium, Trichoderma, Paecilomyces,* or *Aspergillus* re-evalutaed and in most cases confirmed molecularly by a phylogenetic analysis based of internal transcribed spacer (ITS1) sequences.

Key words: *Beauveria, Metarhizium, Paecilomyces, Fusarium, Trichoderma,* ribosomal RNA (rRNA), internal transcribed spacer (ITS), Colorado Potato Beetle (*Leptinotarsa decemlineata*)

Introduction

The deuteromycete form-genera *Metarhizium, Beauveria, Lecanicillium,* and *Paecilomyces* comprise numerous facultative insect pathogens that are of considerable interest with respect to the biological control of pest insects. Evidence (Fukatsu et al., 1997) suggests that these imperfect fungi are closely related to entomopathogenic ascomycetes of the genus *Cordyceps* (*Pyrenomycetes, Clavicipitales*). Nevertheless, as no teleomorphs are obtained, morphological classification relies entirely on relatively undifferentiated anamorphic structures. Recently, the application of molecular phylogenetic studies based on ribosomal DNA sequences have led to a reorganization of the form-genus *Verticillium* and its subdivision into four distinct clades, with entomopathogenic and mycoparasitic isolates clustering in clade B being organized in the new form-genus *Lecanicillium* (Gams and Zare, 2001). In view of an application of imperfect fungi as insect biocontrol agents, reliable classification criteria are even more important as other fungi frequently isolated from the same host, e.g. *Aspergillus flavus,* may also be pathogenic to higher animals, including humans (St. Leger et al., 2000).

Departing from the assumption of an evolutionary early „RNA world", the sequences of rRNA encoding genes have become prominent molecular phylogenetic markers. In fungi, these genes are organized in a complex transcription unit containing the 18S, the 5.8S and the 28S rRNA genes, separated by two internal transcribed spacer (ITS) sequence elements that are eliminated during processing of the primary RNA transcript and therefore are thought to be subject to low selection pressure. ITS elements have been widely used as markers in phylogenetic studies applying RFLP or AFLP **(**Neuvéglise et al., 1994, Coates et al., 2002, Aquino de Muro et al., 2003**)** as well as DNA sequencing approaches (Shih et al., 1995) to entomopathogenic and related deuteromycetes.

Within the wider framework of a research project dealing with the isolation of fungal entomopathogens from agricultural pests, namely the Colorado potato beetle, *Leptinotarsa decemlineata*, in Central Asia, we have used one of both ITS elements to characterize at the genus level fungal isolates from Uzbekistan.

Material and methods

Fungal isolates

The thirteen fungal strains from Uzbekistan used in this study (Table 1) were isolated from moribund or dead mycosed insects found in the field. Single spore-derived colonies were selected on Czapek medium containing varying concentrations of dodine and the dye Bengal Rose (Goettel & Inglis, 1997). The morphology of sporulating pure cultures as revealed by light-microscopy was taken as the criterion for preliminary taxonomic classification.

Table 1. Fungal strains from Uzbekistan investigated in this study, their previous taxonomic classification based on morphology criteria, and a rough estimate of their pathogenicity for the respective host using a descriptive scale ranging from non-pathogenic (-) to highly pathogenic (+++).

Isolate	Original host	Morphological classification	Pathogenicity
UZB #1	*Leptinotarsa decemlineata*	*Beauveria bassiana*	+++
UZB #2	*Eurygaster integriceps*	*Beauveria bassiana*	++
UZB #3	*Leptinotarsa decemlineata*	*Beauveria bassiana*	+++
UZB #4	*Leptinotarsa decemlineata*	*Beauveria bassiana*	++
UZB #5	*Leptinotarsa decemlineata*	*Beauveria bassiana*	+++
UZB #6	*Leptinotarsa decemlineata*	*Metarhizium anisopliae*	+++
UZB #7	*Leptinotarsa decemlineata*	*Metarhizium anisopliae*	++
UZB #8	*Leptinotarsa decemlineata*	*Metarhizium anisopliae*	++
UZB #9	*Leptinotarsa decemlineata*	*Fusarium* sp.	+
UZB #10	*Leptinotarsa decemlineata*	*Fusarium* sp.	+
UZB #12	*Leptinotarsa decemlineata*	*Trichoderma* sp	++
UZB #14	*Leptinotarsa decemlineata*	*Paecilomyces* sp.	++
UZB #15	*Leptinotarsa decemlineata*	*Beauveria bassiana*	+++
UZB #16	*Leptinotarsa decemlineata*	*Aspergillus* sp.	++

PCR and sequence analysis

Fungal total DNA used as PCR template was isolated from ground mycelium and purified by passage over an AXG NucleoBond column. PCR reactions were performed using Taq DNA polymerase and primers its1 and its4 (White et al., 1990) in a reaction running over 25 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. Independent reactions were performed in triplicate, and sequences of three corresponding products used to generate strain specific consensus sequences submitted to the GenBank database under accession numbers AY755502 through AY755519. Consensus sequences were compared using the ClustalX software tool. Phylogenetic analyses including searches for maximum parsimony trees, confidence limit assessments based on the bootstrap resampling algorithm, and construction of sequence distance matrices were carried out using PAUP* 4.0 beta 10.

Results and discussion

Morphological classification and entomopathogenicity data for isolates UZB#1-16 are presented in figure 1.

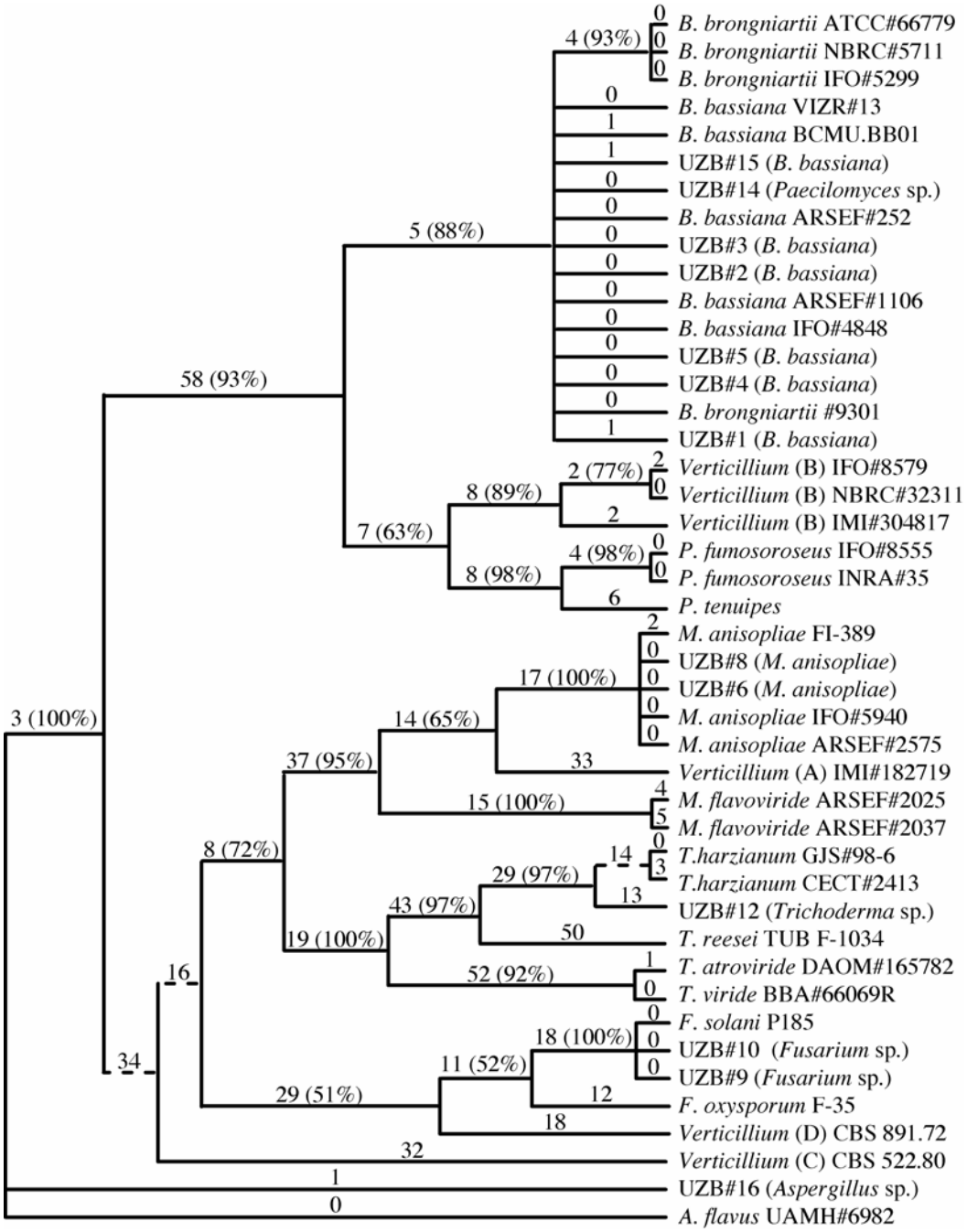

Figure 1. Maximum parsimony tree based on a comparison of ITS1 sequences. The cladogram shown is the 50% majority rule consensus of 2248 most parsimonious trees (tree length 476, CI=0.6828, HI=0.3172, RI=0.8692) generated in a heuristic search using the TBR branch swapping algorithm. Dashed lines indicate branches that collapse in the corresponding strict consensus tree. Branch lengths are indicated on top of branches, with the bootstrap support values in brackets. 1000 bootstrap replications were performed with the same search settings, generating a total of 611.329 trees.

Using primers its1 and its4, PCR products containing the ITS element separating the 18S and 5.8S rRNA encoding genes were amplified from genomic fungal DNA preparations, cloned, and sequenced. The maximum parsimony phylogeny generated by heuristic (TBR) searches on the basis of these and further ITS1 sequences is presented in Figure A. Outgroup rooted by the respective sequence from *Aspergillus flavus*, the tree shows mostly well supported generic clades of standard strain and confirms the morphological classification for all but two of the fungal isolates from Uzbekistan. For one of these, strain UZB#16, the position close to the root of the tree is consistent with its former assignment to the genus *Aspergillus*, but, due to the set of standard strains chosen, cannot be interpreted in terms of a positive corroboration of this classification. More interestingly, isolate UZB#14, originally classified as a *Paecilomyces* species, clusters within a little compact *Beauveria* clade and is thereby clearly separated from a firmly supported clade comprising all standard sequences from *Paecilomyces* strains. According to this genetic analysis, isolate UZB#14 should therefore be considered a *Beauveria* species, most likely a strain of *B. bassiana*.

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Mycotal, a microbial insecticide recently registered in Spain to control whiteflies in IPM programs

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Abstract: Mycotal is a microbial insecticide, formulated with a specific strain of the entomopathogenic fungus *Lecanicillium muscarium* (Petch), Zare & Gams, 2001. This biocontrol agent is registered in Europe since 1986, first in United Kingdom to control whiteflies in greenhouse crops, following other European countries as Holland in 1992, France in 2004 and finally in Spain where recently has been registered (R.O.P.M.F. no. 24,435) for the control of *Bemisia tabaci* Gennadius 1889 and *Trialeurodes vaporariorum* Westwood 1856 in protected crops. The results of different efficacy trials show that this product in Spain is a valuable tool for the control of insect pests in IPM programs due to its specificity, usefulness for insect resistance management strategies, very low side-effects on beneficials and friendly profile for the environment.

Key words: Biological control, insecticide, IPM, *Lecanicillium muscarium*, protected crops, whiteflies

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Occurrence and distribution of entomopathogenic fungi in Moroccan endemic forests of *Argania spinosa*

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Abstract: The fungi *Beauveria* and *Metarhizium* have a world-wide distribution. These species are frequently found in the soil and are known by their ability to control a wide range of insects. The use of local entomopathogenic fungi will be appropriate to control insects since these strains may be better adapted to local environmental conditions. Our study aimed to analyze the occurrence and the natural abundance of *Beauveria* and *Metarhizium* spp. in Moroccan forests of *Argania spinosa* (endemic Moroccan tree) and to isolate and select potential strains for control of insects. The presence of entomopathogenic fungi was examined by using selective media for *Beauveria* and *Metarhizium* spp. and by the *Galleria* baiting methods. In approximately 55% of soil samples the occurrence of *Beauveria* on selective medium was recorded, whereas *Metarhizium* spp.were found only at low density in only three regions. The baiting method revealed the presence of entomopathogenic fungi in all soil samples. *Beauveria bassiana* was dominant in all soil samples, whereas *Metarhizium* spp. was recovered mainly from soil collected in South-Western Morocco. More than 400 isolates were identified and constitute the first Moroccan collection of entomopathogenic fungi. In addition, isolates were characterized for their temperature tolerance and potential to produce cuticle-degrading enzymes.

Key words Biological control, *Beauveria* ssp., *Metarhizium* ssp., natural occurrence, Moroccan Argane forests

Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Diptera: Tephritidae), is a polyphagous fruit fly attacking over 300 different fruits, vegetables and nuts and causing important economic losses to the cosmopolitan agriculture. In Morocco, the medfly is widely distributed all over agricultural regions and its management relies only on chemical insecticides. Besides the environmental and human health problems caused by pesticides, the insects may become resistant to insecticides. One of the most efficient control methods used against fruit flies is the Sterile Insect Technique (SIT). Although the autocide strategy has been widely used to eradicate the medfly from new invaded regions, it is not effective when applied against large target populations. Therefore, alternative pest management strategies such as entomopathogenic fungi should be tested to reduce efficiently the medfly populations.

The fungi *Beauveria* and *Metarhizium* are frequently found in the soil and are known by their ability to control a wide range of insects (Jackson et al., 2000). The use of local entomopathogenic fungi might be most appropriate to control insects since these strains may be better adapted to local environmental conditions (Carruthers & Onsager, 1993). The distribution of these species is essential to understand the pathogen ecology and to improve insect pest management. The aim of our investigation was to analyze the occurrence and the distribution of species within two genera of entomopathogenic fungi, *Beauveria* and *Metarhizium*, in the Moroccan Argan forest. Localized approximately in the Western regions of Morocco with 800.000 hectares and 21 millions Argan tree, they are hardly affected or modified by human activity. These endemic forests have been chosen since they are known to

be a large refuge of medflies in Morocco and may contain fungi, which are pathogenic to the medfly. Furthermore, much emphasis has been placed on the search of suitable genotypes in those environment where the insects will be controled in the future (Prior & Greathead, 1989).

Material and methods

Sampling and isolation

Sampling was realized in Moroccan Argan forests. A total of 83 soil samples were collected at 11 geographically distant locations (maximum 1000 km) and localized at altitudes ranging from 40 to 1200 m above sea level. Samples were collected with a cylindrical probe to a depth of 25 cm after removal of surface litter. At every site, each sample was a mix of five combined soil samples, collected randomly from five points in an area of 10 m around an Argan tree. 60g of each sample was baited with four fifth instar *Galleria mellonella* larvae as decribed by Zimmermann (1986). Three repetitions were realized independently. Larvae were inspected daily for infection. The cadavers were removed, disinfected and placed at room temperature. The mycelium, which developed on the larvae, was identified *in situ* according to mycological criteria (Humber, 1997). All samples were also plated on to selective media plates with the following composition:1% peptone, 1% yeast, 2% glucose, 1,5% agar, all dissolved in 1 liter destilled water and autoclaved at 120°C for 20 minutes. At a temperature of 60°C 0,5g chloramphenicol and 200 mg of cycloheximide per litre were added (Veen & Ferron, 1966). 20 g of a soil sample was resuspended in 200 ml sterile Tween 80 and shaken in a water bath for 30 min. at 22°C. The soil suspensions were diluted and three replicates of each dilution were plated on selective media. The number of colonies of *Beauveria* and *Metarhizium* spp. was determined on every plate and the CFU g^{-1} of soil was calculated.

Molecular identification

Beauveria isolates from different regions of the Argan forest were grown in sterile liquid medium. DNA was extracted from freeze-dried mycelium and was amplified using ITS1 and ITS4 primers. The amplified ITS regions were digested with restriction enzymes and compared on agarose gels with *Beauveria bassiana* strains obtained from the ARSEF collection, China and Brazil. Sequencing of the amplified ITS regions was carried out for 15 isolates using an automated sequencer. The ITS regions sequences were compared with the GenBank Nucleotide Database using the algorithm Blastn.

Results and discussion

According to mycological criteria (Humber, 1997), the mycelia which developed either on *Galleria mellonela* larva or selective media were identified as *Beauveria* or *Metarhizium* ssp. The comparison of the ITS1-5.8S-ITS2 region sequences of 15 *Beauveria* isolates with Genbank data revealed that all our isolates are *Beauveria bassiana* with a high degree of identity.

The occurrence of both species was analyzed either by selective media plating or *Galleria* baiting. *Beauveria* ssp. were found in approximately 55 % of soil samples, plated on selective medium. Its density varied in the Argan forest soil between 26 conidia g^{-1} soil and 1962 conidia g^{-1} soil. *Metarhizium* colonies were not found in any soil sample. It is known that it is difficult to detect in selective medium any fungi at a concentration below 10^3 conidia/g soil. Therefore, samples without colonies of entomopathogenic fungi, does not mean an absence of these fungi in the soil. Furthermore, *Beauveria* ssp. occur more often at the Northern locations than in the others.

Galleria baiting is more efficient than the selective media method, thus *Beauveria* ssp. were detected in all analyzed samples and *Metarhizium* ssp. were detected less often (four locations). More than 400 isolates belonging to the Deuteromycetous fungi were identified. The *Galleria* bait method is strongly recommended to study the distribution of entomopathogenic fungi, but the number of larva used to bait a sample vary mostly from one (Chandler et al., 1997) to ten larvae (Quesada-Moraga et al., 2007). Therefore, it will be difficult to compare the published studies. However, the relative abundance of entomopathogenic fungi was 96 % in Switzerland (Keller et al., 2003), 91% in Ontario (Bidochka et al,. 1998), 52 % in the Pacific Northwest (Bruck, 2004) and 17.5 % in United Kingdom (Chandler et al., 1998). In the Moroccan endemic Argan forests entopathogenic fungi were found in 100% of the soil samples.

Beauveria ssp. occur more frequently in forested habitats than *Metarhizium*, which confirms data reported by Vanninen (1996) and Bidochka et al. (2002). Furthermore, *Beauveria* and *Metarhizium* spp. have been shown to have different environmental habitat preference. *Beauveria bassiana* prefers forest habitats whereas *Metarhizium anisopliae* is more abundant in agricultural and cultivated habitat (Rath et al., 1992; Vanninen, 1996).

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Assessment of *Metarhizium anisopliae* for control of the Japanese Beetle on the Azores

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Abstract: *Popillia japonica* (Coleoptera; Scarabaeidae), the Japanese Beetle, is an important pest on some of the Açores islands, attacking a variety of crops as well as pastures. The beetle first appeared on Terceira Island in 1970 and subsequently has spread to other islands on the archipelago. In 2003, the insect was observed for the first time in a small area on the island São Miguel. The regional authorities are interested in taking several measures to limit or delay the distribution of this insect on and between the islands and also to avoid its introduction to mainland Europe. Our strategy is to use autodissemination of *Metarhizium anisopliae* conidia through the adult population via modified Japanese Beetle traps. During our 2008 Japanese beetle larvae survey we found several larvae with *M. anisopliae* and isolated the fungus from these cadavers. These isolates, along with several isolates made from beetles on Terceira in the 1990s, are currently under evaluation. Data regarding conidial production by the candidate isolates via solid substrate fermentation will be presented.

Key words: *Metarhizium*, mass production

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Entomopathogenic fungi isolated from soil in the vicinity of *Cameraria ohridella* infested horse chestnut trees

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Abstract: Occurrence of entomopathogenic fungi in soil samples collected near horse chestnut trees infested by its pest, *Cameraria ohridella*, was surveyed using the modified Galleria bait method. The results revealed that entomopathogenic fungi frequently occur in the soil collected from *C. ohridella* habitats. Dominant species found were *Isaria fumosorosea* (Wize) Brown et Smith and *Beauveria bassiana* (Balsamo) Vuillemin. The isolated strains are deposited in the CCEFO (Culture Collection of Entomopathogenic Fungi Olešná) in the Czech Republic. The virulence of 100 native strains was tested using standardised biotests.

Key words: Horse chestnut leaf-miner, *Cameraria ohridella, Aesculus hippocastanum*, soil samples, biological control, entomopathogenic fungi, *Isaria fumosorosea, Isaria farinosa, Beauveria bassiana, Metarhizium anisopliae,* virulence

Introduction

The horse chestnut, *Aesculus hippocastanum* L., is an important ornamental tree in Europe. It is attacked by the horse chestnut leaf-miner, *Cameraria ohridella* Deschka et Dimic, an important invasive lepidopteran pest. Besides an aesthetic damage, injury inflicted to the leaves can result in weakening of infested trees and reduction of their growth.

Present methods of *C. ohridella* control are based on the application of non-selective insecticides and composting or burning of leaf litter, in which *C. ohridella* overwinters as pupa. However, these methods also kill beneficial organisms including natural enemies of *C. ohridella*. Biological control of *C. ohridella* has not been implemented yet. There are several studies on the impact of parasitoids and predators like beetles, spiders and birds attacking leaf-mining larvae and pupae. Our project is aimed at the evaluation of entomopathogenic fungi to control *C. ohridella*. There is only one report on the infection of *C. ohridella* by *Lecanicillium* (*Verticillium*) *lecanii* (Zimmermann) Gams & Zare (Samek et al., 2006). Obtaining new native strains of entomopathogenic fungi was the first step of our project. It is known that native „vital" isolates of fungi are more virulent compared to collection strains kept continuously on artificial medium (Kawakami, 1960; Schaerffenberg,1964; Morrow et al., 1989). In the present paper we report the results of a survey of the occurrence of entomopathogenic fungi in soil samples collected in the vicinity of horse chestnut trees.

Material and methods

Soil samples

The occurrence of entomopathogenic fungi was surveyed in soil samples collected on 12 localities in the Czech Republic (districts Písek, České Budějovice and Plzeň) in autumn 2006. The soil was sampled in the vicinity of horse chestnut trees heavily infested by *C.*

ohridella. The ground around these trees was natural, i.e. without asphalt or paving. At each locality, the samples were taken from four biotopes differing in management and pollution: (1) a city park, (2) trees in gardens outside the city, (3) an alley at a heavy traffic road, and (4) an alley at a low traffic road. Totally, 48 soil samples were collected and processed. Native entomopathogenic strains were obtained from the samples by „Galleria bait method" (Zimmermann, 1986; Mietkiewski et al., 1992, 1997, 1998; Vanninen, 1996; Vanninen et al., 1989; Kleespies et al., 1989; Landa et al., 2002; Keller et al., 2003) using the great wax moth, *Galleria mellonella* L. larvae.

Isolation of entomopathogenic fungi

All 48 collected soil samples were processed as follows: (1) the sample was sieved and if necessary, the soil was moistened with water, (2) twenty *G. mellonella* larvae were placed on the bottom of a plastic vial of 300 ml volume and the tested soil was added up to the volume of 200 ml above the caterpillars, (3) the vial was closed with a lid and incubated at room temperature for period of 1 to 2 months. The procedure was replicated four times with each soil sample giving totaly 80 larvae in four vials.

We made two substantial changes in the live-bait method used so far. First, the caterpillars were placed on the bottom of the vial and then covered by the substrate instead of placing them on the surface of the substrate. Majority of the caterpillars dig through the substrate upwards to the surface and this increases their contact with the tested soil and enhances probability of receiving more infection units of the entomopathogenic fungi. If the caterpillars are placed on the soil surface under the lid, they stop moving and start to spin cocoons in which they pupate. This minimizes their contact with the soil. Second, the incubation time the caterpillars of *G. mellonella* were kept in contact with the samples of the tested soil was extended from one to two months. The period was deliberately chosen that long in order to allow for formation of the fructification organs of the fungus. We assumed that isolation of entomopathogenic fungi by means of inoculation of spores of these organs could be more successful than isolation from early stages of the fungal infection.

Strains of entomopathogenic fungi that infected larvae were species identified according to their morphological characteristics (shape and size of conidiospores and structure of conidiophores) using mycological identification keys (Samson et al., 1988; Lacey, 1997).

Results and discussion

Occurrence of entomopathogenic fungi in soil samples

The adapted live-bait method turned out to be very successful in obtaining native strains of entomopathogenic fungi. Totaly, 1741 out of 3840 *G. mellonella* larvae, i.e. 45.3%, were infected with entomopathogenic fungi. Dominant species found were *Isaria fumosorosea* (Wize) Brown et Smith and *Beauveria bassiana* (Balsamo) Vuillemin (Table 1). Species *Isaria farinosa* (Holm ex S. F. Gray) Brown et Smith and *Metarhizium anisopliae* (Metschnikoff) Sorokin occurred rarely.

Results of statistical analysis revealed highly significant effects of district on the occurrence of entomopathogenic fungi (contingence tables, X^2=129, df=8, P<0.0001). Distribution of individual fungal species among infected larvae was not uniform (X^2=2750, df=3, P<0.0001). The dominant species was *I. fumosorosea* (77.6%) followed by *B. bassiana* (20.6%). The other species, *I. farinosa* and *M. anisopliae* occurred rarely (1.7% and 0.1%, respectively).

The effect of the biotop on occurrence of entomopathogenous fungi was statistically significant in the district Písek (X^2=142, df=12, P<0.0001), district Plzeň (X^2=81,5, df=9,

P<0.0001) and in the region Česke Budějovice (X^2=194, df=9, P<0.0001). The lowest number of infected larvae were found in soil samples collected in biotop with the lowest impact of human activity, i.e. pollution from cars etc.

Table 1. Numbers of *Galleria mellonella* larvae infected by individual species of entomopathogenic fungi.

District	Species	Biotop				Total
		City park	Garden outside the city	Alley at a heavy traffic road	Alley at a low traffic road	
Písek	*I. fumosorosea*	102	113	109	44	368
	I. farinosa	0	0	0	1	1
	B. bassiana	50	16	14	0	80
	M. anisopliae	0	2	0	0	2
	Total	**152**	**131**	**123**	**45**	**451**
Plzeň	*I. fumosorosea*	109	99	155	65	428
	I. farinosa	13	0	4	3	20
	B. bassiana	36	38	19	45	138
	M. anisopliae	0	0	0	0	0
	Total	**158**	**137**	**178**	**113**	**586**
České Budějovice	*I. fumosorosea*	178	54	185	138	555
	I. farinosa	2	1	3	2	8
	B. bassiana	9	83	12	37	141
	M. anisopliae	0	0	0	0	0
	Total	**189**	**138**	**200**	**177**	**704**

Collection of isolated strains

It was not necessary to single out all strains; only 150 strains were isolated by purification on synthetic medium (Sabouraud´s agar, IMUNA) and deposited in the CCEFO (Culture Collection of Entomopathogenic Fungi Olesna) in the Plant Protection Laboratory, Olešná, the Czech Republic. The collection was thus expanded to 250 native strains. For objective reasons (time demanding activity, limited number of pupae, limited space in thermoboxes) only 100 native strains of entomopathogenic fungi were chosen for the tests of virulence. These include 20 strains of *B. bassiana*, 2 strains of *I. farinosa*, 1 strain of *M. anisopliae* a 77 strains of *I. fumosorosea*, all showing better growth characteristics than the remaining strains. Standardised biotests were employed. The results of the tests are currently undergoing statistical analyses.

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Microbiological control of the leaf-footed bug *Leptoglossus occidentalis*

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Abstract: The leaf-footed bug *Leptoglossus occidentalis* Heidemann is one of the many phytoparasites infesting pine trees in several forest areas of Italy. This species has shown strong development in recent years, seriously compromising pine-seed production. To assess the effectiveness of microbiological control of the pest, we conducted laboratory trials in 2006-2008 using isolates of the entomopathogenic fungi *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorok. collected from Italian agro-forest environments. In preliminary trials, apical sprigs of *Pinus nigra* Arn. and adult specimens of *L. occidentalis* were placed in plastic boxes and sprayed with conidial suspensions of the fungi. Further treatments were carried out directly on 1-year-old, 50-cm-high *Pinus nigra* plantlets sprayed with suspensions of the entomopathogenic fungi. The results showed that the leaf-footed bug is sensitive to infection by the fungal isolates, some of which killed the treated individuals within 10 to 30 days.

Key words: Microbiological control, *Leptoglossus occidentalis*, *Beauveria bassiana*, *Metarhizium anisopliae*, conifers

Introduction

The leaf-footed bug *Leptoglossus occidentalis* Heidemann (Hemiptera Heteroptera Coreidae) is native to North America. Linked to conifers, it has become widely distributed and naturalized in Italy in recent years (Villa *et al.*, 2001). In the larval and adult stages, it can attack numerous *Pinus* species, seriously compromising the production of seeds which abort and fall, reducing reproduction of both spontaneous and cultivated trees. The resulting environmental and economic damage is significant.

Being a serious forest pest, the leaf-footed bug requires adequate control measures, but the control strategies must be linked to environmental respect and biodiversity conservation. In association with silvicultural interventions, microbiological control can play an important role in the control of leaf-footed bug populations. For this reason, we tested the use of entomopathogenic fungi (Grimm and Guharay, 1998), carrying out laboratory trials to assess the pathogenicity of isolates of *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metsch.) Sorok. collected from different substrata in Italian agro-forest environments.

Materials and methods

In 2006-2008, we conducted laboratory trials using isolates of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* collected in Italian forests. Adult specimens of *L. occidentalis* collected in the Apennine or raised in our institute were used for the trials. For preliminary laboratory trials in 2006-2007, apical sprigs of *Pinus nigra* Arn. and adult specimens of *L. occidentalis* were placed in plastic boxes and sprayed with conidial suspensions of the fungi. Control specimens were sprayed with water alone. Five *B. bassiana* isolates were tested

(Bba01/T01, Bba01/T02, Bba01/T06, Bba01/T08, Bba01/I16). In both trials, two treatments were carried out one week apart. Dead specimens were constantly counted during the trials.

In another two trials (2008), treatments were carried out directly on 1-year-old, 50-cm-high *Pinus nigra* plantlets caged in fine-mesh nets. For each plant, 5 adult specimens of *L. occidentalis* were used. The plants were sprayed with conidial suspensions (1.0-2.0 x 10^7 conidia/ml) of our fungal isolates (Bba01/T02, Bba01/T06, Man05/X03, Man08/I05) and of a commercial formulation (Naturalis, Intrachem Bio Italy), while controls were treated with water alone. The first treatment was applied to the plants prior to the infestation with insects. After 10 days, a second treatment was applied in the presence of the insects. Each trial was repeated four times. The plants were maintained in a greenhouse under controlled conditions. The mortality was controlled periodically and moist chambers were used to verify the presence of the entomopathogenic fungi on the body of the insects.

Results

Results indicted that the leaf-footed bug was sensitive to infection by some of the fungal isolates, even though the response was not immediate (treated insects died after 10 - 30 days). The trials conducted in 2008 (Fig. 1) were particularly interesting since they clearly demonstrated the efficacy of two isolates (Bba01/T02 and Man08/I05). Their strong activity opens promising perspectives for use of autochthonous micro-organisms in Italian forests.

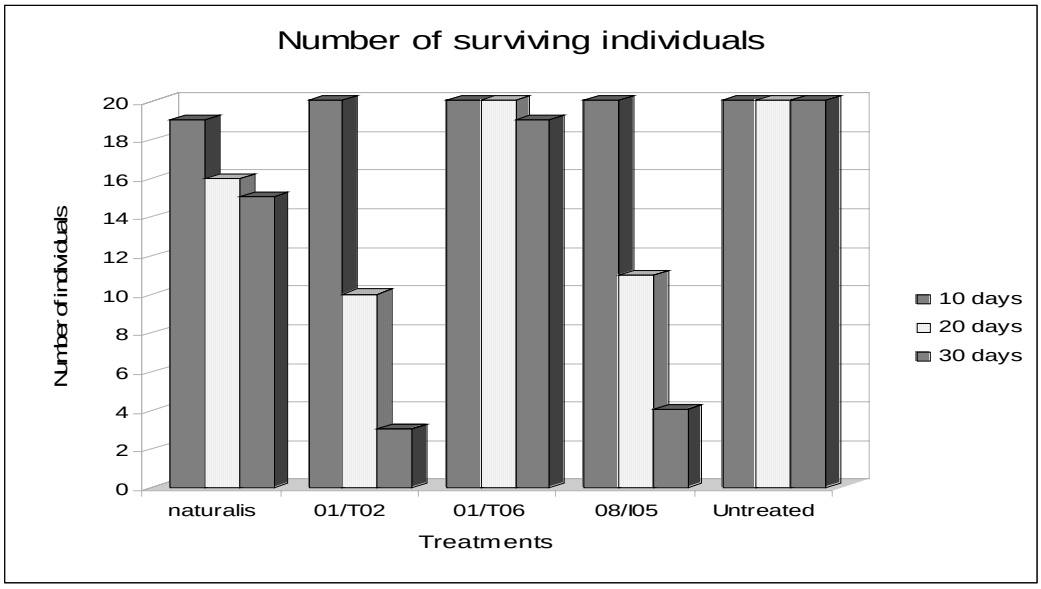

Figure 1. Survival of adult *Leptoglossus occidentalis*

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Evaluation of some parameters influencing the activity of a fungal biocontrol agent used for *Bemisisa tabaci* control

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Abstract: Some problems with using biological agents under field conditions are their susceptibility for sun radiation and their incompatibility with commonly used chemical pesticides. This is often due to the use of no or poor formulations. When photostability of a previously developed biocontrol product based on a *Lecanicilllium lecanii* isolate was evaluated by exposure to sun radiation, a reduction of 69.7% in the germination of non-formulated *L. lecanni* (VL026) conidia was obtained, compared with 37.4% reduction observed with the formulated conidia, six hours after exposure to radiation. Chemical pesticides, like Chlortalonyl, Diphenoconazole, Carbofuran and Dimethoate were not compatible with the fungus, whereas Sulfur, Cooper Oxichloride, Propiconazole and Carboxyn-Captan showed some compatibility. Under laboratory conditions, formulated and non-formulated conidia produced a80.6% and 69.1% mortality of *B. tabaci* nymphs, respectively.

Key words: UV-radiation, compatibility, pesticides, biological control, *Lecanicillium lecanii*

Introduction

The whitefly *Bemisia tabaci* causes very important losses in more than 500 species of plants as tomato, cucumber, beans, potato, cotton, melon, peanut, soybean, flower and others (Rodríguez & Cardona, 2001). Damages caused by this insect can be direct due to plant sap suction and toxin injection by the insect saliva. However, indirect damages are the most important, which consist in pathogenic virus transmission that can result in the 100% of losses (Faria & Wraight, 2001). Biological control by using the entomopathogenic fungus *Lecanicillim lecanii* is an environmental alternative for managing this pest as it has been demonstrated by Meade and Byrne (1991) for *B. tabaci* nymphs. Biological control research group of Corpoica developed a biopesticide formulation based on the native strain of *L. lecanii*, which includes sunscreens for protecting the fungus conidia against sun ultraviolet radiation (UV), with 100% of protection against UV radiation type C (254nm), under laboratory conditions. In the present work, biological activity of the formulation was determined over *B. tabaci* under laboratory conditions. Additionally, in order to determine the potential of developed biopesticide for being applied under field conditions, the protection against sun radiation and fungus compatibility with chemical pesticides were also evaluated.

Materials and methods

Fungus and biopesticide production

Based on standard procedures developed to produce a *L. lecanii* biopesticide, conidia were obtained by solid state fermentation and harvested by using washing and centrifugation. Then pure conidia were formulated as wettable powder (WP), which presented following characteristics: 1 x 10^{10} conidia/g of final product concentration, 5% of moisture content, and less

than 100µm of particle size. Non formulated fungus was obtained by drying centrifuged biomass.

Evaluation of biological activity

Biological activity of formulated and non-formulated *L. lecanii* was evaluated by a previously standardized bioassay under laboratory conditions (Espinel *et al.*, 2008). Red bean plants with the first formed leaf, individually planted in plastic pots, were infested with 30 adults of *B. tabaci* adults confined inside a pincers cages for 30 hour. After this time, adults were removed and plants were maintained until obtaining second instar nymphs, which were counted and this result was considered as initial population. Formulated and non-formulated conidia suspensions adjusted to 1 x 10^7 conidia/mL, were sprayed on second instar nymphs by using 2mL per plant. Total and infected nymphs were evaluated 14 days after application. Mortality was used to calculate efficacy percentage by using Schneider Orelli equation (Zar, 1999). An untreated control treatment consisting in non-applied insects was considered. Experimental design was arranged in a completely randomized design with four replications per treatment and results were analyzed by ANOVA and LSD test (95%).

Photostability of formulated fungus

Samples of 0.1g of formulated and non-formulated *L. lecanii* conidia were spread over tomato leaves, which were individually placed on the surface of Agar-Agar medium contained in Petri dishes. Uncovered plates were exposed at high sun radiation (25ºC – 36ºC), with intensity (4,5 – 6,0 kWh/m^2), during six hours. The experiment was conducted in Espinal (Colombia), where *B. tabaci* is considered the most limiting pest for several crops. One sample of each treatment was removed each two hours from sun exposition and conidia germination was determined on Malt Extract Agar medium. Experiments were arranged in a completely randomized design with three replications per treatment and results were analyzed by ANOVA and Tukey test (95%).

Compatibility* of *L. lecanii with chemical pesticides

Saboureaud medium supplemented with different chemical pesticides in three different concentrations (commercial recommended dose, a half and a quarter) contained in Petri dishes was inoculated with *L. lecanii* conidia suspension. After 24 hours of incubation at 25˚C, germination was evaluated by light microscope. Conidia germination in non-supplemented Saboureaud medium was used as control treatment. Experimental design was a completely randomized design with three replications per treatment and results were analyzed by ANOVA and Tukey test (95%). Evaluated chemical pesticides were the fungicides Sulphur (T1), Diphenoconazole (T2), Chlorotalonyl (T3), Copper Oxichloride (T4), Carboxym-Captam (T5) and Propiconazole (T6), and the insecticides Dimetoate (T7) and Carbofuran (T8).

Results and discussion

Evaluation of biological activity

Formulated and non-formulated conidia of *L. lecanii* produced 81.8% and 71.7% of nymph mortality respectively (Table 1), while the mortality in the control treatment was significantly lower ($P<0.05$) with a value of 8.5%. This result suggests that nymph mortality in the treatments was due to *L. lecanii* effect.

Obtained efficacy for formulated and non-formulated fungus did not present statistical differences, allowing to conclude that formulation does not negatively affect biocontrol activity. Although numerical differences were obtained in efficacy of formulated and non-

formulated fungus, these were not significant. Considering that formulated conidia efficacy was numerically higher than obtained for non-formulated fungus in laboratory evaluation, this difference could be more important under field conditions, where conidia will be exposed to detrimental environmental factors as temperature and UV-radiation.

Table 1. Mortality and efficacy of formulated and non-formulated *L. lecanii* over *B. tabaci* nymphs

Treatment	Initial population	Emerged adults	Dead individuals	Mortality (%)	Efficacy (%)
Formulated	147.0	28.0	119.0	81.8	80.6
Non-formulated	159.0	47.0	112.0	71.7	69.1

Photostability of formulated fungus

Non-formulated conidia reduced germination capacity in 69.7%, compared with the 37.4% of reduction observed in formulated conidia, after six hours of irradiation. Final germination of non-formulated conidia was significantly lower ($p<0.05$) than obtained for formulated conidia (Figure 1a), suggesting that formulation could reduce the damage caused by sun radiation. Photostabilizing effect was attributed to the sunscreen added to the formulation, which can absorb a portion of UV-radiation emitted by the sun, which causes DNA damage, protein denaturizing and free-radicals oxidation (Devotto & Gerding, 2003), to maintain the conidia viability.

Although formulated product was more stable than non-formulated conidia, a significant conidia viability reduction was observed when they were exposed to direct sun. This result could be related with the wavelength spectrum absorbed by the sunscreen used in the formulation, which does not cover the entire UV sun spectrum able to cross the atmosphere (300 – 400 nm), suggesting the necessity to include alternative sunscreens in this formulation.

Compatibility* of *L. lecanii with chemical pesticides

Conidia germination was not affected by the fungicides Sulphur and Copper Oxichloride at three evaluated concentrations, with values higher than 95% after 24 hours of incubation, with non significant differences among them (Figure 1b) in comparison with the control treatment, allowing to conclude that fungus was compatible with both fungicides. This result is interesting considering that numerous laboratory studies have shown that many common fungicides can be highly antagonistic toward beneficial insect fungi (reviewed by Roberts and Campbell, 1977; Glare and Milner, 1991).

Less than 5% of germination was obtained with the fungicides Clorotalonyl and Difenoconazole at all evaluated concentrations, probably due to their low specific host spectrum. Based on those results, these chemical products are not recommended for using with *L.lecanii.*

The fungicides Carboxym-Captam and Propiconazole did not allow conidia germination when commercial dose and half of it were evaluated, instead the lower concentration allowed a germination higher than 95%, which was not significantly different from control treatment ($p>0.05$).

The insecticides Carbofuran and Dimetoato also drastically affected conidia germination, with values lower than 7%, classifying both insecticides in the group of *L. lecanii* non-compatible pesticides.

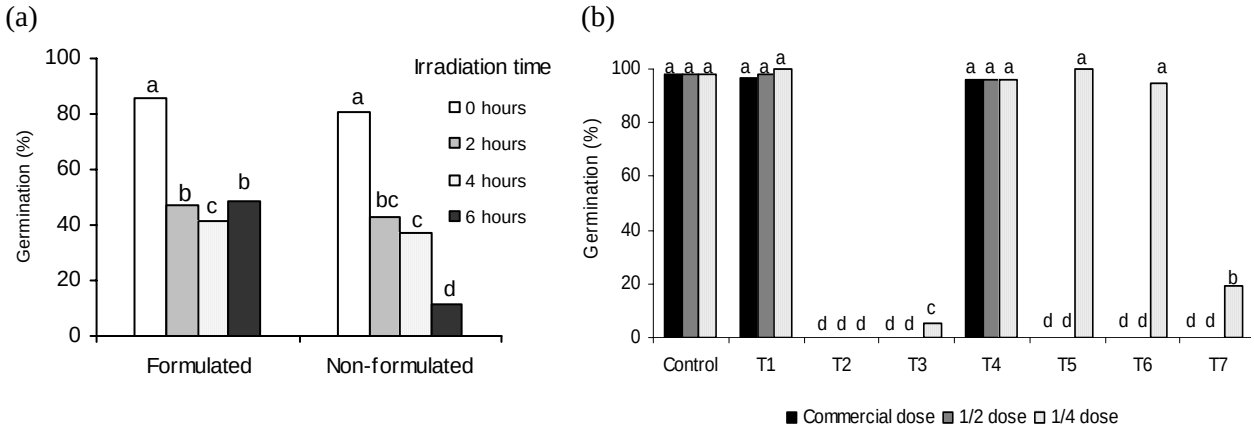

Figure 1. (a) Photostability of formulated and non-formulated conidia exposed to direct sun radiation (b) Germination (%) of L. *lecanii* conidia exposed to chemical pesticides

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Possibility of microbial control using entomopathogenic fungi *Lecanicillium* spp. hybrid strains and *Beauveria bassiana* against the diamondback moth

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Abstract: For control of the diamondback moth (*Plutella xylostella*), the pathogenicities of *Lecanicillium* sp. and *Beauveria bassiana* were investigated. Conidial suspensions of 1×10^6/ml of 13 hybrid isolates of *Lecanicillium* spp. were used in bioassays against second-instar larvae. Of these isolates, 2aF27 was selected based on the low LT_{50} value and the mortality 6 days after inoculation.Then suspensions of 2×10^7/ml of 2aF27 and *Beauveria bassiana* (strain MG-Bb-1) were sprayed on a cabbage field. MG-Bb-1 treatment provided high control of the *P. xylostella* population, whereas Bt. 2aF27 was less effective. Results suggest that entomopathogenic fungi qualify as candidates for microbial control of *P. xylostella*.

Key words: *Beauveria bassiana*, diamondback moth, fungi, *Lecanicillium* spp.

Introduction

The diamondback moth (DBM) *Plutella xylostella* (L) (Lepidoptera: Yponomeutidae) is one of the most destructive cosmopolitan insect pests of brassica crops. It has developed significant resistance to almost every synthetic insecticide applied in the field (Talekar and Shelton, 1993). Consequently, increased efforts worldwide have been undertaken to develop integrated pest management (IPM) programmes, including biological control (Sarfraz et al., 2005). Currently, products based on *Bacillus thuringiensis* (Bt) are applied against *P. xylostella*, which has now evolved resistance even against Bt in open field populations. Therefore, we investigated the possibility to use the entomopahogenic fungi *Lecanicillium* and *Beauveria bassiana* against diamondback moth. The *Lecanicillium* strains are the result of protoplast fusion and selection for high virulence.

Material and methods

Insect rearing and fungal cultures

Plutella xylostella second instars and *Beauveria bassiana* strain MG-Bb-1 were obtained from Miyagi prefecture horticulture experimental station maintained by T. Masuda (Japan). Larvae were reared on radish germ diet and were maintained at 25°C and 16:8 h L:D until use in this study. Thirteen *Lecanicillium* hybrid strains were selected from 174 strains as useful candidates for microbial control (Aiuchi et al., 2007). Fungal strains were cultured for 10 days on potato dextrose agar (PDA) at 25°C and 16:8 h L:D. The concentration of the spore suspension was adjusted to 1×10^6 spores/ml using an improved haemocytometer.

Bioassay with* Lecanicillium *hybrid strains

Thirteen strains of *Lecanicillium* hybrid strains were screened against *P.xylostella* larvae. The control larvae were treated with sterile 0.1% Tween using the same procedure. This experiment was repeated five times. The chambers were kept at 100% relative humidity. Suvival of larvae was checked daily for 6 days.

Experiment in cabbage field

A cabbage field was grown in Obihiro University of Agriculture and Veterinary medicine during June to July 2007. Hybrid 2aF27 (*Lecanicilium* spp.) and MG-Bb-1 (*Beauveria bassiana*) were sprayed every week. Conidial suspensions were adjusted to 2×10^7 spores/ml. For comparison, a Bt product was used at recommended concentration and controls received destilled water only. All spray solutions were supplemented with the spreader Tween. One division consisted of 18 cabbage plants, four of them inspected at each of three sites in the field for a total of 12 plants per sample date. *P. xlostella* population was counted every week. The population was classified into eggs, early and late instar larvae and pupae.

Results and discussion

Bioassay with* Lecanicillium *hybrid strains

Of 13 *Lecanicillium* hybrid isolates tested, 2aF27 was most virulent with 84% mortality after six days and it provided the shortest LT_{50} value (2.5 days). Damage was recorded and 2aF27 reduced feeding most of all 13 strains.

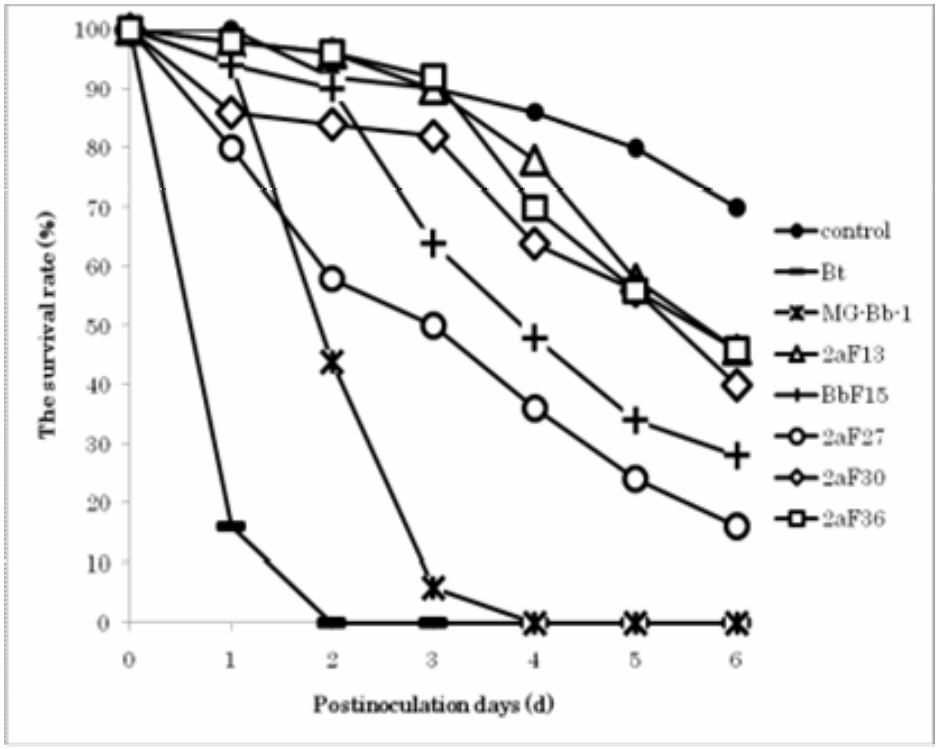

Figure 1. Survival of second instar larvae diamondback moth, *Plutella xylostella*, inoculated with the *Lecanicillium* hybrid isolates and *Beauveria bassiana* strain MG-Bb-1

On the other hand, larval mortality reached 100% four days after inoculation in treatments with *Beauveria bassiana* strain MG-Bb-1. This is the first report of using *Lecanicillium* against DBM.

Experiment in the cabbage field

Using entomopathogenic fungi in the field is said to be difficult because they are sensitive to environmental condition, especially humidity. However, MG-Bb-1 provided good control of *P. xylostella*, particularily against later-instars. Two applications were sufficient to achieve comparable control results like those recorded for the Bt treatment. Hybrid *Lecanicillium* strain 2aF27 was the least effectived of all three treatments against eggs, instars and pupae, but usually superior to the untreated control (Figure 2). Lots of the sporulating cadavers of later-instar larvae and pupae were detected on cabbage plants. These cadavers play an important role as a source of secondary infection. At the end of the investigation, the population level of *P. xylostella* had increased again. The experiment demonstrated that entomopathogenic fungi have a considerable potential for microbial control of *P. xylostella.*

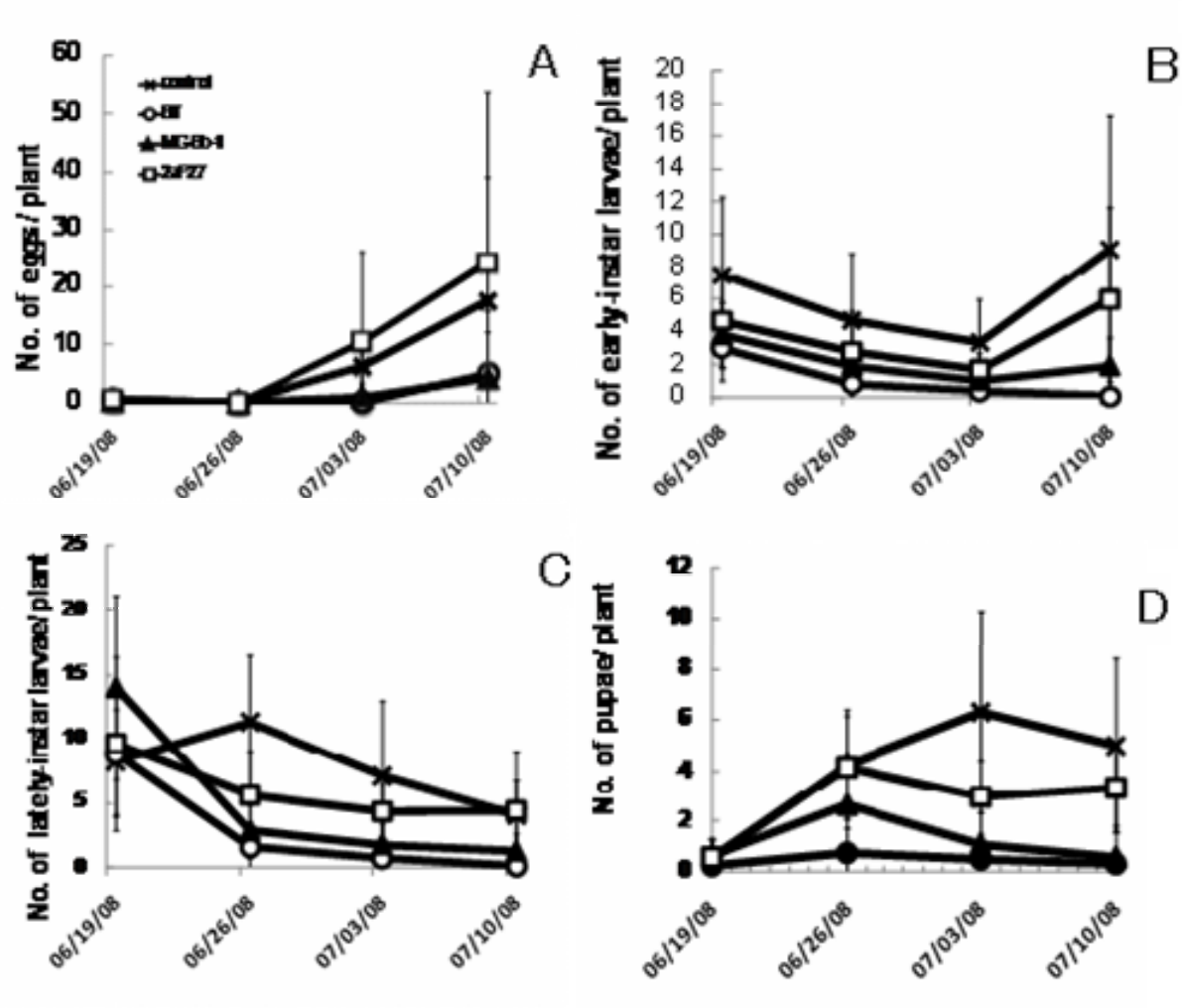

Figure 2. Number of A) eggs, B) early-instar, C) later-instar larvae, D) pupae of *Plutella xylostella* on cabbage plants in the field treated with Bt, *Beauveria bassiana* (MG-Bb-1) and *Lecanicillium* spp. (Hybrid strain 2aF27).

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Nematodes

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Identifying genes that are involved in the recovery process of the entomopathogenic nematode *Heterorhabditis bacteriophora* TTO1 strain

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Abstract: Characterizing the process of recovery in *Heterorhabditis bacteriophora* was done by identifying genes that are putatively involved in this process. For this purpose, a large scale bioassay for recovery was established and two subtraction libraries of recovered IJs subtracted by arrested IJs were constructed. Six hundreds expressed sequence tags (ESTs) were sequenced and annotated resulting in 300 useful ESTs that were compared to the *C. elegans* Wormbase and categorized into functional categories according to gene ontology. Of these, twenty three genes were chosen for further analysis. These genes were examined for their expression in the recovery process by quantitative (q) RT-PCR. The results of the RT-qPCR supported the results obtained from the subtraction libraries. Further analysis to these genes is being done by RNAi-based functional analysis in *H. bacteriophora*.

Key words: *H. bacteriophora*, nematodes, recovery, RT-qPCR, RNAi.

Introduction

Nematodes of the genus *Heterorhabditis* are widely used in biological control (Grewal et al., 2005). In *H. bacteriophora* the infective stage is the third juvenile; it is developmentally arrested and well adapted to long-term survival in the soil. When the infective juvenile (IJ) infects a suitable host, it recovers from this stage and resumes growth and development. This process is called recovery (Golden and Riddle, 1982). The recovery process is the first outcome of the host-parasitic interaction. Recovery is also a very important process from a commercial point of view: nematodes are produced for biocontrol purposes in industry-scale bioreactors (Ehlers, 2001). Recovery in liquid culture varies from 25-40% in few days. Low recovery leads to a non-synchronous population development, prolonged culture duration and unstable yields (Ehlers et al., 1998). Consequently, the key to a successful and cost-effective liquid culture process is through the management of IJ recovery. Tracking the process of recovery in *H. bacteriophora* is important for characterization of the host-parasite interaction. Only little is known about the molecular mechanism of recovery in *H. bacteriophora*. Understanding the molecular mechanism of the recovery will improve the recovery in vitro and thus the production efficiency of these nematodes.

Material and methods

Nematodes strain and recovery bioassay

Nematode strain used was *Heterorhabditis bacteriophora* TT01 and its natural bacterial partner *P. luminescens* TT01. Recovery bioassays were done in 9 cm plates, on 1.5% double destilled water agar at 28°C with 3 weeks old sterile IJs. The assay plates were covered with

hemolymph diluted with PBS 1:1 and treated with PTU in order to prevent melanization. The control plates were covered with the same concentration of PBS and PTU. The plates were inoculated with sterile IJs and incubated in 28°C. Recovery was determined by detecting secreted *P. luminescens* bacteria from IJs under the inverted microscope.

Construction of subtraction expressed sequence tag (EST) libraries

RNA was extracted from IJs subjected to hemolymph and control IJs. Subtraction hybridization was performed with the Clontech PCR-Select cDNA subtraction kit (Clontech, Palo Alto, USA). Random hexamer primers were used to convert mRNA into cDNA, which was subjected to subtractive hybridization against cDNA from control nematodes.

ESTs analysis and real time PCR (RT-qPCR)

The sequences were searched against existing protein databases using BLASTX (Altschul et al., 1990) in the databases wormpep131 (*C. elegans* protein database at The Wellcome Trust Sanger Institute, Cambridge, UK) (Apweiler et al., 2004) and the non-redundant (nr) protein database at the National Center for Biotechnology Information (NCBI). Sequence similarities with e-value less than or equal to 10^{-5} were considered significant.

Gene-specific primers were designed with Primer Express Software (Applied Biosystems) to amplify 100-bp sequences from each gene. 18S ribosomal DNA was used as a reference gene. RT-qPCR was done with Absolute™ QPCR Mix (ABgene, UK); 250 or 350 nM forward and reverse primers were used for each gene. The amplification conditions were: 95°C for 15 m, and 45 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Annealing temperature for all primers was 60°C. RT-qPCR was preformed with Mx3000p™ (Stratagene, USA). The level of target gene expression was calculated according to the ΔΔCT method.

Results and discussion

Phenotype characterization of P. luminescens regurgitation

Regurgitation of *P. luminescens* bacteria (Ciche and Ensign, 2003) is one of the first characterized signs in the recovery process. The recovery bioassay enables us to induce the recovery process *in-vitro*, in large scale. Regurgitation of *P. luminescens* was observed in 70% of the IJs 6-8 h after exposure to hemolymph. IJs that were not subjected to hemolymph did not regurgitate the symbiontic bacteria. At this time point, the IJs were harvest for the first subtraction library, the second library was done after 3 hours exposure to hemolymph.

ESTs analysis

A total of 600 ESTs were sequenced from the two cDNA subtraction library. Of which 421 sequences were searched against existing protein databases.The BLAST searches showed that 117 ESTs (27%) did not match any protein in the databases and were hence novel. A total of 304 ESTs (72%) matched to one or more known sequences in all databases. Of these, 109 from the 6h library and 161 from the 3h library had significant similarities to *C. elegans* proteins. The remaining 90 were similar to the mammalian parasite *Brugia malai* or eukaryotes like *Danio rerio* (zebra fish) and *Drosophila melanogaster* (fruit fly).

Categorized into functional groups

To categorize the transcripts by function, ESTs were assigned to metabolic pathways following gene ontology GO. ESTs with the same hit were grouped. The 270 ESTs that showed similarities to *C. elegans* proteins were categorized into functional groups (Table 1).

Table 1. Functional categorization of 271 ESTs of *Heterorhabditis bacteriophora* with significant similarity to *Caenorhabditis. elegans* proteins.

Regulatory pathway	**Number of ESTs 3h**	**Number of ESTs 6h**	**Percentage of ESTs 3h**	**Percentage of ESTs 6h**
1. Metabolism	**86**	**41**	**53**	**38**
1.1. Carbohydrate metabolism	2	11	1	10
1.2. Energy metabolism	73	7	45	6
1.3. Lipid metabolism	3	4	2	4
1.4. Nucleotide metabolism	1	3	1	3
1.5. Amino acid metabolism	4	8	2	7
1.6. Metabolism of other amino acids	0	6	0	6
1.7. Glycan biosynthesis and metabolism	1	1	1	1
1.8. Biosynthesis of polyketides and non ribosomal peptides	0	0	0	0
1.9. Metabolism of Cofactors and Vitamins	1	1	1	1
1.10. Biosynthesis of secondary metabolites	1	0	1	0
1.11. Biodegradation of xenobiotics	0	0	0	0
2. Genetic information processing	**27**	**17**	**17**	**15**
2.1. Transcription	10	2	6	2
2.2. Translation	8	12	5	11
2.3. Folding, Sorting and Degradation	9	3	6	3
3. Environmental information processing	**15**	**9**	**9**	**8**
3.1 Membrane transport	2	2	1	2
3.2. Signal transduction	7	5	4	5
3.3. Ligand-receptor interaction	6	2	4	2
3.4. Immune system	0	0	0	0
4. Cellular processes	**9**	**15**	**6**	**14**
4.1 Cell motility	1	2	1	2
4.2 Cell growth and death	2	2	1	2
4.3 Cell communication	6	9	4	8
4.4 Development	0	2	0	2
5. Unasigned	**13**	**19**	**8**	**17**
6. Hypothetical proteins	**11**	**9**	**7**	**8**
Total	**161**	**109**	**100%**	**100%**

In the 3 h library most ESTs (53%) in recovered IJs belonged to the metabolism category, to the sub category of energy metabolism, for example, mitochondrial genes that take part in the electron chain during respiratory processes. In the recovery process the infective juvenile has to undergo multiple changes as a response to the changing environment, in order to cope and adapt successfully. In the 6 h library the lipid metabolism pathway is the most active pathway. For example, fat reserves are the main reservoirs for energy (Selvan et al., 1993), and need to be degraded. Our results support high level of energy metabolism in the recovering IJs, which probebly provides the energy reqaired for the process. Those results were also supported by previous study made by Wang and Kim (Wang and Kim, 2001). Cellular processes category in 6h is more abundant compared to 3h, due to genes that take part in cell communication. We observed more regulatory genes in the 3 hours library comparing to the 6 hours library.

RT-qPCR analysis

For further analysis genes were chosen according to the number of times they appeared in the libraries. Gene were also selected according to RNAi phenotype in *C. elegan*as published in

wormbase (*C. elegans* database) and according to wormbase expression and interactions information. We selected genes that appeared to be known in *Brugia malayi* and not in *C. elegans* assuming these genes will express aspects that are only common to the two parasitic nematodes. We selected genes that are involved in signal transduction, in intracellular signaling, in cellular processing and signaling, and in metabolism processes.

RT-qPCR enabled us to detect genes expression differentially at 3 or 6 hours following exposure to hemolymph (Figure 1). To conclude analysis of the 23 genes expression pattern by RT-qPCR supported the subtraction libraries results. Further analysis to these genes is being done by RNAi-based functional analysis in *H. bacteriophora*.

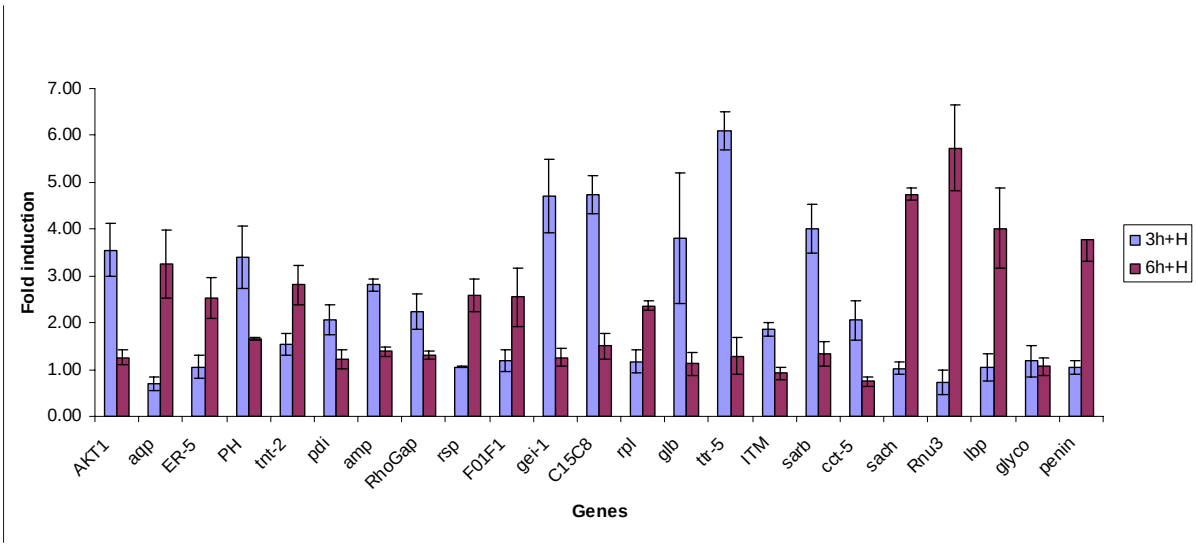

Figure 1: RT-qPCR results 3 and 6 hours post exposure to insect hemolymph: The ratio of normalized expression of treated IJs to non-treated is presented for each gene. The ratios are average values of 3 biological repeats. Standard error means are presented as error bars.

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Improving application to enhance pest control with entomopathogenic nematodes.

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Abstract: Entomopathogenic nematodes (EPN) (Nematoda: Rhabditidae) have been commercially manufactured for over 20 years. Their use, as with other biological control agents, has increased in recent years due to desire for more environmentally sensitive growing and legislative restrictions on chemical use. Beneficial nematodes offer high levels of pest control which enable them to compete in conventional pest control markets against a number of chemical insecticides. In order to obtain the highest levels of control with these biological control agents, targeted application is very important. This paper will discuss the advances in understanding in application of *Steinernema feltiae* (Nemasys™) to control sciarid larvae (*Bradysia* spp.) and Western flower thrips (*Frankliniella occidentalis*) to a number of growing systems which has helped to improve the commercial use of EPN.

Key words: Steinernematidae, Heterorhabditidae, entomopathogenic nematode, application technology, pest location.

Introduction

Entomopathogenic nematodes (EPN) (Nematoda: Rhabditidae) have been commercially manufactured for over 20 years. Their use, as with other biological control agents, has increased in recent years partly due to a desire for more environmentally sensitive growing and legislation such as EC/91/414 restricting chemical usage. Nine species of EPN are currently manufactured on a commercial scale (*Steinernema feltiae, S. carpocapsae, S. kraussei, S. riobrave, S. scapterisci, S. glaseri, Heterorhabditis megidis, H. bacteriophora* and *Phasmarhabditis hermaphrodita*), six of these are currently available in Europe.

Under laboratory conditions EPN have been shown to be able to kill a high number of pest insect species, for example *S. carpocapsae* has been shown to kill 250 insect species in the laboratory (Poinar, 1979). However, when these biological control agents are taken into the field the range and degree to which they can be controlled is often greatly reduced. Application of beneficial nematodes has been highlighted as arguably the most important factor in determining the success of their application (Matthews, 2000; Grewal, 2002).

The first true success of commercial use of EPN was the use of *S. feltiae* (Nemasys M) to control sciarid fly larvae in mushroom production. Nematode drenches are now a standard practice in the UK mushroom industry replacing chemical treatments. This success has expanded into soil drenches to control sciarid fly in a number of horticultural crops. *Steinernema feltiae* (Nemasys) is also used for control of Western flower thrips (*Frankliniella occidentalis* Pergande). Applications can be made as either foliar applications against adult females or soil drenches against pupae. For both methods of application targeted application is important in what are often challenging glasshouse environments.

Material and methods

Sciarid location in potted plants

Parsley, Begonias and Cala lillies are three examples of crops which have regular soil drenches of *S. feltiae* in order to control sciarid fly larvae. This investigation looked at the location of the sciarid fly larvae within the growing compost. For each type of plant between 8-10 plants were analysed. The plants had their rot ball cut into three depths. Soil was kept in clear plastic containers (23x12x7.5cm) at 22°C. Emerging flies were trapped on sticky cards (5x9.5cm) and identified.

Foliar deposition* S. feltiae *(Nemasys) on different varieties of potmum

Steinernema feltiae (Nemasys) was applied to a glasshouse of potmums at a rate of 121,000 nematodes/m^2 with Agral (0.003%) used as a foliar adjuvant. Application was made through a 7.6 m boom fitted with 16 nozzles (Turbo Teejet 0.3 l/h). Spraying was carried out at 0.75 bar and the boom was travelling over the crop at 40 m min^{-1}. Plants of the same age from the eight varieties to be investigated were placed on a bench under the central nozzle of the boom. Following application, four leaves were collected from the top of each plant, washed in 15 ml of water and number of nematodes present counted. Leaf area was calculated using ImageJ.

Effect of age of cut chrysanthemum crop on soil cover

In glasshouses in the UK and Netherlands a growing cycle for cut chrysanthemums is between 10-12 weeks depending on the time of year. Photographs were taken in a glasshouse in the Netherlands and then analysed with computer software to determine the percentage ground cover. Only one variety (Zembia) of cut chrysanthemum was grown in this 2.5 ha glasshouse. Three photgraphs were taken from each growing bay. Photographs were taken from 1.0 m above the ground of the crop growing next to the central walkway through the glasshouse. A Fujifilm Finepix A340 camera was used. Photographs were analysed using SigmaScan Pro software (SPSS, 1998; Karcher and Richardson, 2003). A turf grass analysis macro with colour settings of saturation 1:100 and hue 34:100 was used to differentiate soil and canopy cover as decribed by Karcher and Richardson (2005).

Results and discussion

Sciarid location in potted plants

Sciarid larvae were located at all depths of the potting media (Figure 1). Analysis over time showed that there was no difference in age of the larvae from different depths. Eggs have to be laid either on the upper surface or in the drainage holes on the underside of the pots. This work shows that sciarid larvae move throughout the growing media. These results highlight the importance of correct application of *S. feltiae* in a high volume of water (2 l/m^2) in order to distribute the EPN completely through the compost.

Foliar deposition* S. feltiae *(Nemasys) on different varieties of potmum

This work shows that for the majority of potmum plant varieties investigated there was no effect on the deposition of *S. feltiae* following application through this boom setup (Figure 2). This is important as commercial growers will often grow a number of varieties of potmums or chrysanthemums in the same glasshouse. This work has shown that for these varieties of potmum, differences in leaf wax covering, size, growth angle and hairiness have no effect.

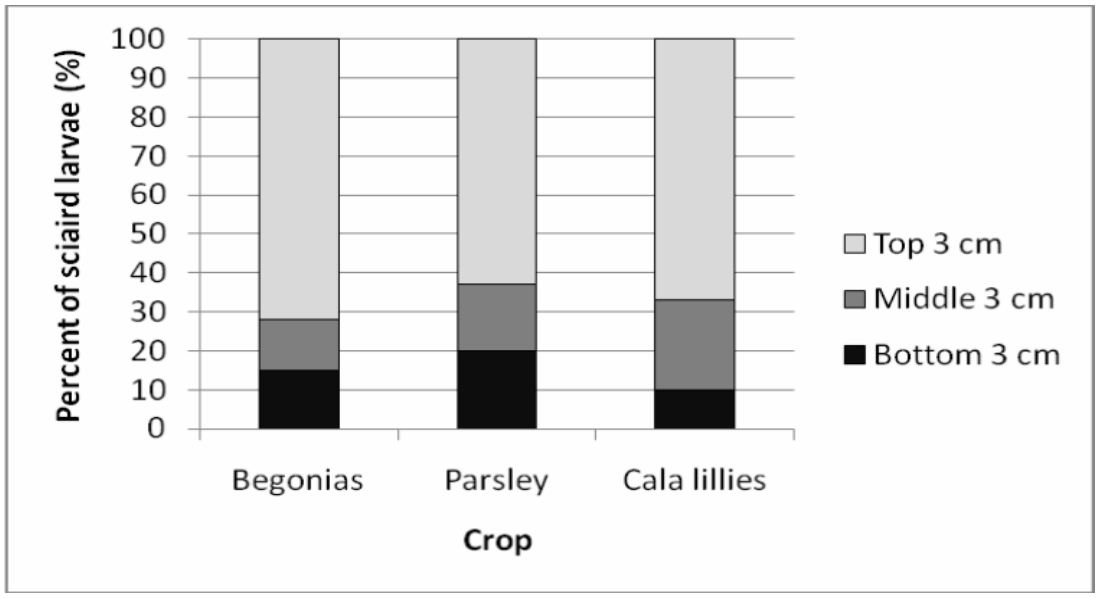

Figure 1. Percent distribution of sciarid larvae in a) begonias, b) parsley, c) cala lillies.

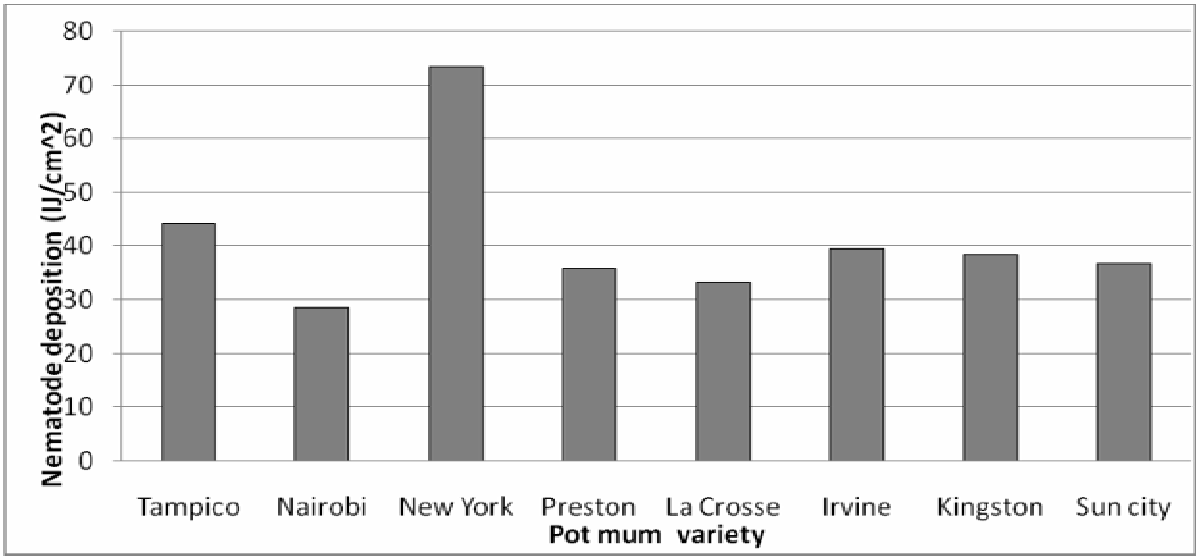

Figure 2. Effect of potmum variety on foliar deposition of *S. feltiae* (Nemasys) applied through a 7.6 m boom fitted with Turbo Teejet 0.3 l/h nozzles sprayed at 0.75 bar.

Effect of age of cut chrysanthemum crop on soil cover

Applications of *S. feltiae* can be made to the soil in order to control WFT pupae (Helyer *et. al.,* 1995). However in order to do so it is important that there are enough gaps in the crop canopy to allow the nematodes to reach the soil. For this variety of chrysanthemum there was complete canopy coverage after 26 days (Figure 3). Soil applications should be made before 66% canopy cover is reached in order to deliver the EPN to the soil with an even distribution. For this variety of chrysanthemum planted at 45 stems m^{-2} this occurred 15 days after planting. Application of EPN in a high volume of water or post-application irrigation can be used to increase canopy penetration. As with deposition on leaf surface, different nozzle types will affect canopy penetration to the soil and should be investigated.

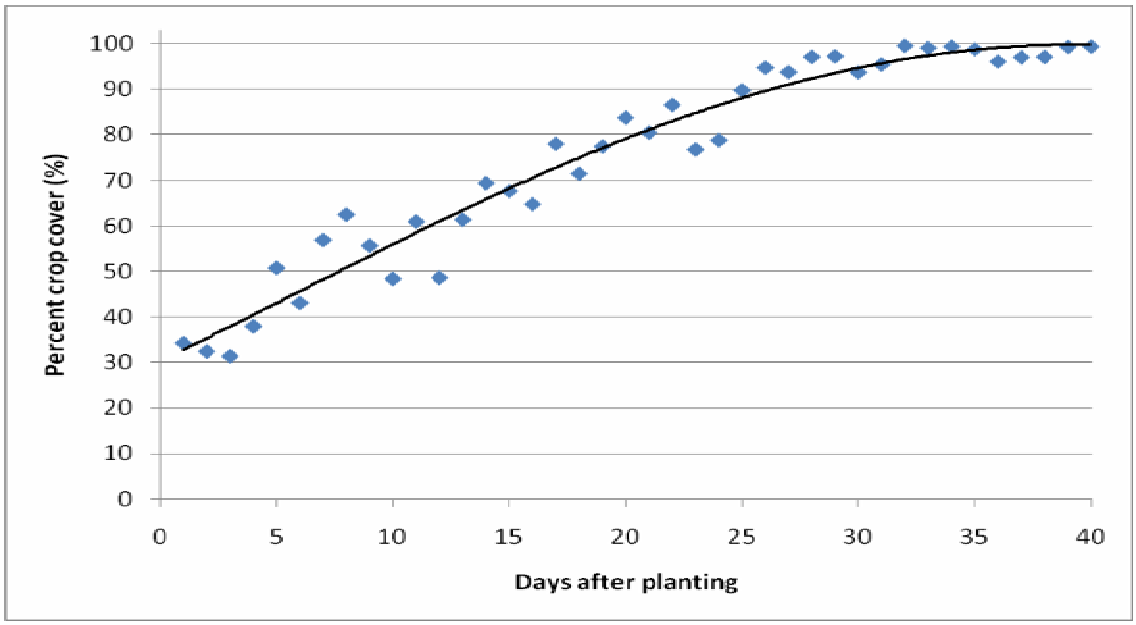

Figure 3. Effect of age of cut chrysanthemum plants (var. Zembia) on percentage soil cover.

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Control of carpophagous Lepidoptera in chestnut by means of entomopathogenic nematodes

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Abstract: *Cydia splendana* and *C. fagiglandana* are the main insect pests of chestnut groves in the Emilia-Romagna (Northern Italy). They damage fruits and cause sizeable quantitative and economic losses. A biennial investigation was carried out in some chestnut groves in the provinces Bologna and Ravenna with the aim of evaluating sustainable strategies for the control of both moth species with entomopathogenic nematodes (EPN). The effectiveness of different EPN species on moth larvae was tested in experimental trials. The application of EPN suspension on the ground showed encouraging results in decreasing the fruit damage at harvest.

Key words: *Steinernema*, *Heterorhabditis*, chestnut, *Cydia splendana*, *Cydia fagiglandana*, entomopathogenic nematodes

Introduction

Among the arthropods able to damage chestnut, the carpophagous species, chestnut weevil - *Curculio elephas* (Gyll.), chestnut leaf roller - *Pammene fasciana* (L.), beech seed moth - *Cydia fagiglandana* (Zel.) and chestnut moth - *Cydia splendana* (Hb.) (Bovey, 1966) can cause either a premature fruit drop or a direct damage of the growing and ripening fruits with heavy economic losses (Bariselli *et al.*, 2008). In particular *Cydia splendana* and *C. fagiglandana* can be considered the main insect species of chestnut groves in the Emilia-Romagna (Northern Italy) (Martini *et al*., 1998). The management of these pests needs to pay attention to the peculiar forest environment, a complex ecosystem, where each human intervention implies more or less heavy disturbance in its stability. In 2007-2008 experimental trials were carried out in chestnut groves with the aim of verifying both various methods to apply EPN suspensions to the soil and EPN effectiveness in the control of moth infestations.

Material and methods

In Spring 2007, five chestnut groves heavily infested by *C. splendana* and *C. fagiglandana* (in 2006, more than 50% decrease of yield at harvest), were chosen in the Emilia-Romagna (Northern Italy), in the provinces of Bologna (Monte S. Pietro, Castel del Rio, Loiano) and Ravenna (Casola Valsenio). In spring 2008, another five chestnut groves with the same characteristics of the previous year where chosen in the Ravenna province (Casola Valsenio).

In every chestnut grove, except in one case in 2007, a surface of 10,000 m^2 was defined and split in two large plots of 5,000 m^2: One plot was watered (untreated control) and the other one was treated with EPN suspensions of either *Heterorhabditis bacteriophora* or *Steinernema feltiae* + *H. bacteriophora* in 2007 and *H. bacteriophora*, *S. feltiae* or *S. carpocapsae* in 2008 at the rate of 1.5 x 10^9 infective juveniles (IJ) ha^{-1}.

The treatments were carried out by the end of May, at the first useful rainfall and with temperature >12 °C, using as equipment either a hand nozzle (launch) connected with a tractor power pump or a back power pump KWH, with pressure <15 bars and nozzles of Ø > 0.5 mm without filters, or a hand launch connected to a water tank placed aloft. EPN were distributed in various water volumes depending on the rain intensity (Tables 1 and 2).

Assessments

Pheromone traps (mod. Traptest®) baited with the synthetic sexual pheromone of *C. splendana* and *C. fagiglandana* (Rotundo & De Cristofaro, 1993) were hung up in the chestnut trees. The traps were inspected every 10-15 days, the moths were counted and removed at every check and the lure was replaced every 4 weeks. At chestnuts harvest, the undivided burrs were randomly collected from three trees located in the middle of each plot. Afterwards, the chestnut cupules were examined in the laboratory, assessing the percentage of damaged burrs and damaged fruits. The species of moth larvae were identified during the fruit check.

Table 1. Year 2007 - Main features of each EPN application

2007	Location:	Loiano (Bologna)
	Date of treatment:	2007/05/17
	EPN specie	*Heterorhabditis bacteriophora* + *Steinernema feltiae*
	Equipment:	hand nozzle (launch) connected with a tractor power pump
	Water volume:	18 hl in 5,000 m^2
	Weather conditions:	EPN application in a great water volume, 24 hours after a light rainfall; cloudy sky
	Location:	Monte San Pietro (Bologna)
	Date of treatment:	2007/05/29
	EPN specie	*Heterorhabditis bacteriophora*
	Equipment:	hand nozzle (launch) connected with a tractor power pump
	Water volume:	2 hl in 5,000 m^2
	Weather conditions:	EPN application under a pouring rainfall
	Location:	Castel del Rio (Bologna)
	Date of treatment:	2007/05/30
	EPN specie	*Heterorhabditis bacteriophora*
	Equipment:	back power pump KWH
	Water volume:	1 hl in 5,000 m^2
	Weather conditions:	EPN application 24 hours after a light rainfall; cloudy sky
	Location:	Sommorio of Casola Valsenio (Ravenna)
	Date of treatment:	2007/05/16
	EPN specie	*Heterorhabditis bacteriophora*
	Equipment:	hand nozzle (launch) connected with a tractor power pump
	Water volume:	12 hl in 5,000 m^2
	Weather conditions:	EPN application with cloudy sky
	Location:	Monte Battaglia of Casola Valsenio (Ravenna)
	Date of treatment:	2007/05/17
	EPN specie	*Heterorhabditis bacteriophora*
	Equipment:	back power pump KWH
	Water volume:	0.8 hl in 1,000 m^2
	Weather conditions:	EPN application under a pouring rainfall

Table 2. Year 2008 - Main features of each EPN application

2008	Location:	Sommorio 1 of Casola Valsenio (Ravenna)
	Date of treatment:	2008/05/18
	EPN specie	*Steinernema feltiae*
	Equipment:	hand nozzle (launch) connected with a tractor power pump
	Water volume:	6 hl in 5,000 m^2
	Weather conditions:	EPN application just before a pouring rainfall
	Location:	Banzuole of Casola Valsenio (Ravenna)
	Date of treatment:	2008/05/19
	EPN specie	*Heterorhabditis bacteriophora*
	Equipment:	treatment by hand nozzle (launch) powered by freefall water from a tank
	Water volume:	6 hl in 5,000 m^2
	Weather conditions:	EPN application under a pouring rainfall lasted three days
	Location:	Prugno of Casola Valsenio (Ravenna)
	Date of treatment:	2008/05/20
	EPN specie	*Steinernema carpocapsae*
	Equipment:	hand nozzle (launch) connected with a tractor power pump
	Water volume:	6 hl in 5,000 m^2
	Weather conditions:	EPN application before and after a pouring rainfall
	Location:	Sommorio 2 of Casola Valsenio (Ravenna)
	Date of treatment:	2008/05/20
	EPN specie	*Steinernema carpocapsae*
	Equipment:	hand nozzle (launch) connected with a tractor power pump
	Water volume:	6 hl in 5,000 m^2
	Weather conditions:	EPN application before and after a pouring rainfall
	Location:	Sommorio 3 of Casola Valsenio (Ravenna)
	Date of treatment:	2008/05/21
	EPN specie	*Steinernema feltiae*
	Equipment:	hand nozzle (launch) connected with a tractor power pump
	Water volume:	6 hl in 5,000 m^2
	Weather conditions:	EPN application before and after a pouring rainfall

Results and discussion

The initiative of the farmers allowed fitting the available equipments for EPN application even in traditional chestnut groves, where the water supply was quite difficult. The planting layout is irregular and the slopes may be steep. In some situations emerged the impossibility to take capacious water containers to the select places. In these cases the EPN treatment was closely bound to rainfall events.

The EPN applications were carried out by the end of May (2007 and 2008) when the moth populations (*C. fagiglandana* and, above all, *C. splendana*) were still found as wintering mature larvae in the soil. In 2007 the springtime was particularly dry and the rainfalls in April-May were lacking except at Monte San Pietro. Despite the lack of rain the treatments were carried out also on the other sites, distributing high water volumes before the moult of larvae to chrysalis, a stage unsusceptible to EPNs. In 2008 all treatments were carried out under optimal weather conditions.

The pheromone traps recorded in most cases a higher number of catches in the EPN treated areas than in the control. The burr and fruit assessments, however, indicated a significant effectiveness of *H. bacteriophora* in decreasing the moth damage. The best results

were achieved where the treatment was carried out at rainfall (Table 3). Sometimes the burrs were damaged only by superficial erosions, while the chestnuts were uninjured. *C. splendana* was found with greater frequency inside of the worm-eaten fruits. The larvae and the damage of the chestnut weevil were observed only sporadically.

This study has confirmed the feasibility of chestnut pest biocontrol by means of EPNs as other researches already suggested (Kuske *et al*., 2005; Vinciguerra & Clausi, 2006).

Table 3. Percentage of burrs and chestnuts damaged by moths at harvest

EPN Species	**Location**	**Damaged burrs (%)**		**Damaged chestnuts (%)**	
		Control	**EPN treated**	**Control**	**EPN treated**
H. bacteriophora	Casola Valsenio – Banzuole	45.6	17.0	38.2	11.0
	Monte San Pietro	59.9	37.3	50.0	27.5
	Castel del Rio	73.5	50.0	59.4	38.9
	Casola Valsenio - Sommorio	75.0	25.0	58.3	26.7
	Casola Valsenio – Monte Battaglia			70.0	14.0
	Standard dev.	13.7310	14.4415	11.8567	11.2897
	t Student	**3.1289 ***		**4.3105 ****	
S. feltiae	Casola Valsenio – Sommorio 1	56.0	41.0	45.0	30.0
	Casola Valsenio – Sommorio 3	32.2	33.0	36.3	27.3
	Standard dev.	16.8291	5.6569	6.1518	1.9092
	t Student	**0.5655 N.S.**		**2.6347 N.S.**	
S.carpocapsae	Casola Valsenio – Prugno	65.8	52.1	54.0	40.4
	Casola Valsenio – Sommorio 2	48.9	44.3	41.9	34.7
	Standard dev.	11.9501	5.5154	8.5560	4.0305
	t Student	**0.9832 N.S.**		**1.5551 N.S.**	
S.feltiae + H. bacteriophora	Loiano	76.1	50.0	62.3	40.0

N.S. = not significant * significant P≤0.05 ** highly significant P≤0.01

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Conditioning of *Steinernema kraussei* and *S. carpocapsae* through storage: Enhancing their performance over wide temperature ranges

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Abstract: The influence of conditioning of entomopathogenic nematodes *Steinernema kraussei* and *S. carpocapsae* by storage at low temperature was assessed. *S. kraussei* virulence greatly decreases between 15 and 20°C suggesting that it is a poor biocontrol agent in warm conditions; *S. carpocapsae* shifted from showing poor to higher virulence between these temperatures. Virulence of both EPN species can be enhanced, at times to over 90% host death, at both sub-optimal and optimal temperatures following storage at low temperatures, this being greatest following 9-12°C storage. The greatest conditioning cases occurred for *S. kraussei* bioassayed at 27°C, and *S. carpocapsae* bioassayed at 9-15°C. Results indicate that low temperature storage causes major conditioning effects in terms of EPN virulence.

Key words: Virulence, low temperature activity, storage temperature storage

Introduction

Entomopathogenic nematodes (EPN) can provide effective pest control over narrow temperature ranges and these vary between EPN species (Long et al., 2003). One example where ambient temperature currently hinders biological control by EPN is the management of *Otiorhynchus sulcatus*, black vine weevil. While *Heterorhabditis megidis* is a potential biological control agent of the pest, its performance is poor at low temperatures (Long et al., 2000). EPN infectivity can be enhanced, which may increase their biocontrol potential at both optimal and sub-optimal temperatures (Griffin et al., 1996; Fitters et al., 2001). These 'conditioning' effects occur following low-temperature storage of EPN. This study examined the potential of conditioning on EPN virulence, which was assessed over a much wider set of storage and bioassay temperatures than previous studies for *Steinernema kraussei* and *S. carpocapsae*. We aim to identify how the largest conditioning effects can be achieved.

Materials and methods

S. kraussei and *S. carpocapsae* were obtained from Becker Underwood Ltd (Littlehampton, UK) and their virulence was assessed at 4, 7, 9, 12, 15, 20, 27 and 32˚C. The EPN were then stored in sealed plastic circular containers (200 ml, 9 cm diameter) at the concentration 1,000 ±100 EPN/ml tap water, with 20 ml of suspension per container, at 4, 9, 12 and 15˚C. They were bioassayed at 4, 9, 12, 15, 20 and 27˚C following 3, 6, 9 and 12 weeks of storage. Mid-instar mealworms *Tenebrio molitor* were used for the bioassays in 24-well plates. Each well contained 0.12 g coconut coir soaked with 2.5 times (w/w) tap water. Twelve wells per plate were controls with 20 µl distilled water added to each well. The remaining 12 wells per plate

had 10 µl of EPN suspension (containing 10 ± 1 EPN) added and 10 µl of tap water. A *T. molitor* larva was then added to each well, and mortality was assessed after 5 days.

Results and discussion

Bioassay temperature affected the virulence of both EPN species against *T. molitor* larvae, with *S. kraussei* and *S. carpocapsae* having contrasting temperature ranges where they are most virulent (Fig. 1). The temperature ranges where the EPN were most virulent were 7-15˚C for *S. kraussei* and 20-27˚C for *S. carpocapsae*.

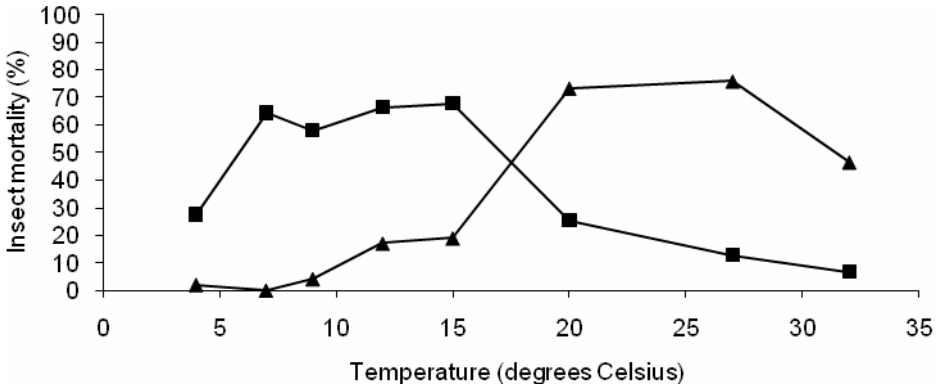

Figure 1. Effect of bioassay temperature on insect mortality (%) following application of pre-stored *S. kraussei* (■) and *S. carpocapsae* (▲).

Both EPN species showed some evidence of virulence increases at all bioassay temperatures except 4˚C following storage (Fig. 2, 3). These enhancements were greatest and persisted for the whole storage period for EPN stored at 9-12˚C. Virulence of both species was enhanced at optimal and sub-optimal bioassay temperatures, with these effects greatest at previously sub-optimal temperatures (27˚C for *S. kraussei*, 9-15˚C for *S. carpocapsae*).

This study has emphasised how *S. kraussei* and *S. carpocapsae* are most virulent over distinct thermal niches, influencing where and when they can be utilised as biocontrol agents. *S. kraussei* virulence greatly decreases between 15 and 20˚C suggesting that it is a poor biocontrol agent in warm conditions. Virulence of both EPN species can be enhanced, at times to over 90% host death, at both sub-optimal and optimal temperatures following storage at low temperatures, this being greatest following 9-12˚C storage. The greatest conditioning cases occurred for *S. kraussei* bioassayed at 27˚C, and *S. carpocapsae* bioassayed at 9-15˚C. This shows that low temperature storage causes major conditioning effects in terms of EPN virulence. Further research is ongoing, including identification of how the conditioning alters EPN virulence and assessment of how conditioned EPN perform in field situations.

Acknowledgements

This study was financed by funding from both Teagasc and the IRCSET.

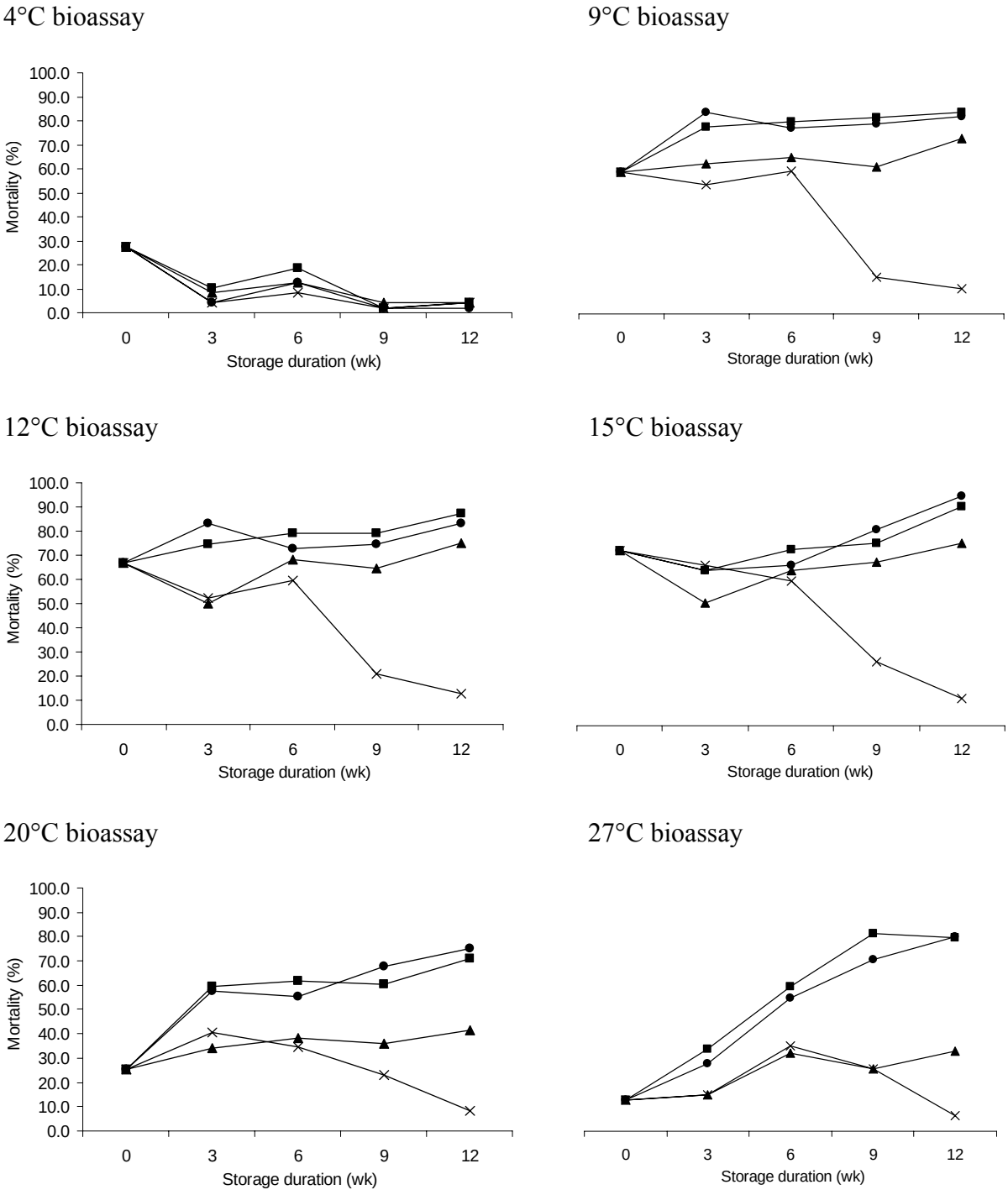

Figure 2. Effect of storage temperature and duration on virulence of *S. kraussei* at various bioassay temperatures (all graphs, ▲ = 4°C storage of EPN; ■ = 9°C; ● = 12°C; X = 15°C).

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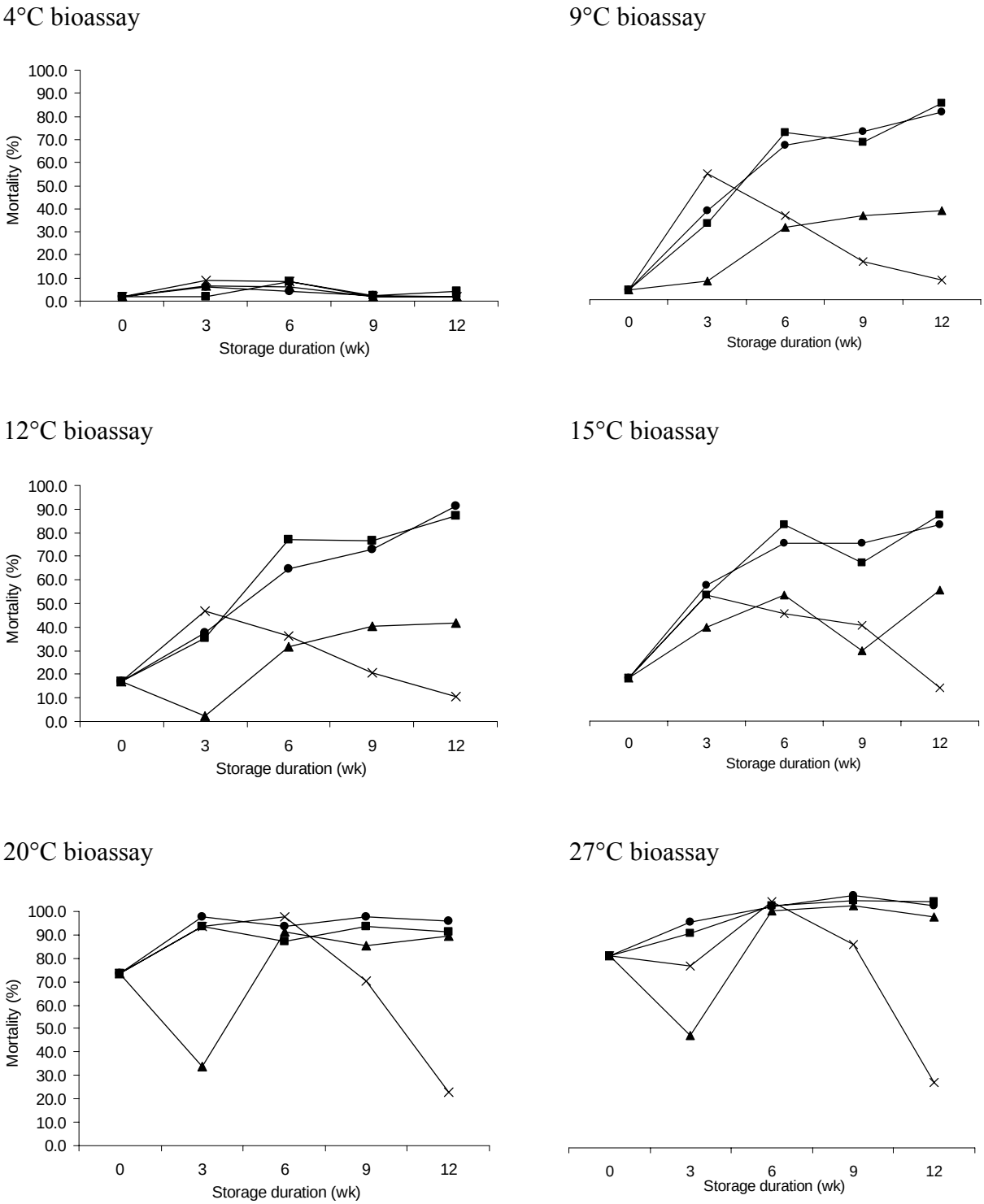

Figure 3. Effect of storage temperature and duration on virulence of *S. carpocapsae* at various bioassay temperatures (all graphs, ▲ = 4°C storage of EPN; ■ = 9°C; ● = 12°C; X = 15°C).

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Population dynamics of *Steinernema carpocapsae* and *S. feltiae* in liquid culture

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Abstract: *Steinernema carpocapsae* and *S. feltiae* were cultured with their symbiotic bacteria in liquid media. Although these two species are broadly used as biological control agents, the life cycle in liquid culture has not been described well. In this study, the population dynamics of both *Steinernema* species in liquid culture was observed in relation to the development of their symbiotic bacteria in the monoxenic cultures.

Key words: entomopathogenic nematode, population development, *Xenorhabdus* spp.

Introduction

Entomopathogenic nematodes, *Steinernema carpocapsae* and *S. feltiae*, are symbiotically associated with the bacteria *Xenorhabdus nematophila* and *X. bovienii*, respectively. For commercial production, nematodes are produced in large scale bioreactors (Ehlers, 2001). The life cycle of nematodes are basically similar to that in insect with few exceptions. For example, in order to assist nematode development in liquid culture, the symbiotic bacteria are inoculated into liquid medium at least one day before DJ inoculation. Pre-cultured bacteria produce chemical cues triggering DJ recovery (Aumann and Ehlers, 2001). However, DJ recovery can vary among cultures. Low DJ recovery causes non-synchronous population development resulting in additional generations and prolonged process time to reach maximum population density (Ehlers, 2001).This study describes the population dynamics of *Steinernema carpocapsae* and *S. feltiae* in liquid culture.

Material and methods

Nematodes and symbiotic bacteria

The All strain of *Steinernema carpocapsae* was used for all experiments. The DJs were inoculated in liquid medium at the density of 4,000 DJs ml^{-1} and incubated on a shaker at 180 rpm at 25^{o}C in the dark for 14-16 days. Then they were washed with sterile Ringer solution and the active DJs were selected by migration of DJs through 0.03 mm sieves. Their symbiotic bacterium, *Xenorhabdus nematophila*, was isolated from nematode-infected *Galleria mellonella* larvae and propagated in YS medium. The stock solution supplemented with glycerol (15% ^{V}/v) was stored at -20^{o}C until use. The EN02 hybrid strain of *S. feltiae* and their symbiotic bacteria, *X. bovienii*, were prepared as described above.

Bacterial pre-culture and nematode flask cultures

Bacterial pre-culture and flask cultures were carried out according to Johnigk et al. (2004). A 10 l bioreactor was filled with pH adjusted artificial liquid medium, sterilzed and then

inoculated at 1% of its total volume with a symbiotic bacterial culture of 10^9 cells ml^{-1}. The bacteria were pre-cultured for 12-18 h until a cell density of *X. nematophila* of 2 x 10^{10} cells ml^{-1}. The cell density was adjusted at 10^{10} cells ml^{-1} by dilution with culture supernatant, from which cells were removed by centrifugation. Pre-culture of *X. bovienii* reached a cell density of 10^{10} cells ml^{-1}. The cultures were then transferred into sterile 200 ml flasks and DJs were inoculated at a density of 4,000 DJs ml^{-1}. Samples were collected from flask cultures every day and examined under an invert microscope for nematode recovery and development of the population dynamics.

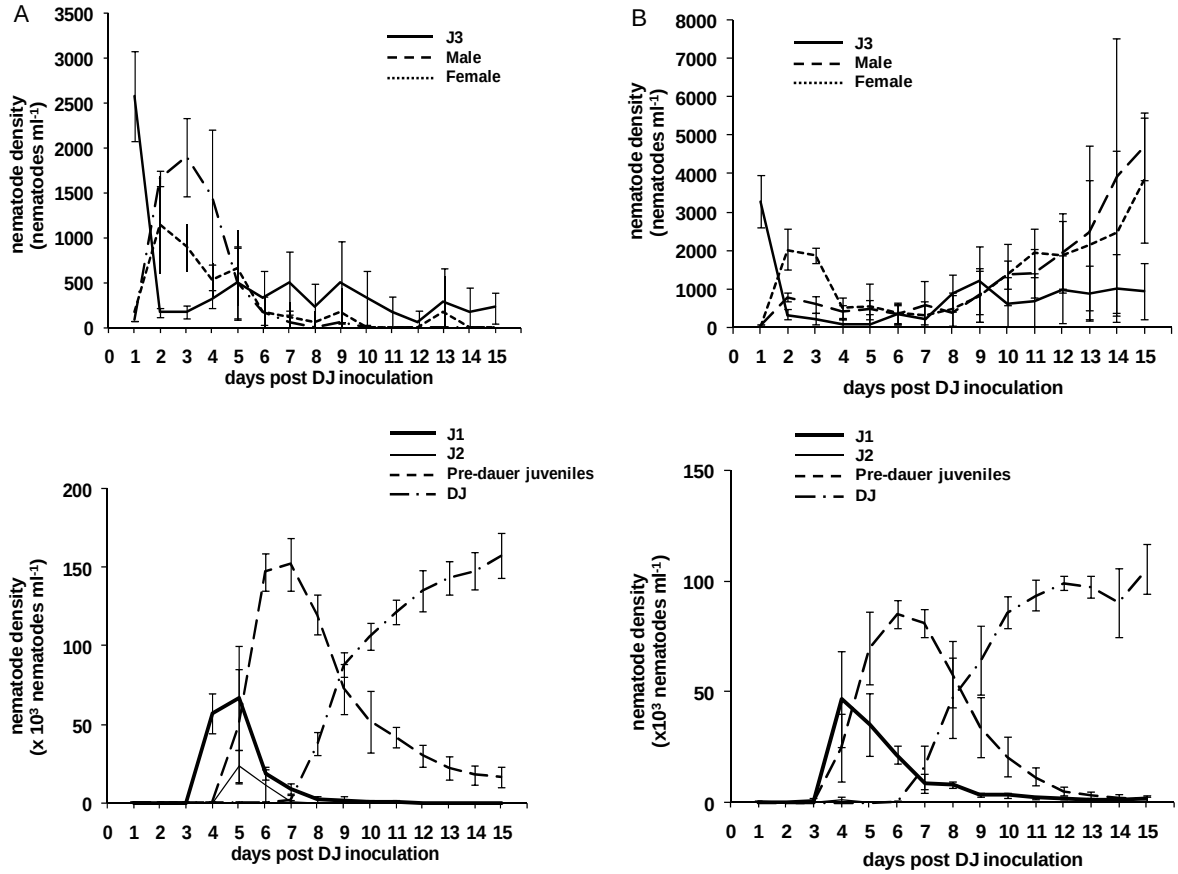

Figure 1. Population dynamics of *Steinernema carpocapsae* (A) and *S. feltiae* (B) in liquid culture recorded from DJ inoculation to 15 days post DJ inoculation at 25°C. Density of 3rd stage juvenile (J3), male and female are shown in upper figures. Density of first and second stage juvenile (J1 and J2), pre-dauer juvenile, DJ are shown in the bottom figure. Bars in lines indicate upper and lower 95% confidence interval.

Results and discussion

Population dynamics of Steinernema carpocapsae and S. feltiae

The population dynamics of *Steinernema carpocapsae* and *S. feltiae* are presented in Fig. 1. DJ recovery was assessed one day before newly hatched progeny was observed. DJ recovery of *S. carpocapsae* was 70.6%, while DJ recovery of *S. feltiae* was 82.4%. Newly hatched progeny was observed on 4 days post DJ inoculation (dpi) in *S. carpocapsae*, while first

progeny of *S. feltiae* was observed on 3 dpi. The maximum population density of *S. carpocapsae* reached 180 x 10^3 nematodes ml^{-1} on 6 dpi, while the maximum density of *S. feltiae* was observed on 8 dpi, at a density of 115 x 10^3 nematodes ml^{-1}. Newly developed DJs of *S. carpocapsae* were observed on 7 dpi, while new DJs of *S. feltiae* were observed on 6 dpi. On 15 dpi, the proportion of DJs reached approximately 90% in both species. Second and third adult generations of *S. feltiae* increased from 8 dpi, while further generations of *S. carpocapsae* were rarely observed.

Symbiotic bacteria development

Cell density dynamics of *Xenorhabdus nematophila* and *X. bovienii* are presented in Fig. 2. The cell density of *X. nematophila* and *X. bovienii* had increased until 1 dpi, then decreased. *Xenorhabdus nematophila* cell density rapidly decreased from 1 dpi to 5 dpi, and continued to decrease slightly. The cell density reached the minimum on 11 dpi, at a density of 4.1 x 10^8 cells ml^{-1}, then slightly increased until 15 dpi. The cell density of *X. bovienii* had decreased 1 dpi to 4 dpi, reaching the minimum at a density of 9.8 x 10^8 cells ml^{-1}. The cell density increased after 4dpi, and reached 1.2 x 10^{10} cells ml^{-1} on 12 dpi.

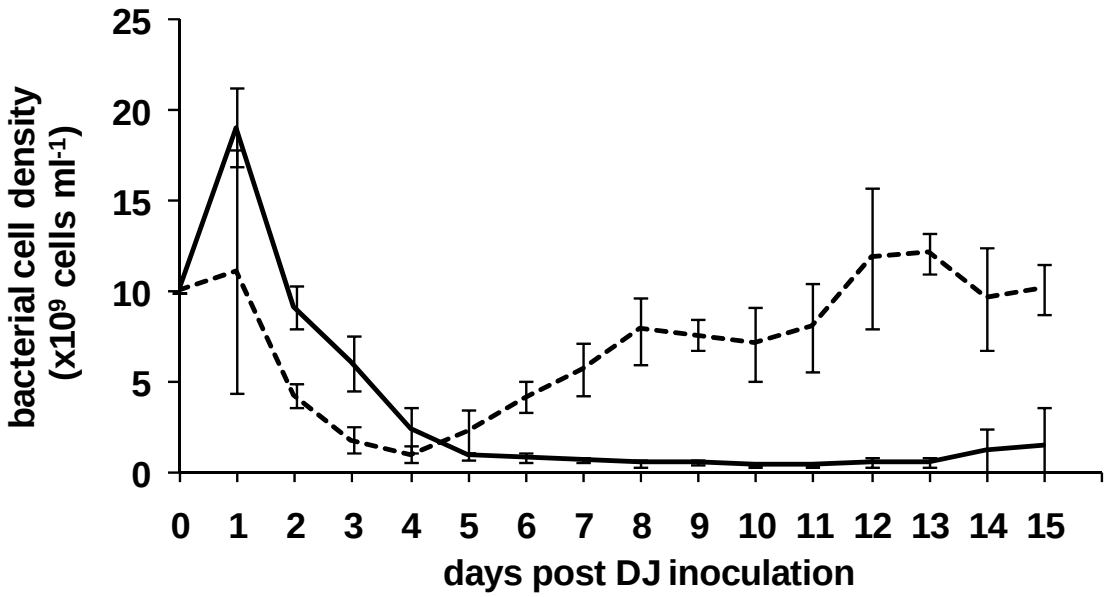

Figure 2. Cell density dynamics of *Xenorhabdus nematophila* (solid line) and *X. bovienii* (dotted line) in liquid culture from the day of DJ inoculation to 15 days post DJ inoculation at 25°C. Bars on lines indicate upper and lower 95% confidence interval.

In this study, a high DJ recovery was induced in bacterial pre-culture at a density of 10^{10} cells ml^{-1}. DJ recovery of *S. carpocapsae* reached 70% on 1 dpi and it had not much increased afterwards, while DJ recovery of *S. feltiae* continued to increase after newly hatched juveniles were observed and no DJs could be determined on 4 dpi, indicating an asynchronous development in the first generation. The *S. carpocapsae* population developed more synchronously and almost all progeny developed to DJs. The DJ density, which increased continuously until 15 dpi indicated that the progeny of second and third generations also developed to DJs. Their symbiotic bacteria, *X. nematophila,* were consumed until 5 dpi and the density remained lower than 10^9 cells ml^{-1} until 15 dpi. This nutritional limit might induce

newly hatched progeny of *S. carpocapsae* to form DJs. Most of the inoculated DJs of *S. feltiae* recovered early and developed synchronously. Then the progeny of these nematodes was induced to develop to DJs. The increase of adult density after 8 dpi indicates that the progeny of late recovered DJs and progeny in second and third generation were induced to develop to adults instead of DJs. The cell density of *X. bovienii* reached the lowest density between 3 to 4 dpi. However, the cell density increased again. The increase of the cell density prevented starvation condition and thus cues inducing development to DJs were missing. Nutritional effects on development either to DJs or adults was observed also in *H. bacteriophora* (Strauch et al., 1994). The factor which supported the development of *X. bovienii* could not be determined in this study. To obtain high number of DJs within a short process time, the control of the bacterial cell density is necessary at the moment of F1 generation progeny hatching, especially for development of *S. feltiae* DJs.

Acknowledgements

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Optimal incubation temperature for *Steinernema carpocapsae* and *S. feltiae* in liquid culture

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Abstract: Mass production of *Steinernema carpocapsae* and *S. feltiae* is done in liquid culture by pre-culturing the symbiotic bacteria of genus *Xenorhabdus* spp. for 1 to 2 days prior to dauer juveniles (DJ) inoculation. Major problems for commercial production can originate from variable progeny DJ density. This study aimed to determine optimal incubation temperature for DJ reproduction to reduce process duration. Temperatures tested were 20, 23, 25, 27 and 29°C. The population development was observed. The onset of development of DJs (termed 'recovery') and the fecundity in *S. carpocapsae* were significantly reduced at 29°C. Only the fecundity but not recovery of *S. feltiae* was reduced at high temperature (27°C). The DJ yield was lower at high temperature and the development was delayed at low temperature in both *Steinrenema* spp. Optimal growth temperature for both nematodes is 25°C. Highest DJ densities were obtained after 10 days incubation at 25°C.

Key words: *Steinernema spp.*, temperature, dauer juvenile, recovery, fecundity, liquid culture

Introduction

The dauer juveniles (DJs) of entomopaghoenic nematodes, *Steinernema carpocapsae* and *S. feltiae*, are used as biological control agents against a variety of pest insects (Grewal et al., 2005). The mass production in liquid culture begins with the pre-culture of symbiotic bacteria, *Xenorhabdus nematophila* and *X. bovienii*, in artificial media prior to DJ inoculation. During the pre-culture, the bacteria produce secondary metabolites which induce DJ recovery, called 'food signal' (Strauch and Ehlers, 1998). Inoculated DJs are triggered by the food signal and recover development. The new progeny develops either to the reproductive adults or to DJs, depending on the nutritional condition (Golden and Riddle, 1984). Cost effective DJ production is determined by progeny DJ density and culture duration. Longer process duration and variable progeny DJ density are the main problems, often caused by variable DJ recovery, reduced female fecundity and variable DJ formation.

In this study, the optimal culture temperature was determined. The effect of different temperatures on population development, DJ recovery, fecundity and DJ yield in liquid culture was assessed. The influence of incubation temperature on the infectivity of progeny DJ was also determined.

Material and methods

Nematodes and symbiotic bacteria

The All strain of *S. carpocapsae* and the EN02 hybrid strain of *S. feltiae* were used for all experiments. The DJs were inoculated in liquid medium at the density of 4,000 DJs ml^{-1} and incubated on shaker at 180 rpm at 25°C in the dark for 14-16 days. Then they were washed with sterile Ringer solution and the active DJs were selected by immigration though 0.03 mm

sieves. Their symbiotic bacteria, *Xenorhabdus nematophila* or *X. bovienii* were isolated from nematode infected *Galleria mellonella* larvae and propagated in YS medium.

Bacterial pre-culture and nematode flask cultures

A bioreactor of 10 l volume was filled with pH-adjusted artificial liquid medium, and sterilized. A bacterial culture in YS medium at a cell density of 10^9 cells ml^{-1} was transfered at 1% volume to the bioreactor. When the bacterial pre-culture had reached a cell density of 10^{10} cells ml^{-1}, the pre-culture was distributed among several sterile flasks and inoculated with 4,000 DJs ml^{-1}. These flask cultures were incubated at 23, 25, 27 and 29°C for *S. carpocapsae*, and 20, 23, 25 and 27°C for *S. feltiae*. Samples were collected from flask cultures every day and at least 100 nematode individuals were examined under an invert microscope. DJ recovery, the day on which newly hatched F1 progeny was observed, fecundity of females developed from inoculum DJs and progeny DJ density on the 10^{th} day after DJ inoculation (dpi) were recorded. The experiments were repeated twice.

Influence of incubation temperature on the infectivity of progeny DJs

The infectivity of progeny DJs harvested on 10 dpi was assessed by one-on-one bioassays using final instars of *G. mellonella* larvae for *S. carpocapsae*, and using sand buried *Tenebrio molitor* larvae for *S. feltiae*. The insect mortality was checked on 5 days after infection.

Statistical analysis

All experiments had three replications and each experiment was repeated twice. Percentage values for DJ recovery and infectivity of progeny DJs were arcsine-transformed. Influence of incubation temperature was analysed using ANOVA and the Tukey HSD test.

Results and discussion

Effect of different temperatures on nematode population development

DJ recovery, the day of first F1 progeny observed, fecundity of female developed from inoculum DJs and DJ yield on 10 dpi at different incubation temperatures are presented in Table 1. DJ recovery of *S. carpocapsae* was significantly reduced at 29°C, while DJ recovery at 23, 25 and 27°C was not significantly different. DJ recovery of *S. feltiae* was over 90% in all cultures. The developmental speed from inoculum DJs to the next generation was also affected by the incubation temperature.The DJ yield on 10 dpi was highest at 25°C in *S. carpocapsae*, while the optimal incubation temperature for DJ reproduction ranged from 23 to 25°C in *S. feltiae*.

Effect of different temperatures on infectivity of progeny DJs

The infectivity of progeny DJs harvested on 10 dpi is presented in Figure 1. There was a significant effect of the incubation temperature on the infectivity of *S. carpocapsae* DJs to *G. mellonella* larvae and *S. feltiae* DJs to *T. molitor* larvae. The infectivity of *S. carpocapsae* DJs varied between 22.2 and 37.5 %, with the highest infectivity observed at 27°C. The infectivity of *S. feltiae* incubated at 23, 25 and 27°C ranged between 40.3 and 45.8%, and there was no significant difference. The DJs developed at 20°C represented significantly lower infectivity at 16.6 %.

Table 1. Influence of different incubation temperature on dauer juvenile (DJ) recovery, first occurrence of F1 in days post inoculation (dpi), fecundity of females (eggs per female) and DJ yield (x10^3 DJs ml^{-1}) on 10 dpi. Different letters indicate significant differences between treatments (small letters for *S. carpocapsae*, capital letters for *S. feltiae*) (Tukey HSD test, P≤ 0.05).

	S. carpocapsae			
°C	**23**	**25**	**27**	**29**
Recovery (%)	$64,8^{a}$	$58,8^{a}$	$65,1^{a}$	$49,8^{b}$
F1 (dpi)	5,8	4,5	5,0	6,8
Fecundity (eggs/♀)	$100,1^{b}$±8,1	$99,8^{b}$±6,0	$130,8^{a}$±14,8	$75,2^{c}$± 8,4
Yield (10^3 DJs ml^{-1})	$2,6^{c}$±1,4	$103,1^{a}$± 8,7	$54,3^{b}$±32,9	$19,5^{c}$±18,4
	S. feltiae			
°C	**20**	**23**	**25**	**27**
Recovery (%)	$94,3^{A}$	$93,5^{A}$	$94,4^{A}$	$94,3^{A}$
F1 (dpi)	4,6	3,5	3,5	4
Fecundity (eggs/♀)	$99,6^{B}$±10,3	$146,5^{A}$± 9,6	$141,7^{A}$±10,2	$112,3^{B}$± 8,7
Yield (10^3 DJs ml^{-1})	$14,2^{B}$±14,0	$61,7^{A}$±14,1	$66,2^{A}$± 4,3	$38,2^{B}$±13,9

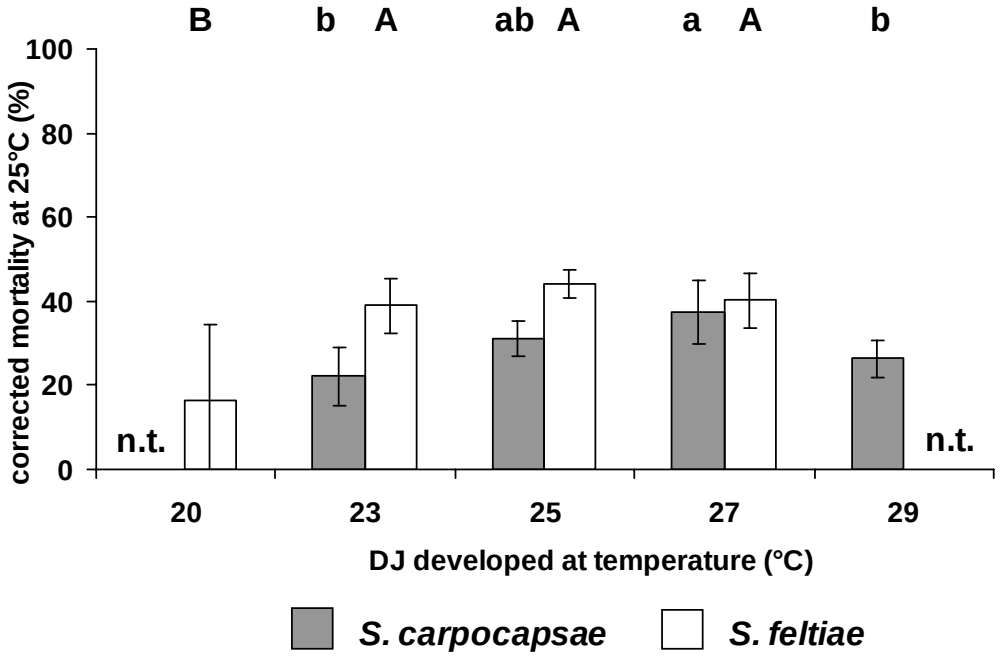

Figure 1. Infectivity of dauer juveniles (DJ) of *S. carpocapsae* (grey bar) and *S. feltiae* (white bar) at 25°C. DJ of *S. carpocapsae* developed at 23, 25, 27, 29°C, *S. feltiae* at 20, 23, 25, 27°C. Bars on columns indicate 95% confidence intervals. Different letters on bars indicate significant differences between treatments (small letters for *S. carpocapsae*, capital letters for *S. feltiae*) (Tukey HSD test, P ≤ 0.05).

The optimal culture temperature is 25°C for *S. carpocapsae* and 23° to 25°C for *S. feltiae*. Incubation at 29°C reduced DJ recovery and reproduction of *S. carpocapsae* significantly. Fecundity was low and the development took longer (6.8 days for occurrence of

first F1 progeny). However, DJ density on 10 dpi was higher than the cultures incubated at 23°C, indicating that the incubation temperature at 29°C had no negative influence on DJ formation. *Steinernema feltiae* development was longer at 20°C. Although the DJ recovery reached over 90%, developmental speed and fecundity were reduced and DJ yields were significantly lower. The quality (infectivity at 25°C) of DJ, which had developed at different temperatures, was variable. The DJs of *S. carpocapsae* incubated at 23 and 29°C had significantly lower infectivity. Whereas the DJs of *S. feltiae* represented similar infectivity except for those which had developed at 20°C.

This study determined the optimal temperature which supports the development of progeny DJ. The results also indicate that culture temperature can influence other traits than just the reproduction process. E.g., the influence of the incubation temperature on fatty acid composition was reported by Fodor et al. (1994). A detailed analysis for the correlation between culture temperature and nutritional condition is necessary for further improvment of the culture conditions.

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First field experiment with entomopathogenic nematodes in Slovenia

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Abstract: Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) was the target pest in our research. The experiment had 6 different treatments: *S.feltiae* B30 lconc., *S. feltiae* B30 hconc., Entonem lconc., Entonem hconc., Actara and control all repeated in 4 blocks. Observing the population dynamics of CPB we conclude, that entomopathogenic nematodes have a big influence on larval stages but on the other hand no effect on egg and adults in field experiment. Insecticide Actara showed the best results among the observations. Here the mortallity of the insect was the highest. We also studied the effect of controling CPB on the yield of potato. There were no differences beetwen EPN treatments, however the results using EPN were better compared to control treatments and less evident as the results of insecticide Actara.

Key words: Entomopathogenic nematodes, Colorado potato beetle, field experiment, biological control

Introduction

The Colarado Potato Beetle (CPB) (*Leptinotarsa decemlineata* Say) (Coleoptera: Chrysomelidae) is an economicaly important pest in potatos. Full defoliation can reduce the yield for more than 50 % (Hare, 1990). Control is usualy with insecticide, but some populations have already developed resistance (Forgash, 1985) and even to *Bacillus thuringiensis* Berliner subsp. *tenebrionis* resistance is possible (Whalon *et al*., 1993). Therefore solution for potato protection using predators and parasitoids are needed (Armer *et al*., 2004).

Entomopathogenic nematodes (EPNs) are natural enemies of pest (Kaya, 1990). *S. feltiae* strain B30, used in our experiment, was isolated in Slovenia (Cerknica area). The aims of our research were: (1) to compare the efficacy of *S. feltiae* B30 with the commercial product Entonem and the insecticide Actara against all developmental stages of CPB in the field, (2) to establish the influence of controling CPB on potato yield, (3) to study the concentration effect on control efficacy and (4) to investigate the effect of double applications.

Material and methods

The potato variety Kondor was planted in the field (45 x 11 m), which was then divided into 4 blocks and each of them with 6 treatments: control, SfB30 (produced in liquid culture at the Research and Extension Centre for Fruitgrowing, Hungary), Entonema (Koppert B.V., The Netherlands, through Zeleni hit d.o.o., Ljubljana) or the insecticide Actara (Syngenta, a.i. Thiametoxam). Atara was applied on June 16, 2008 at the dose of 60 g/ha. EPNs were applied on June 10 with a backpack spayer (Solo 425), using a jet stream nozzle number 04F110 while a pressure of 2 bars. Two concentrations of 250.000 IJ/m^2 and 500.000 IJ/m^2 were used. All treatments were repeated on June 26, 2008, but only with half the concentration of the EPNs. For EPN application 0.05 % of the surfactant Nu-Film-17 (a.s. di-1-p-methene, 96 %;

Lances Links SA, Geneva, Switzerland) was added. We observed population dynamics of CPB on 3, 10, 16, 19 and 26 DAT. Potatos were harvested on August 12, 2008. Differences in population dynamics of all CPB stages between treatments as well potato yields were analysed by ANOVA and Student-Newman-Keuls's test (Statgraphics Plus Windows 4.0, Statistical Graphics Corp., Manugistics, Inc.).

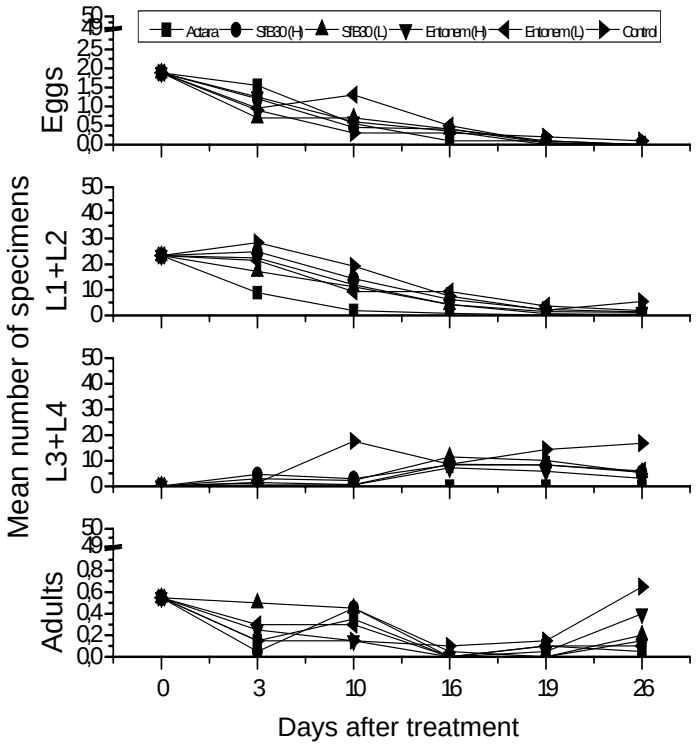

Figure 1. Population dynamics of CPB (egg clusters, L1+L2, L3+L4 and adults) according to different treatments in an experiment.

Results and discussion

Results indicate that both EPN strains are effective in control of particularily larval stages on the foliage (Figure 1). Stewart *et al*. (1998) reported 100 % mortality of different stages of CPB in laboratory conditions, meanwhile the efficacy of *S. carpocapsae* in the open field was only 31 %. Welch and Briand (1961) reported that foliar application of EPNs is not recommended due to loss of effectivity by fast drying of EPN on the leaves. Because the relative humiditiy in our experiment during the time of application at night amounted 66.7 % we consider that low efficacy was caused by low temperature. Average temperature during the night at the first application (June 10) was 14ºC and at the time of the second application (June 26) 16 ºC. Better efficacy of EPNs against adults of *Phyllotreta undulata* was attained at temperatures between 20 and 25ºC (Trdan *et al*., 2008). EPNs did not have any effect on egg clusters. Satisfactory efficacy on larval stages was also reported by Armer *et al*. (2004). Statistical analysis indicated that Slovenian strain B30 was even better from the commercial product. Insecticide Actara performed at best as no older larva was found on the plants. Meanwhile in average 5 older larvae per plant was found at the end of our experiment in treatment with strain *S. feltiae* B30 (high concentration of nematode suspension). Different

concentrations of nematode suspension did not have effect on mortality of developmental stages of insect which gives from the economical point of usage of biological agents in integrated agriculture better prospect as cost of plant protection as highly linked with the quantity of applied EPNs. In a connection with population changes of CPB in our experiment we studied also their influence on potato yield (Figure 2). We established that between EPNs treatments there were no differences. So potato yield was not affected by the different concentrations of nematode suspension. Potato from the plots of 20 m^2 in size, treated with EPNs, yielded in average 34.71 kg (17.33 t/ha). At the same time plants treated with Actara gave yield of 48.75 kg (24.37 t/ha). The lowest yield was on control plots, namely only 23.12 kg (11.56 t/ha).

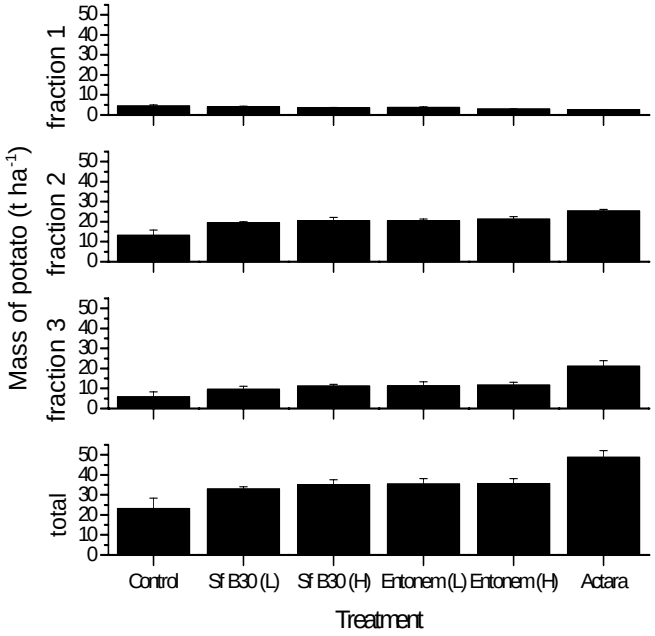

Figure 2. Potato yield (total and in fractions) according to treatments. In fraction 1 tubers smaller than 4 cm are included, in fraction 2 are tubers from 4 to 5 cm and in fraction 3 are tubers which are larger than 5 cm.

Acknowledgements

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Dosage screening with *Heterorhabditis indica* for grub control in peanut fields

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Abstract: A trial with the entomopathogenic nematode（*Heterorhabditis indica* in dosages of 2,000, 3,000, 4,000, 5,000 and 10,000 nematode/peanut plant was conducted for selecting an effective dosage to control grubs. Damaged plants (%) were significantly less in all treatments compared to the control treated with water only. The dosages of >4,000/plant resulted in 4.1%, 5.7% and 7.5% damage, respectively, which was significantly lower than 10.2% damage recorded in the chemical control treated with 40% isofenphos-methyl. A 95.7% decrease in number of grubs was achieved with 4,000 and 5,000 nematodes/plant, which was significantly higher than in the chemical control (82.7%). Peanut yields at 4,000 nematodes/plant were 327.6 kg/667m^2, significantly higher than in the chemical control (292.6 kg/667m^2). The results demonstrate that *H. indica* can successfully control chafer grub in peanuts and subsequently increase peanut yields and is superior to the chemical control strategy. The dosage of 4,000 nematodes/plant can be recommended.

Key words: *Heterorhabditis indica*; isofenphos-methyl; chafer grubs; *Holotrichia parallela*, *Holotrichia oblita*, *Holotrichia trichophora*, *Anomala corpulenta*, *Arachis hypogaea*

Introduction

Peanut is one of the major oil crops in China. White grubs (Scarabaeidae) are a serious pest in peanut fields. They generally reduce peanut yield by 25 to 35%. Chemical pesticides were used conventionally to control grubs. However, they were only moderatley effective, induced grub resistance, produced chemical residues and gradually led to environment pollution.

Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are insect parasites characterised by their symbiotic relationship with entomopathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively. Because they have a broad host range and can be applied conveniently via irrigation systems or by spraying even combined with chemical and biological pesticides, EPNs have been used to control various soil living and concealed pests for many years (e.g. Karunakar *et al*., 2000; Liu *et al.*, 2002; Yadav *et al*., 2004; Sankaranarayanan *et al*., 2006).

Only few papers have been published on the control of chafer grubs in peanut fields (Li *et al*., 1993; Liu *et al.*, 2007). To evaluate the effect of EPNs, *Heterorhabditis indica* was applied to control the grub complex (*Holotrichia parallela* Motschulsky, *H. oblita* Faldermann, *H. trichophora* Fairmaire and *Anomala corpulenta* Motschulsky) in peanut fields at various dosages.

Material and methods

Heterorhabditis indica infective juveniles (IJs) were produced in liquid culture in Germany by *e-nema* GmbH. The insecticide isofenphos-methyl EC (40% emulsifiable concentrate) was produced in China by Luxi-yinong Chemical Co., Ltd.. The field trial was conducted in a peanut field seriously infected with grubs in the Henan province of China. Peanuts (varienty Yuhua 15) were planted on May 11, 2008 in the density of 10,000 pits/667m^2, (one plant in one pit). *H. indica* was applied at 2,000, 3,000, 4,000, 5,000 and 10,000 IJs/pit and 40% isofenphos-methyl EC at 400ml/667m^2 was used as a chemical pesticide control (CK1). Tap water was applied as a negative control (CK2). Application was on July 19, 2008 after peanut bloom period, with flooding irrigation. Each treatment was conducted on three plots. One plot (60 m^2) had 900 pits (peanut plants). The data were collected two days before harvest (September 22-23, 2008). In each plot, 15 x 5 plants were collected from 5 patches of 1 m^2 along a Zigzag line transecting the plot. The peanut legumes and the soil around the roots (15 x 15 x 20 cm depth) were digged out and the number of living grubs, the number of damaged legumes and the fresh-weight of the peanuts per plant were recorded. From these values, the proportion of damaged legumes, number of grubs, reduction in grub numbers and peanut yield were calculated followed the formula of Liu *et al.* (2007).

Results and discussion

Effect of different dosages of H. indica on damaged legumes

The proportion of damaged legumes was significantly lower in all treatments compared to the untreated control (Fig.1). Dosages of 4,000 and 5,000 IJs/pit were most effective resulting in significantly lower damage than in plots treated with 40% isofenphos-methyl control (damage 10.2%). Among the five dosages of *H. indica*, 4,000 IJs/pit had the lowest damage. Considering grub control and economic aspects, the doses of 2,000 and 10,000 IJs/pit are least promising.

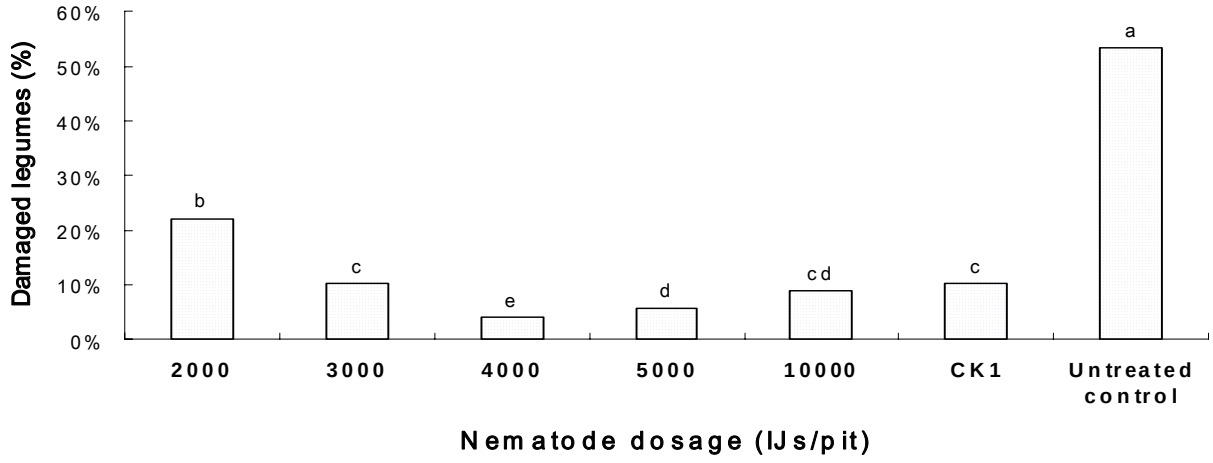

Figure 1. Proportion of damaged legumes in peanuts treated with different dosages of *Heterorhabditis indica* and 40% Isofenphos-methyl EC (CK1)

Effect of different dosages of H. indica on grub numbers

The average number of grubs in all treatments were significantly lower than in the untreated control. On average the grub number was reduced by 95.7% (Fig. 3) to 0.33 individuals/m^2 (Fig. 2). Dosages of 4,000 and 5,000 IJs/pit reduced grub numbers the most, which is reflected by the best protection of legume damage at these dosages. In the plots treated with 40% isofenphos-methyl EC, grub reduction was 82.7%.

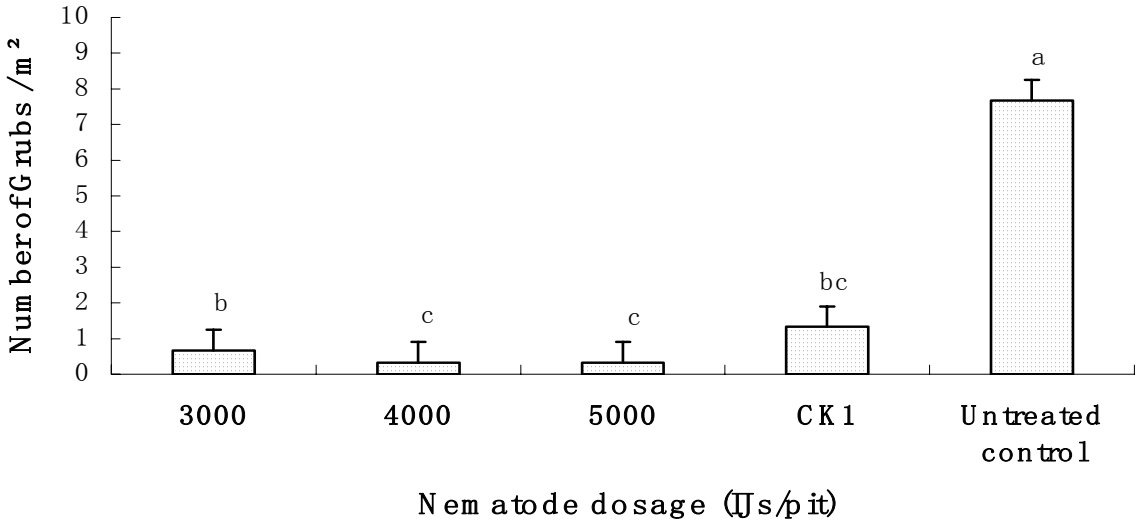

Figure 2. Number of grubs after treatment with different dosages of *Heterorhabditis indica* and 40% Isofenphos-methyl EC (CK1).

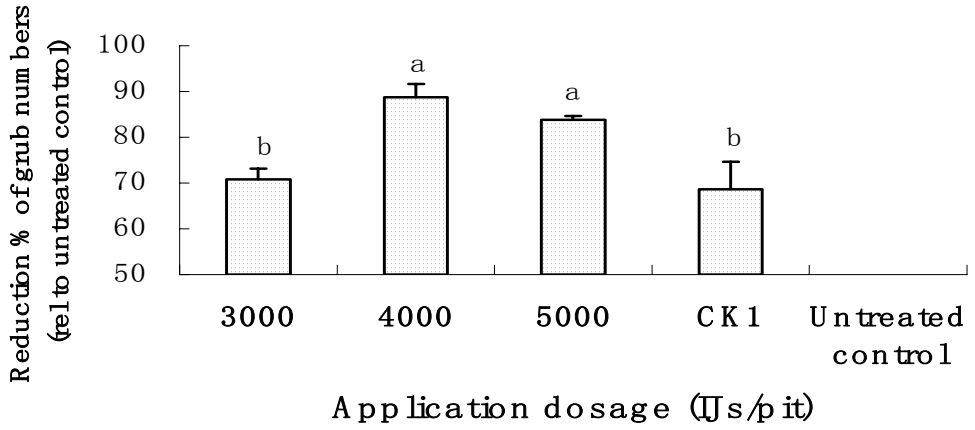

Figure 3. Reduction of grub numbers after treatment with different dosages of *Heterorhabditis indica* and 40% Isofenphos-methyl EC (CK1)

Effect of different dosages of H. indica on peanut yield

The peanut yields (Fig. 4) was 327.6 and 319.0 kg/667m^2, respectively at the dosages of 4,000 and 5,000IJs/pit. Both were significantly higher than the yield in the plots treated with 40% isofenphos-methyl EC control (292.6kg/667m^2) and in the untreated tap water control (173.5kg/667m^2) ($p<0.05$).

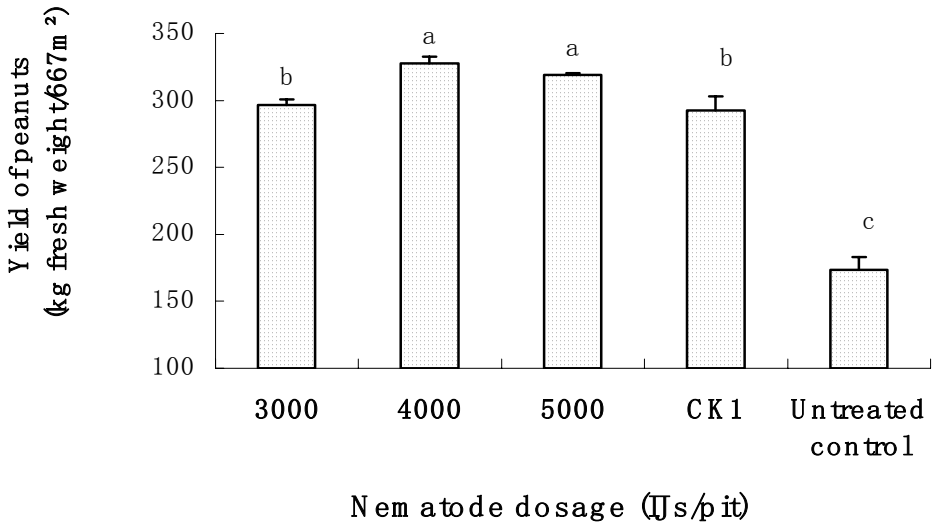

Figure 4. Peanut yield after treatment with different dosages of *Heterorhabditis indica* and 40% Isofenphos-methyl EC (CK1)

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Use of entomopathogenic nematodes and chitosan against *Rhynchophorus ferrugineus and Paysandisia archon* in *Phoenix canariensis, P. dactilifera* and *Chamaerops humilis* in Spain

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Abstract: The nematode *S. carpocapsae* was tested in combination with chitosan (Biorend R®) against palm tree pest insects. Biorend R® contains an organic biodegradable plant growth promoter with the active ingredient N-acetyl-glucosamine. It activates defence mechanisms in plants (Hadwiger and Loschke, 1981), increases lignification and promotes the development of roots (Ait Barka et al., 2004). The use of nematodes with chitosan is patented (Martinez Peña, 2002) and has already been used in other systems (e.g., Martínez de Altube et al., 2007). Preventive and curative field trials against *R. ferrugineus* were carried out at the "Instituto Valenciano de Investigaciones Agrarias" (IVIA) in smaller *Phoenix canariensis* trees in Spain during the months of June, July, and August 2007. The efficacy of *S. carpocapsae* (Weiser) in a chitosan formulation (product name Biorend R®) was studied. Efficacy of 80% were obtained in the curative application and up to 98% in the preventive treatment with doses of 3.6 $x10^6$ dauer juveniles (DJs) per tree (Llacer et al., 2008).

Studies about persistence of *S. carpocapsae* in a chitosan formulation after preventive and curative treatments against *R. ferrugineus* in *Phoenix dactilifera* with a dose of 2.5$x10^6$ DJ per palm tree were carried out at the "Estacion Phoenix", Elche, Alicante, from May to June, 2007. In spite of nematode population decrease over time, it was possible to detect DJs until 21 days after application and the efficacy was between 66 and 100% in the preventive treatment and up to 83% in the curative treatment (Gomez Vives et al., 2008).

Another field trial was carried out by the University Barcelona and the "Servicio de Sanidad Vegetal de la Generalitat Cataluña". The evolution of *R.. ferrugineus* populations in of 109 years old *P. canariensis* was recorded. Palm trees within a high infestation level of the Red Palm Weevil were applied monthly with *S. carpocapsae* and chitosan. Crowns of trees were dissecting every month. The population decreased by 68% after the first treatment, another 36% after the second treatment and only 8% of the initial population survived the third treatment.

Results obtained by the Research Institute of the "Junta de Andalucia" (IFAPA) were presented at the internacional fair Expoagro in Almeria in 2008. An application of *S.carpocapsae* alone reached 43% control, whereas the combination of *S..carpocapsae* with chitosan reached 80% RPW control in *P. canariensis*.

In laboratory trials the effect of *S. carpocapsae* in the chitosan formulation against *Paysandisia archon* in *Chamaerops humilis* was recorded. Efficacy ranged between 66.6 to 100% depending on the DJ dose. In a palm nursery a trial was carried out by the "Universidad Politécnica de Valencia". An efficacy of 80% applying 0,3$x10^6$ DJs and 100% applying 1$x10^6$ DJs were reported.

Key words: Entomopathogenic nematodes, chitosan, *Rhynchophorus ferrugineus, Paysandisia archon,* biological control

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Genetic breeding for heat tolerance of *Heterorhabditis bacteriophora*

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Abstract: High temperatures > 30°C can occur during transportation of entomopathogenic nematodes to the user and result in loss of viability and quality of nematode products. This study investigated the possibilities to improve heat tolerance of the nematode *Heterorhabditis bacteriophora.* The increase of heat tolerance in *H. bacteriophora* through genetic selection has been reported previously. This contribution screened sixty *H. bacteriophora* strains for their survival at high temperatures and subsequently crossed the most heat tolerant strains to further improve the tolerance. There were significant differences in heat tolerance among the *H. bacteriophora* strains. Adaptation to higher temperature for 3 h could not increase heat tolerance and no correlation was recorded for tolerance with or without adaptation. The mean tolerated temperatures ranged from 33.3°C to 40.1°C for non-adapted populations and from 34.8°C to 39.2°C for adapted populations. Prior to crossing the most tolerance strains, only the 10% with the highest tolerance of one population were propagated. The mean heat tolerance of hybrid strains was 40.3°C for non-adapted and 39.9°C after adaptation. These results confirmed superior performance of hybrid strains over parental nematodes.

Key words: Heat tolerance, breeding, genetic improvement, biocontrol, *Heterorhabditis bacteriophora*

Introduction

Short-term exposure of entomopathogenic nematode dauer juveniles (DJs) to temperatures above 35°C, for instance during transportation, can hamper reproduction, activity and viability and thus spoil nematode products. An increasing tolerance of nematodes to higher temperatures (>35°) can help to improve quality and shelf-life of DJs and ease the commercial handling of nematode-based products.

Heat tolerance is a heritable trait in *Heterorhabditis* spp. (Glazer et al., 1991). Tolerance to high temperature can be improved by genetic selection. Ehlers et al. (2005) calculated a heritability of the trait "heat tolerance" of $h^2 = 0.68$ and assessed a heat tolerance of inbred lines between 38.3° and 39.3°C (mean temperature tolerated by 50% of the population). This study has screened heat tolerance of 61 *H. bacteriophora* strains from different geo-climatic regions. The most heat tolerant strains were crossed and the heat tolerance of the hybrids evaluated.

Materials and methods

Nematode strains from the Middle East, China, India, Iran, Italy, Germany, Czech Republic, Australia and USA were collected from liquid nitrogen and cultured *in vivo* using *Galleria mellonella* (Lepidoptera, Pyralidae). The strains were passed twice through *G. mellonella* before experimentation. Emerging DJs from the insect cadaver were kept at 10°C and were used within one week after emergence.

To determine the heat tolerance, batches of 200 DJs of each strain were transferred into cover-slide chambers containing 5 ml tap water and were exposed to five different temperatures between 32°C and 40°C on a temperature gradient, generated on an aluminium bar for 2 h (Ehlers et al., 2005). The temperature on the bottom of each chamber was recorded with a platinum Pt 100 thin layer sensors (M-FK 422, Heraeus Sensor-Nite GmbH, Kleinostheim, Germany). The same strains were also tested after adaptation to heat at 35°C for 3 h. Afterwards, the DJs were left to recover for 1 h at 25°C and then exposed to five different temperatures between 33°C and 40°C as described above. After exposure to heat treatment, active and inactive nematodes were separated using a water trap (Strauch et al., 2004). The experiments were repeated twice for each strain using freshly propagated DJs.

Genetic crosses of the most tolerant parental nematode strains were conducted according to Iraki et al. (2000). For crosses, those 1,000 individuals of each batch were used, which had survived the highest temperatures (best 10%). They were propagated in about 20 larvae of *G. mellonella*. The heat tolerance for non-adapted (HH1 and HH2) and adapted (HA1 and HA2) hybrids was assessed as described above with five different temperatures on a gradient ranging between 37.5°C and 42°C.

Significant differences between strains were determined using student's t-test at 5% confidence level. The correlation between non-adapted and adapted populations was determined using Pearson's correlation coefficient ranking at 5% confidence level.

Results and discussion

The mean tolerated temperature for non-adapted populations ranged from 33.3°C to 40.1°C and for adapted populations from 34.8°C to 39.2°C. Increasing heat tolerance is also influenced by the production of heat-shock-proteins, which are synthesized as a response to adaptation. Surprisingly, the non-adapted tolerance was higher than the tolerance measured after adaptation.

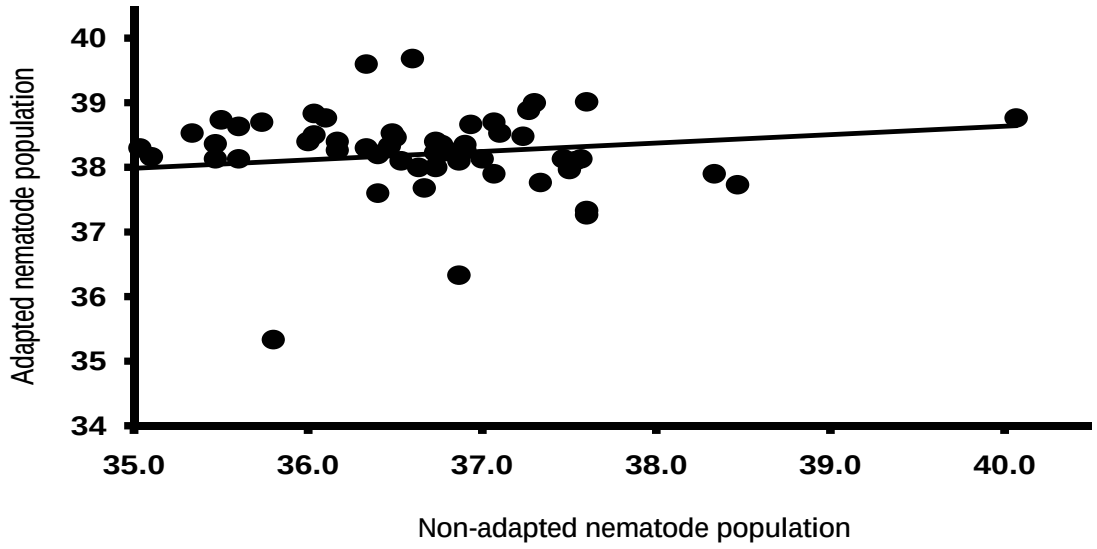

Figure 1. Correlation between mean tolerated temperature of each strain assessed with and without adaptation ($r = 0.18$, not significant at $\alpha \leq 0{,}05$).

Significant differences were recorded among the *H. bacteriophora* strains. The correlation between non-adapted and adapted population was low (Figure 1), probably genes for heat tolerance among the nematode strains are segregating for identical alleles.

There were significant differences of the heat tolerance between parental and hybrid strains according to student t-test ($\alpha \leq 0{,}05$): The hybrid strain HH2 had the highest heat tolerance (Fig. 2). The mean tolerated temperatures for the hybrid strains ranged from 38.9°C (HH1) to 41.6°C (HH2) for non-adapted populations and from 39.2°C (HA1) to 40.5°C (HA2) for adapted population. The mean tolerated temperature for parental strains was 38.1°C and for hybrid strains was 40.1°C, indicating a substantial increase of 2°C.

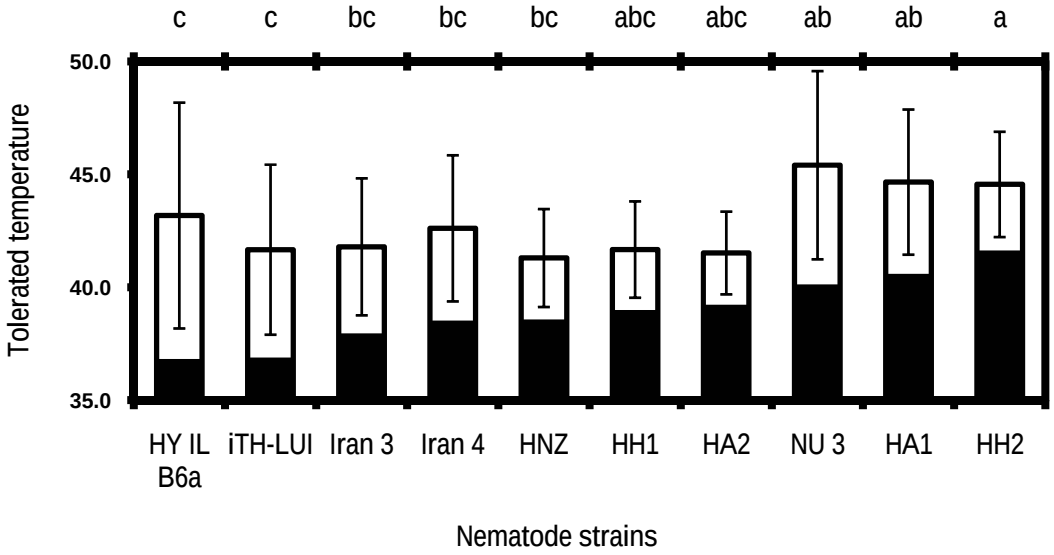

Figure 2. Mean temperature tolerated by 50% of the populations (black bars) and highest mean temperature tolerated by 10% of the populations (white bars) for parental strains (HYIL B6a, iTH-LUI, Iran 3, Iran 4, HNZ and NU3) and hybrid strains (HH1, HH2, HA1 and HA2). Error bars on the columns indicate standard deviation of the mean and different letters above the bars indicate significant differences between the means for the 10% most tolerant individuals at $\alpha \leq 0{,}05$.

The investigation could identify strains with a higher tolerance to high temperature than assessed previously among inbred lines of a hybrid, which was the result of crossing 7 strains from Europe, North America and China (Ehlers et al., 2005). The selection of only those 10% of a population which tolerated the highest temperature for crosses resulted in an additional increase in tolerance. The availability of hybrid strain with a higher heat tolerance will increase the ability for prolonged storage and enhance the quality of commercial nematode products.

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Use of entomopathogenic nematodes for the control of *Paysandisia archon* Burmeister

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Abstract: A preventative and a curative field trial were carried out in 2008 on in-ground and potted *Trachycarpus fortunei* (Hooker) Wendland palms in order to evaluate the efficacy of two different formulations of the entomopathogenic nematode *Steinernema carpocapsae* (Weiser) in respectively preventing new *Paysandisia archon* Burmeister infestations and controlling the palm pest on already infested plants. The tested *S. carpocapsae* formulations showed very high efficacy (close to 100%) in both preventing new infestations and controlling *P. archon*. The entomopathogenic nematode can thus be considered a highly valuable tool for the control of this target pest.

Key words: *Paysandisia archon*, palms, entomopathogenic nematodes, biological control

Introduction

The South American palm borer *Paysandisia archon* Burmeister (Lepidoptera: Castniidae) is native to Uruguay and central Argentina. It has been accidentally introduced to Europe, where it is spreading rapidly. In the EPPO (European and Mediterranean Plant Protection Organization) region it was first found in 2001 in Spain and France. The presence of *P. archon* has been reported in Spain, France, UK, Greece and Italy, where the moth is present in the following regions: Campania, Marche, Tuscany, Sicily, Apulia, Liguria, Emilia Romagna, Abruzzo and Lazio (OEPP/EPPO, 2008; Nardi *et al.*, pers. comm.). The moth is currently listed in EPPO A2 List of "Pests recommended for regulation as quarantine pests". *P. archon* lives on a wide range of palms (Arecacae family). Most common infestation symptoms of the pest on palms are: Presence of debris (fibrous material) discarded by the boring larvae on top of the stem close to the crown, wilting and chlorosis of the crown, pupal exuviae visible outside on the stem, galleries along the longitudinal axis of the leaf petiole, presence of perforated or nibbled leaves and eventually perishing of the palm.

In the Marche region, located in central-eastern Italy, ornamental palm species are not only an extremely important part of the landscape, but also of high economic importance, because many nurseries are specialized in their commercial cultivation.

In the Marche, almost all common palm species are susceptible to *P. archon* attack, but the most severe infestations occur on *Chamaerops humilis* Linnaeus and *Trachycarpus fortunei* (Hooker) Wendland. In this region *P. archon* probably has one generation per year, but the occurrence of different larval instars throughout winter and spring suggests that a small part of the moth population may have one generation every two years (Riolo *et al.*, 2005).

The Marche Plant Protection Service, over a period of three years (2005-2007), carried out several trials with chemical insecticides to prevent the spread of *P. archon* and to limit damage on palms (unpublished data). Both foliar and soil applications of different products were tested. Foliar chemical insecticide applications showed difficulties in reaching endophagous *P. archon* larvae and preventative treatments proved to be more effective than curative treatments. Given these results and those obtained in previous preliminary studies with entomopathogenic nematodes (henceforth EPNs) (Sanches & Clemente, 2007), in 2008 we decided to evaluate the efficacy of both preventative and curative applications of *Steinernema carpocapsae* (Weiser) (Nematoda: Steinernematidae), mutualistically associated with the bacterium *Xenorhabdus nematophila* (Enterobacteraceae), for the control of *P. archon*.

Material and methods

Tested *S. carpocapsae* formulations

In both the preventative and the curative trial the following two formulations of *S. carpocapsae* were tested: Nemasys C, produced by Becker Underwood (UK) and distribuited in Italy by Intrachem Bio Italia S.p.A. and Nemopak SC Palme, produced by Idebio S.L. (Spain) and distribuited in Italy by Bioplanet s.c.a.. As recommended by the producer, at the moment of the application, a liquid chitosan-based adjuvant (a.i. N-acetyl-glucosamine) was added to the Nemopak SC Palme tank mixture at a rate of 10 ml/10^6 EPNs, while no adjuvant was added to Nemasys C.

Preventative trial

The trial was carried out in a palm nursery in Massignano (AP, Italy) in July-October 2008, where the target pest was present for several years. Prior to beginning of the trial the mean palm infestation level, assessed on a sample of 100 randomly selected plants, was 9%.

Sixty two-year old uninfested *T. fortunei* palms were transplanted into the ground in the study area just prior to the beginning of the trial. A randomized complete block design with four replicates of 5 plants per plot was used to evaluate the efficacy of the two *S. carpocapsae* formulations in preventing *P. archon* infestations. An untreated control treatment was also included. All treatments were applied three times (July 3, July 24, and August 28) during the flight period of ovipositing *P. archon* females. In the successive applications, according to the recommendations of the distributors, Nemasys C was used at a rate of $6.3x10^6$, $6.6x10^6$, and $6.8x10^6$ ENPs/plant (spray volume: respectively 0.63, 0.66, and 0.68 l/plant), while Nemopak SC Palme was used at a rate of $7.4x10^6$ EPNs/plant (spray volume: 0.5 l/plant). To exclude the possible influence of water on new *P. archon* infestations, every time EPNs sprays were applied to the treated plots, the plants of the untreated control were sprayed with 0.5 l/plant of water only.

In order to determine the percentage of new *P. archon* infestations, all plots were checked for infestation symptoms at 20 day intervals throughout the study period and 20 days after the last treatment application (intermediate assessments). The final assessment was performed at the end of October.

Curative trial

The trial was carried out in a palm nursery in Grottammare (AP) in October 2008 on 2-3-year old potted *T. fortunei* plants, which all showed obvious signs of *P. archon* infestation. A randomized complete block design with 4 replicates of 5 plants per treatment was used. The products were applied to the treated plots on October 2. According to the recommendations of

the distributors, Nemasys C was applied at a rate of $8x10^6$ ENPs/plant (spray volume: 0.8 l/plant), and Nemopak SC palme at $10x10^6$ ENPs/plant (spray volume: 1 l/plant). To exclude the possible influence of water on *P. archon* mortality, untreated control plots were treated with 1 l/plant of water only.

Twenty days after the treatment applications, all palms were dissected, and the number of living and dead *P. archon* specimens per plot was recorded. To verify whether the EPN was the cause of the death, dead specimens were brought to the laboratory, dissected and observed under the microscope to detect the ENPs. For each plot percent mortality due to ENPs was calculated.

Statistical analysis

The percentage of new *P. archon* infestations (preventative trial) and the percentage of mortality due to EPNs (curative trial) were compared across treatments using 1-way ANOVA, followed by Tukey's test for post-hoc comparison of means. To improve homoscedasticity, data were arc sin square root (x/100)-transformed.

Results and discussion

Preventative trial

Throughout the study period and at the final assessment no new *P. archon* infestations were observed in the treated plots. Both *S. carpocapsae*-based products were highly effective in preventing new infestations. Differences among treated plots failed significance (1-way ANOVA: $F_{(2, 9)}=1.00$; $P=0.41$) (Table 1), because also in the untreated control the infestation level at the final assessment was extremely low (5.0 %). However, the mean infestation level in the nursery had increased from 9% at the beginning of the trial to 27%. Low infestation levels in untreated control plots have been observed in other preventative small-plot trials in the Marche region (unpublished data). This may be due to the behaviour of the target pest: *P. archon* females apparently prefer to colonize already infested palms and those located in their immediate surroundings prior to moving to plants which are farther away (Sarto et al., 2005).

Table 1. Percentage of new *P. archon* infestations (m±s.d.) in the different treatments at the final assessment. Different letters indicate statistically significant differences (Tukey's test: $P<0.05$).

No.	Treatment	New *P. archon* infestations (%)
1	Nemasys C	0.0±0.0 a
2	Nemopak SC Palme	0.0±0.0 a
3	Control	0.5±10.0 a

Curative trial

In the curative trial highly significant differences among treatments emerged (1-way ANOVA: $F_{(2, 9)}=142.51$; $P<0.0001$). Both *S. carpocapsae*-based treatments were highly effective in controlling *P. archon* infestations (Table 2). All *P. archon* specimens found inside palms were in the larval stage, and observations under the microscope confirmed that dead larvae had been infested by EPNs.

In the Nemopak SC Palme-treated plots (applied rate: $10x10^6$ EPNs/plant), a mean number of 6.5±1.3 *P. archon* larvae was retrieved from the palms, and mortality due to *S. carpocapsae* was 100% in all plots. In the Nemasys C-treated plots (applied rate: $8x10^6$ EPNs/plant), the infestation level was slightly higher than in the other treatments (mean no. larvae: 11.8±4.6), and mortality due to EPNs was slightly, though not significantly lower (94.5%; 100% in 3 out of 4 plots). No dead *P. archon* larvae were found in the untreated control plots (mean no. larvae: 9.0±4.3), confirming the absence of natural antagonists and/or other abiotic factors, which may limit the spread of the pest in the Marche region.

Table 2. Number of living and dead *P. archon* larvae (m±s.d.) and mortality due to EPNs in the different treatments. Different letters indicate statistically significant differences (Tukey's test: P<0.05).

No.	Treatment	No. living larvae	No. dead larvae	Mortality due to EPNs (%)
1	Nemasys C	1.0±2.0	10.8±3.3	94.1±11.8 b
2	Nemopak SC Palme	0.0±0.0	6.5±1.3	100.0±0.0 b
3	Control	9.0±4.3	0.0±0.0	0.0±0.0 a

Our results confirm the preliminary results obtained by Sanchez & Clemente (2007). Irrespective of the tested formulation,, *S. carpocapsae* proved to be highly effective in both preventing new infestations of *P. archon* and limiting the spread of the pest via curative treatments. EPNs are safe for non-target vertebrates and arthropods and the environment and thus offer an interesting alternative to chemical insecticides, especially in public areas. *S. carpocapsa*e can be considered a valuable tool for the control of endophagous *P. archon* larvae.

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Analysis of secreted/excreted products of *Steinernema carpocapsae* (Nemata)

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Abstract: A genomic and proteomic approach was used to identify transcripts of the parasitic phase of *Steinernema carpocapsae*. Among the 1,592 unique sequences obtained, 119 were predicted to be secreted and a few were hypothesized to be interacting with insect host. Four secreted/excreted serine-proteases were proved to cause disruption in insect host likely facilitating invasion and evasion.

Key words: *Steinernema carpocapsae*, EST, pathogenicity, virulence factors, serine-proteases.

Introduction

Steinernema carpocapsae forms an association with the enterobacteriaceae *Xenorhabdus nematophila*, which is highly virulent against insects. Each organism is expressing virulence factors. The bacterium releases enzymes and toxins (Brown et al., 2004; Caldas et al., 2002; Forst et al., 1997; Sergeant et al., 2003); whereas the axenic nematode produces proteinaceous compounds that induce insect lethality (Boemare et al., 1982; Burman, 1982; Laumond et al., 1992). In this presentation we describe genomic and proteomic approaches that allowed the identification of a few putative virulence factors expressed during the nematode´s parasitic phase. Furthermore we show that 4 of these enzymes must be related with host invasion and evasion.

Materials and methods

Nematodes and parasitic stage induction

In this study we used *S. carpocapsae* Breton strain produced in vitro solid media. IJs were conserved in tap water inside plastic boxes at 10ºC. For described assays IJs were conserved for 2 – 4 months. To induce the parasitic phase, IJs were surface disinfected with 0.5 % sodium hypochlorite for 6 m, rinsed, transferred to 10 ml Tyrod's with 10 % haemolymph of *G. mellonella* larvae and incubated at 23 ºC under rotation for the desired time.

EST and secreted/excreted peptide mass fingerprint databases

EST sequences were obtained from a cDNA library of parasitic phase of *S. carpocapsae* as described by Hao et al. (2009). Database in secreted proteins was obtained from secreted/ excreted products of the parasitic phase as described by Toubarro et al. (2009).

Biological assays

Biological assays were performed using purified serine-proteases obtained from secreted/excreted products of the parasitic phase. Assays were conducted with *Galleria mellonella* larvae and sf9 cell cultures as described previously (Balasubramanian et al, in press).

Results and discussion

EST analysis

2,500 clones were sequenced, 2,180 high-quality ESTs were generated and 1,592 unique sequences obtained (199 contigs and 1,393 singletons). Just 62.8% of the unique sequences had significant hits to the non-redundant protein database. Predicted proteins classification show they are involved in diverse cellular, metabolic and extracellular functions. 119 unique sequences were predicted to encode putative secreted proteins (Table 1). A few of these excreted proteins must be interacting with the host (Hao et al., in press).

Table 1. Number of cluster found in each putative secreted / excreted protein family

Protein	**Nº of clusters**
Serines	9
Cathepsins	3
Aspartic	5
Metallopeptidases	5
Serine-protease inhibitors	10
Cysteine-protease inhibitors	3
Acethylcholinesterase	4
Lectins	5
FAR	2
Saposins	2

Secreted / excreted products analysis

Using 2DE we were able to detect around 200 spots in SP of parasitic phase (Fig. 1). 10 different spots represent 80% of the total protein and were identified as serine-proteases. 100 spots analysed by LTQ/MS-MS have blasts in our EST database. About 30% of these spots represent isoproteins.

Biological activity of S/E proteins

Four serine proteases that were obtained in the SP with sufficient amount to perform purification and biochemical characterization were used to perform studies on biological activities. Other 12 genes were selected based in the identification of signal P in EST and in the match with a spot in 2DE, to be expressed in *E. coli* system in order to investigate their functionality.

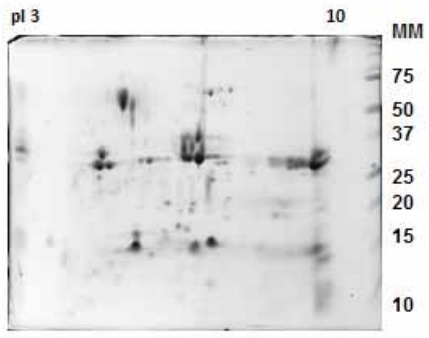

Figure 1. Protein profile in 2DE of S/E products released by induced *S. carpocapsae* (coa-massie stained)

Sc-SP-1 and Sc-SP-3 are interacting with insect mid-gut putatively enabling host invasion. Sc-SP-1 cause evident disruption of the basal lamina of epithelial tissues and Sc-SP-3 is inducing cell apoptosis, thus also opening entries to the parasite (Fig. 2). Sc-SP-1 (Acc Nº AAT27470) is a serine protease belonging to the catepsin G group with a Mr of 27 kDa and pI of 9 and Sc-SP-3 (Acc Nº FJ152416) is a chymotrypsin-like serine protease with a Mr of 30 kDa and a pI of 7.

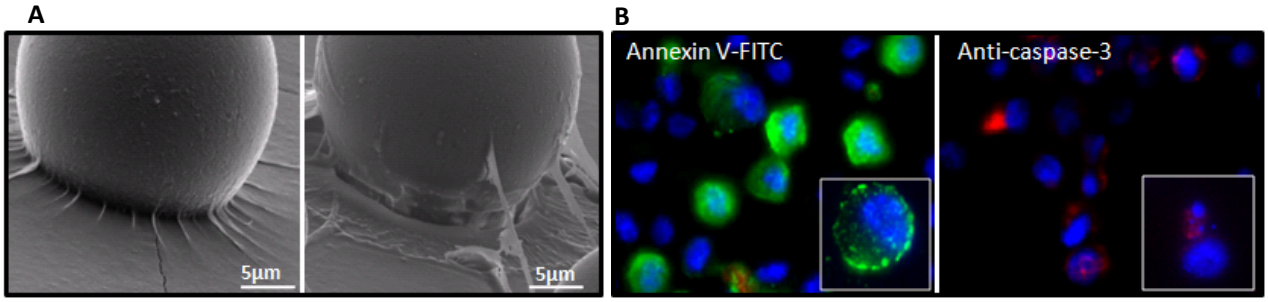

Figure 2. A - Sc-SP-1 effect in basal lamina (left, control; right, treated). B - Induction of apoptosis by Sc-SP-3 in insect cells

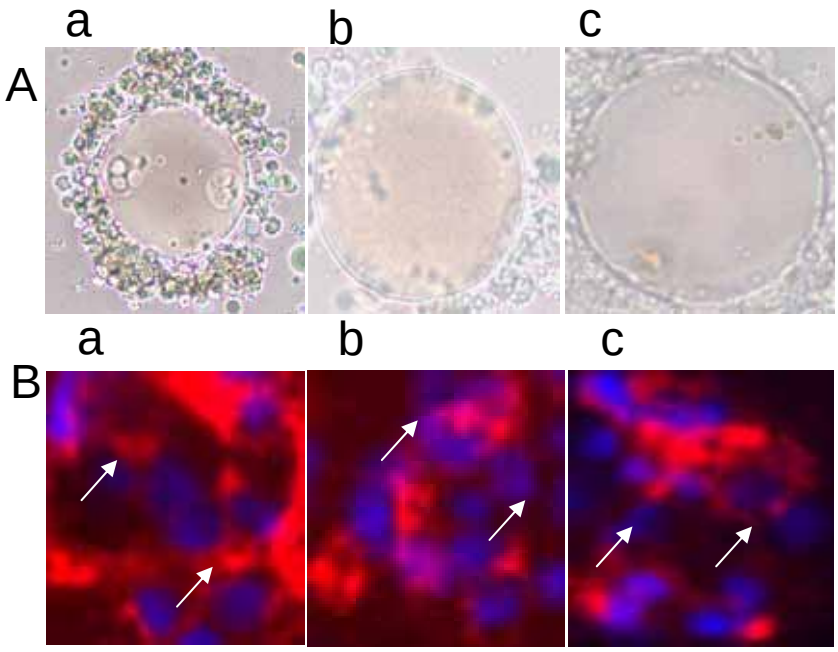

Figure 3A. Effect of chymotrypsin on encapsulation and melanization in *Galleria mellonella* in vivo. DEAE Sepharose CL-6B (12 h post injection) in saline buffer (a), in TPCK (tosyl phenylalanyl chloromethyl ketone) inhibitor (b), in 64 µg of purified chymotrypsin (c). Magnifications are 200 ×. B. Effect of purified trypsin on *Galleria mellonella* haemocytes cytoskeleton F-actin filaments. Haemocytes treated with PBS (a), APMSF treated haemocytes (b), Purified trypsin treated haemocytes (c). Blue colour is DAPI stained nucleus, arrows indicate organized (a) and disorganized actin filaments (b and c). Magnification is 200 ×. Scale bar = 50 µm.

Sc-Chym (Acc Nº ABY74341) belongs to chymotrypsin protease and has a Mr of 30 kDa and a pI of 5.9. Sc-Tryp is a trypsin with a Mr 29 kDa and a pI of 6.3. Sc-Chym and Sc-Tryp are interacting with insect defences. Sc-Chym causes an important reduction of PPO activity and blocks the recognition of foreign bodies whereas Sc-Tryp is reducing the ability of haemocytes to recognize invaders by modifying the cytoskeleton (Fig. 3).

Using real time PCR we proved that parasitic nematodes collected inside the mid-gut have higher expression levels of *sc-sp -1* and *-3* than nematodes already in the haemocoel, thus supporting our concern that these proteases are participating in the initial phase of parasitism, probably in invasion. *sc-chym* was shown to be expressed a little after the other two proteases.

In addition to these proteases other proteins detected in SP are being shown by protein-protein interaction to bind with insect cells membrane proteins (data not shown), probably inducing a specific response from the insect.

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Contribution to the knowledge of insect-nematode associations

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Abstract: Among nematodes the species associated with insects are present in more than 30 families. During our research activities we found the following insect-nematode associations: *Eudiplogaster aphodii* Bovien (Rhabditida, Diplogasteridae) with *Aphodius fimetarius* Linnaeus, 1758 (Coleoptera, Scarabaeidae); *Oryctonema pentodonis* Poinar & Triggiani, 1979 (Rhabditida, Rhabditidae) with *Pentodon punctatus* Villers, 1789 (Coleoptera, Scarabaeidae); *Hexamermis albicans* von Siebold, 1848 (Mermithida, Mermithidae) in larvae of *Aleimma loeflingiana* Linnaeus, 1758, *Tortrix viridana* Linnaeus, 1758, *Archips crataegana* Hübner, 1799 (Lepidoptera, Tortricidae) and unidentified species of Geometridae; *Hexamermis* sp. in *Traumatocampa pityocampa* Denis & Schiffermüller, 1775 (Lepidoptera, Thaumetopoeidae) eopupae; *Sphaerularia bombi* Dufour, 1837 (Thylenchida, Sphaerulariidae) inside queens of *Bombus terrestris* Linnaeus, 1758, *Megabombus pascuorum* Scopoli, 1763 and *Pyrobombus sicheli* Radoszkowski, 1859 (Hymenoptera, Apidae). From soil samples of different biotopes 22 strains of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida, Heterorhabditidae), 25 *Steinernema feltiae* Filipjev, 1934, 11 *S. affine* Bovien, 1937, 8 *S. apuliae* Triggiani, Mráček & Reid, 2004, 3 *S. ichnusae* Tarasco, Mráček, Nguyen & Triggiani, 2008, 1 *S. carpocapsae* Weiser, 1955 and 1 *Steinernema* sp. of *S. arenarium* Artyukhovsky, 1967 group (Rhabditida, Steinernematidae) were isolated. *O. pentodonis*, *S. apuliae* and *S. ichnusae* are new species so far only reported from Italy.

Key words: parasitic nematodes, Diplogasteridae, Mermithidae, Sphaerulariidae, Steinernematidae, Heterorhabditidae

Introduction

In the *Phylum* Nematoda the species associated with insects are present in more than 30 families. The research focused mainly on the parasitic species and we report here contributes related to the insect-nematode associations present in these families: Mermithidae, Diplogasteridae, Sphaerularidae, Rhabditidae, Steinernematidae and Heterorhabditidae, found during our research activities in southern Italy.

Results and discussion

Juvenile stages of *Eudiplogaster aphodii* Bovien (Rhabditida, Diplogasteridae) were recovered in the haemocel of *Aphodius fimetarius* Linnaeus, 1758 (Coleoptera, Scarabaeidae) larvae, pupae and adults in the Puglia Region (Poinar *et al.*, 1976) (Fig. 1A).

Oryctonema pentodonis Poinar & Triggiani, 1979 (Rhabditida, Rhabditidae) is a species closely related to *O. genitalis* found by Poinar in 1970 inside the reproductive system of tropical Dynastinae (Coleoptera). *O. pentodonis* was recovered inside the edeagus and bursa copulatrix of *Pentodon punctatus* Villers, 1789 (Coleoptera, Scarabeidae) individuals, collected in Basilicata and the Puglia Regions Pollino Mountains, Bari, Lavello and Bradano River. This species is located in the genital system of both sexes and should be transmitted during mating. *O. pentodonis*, as well as *O. genitalis*, feeds the exudates of the bursa

copulatrix in the female and the sperm of the reproductive system of the male (Poinar and Triggiani, 1979) (Fig. 1B).

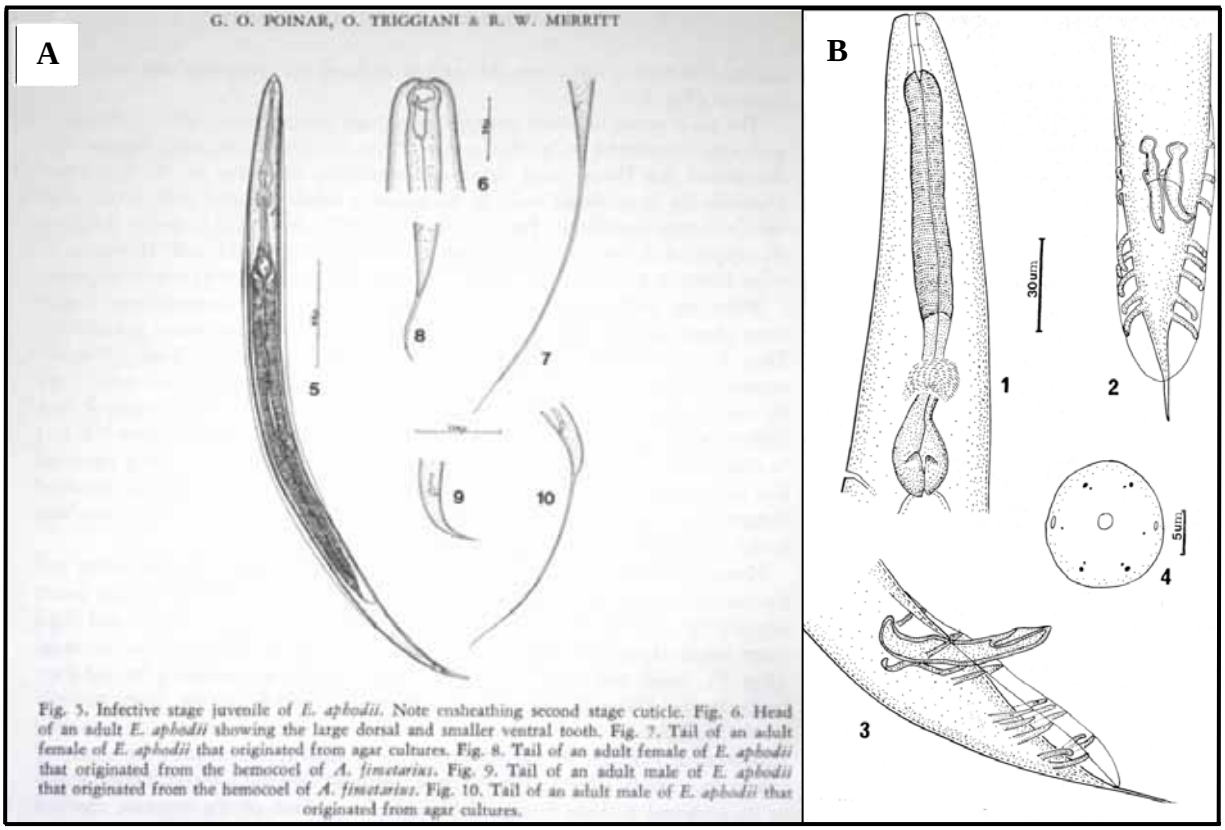

Figure 1. Description of the infective stage of *E. aphodii* (A) and male of *O. pentodonis* (B).

Hexamermis albicans von Siebold, 1848 (Mermithida, Mermithidae) is a species frequent in the *Quercus trojana* Webb. apulian woods infested by defoliator caterpillars, such as *Aleimma loeflingiana* Linnaeus, 1758, *Tortrix viridana* Linnaeus, 1758, *Archips crataegana* Hübner, 1799 (Lepidoptera, Tortricidae) and unidentified species of Geometridae (Triggiani 1984/85; Triggiani 1991; Triggiani, 1992/93). *H. albicans* is cosmopolitan and infects numerous species of different zoological groups including insects, myriapods and gastropods.

In 1992 another *Hexamermis* sp. was found in *Traumatocampa pityocampa* Denis & Schiffermüller, 1775 (Lepidoptera, Thaumetopoeidae) eopupae in a strongly infested pine-wood in the northern part of the Puglia Region in Pietramontecorvino (Tarasco & Triggiani, 1994).

Sphaerularia bombi Dufour, 1837 (Thylenchida, Sphaerulariidae) infects queens of *Bombus terrestris* Linnaeus, 1758, *Megabombus pascuorum* Scopoli, 1763 and *Pyrobombus sicheli* Radoszkowski, 1859 (Hymenoptera, Apidae) and the infections occur when the queens move underground for overwinter. *S. bombi* develops inside the queens' abdomen causing sterility (Triggiani & Tarasco, 1996, 1997) (Fig. 2).

From soil samples of different biotopes 22 strains of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida, Heterorhabditidae), 25 *Steinernema feltiae* Filipjev, 1934, 11 *S. affine* Bovien, 1937, 8 *S. apuliae* Triggiani, Mráček & Reid, 2004, 3 *S. ichnusae* Tarasco, Mráček, Nguyen & Triggiani, 2008, 1 *S. carpocapsae* Weiser, 1955 and 1 *Steinernema* sp. of

S. arenarium Artyukhovsky, 1967 group (Rhabditida, Steinernematidae) (Tarasco & Triggiani, 1997, 2005, 2007; Triggiani & Tarasco, 2000) were isolated (Fig. 3 A, B). *O. pentodonis*, *S. apuliae* and *S. ichnusae* are new species so far reported only from Italy.

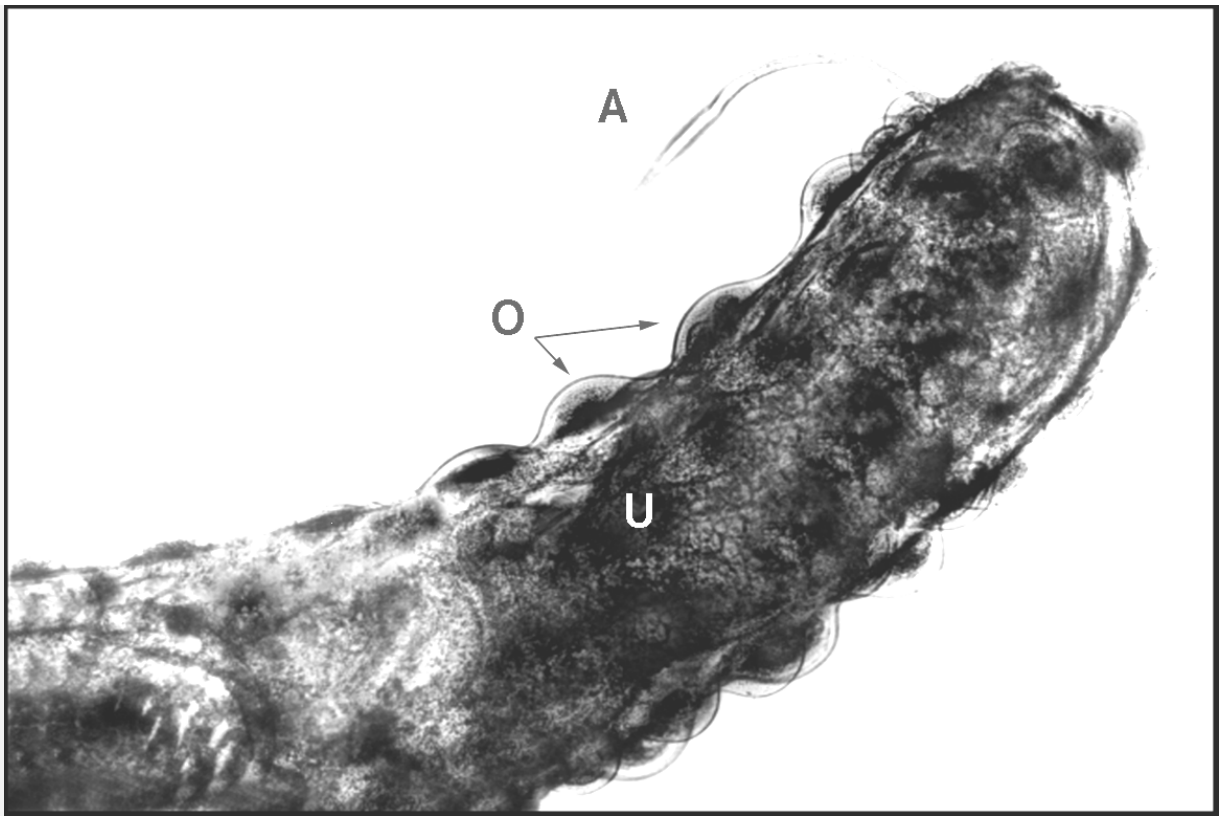

Figure 2. *Sphaerularia bombi*: male (A); egg (O); uterus (U)

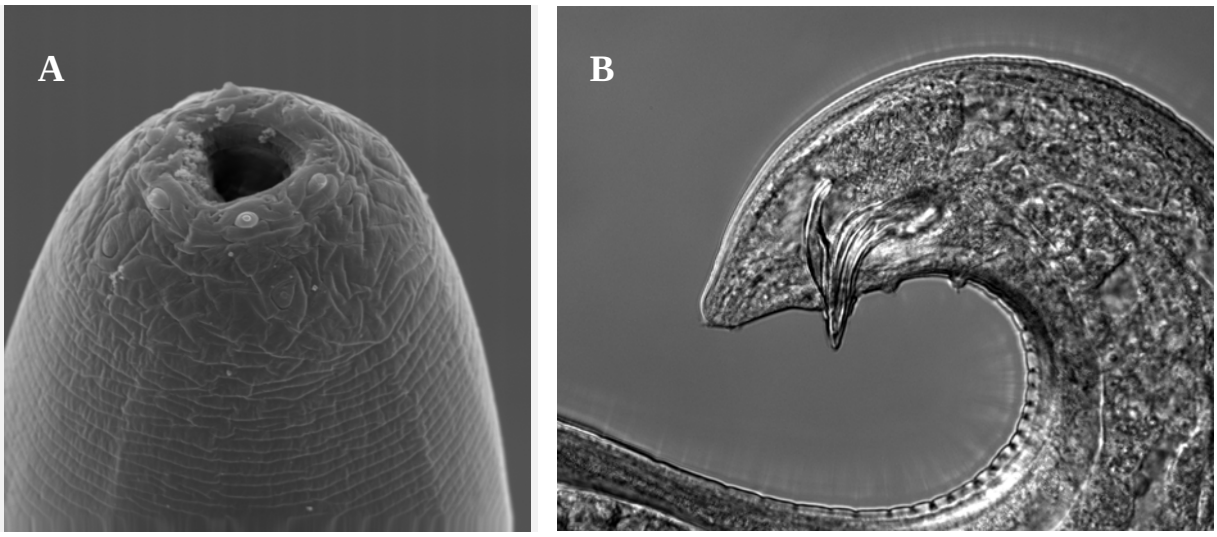

Figure 3. *Steinernema apuliae,* female mouth (A); *Steinernema ichnusae*, male spicule (B)

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Could Italy be considered a favorite place in Europe for EPN biodiversity?

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Abstract: EPN surveys carried out in Italy point out the presence of a high number of species if compared with other European and Mediterranean countries. Adding up the total, 106 EPN isolates belonging to 8 species were collected from about 1,500 soil samples in 20 years: 38 strains of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida, Heterorhabditidae), 39 *Steinernema feltiae* (Filipjev, 1934), 11 *S. affine* (Bovien, 1937), 4 *S. kraussei* (Steiner, 1923), 8 *S. apuliae* (Triggiani, Mráček & Reid, 2004), 3 *S. ichnusae* (Tarasco, Mráček, Nguyen & Triggiani, 2008), 2 *S. carpocapsae* (Weiser, 1955) and 2 *Steinernema* sp. Of the *S. arenarium* (Artyukhovsky, 1967) group (Rhabditida, Steinernematidae). *S. kraussei* was isolated only in the chestnut groves around the Enta Vulcano on Sicily; *S. apuliae* and *S. ichnusae* are new species so far only found in Italy.

Key words: Steinernematidae, entomopathogenic nematode, Heterorhabditidae

Introduction

The distribution of entomopathogenic nematode (EPN) species in natural habitats, the possible presence of endemic species in specific areas, the relationship of single species of EPN to particular environments are still rather poorly known, mainly because of relatively few researches with such a target; however, surveys aimed at improving our knowledge in this field would be certainly useful and would allow to conceive programs of biological control of insect pests both efficacious and not affecting biodiversity and environmental protection. In Italy the research of autochthonous species was started by Deseö *et al.* (1984), who studied some cultivated soils in the Emilia Romagna. Recently, the screening for EPNs has been increased with investigations in numerous areas of southern Italy and Italian islands and a joint research project of the Universities of Bari, Catania and Florence, aimed at a wide recognition of EPN species in Italy, is still in progress. In the present paper the results of 20 years of researches on EPN surveys in Italy are reported.

Materials and methods

The surveys were carried out by the research teams of Bari and Catania Universities. More than 1,500 soil samples have been collected along 20 years from different localities and biotopes in almost all Italian Regions. Three or five soil samples of about 2 kg each were collected form the biotopes investigated (fields, orchards, uncultivated lands, woodlands, river and lake borders, sea beaches, natural areas and coastal zones) and under the crown of trees heavily infested with insect pests. EPN strains were isolated following the procedures of the

"*Galleria* bait method" and kept in refrigerators at different temperatures; some strains have been stocked in nitrogen and stored at -140°C (Cosi *et al.*, 2008).

Results and discussion

A total of 106 EPN strains were isolated and 8 species identified. The first EPN survey was carried out in 1983 in cultivated soils of the Emilia-Romagna Region (Deseö *et al.*, 1984) while the first data on the occurrence of EPNs in Italy were reported some years later, in 1988 (Deseö *et al.*, 1988; Ehlers *et al.*, 1991). Then the presence and distribution of EPNs have been more deeply investigated in southern Italy and some Italian islands (Tarasco & Triggiani, 1997, 2005, 2007; Susurluk *et al.*, 2007; Triggiani & Tarasco, 2000, 2002; Clausi & Vinciguerra, 2005; Vinciguerra & Clausi, 2006). At present the species isolated so far in Italy are: *Heterorhabditis bacteriophora* (38 isolates), *Steinernema feltiae* (39), *S. affine* (11), *S. kraussei* (4), *S. apuliae* (4), *S. ichnusae* (3), *S. carpocapsae* (2) and 2 *Steinernema* sp. (long nematodes belonging to *S. arenarium* group). *S. apuliae* and *S. ichnusae* were described as new species (Triggiani, Mrácek, & Reid, 2004; Tarasco, Mrácek, Nguyen & Triggiani, 2008) and have been reported only from Italy so far; the first one have been collected only in Apulia while the second one only in Sardinia. *S. kraussei* strains were isolated in Sicily from soils of chestnut groves on the slopes of Etna Volcano (Clausi, unpub. data).

The results of the researches show extremely interesting data on the Italian EPNs and represent a specific contribute to the knowledge of EPN biodiversity in Italy.

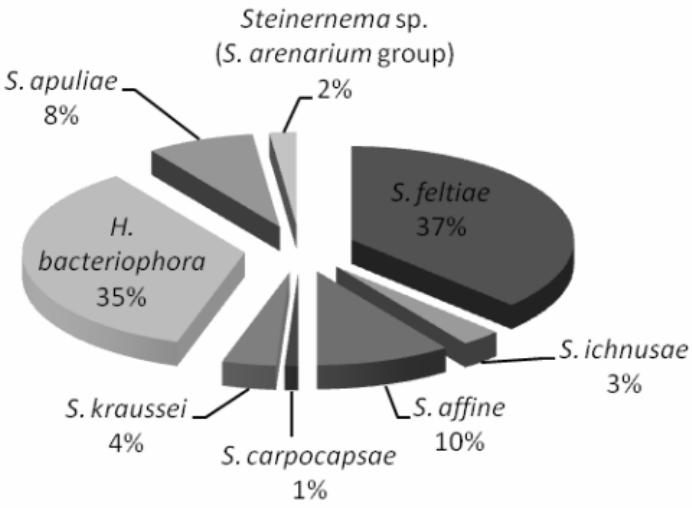

Figure 1. Occurrence (%) of EPNs in Italy

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Biological control of western corn rootworm larvae using nematodes

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Abstract: A three-year project aimed to develop a nematode-based biological control product against the larvae of the western corn rootworm, *Diabrotica virgifera virgifera*, for Europe. All three nematode species tested in field experiments in Hungary significantly reduced the number of *D. v. virgifera*. Particularly the nematode species *Heterorhabditis bacteriophora* showed a promising reduction of damages on maize roots, no matter whether applied at sowing time of maize or later in June. The nematode survived longer than two months in field soils, which is long enough to control *D. v. virgifera.* Moreover, it was able to effectively kill all three larval instars and the pupae of *D. v. virgifera*. At rates of ca 1×10^9 nematodes/ha root damage was reduced by about 50 % when using stream sprays of nematodes into the soil at sowing or narrow flat sprays over young maize plants or stream sprays onto the soil along maize rows early June. Those three nematode applications also reduced plant lodging and thus direct yield losses by up to 62%. Results and knowledge from this project are currently taken further by industry partners and implemented.

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Inoculative release of entomopathogenic nematodes for control of *Phytodecta quinquepunctata* (Coleoptera: Chrysomelidae) and other tree pests in urban green space

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Abstract: *Phytodecta quinquepunctata* is a common leaf-feeding beetle causing extensive damage to bird cherry, black cherry, and rowan in urban parks. Laboratory and field experiments revealed high control potential of entomopathogenic nematodes against this pest during its pupation in the soil. Mass reproduction in the host and good persistence in the soil ensured high efficacy of *S. feltiae* for at least two seasons after introduction. Due to extinction of some infra-populations, *H. megidis* was less effective in the second seasons. Inoculative release of EPNs could be a valuable solution to management of this pest.

Key words: Biological control, *Heterorhabditis megidis*, *Phytodecta quinquepunctata*, *Steinernema feltiae*, urban tree pests

Introduction

Phytodecta quinquepunctata (Coleoptera: Chrysomelidae) is a common leaf-feeding beetle attacking bird cherry (*Prunus padus*), black cherry (*Prunus serotina*), and, occasionally, rowan (*Sorbus aucaparia*). As these trees bloom abundantly and produce pleasant scent, they are frequently planted in urban parks and forests for ornamental purposes. However, the mass presence of *P. quinquepunctata* is usually associated with a heavy perforation of leaves or even complete defoliation and thus with a substantial loss of the trees' aesthetic value. Mature larvae of the beetle descend to the soil for pupation from May till July, and then young adult beetles return to the soil litter for over-wintering.

Due to the intensive recreation activity in urban parks and forests use of chemical insecticides is generally excluded. Other methods are needed to prevent potential damage. Entomopathogenic nematodes are potentially one of the best candidates as biocontrol agents for this environment. Our earlier study has shown that many insects causing damage to the tree foliage can be effectively controlled during pupation in the soil (Tomalak, 2003, 2004). The main objective of the research reported here was to examine the effect of an inoculative application of *Steinernema feltiae* and *Heterorhabditis megidis* as a means for management of *P. quinquepunctata* and potentially other tree pest populations in the urban park.

Material and methods

Nematodes and insects

All insects used in the experiments were collected in the field as mature larvae, shortly before their descending to the soil for pupation. Two local nematode species, i.e. *Steinernema feltiae* (ScP) and *Heterorhabditis megidis* (PL03) were used in the experiments. For the laboratory study the nematodes were reproduced *in vivo*, in *Galleria mellonella* larvae. For the field

experiments both species were reproduced *in vitro* on solid media, according to the Bedding's protocol (1981) with minor modifications.

Laboratory experiments

The nematode infectivity and their ability to complete reproduction in the host insect were examined in the laboratory, in multi-well (12-well) plates, half-filled with a moist steam-sterilized forest soil (sandy loam). The nematode infective juveniles (IJs) at a dose of 10^5 IJ m^{-2} (i.e. 35 IJ per well) were applied in 50 µl distilled water. The insects in the stage of mature larvae, pupae or young adults were individually exposed to nematodes for 3 days, then washed in distilled water, and immediately dissected or incubated until emergence of new generation IJs. Six replicates, with 12 insects each, were performed per experimental variant.

Field experiments

Control effects of the inoculative application of *S. feltiae* and *H. megidis* to the soil on populations of pupating and over-wintering *P. quinquepunctata*, as well as effects of the availability of host insects on persistence of nematode populations, were examined in two field experiments under tree canopies. The experiments were conducted in the green space area of the Institute of Plant Protection (2005) and in the Malta urban park (2006-2007) in Poznan, Poland. Prior to the experiments, samples of the local soil were examined for natural populations of entomopathogenic nematodes with the aid of *G. mellonella* baiting technique (Bedding & Akhurst, 1975). For both experiments only nematode-free sites were selected.

In the first experiment nematode biocontrol efficacy against mature larvae of *P. quinquepunctata* descending to the soil for pupation was examined in Ø16 cm plastic pots inserted into the soil and covered with screened glass cylinders, according to the method described earlier (Tomalak, 2004). The experiment included 5 variants i.e. soil treatment with *S. feltiae* (ScP) at doses of 10^5 (*i.*) and $2.5x10^5$ IJ m^{-2} (*ii.*); with *H. megidis* (PL03) at doses of 10^5 (*iii.*) and $2.5x10^5$ IJ m^{-2} (*iv.*), and water control (*v.*). Thirty mature insect larvae on twigs of bird cherry were placed into each cylinder. The number of insects used was based on the average number of *P. quinquepunctata* larvae falling from the shaken tree canopy. Six replicates (pots) per variant were used. Adult beetles which emerging after pupation were collected in 3-day intervals. After 6 weeks the soil was extracted from pots and all remaining insects counted and examined for nematode infection.

In the second experiment performed in Malta park with heavy natural population of *P. quinquepunctata*, plastic pots were inserted into the soil under infested trees. The soil within 2 m radius around tree trunks was sprayed with nematodes at a dose of 10^5 IJ m^{-2}. The experimental variants were as follows: (1) *S. feltiae* (ScP) and 30 mature larvae of *P. quinquepunctata* added to each pot and screened with a nylon mesh; (2*) H. megidis* (PL03) applied in a similar arrangement; (3*)* water control-1 with a similar insect arrangement, (4) *S. feltiae* (ScP) in pots left open for 2 weeks and then screened as in previous variants; (5) *H. megidis* (PL03) applied in a similar arrangement, and (6) water control-2 with a similar insect arrangement. Each variant was replicated 6 times. Adult insects emerging from the soil after pupation were collected in 3-day intervals. Six weeks after starting the experiment the soil was extracted from pots and examined for the presence of insects and nematodes. The remaining soil was then returned to pots and left unscreened until the next season.

Soil samples were taken again in mid-May of the following season, shortly before descending of the new generation of *P. quinquepunctata* larvae for pupation. After removing all larger debris (i.e. fallen leaves and twigs) from the soil surface in pots, 30 mature larvae of *P. quinquepunctata* were added again into each pot and the pots screened with nylon mesh. Emergence of adult insects after pupation was examined as in the previous season. To

evaluate the survival of nematode infra-populations in the soil, the soil samples were incubated in 9 cm Petri dishes with 3 *G. mellonella*, and the insects dissected after 3 days.

Results and discussion

Nematode infectivity and reproduction in P. quinquepunctata (laboratory experiments)

All the examined developmental stages of *P. quinquepunctata* (i.e. mature larvae, pupae and young beetles) were highly susceptible to nematode infection. Within 3-day exposure to the dose of 35 IJs per insect, respectively, 89, 86, and 94% of insects were infected by *S. feltiae*, and 97, 90 and 97% by *H. megidis*. *S. feltiae* reproduced in the host insects with new generation IJs emerging after 8-15 days. Development of *H. megidis* was much slower (up to 36 days) and erratic, frequently with no production of IJs in spite of all characteristic symptoms of *Heterorhabditis* infection.

Nematode biocontrol potential against P. quinquepunctata (field experiments)

The natural mortality of *P. quinquepunctata* during pupation in the soil ranged between 27 and 63% (mean 49%). After 6-week exposure in field conditions only 12.8 and 11.1% of adult insects emerged from pots treated with *S. feltiae* at 10^5 and $2.5x10^5$ IJ m^{-2}, respectively, and none of the insects emerged as adults from pots treated with *H. megidis*. No significant differences were observed between the nematode doses. Interestingly, after 6 weeks no nematode-infected larvae and pupae were found in the soil treated with *S. feltiae*, while 26.7% of larvae and pupae with clear symptoms of infection and/or developing nematodes were recovered from the soil treated with *H. megidis*. In the soil treated with *S. feltiae* nematode-infected adult beetles constituted much greater part of the exposed insects (i.e. 15.6 and 21.7%) than in the soil treated with *H. megidis* (i.e. 3.3 and 1.1%, respectively).

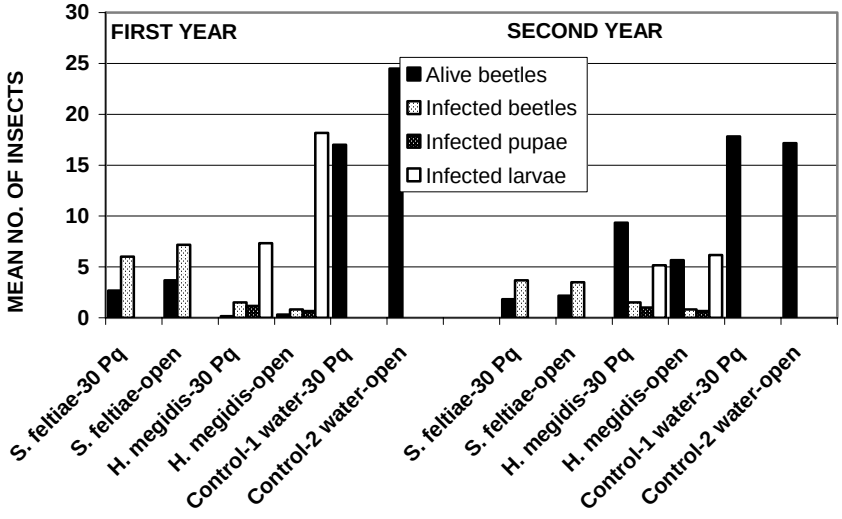

Figure 1. Effect of inoculative release of entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis megidis* on *Phytodecta quinquepunctata* pupating in the soil. Mean numbers of insects recovered in the first and second year after nematode application.

In the first part of the second field experiment results obtained in variants with the fixed number of introduced *P. quinquepunctata* larvae corresponded with those of the first experiment (Figure 1). However, two major differences were recorded in variants with pots left open. Firstly, the number of 30 larvae per pot was somewhat underestimated, as in the Control 2 the number of insects recovered from pots left open for two weeks under the tree canopy was about 30% higher than that from pots of the Control 1 with a fixed number of introduced insects (31.0 and 22.5, respectively). Moreover, the time of the field experiment coincided with maturation of several species of geometrid moths (i.e. *Operophtera brumata, Erannis defoliaria,* and *Agriopis aurantiaria*) which develop on hornbeam (*Carpinus betulus*) and oak (*Quercus robur*) with canopy above the wild cherry floor. These insects also pupate in the soil and have been found to be susceptible to nematode infection (Tomalak, 2003). Some of these geometrids also fell into the experimental pots and their larvae were found infected with nematodes. The mean number of living geometrids recovered from the Control 2 was 2.7 per pot. In variants with *S. feltiae* and *H. megidis* their numbers were significantly reduced to 0.3 and 0, respectively, with a mean of 1.8 dead larvae with clear symptoms of nematode infection in the variant with *H. megidis*.

One year after the original introduction, *S. feltiae* was still present in all pots before and after pupation of the fresh batch of *P. quinquepunctata* larvae and performed as effectively as in the first season (Figure 1.). In contrast, in 5 out of 12 plots with soil treated with *H. megidis* no infection was observed. Although equally numerous in all pots after *P. quinquepunctata* pupation in the first season, examination of the soil samples before and after pupation of the insects in the second season, revealed no nematodes in these 5 pots. This clearly indicates that some infra-populations of *H. megidis* became extinct during the over-wintering period.

Entomopathogenic nematodes can effectively control tree beetles during their pupation in soil. Mass reproduction in the host and good persistence of the new IJ population in the soil ensured high pest control efficacy of *S. feltiae* also in the second season after introduction. Extinction of some *H. megidis* infra-populations made this nematode overall less effective in the following seasons. Inoculative release of entomopathogenic nematodes, particularly that of *S. feltiae,* can be an effective means of long-term management of *P. quinquepunctata* and potentially other soil dwelling pests in urban parks and forests.

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Two different bacterial symbionts of *Heterorhabditis megidis* and *Heterorhabditis downesi* inside one population

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Abstract: During a detailed survey of Hungarian EPN fauna several area were found, from where *H. megidis* or *H. downesi* with two different bacterial symbionts could be collected. The aim of this present study was to estimate the frequency of different *Heterorhabditis/Photorhabdus* combinations in one geographical location. EPN strains were isolated from 20 soil samples collected from an oak forest and a clearing near to the forest. Altogether 44 nematode strains (24 *H. downesi* and 20 *H. megidis*) were isolated from infected *G. mellonella* and *T. molitor* larvae. Two *Photorhabdus* taxa were isolated from IJs of the nematodes: *P. temperata* subsp. *temperata* (24 isolates) and *P. temperata* subsp. *cinerea* (20 isolates). These two bacteria could be isolated both from *H. megidis* and *H. downesi*. Reproductive success of both nematode species associated with *P. temperata* subsp. *cinerea* were higher, than in the case of *P. temperata* subsp. *temperata* symbiont.

Keywords: *Heterorhabditis*, *Photorhabdus*, symbiosis

Introduction

Taxonomic studies have established that each entomopathogenic nematode is associated with a specific bacterial symbiont. In nature, the nematode develops and reproduces in a monoxenic environment that is established by its symbiont within an insect cadaver (Adams et al., 2006). Each steinernematid nematode shares a mutualistic relationship with a single bacterial species. On the contrary, *H. bacteriophora* strains are associated with two different species and two different subspecies of *Photorhabdus*. However, *H. bacteriophora* strains with different *Photorhabdus* symbionts are separeted geographically (Adams et al., 2006).

During a detailed survey of Hungarian EPN fauna (Tóth, 2006) several area were found, from where *H. megidis* or *H. downesi* with two different bacterial symbionts could be collected. The aim of this present study was to estimate the frequency of different *Heterorhabditis/Photorhabdus* combinations in one geographical location.

Material and methods

The soil samples were collected near to the village Dány (Central part of Hungary). 10 samples (each about 3 litres) originated from an 85-years old oak forest (*Quercus robur – Quercus cerris*) and another 10 samples were collected in a clearing (1 km far from the first sampling site). Within 24 hours each soil samples was supplemented with 5 last instar larvae of *Galleria mellonella* and 5 of *Tenebrio molitor* that were caged in Eppendorf tubes with holes. The mortality was recorded after 7 days. The dead insect larvae were checked for bioluminescence in a darkroom and only the bioluminescent cadavers were used for further studies. The cadavers were placed on „white traps" separately and the emerging infective juveniles were counted. The nematodes were identified by PCR-RFLP analysis of the ITS1-ITS2 region of the rDNA gene. *H. downesi* isolate 3107 and 3172 and *H. megidis* isolate 3014

and 3016 with known ITS1-ITS2 DNA sequence (EU921443, EU921444 and EU921442, EU921442, respectively) served as a reference species. The symbiotic bacteria were isolated from the infective juveniles (Gerritsen et al., 1992). Bacterial isolates were identified by morphological characters (Tóth and Lakatos, 2008).

Results and duscussion

Altogether 17 (85%) soil sample were positive for *Heterorhabditis/Photorhabdus*. In these 17 samples, 42 *G. mellonella* larvae were infected and infective juveniles emerged from 39 cadavers. Only 5 nematode isolates originated from *T. molitor* larvae. From the 44 nematode isolates 24 were *H. downesi* (21 from *G. mellonella*, 3 from *T. molitor*), and 20 were *H. megidis* (18 from *G. mellonella*, 2 from *T. molitor*). Both nematode species were found in both sampling sites. In some cases one part of the larvae in one soil sample were infected by *H. megidis*, while the other part was infected by another nematode species. Two *Photorhabdus* taxa were identified: *P. temperata* subsp. *temperata* (24 isolates) and *P. temperata* subsp. *cinerea* (20 isolates). Both of these bacteria were isolated from *H. megidis* and *H. downesi*. The frequency of different nematode/bacteria combinations are shown in Table 1. The reproductive success of nematode/bacteria combinations differed significantly: both nematode species emerged in greater numbers from the infected cadavers with *P. temperata* subsp. *cinerea* symbiont (Table 1).

This study demonstrated that *H. megidis* and *H. downesi* are associated with two different bacterial symbionts, *P. temperata* subsp. *temperata* and *P. temperata* subsp. *cinerea*, moreover, these two bacterial symbionts can be found inside one population. The differences in the reproductive success between different nematode/bacteria complexes suggest that the bacterial symbionts could play a role in the interspecific competition between the two nematode species. Extensive laboratory experiments are in progress aimed to clear this role.

Table 1. Reproductive success of different *Heterorhabditis/Photorhabdus* combinations.

Nematode/bacteria species	**n**	**reproductive success (IJ/infected insect)**
H. downesi/P. temperata subsp. *temperata*	10	42 298 a
H. downesi/P. temperata subsp. *cinerea*	14	75 948 b
H. megidis/P. temperata subsp. *temperata*	14	34 744 a
H. megidis/P. temperata subsp. *cinerea*	6	93 859 b
		$p<0.01$*

* - one-a-way analysis of variance, *ln* transformed data

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Efficacy of entomopathogenic nematodes against caterpillars in greenhouses with climatic conditions of Southern Spain

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Abstract: Some trials were carried out to improve the efficacy of the entomopathogenic nematodes applications to control pests in an IPM program. A bioassay has been carried out to test the most effective dose and a second trial in a commercial greenhouse to evaluate the application in the climatic conditions of Almería (Southern Spain). Some caterpillar pests are difficult to control in these conditions, due to the lack of compatible chemicals with beneficials and the implementation of IPM strategies. The results show that the treatment using *Steinernema carpocapsae* with a co-adjuvant is the most effective control of *Spodoptera littoralis* and the mortality surpassed 80% three days after application.

Key words: Caterpillars, cucumber, nematodes, greenhouse pests, integrated pest management

Introduction

In the last few years, the importance of lepidoptera pests has incressed in the South of Spain because of the reduction of chemical insecticides and the implementation of IPM strategies. Most of these species feed on leaves and sprouts, which at high population density can quickly destroy the crop. Two common species belong to the genera *Spodoptera*, including *S. littoralis* (Boisduval) and *S. exigua* (Hübner) (Lepidoptera: Noctuidae) (Malais & Ravensberg, 2006).

Entomopathogenic nematodes of the Steinernematidae and Heterorhabditidae families are pathogenic for a range of pests belonging to the orders lepidoptera and coleoptera. These nematodes become of importance in horticultural providing an useful alternative treatment for chemical insecticides in IPM and organic agriculture (Pionnier *et al*., 2007).

Steinernema carpocapsae (Weiser, 1955) Wouts, Mrácek, Gerdin and Bedding, 1982, is effective against leptidotera larvae, but dessication of the juveniles after application in the greenhouse or open field decrease their infectivity. Consequently, it is necessary to improve their survival for use on the foilage (Chubinishvili *et al*. 2007).

The focus of this work has been to evaluate the infectivity of *S. carpocapsae* against *S. littoralis* and to improve the control potential by using a co-adjuvant to increase the efficacy of the nematodes under greenhouse conditions in the South of Spain.

Material and methods

Bioassays

Three concentrations of a formulated product containing *S. carpocapsae* (Capsanem®, Koppert Biological Sysems) have been used. 100, 500 and 1,000 juveniles/ml plus an untreated control (1 ml water) have been tested against second and third instars of *S.littoralis.*

In this trial 10 larvae in 10 plastic glasses of 25 ml were used per treatment. Larvae received 1 ml of each nematode concentration or water. Afterwards, the treated larvae and the controls were kept in a climatic chamber (Sanyo MLR 350 H) at 23 ºC, 80% RH and photoperiod of 16:8 (L:D). The mortality was checked at 24 h, 3 and 5 days after the treatment.

Greenhouse trial

For the field trial, a higher concentration of 2,000 juveniles/ml were used under greenhouse conditions with a natural infestation of the pest. The crop was organic cucumber, cv."Borja" The trial started 8 days after transplanting. Three treatments were used: 1) nematodes plus adjuvant, 2) nematodes without adjuvant and 3) control (plants sprayed with water only). The concentration of the oil adjuvant was 0.5 ml/l (0.05% Addit®). Experimental design was a randomized blocks with 3 replicates per treatment. The plants (10 plants/plot) were isolated of the rest of the greenhouse with an insect proof museline tunnel. The treatments were carried out spraying the plants with the equivalent to 1,000 l/ha of water, using a manual backpack sprayer (Matabi 15 L). The evaluation of the treatments was carried out monitoring 10 plants per plot and counting the number of larvae per plant before application and 3 and 5 days after application. Data of number of larvae per plot were transformed by the function log (x + 1) and analysis of variance and mean separation by Tukey (0.05). Morover, the percentage of efficacy was estimated using the Abbot formula (1925).

Results and discussion

Bioassays in the laboratory

The mortality increase with the applied nematode concentration and time after of the treatment. The maximum mortality was observed with 1,000 nematodes/ml 3 days after the application (Table 1).

Table 1. Larval mortality of *S. littoralis* (%) after application of *S. carpocapsae* in the laboratory

Concentration (nematodes/ml)	**Days after application**		
	1	3	5
100 nematodes	50	70	70
500 nematodes	55	73	73
1,000 nematodes	67	100	100
Water	0	0	0

Trial in the greenhouse

The first application was carried out with a high density of *S. littoralis* larvae (3.6 larvae per plant) in first and second instar. The second application was carried out five days after the first. The high density of the pest population allowed to determine the effect of the treatments. Under standard management of the pest in the greenhouse this population is very high, causing considerable damage because of the voracity of the last instar.

No significant differences were observed for the number of S. *littoralis* larvae in the control before and after the first application (Table 2). However, three and five days after the treatment with nematodes, there is a significant difference in the number of *S. littoralis* per plot. No significant differences were recorded between the nematodes treatments, however, the average of

larvae is lower in plots treated with the combination of nematodes and the co-adjuvant. Five days after the first application, the treated plots with co-adjuvant reached a significant lower number of *S. littoralis* larvae per plot (Table 2).

Table 2. Average number of *S. littoralis* larvae per plot, before and after the first application of *S. carpocapsae* [transformed data log (x + 1)] Almería, Spain, 2007.

Treatment	**Days after application**		
	Before	3	5
Control	3.75 a	3.63 a	3.59 a
Nematodes alone	3.51 a	2.37 b	2.41 a b
Nematodes with co-adyuvant	3.57 a	1.82 b	1.80 b

Different letters at the same column indicate significant difference (Tukey, P = 0.05).

Table 3. Average of *S. littoralis* larvae per plot, before and after the second application of *S. carpo-capsae* [transformed data log (x + 1)] Almería, Spain, 2007

Treatment	**Days after application**		
	Before	3	5
Control	3.59	3.77 a	3.31 a
Nematodes alone	2.41	2.60 b	2.91 a
Nematodes with co-adyuvant	1.80	2.27 b	2.11 b

Different letters at the same column indicate significant difference (Tukey, P = 0.05).

The second application conserved the statistical difference between treatments. However, the effect on the mortality was lower than recorded after the first application, probably consequence for the environmental conditions after the second application, which could have affected the survival and infectivity of the nematodes (Table 3).

The percentage of the Abbot mortality of the treatment with the combination of nematodes and co-adjuvant three days after the first application was 81.93%, while without co-adjuvant it reached only 66.65%.

The more effective treatment for control of *S. littoralis* under greenhouse conditions was the combination of *S. carpocapsae* with the co-adjuvant. The efficacy of the nematode treatment is influenced by the weather conditions just after the application.

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Virulence of entomopathogenic nematodes isolated in hazelnut orchards to *Curculio nucum* (Coleoptera: Curculionidae) larvae in the laboratory

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Abstract: The hazelnut weevil *Curculio nucum*, (Coleoptera: Curculionidae) is a major pest of hazelnuts. Entomopathogenic nematodes have been shown to be pathogenic towards the larval stage of this weevil. In this study, the virulence of nine strains of nematodes isolated in hazelnut orchards with presence of this pest was tested in the laboratory against the last instar larvae. Larvae mortality ranged between 15 and 87.5% related to the nematode strain tested. One steinernematid and one heterorhabditid strain with 87.5% mortality could be promising candidates to use in future studies for the control of the hazelnut weevil pest.

Key words: Hazelnut weevil, *Steinernematidae*, *Heterorhabditidae*, virulence, biological control, entomopathogenic nematodes.

Introduction

The hazelnut weevil (*Curculio nucum*) is a key insect pest of hazelnut plantations in Tarragona (Catalonia), the largest producer of hazelnut in Spain (3% of the world production). The weevil female lays single eggs into the hazelnut from end of March to early June. The larvae feed inside the nuts for 4-5 weeks and fall down with the nut from end of July to mid-August. From beginning of August, larvae emerged from hazelnuts and entered the soil, where they overwintered as a full grown diapausing larvae until the following spring. During this period larvae could be parasited by different entomopathogenic agents. Entomopathogenic nematodes (EPN) are one of these biocontrol agents that could be an alternative to the chemical treatments. EPN (*Steinernematidae* and *Heterorhabditidae*) are lethal obligate parasites of a large number of insect species and have been used efficiently against many soil-inhabiting and burrowing insects (Klein, 1990). However, only limited research has been conducted on the susceptibility of *C. nucum* to some commercial strains of entomopathogenic nematodes (Kuske *et al*., 2005; Peters *et al*., 2007). The objective of this study was to screen the virulence of different EPN strains nematodes isolated in hazelnut orchards.

Material and methods

The nematodes used in these experiments were all nematode strains isolated in a survey of 295 hazelnut orchard soils where hazelnut larvae were present, excepting *S. carpocapsae* strain (B14) isolated from an urban garden in Barcelona (Barcelona). Seven undescribed steinernematid and one heterorhabditid strain and one *S. carpocapsae* strain were tested. Nematodes were reared at 25ºC in last instar larvae of the wax moth, *Galleria mellonella* (Lepidoptera: Galleridae), according to procedures described by Woodring and Kaya (1998).

The infective juveniles that emerged from cadavers were recovered using modified White traps (Kaya and Stock, 1997) and stored at 7ºC for 7-14 days before use. Weevil larvae were collected from infested hazelnuts and stored in sterile soil at 4-10ºC before experimentation. Assays were conducted in plastic cups based on procedures described by Shapiro (2001). Cups (3-4 cm diam., 5 cm deep) were filled with 45 g sterile sand moistened with sterile tap water (12%, w/w). In each cup one last instar larva was placed at the bottom of the sand. Nematodes were pipetted onto the soil surface of each cup in 0.5 ml of sterile tap water. The applied dose was 50 nematodes/cm^2 and control treatments received water only. Insects were held in an incubator at 25 ± 2ºC in the dark. Mortality was assessed after 7 and 14 days and presence of nematodes inside the larvae was checked as indicator of nematode infection. Four replicates of 10 cups per treatment (strain) were repeated twice.

Results and discussion

The mortality of hazelnut weevil larvae exposed to the different strains tested is summarized in Figure 1. Larval mortality caused by steinernematid strains ranged between 15% (D-66-S) and 87.5% (D-114-S) and by the heterorhabditid strain (DG-46-H) reached 87.5% mortality.

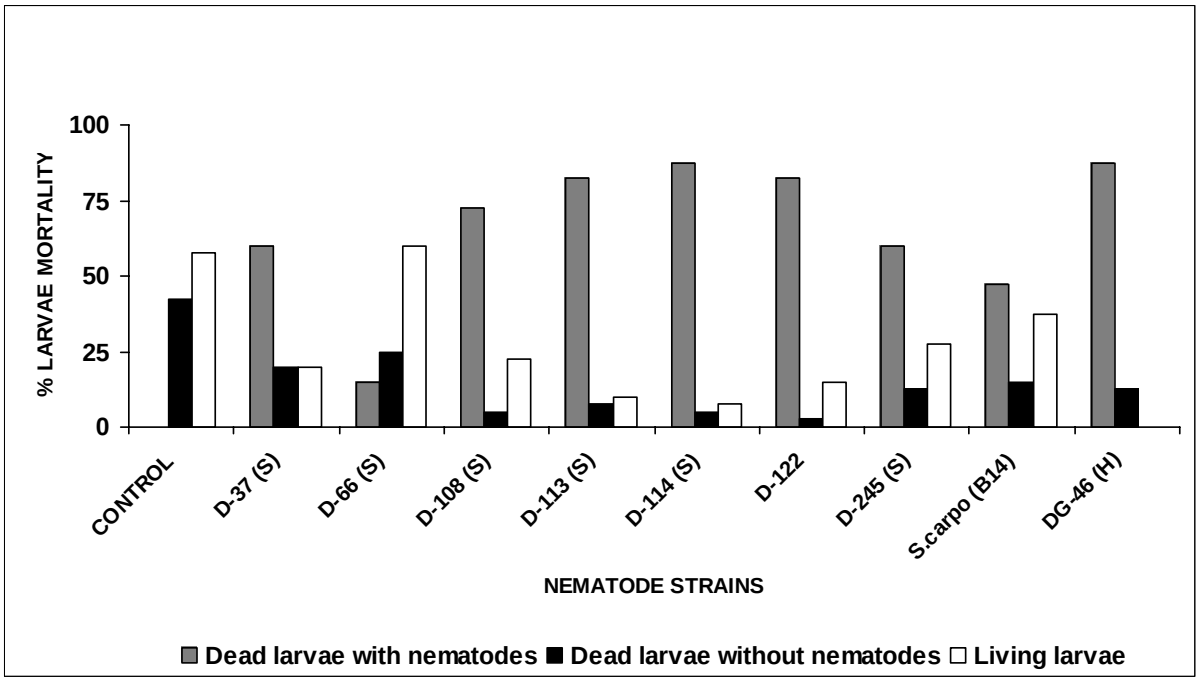

Figure 1. Hazelnut weevil larvae mortality 14 days after exposure to nine entomopathogenic nematode strains. Strains followed by (S) are steinernematids and the strain followed by (H) is a heterorhabditid; S.carpoc (B14) is a *Steinernema carpocapsae* strain isolated in an urban garden.

Mortality of hazelnut weevil larvae caused by nematode infection in the present study (15-87.5%) is similar to mortality reported by Peters *et al.* (2007) in a first laboratory screening with three commercial strains of EPN. These authors showed that with a dose of 100 nematodes per larva *Steinernema carpocapsae* reached the highest mortality (76%) followed by *S. feltiae* (33%) and *Heterorhabditis bacteriophora* (23%). In our study mortality obtained with the *S. carpocapsae* strain isolated in an urban garden (47.5%) was significantly

lower than mortality obtained by Peters *et al*. (2007) with a commercial strain of *S. carpocapsae* (76%). When these authors used *S. feltiae* emerging from infected hazelnut weevil larvae this strain appeared to be more virulent, increasing the mortality from 33% to 88%. Although in the present study all the strains tested (except *S. carpocapsae*) were isolated in fields with presence of hazelnut weevil larvae as a possible host, not all the strains show the same virulence to this insect. Both steinernematid strain D-114 and heterorhabditid strain DG-46 (87.5% mortality) reached mortality reported by Peters et al. (2007) with a *S. feltiae* strain reared in hazelnut larvae (88% mortality). These results could indicate that these two nematodes are adapted to parasitize larvae of hazelnut weevil in natural conditions and could be the most promising candidates to use in future studies for controlling the hazelnut weevil pest.

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Pathogenicity of *Steinernema feltiae* in oil-polluted soil

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Abstract: Abiotic factors, such as heavy metal ions and oil fractions can reduce pathogenicity of entomopathogenic nematodes. Experiments conducted on wasteland contaminated with 2 dcm^3/m^2 of oil derivatives revealed significant decrease in pathogenicity and reproduction of *S. feltiae* lasting throughout 2 month observation. Petrol was more toxic than motor oil and diesel fuel.

Key words: diesel fuel, motor oil, pathogenicity, petrol, reproduction, *S. feltiae*

Introduction

Entomopathogenic nematodes (EPN) occur in soil of the majority of ecosystems controlling population of harmful insects. Pathogenicity of EPN can be significantly reduced by heavy metal ions (Jaworska et al., 1996, 1997a, 1997b), but they were detected even in heavily contaminated soils (Jaworska et al., 1997c). Data on the effect of oil fractions upon EPN are scarce. Diesel fuel, motor oil waste and petrol are the most frequently encountered polluting oil fractions.

Materials and methods

Microplot experiments were conducted on wasteland with leached acid brown soil. Single square meters of 30 cm thick soil layers isolated with waterproof foil from the ground were situated 2 m away from each other. Pollutants (2 dm^3/m^2) were poured on each microfield. The experimental objects were: soil contaminated with commercial petrol (Euro 95), soil polluted with diesel fuel, soil polluted with motor oil waste and non-polluted soil as control. The experiment was set up involving randomized block method in three replications.

In order to test the oil derivative effect on pathogenicity and reproduction of EPN, a suspension of 0.5 million infective juveniles of *Steinernema feltiae* (Owinema Sc., Poland) were applied to each plot a week prior to contamination. Soil samples were collected after one week, one month and two months after contamination. Trap insect larvae (*Tenebrio molitor* L.) were stored under laboratory conditions in the presence of the contaminated soil and their mortality was checked everyday. Dead larvae were then transferred to Petri dishes to examine nematode reproductive abilities according to the Bedding and Akhurst (1975) method. Data were analyzed using the one-way ANOVA and Duncan´s test at $p<0.05$.

Results and discussion

The applied level of soil contamination with oil derivatives reduced pathogenicity and reproduction ability of the nematodes, but it did not extinct them. After one week from administration of the pollutant to the soil, petrol appeared to be least harmful. On the 6th day

mortality of *T. mollitor* larvae was twice as low as in the control (Table 1). A contact of nematodes with oil fractions resulted in decreased reproduction (Table 2).

Table 1. Mortality (%) of *Tenebrio mollitor* L. larvae stored in the presence of soil contaminated with oil fractions and control soil on the 6th day after release of the insect

Time since contamination	Insect mortality (%) in the presence of pollutants*			
	Petrol	Diesel fuel	Motor oil waste	None
One week	29.3 a	81.3 b	74.0 b	77.6 b
One month	23.1 b	59.7 c	63.4 c	52.4 c
Two months	16.7 ab	13.3 ab	26.7 b	60.0 c

* Means followed by the same letters within lines are not significantly different ($p<0.05$).

Oil fractions under study differed from one another in their physical and physiological properties. Petrol is a blend of low alkanes and, therefore, it has a low-viscosity, is a mobile and readily volatile liquid. Usually it contains a small fraction of higher alkanes of lower vapor pressure. Because of its mobility, petrol can readily penetrate experimental soils up to the bottom layers. Petrol, particularly its higher-molecular fraction, is absorbed by soil particles and therefore resides in the soil for longer time. Diesel fuel is composed of higher, less volatile alkanes. Its viscosity is essentially lower than that of petrol. Therefore, it permeates soil much slower. Higher alkanes sorb on soil particles to a lower extend and can envelop them in form of thin films. Motor waste oil contains still higher hydrocarbons and therefore their volatility under experimental conditions may be negligible. However, on its use as lubricating oil the alkanes undergo several chemical changes, among them fragmentation and oxidation to carboxylic acids.

Table 2. Reproduction of *Steinernema feltiae* nematodes in *Tenebrio molitor* larvae maintained in the presence of soil contaminated with oil fractions and non-polluted soil.

Time since contamination	Count of infective juveniles per 1 test insect in the presence of pollutants*			
	Petrol	Diesel fuel	Motor oil waste	None
One week	2870 c	1772 a	2108 b	7502 d
One month	2620 b	1582 a	2306 b	5480 c
Two months	2152 c	1643 b	1120 a	3354 d

* Means followed by the same letters within lines are not significantly different ($p<0.05$).

Physiological action of all three oil fractions are also different. In respect to mammals and insects, petrol is harmful for its regular narcotic properties and isolation of the organism from atmospheric oxygen. Frequently, petrol is used as a solvent for lipids. Thus, on contact with petrol lipid components of membranes can be washed out and its manner physiological properties of membranes can be perturbed. Narcotic properties of the components of diesel fuel are significantly reduced in respect to those of petrol but they may not be neglected.

When isolating nematodes from contaminated environments they can be enveloped with thin, hydrophobic, non-permeable hydrocarbon film.

All observed physiological effects of oil fractions (Table 1) can readily be explained in terms of above arguments. Petrol readily penetrated experimental soil up to its bottom causing fairly rapid either toxic or irritating effect on the nematodes. This effect increases in time, likely because gradual evaporation of lower petrol fractions to the atmosphere leave higher molecular fractions acting through the formation of thin films around the nematodes body. Within one week and one month after pollution, motor oil waste and diesel fuel caused mortality of *T. molitor* larvae similar to that recorded in the control. The low toxicity of diesel fuel might reflect its slow penetration from the top to the bottom of the experimental soil layer. In such manner only part of the nematodes present in the soil were exposed to the hydrocarbon pollution. Differences in the time dependent toxicity of oil waste and diesel fuel can be explained by the toxic effect of admixtures present in the oil waste.

Prihonen and Huhta (1984) who applied a three times higher dose (6.25 dm^3/m^2) of light heating oil and hydraulic oil upon deciduous forest soil noted a total extermination of the micro-arthropod population. Wild nematode population counts decreased immediately after application of heated oil, but two months later it was twice as high as in control. Similarly, as in the case of contamination with hydraulic oil, the nematode population grew, but not as rapidly as after contamination with light oil. Within the second year of observation, the nematode population decreased (Prihonen and Huhta 1984).

Differences in the results point to petrol contamination as lest harmful and that effect can be caused by slow evaporation of that fraction from the soil. Hydrocarbons gradually evaporated to the air environment. First directing their migration to the bottom of the experimental soil the movement is then reversed and hydrocarbons slowly migrated to the soil surface. In contrast to the contamination with petrol, which can be considered reversible in time, the soil contamination with diesel fuel could be considered reversible in a long period and the contamination of the soil with motor fuel waste shows no symptoms of reversibility in the period under study.

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Insect pathogenic nematode, *Steinernema feltiae*, from Iran

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Abstract: A survey of entomopathogenic nematodes was conducted in the Iran. Out of a total of 194 soil samples, 9 were positive for an isolate of entomopathogenic nematodes of the genus *Steinernema*. Morphological and molecular studies as well cross hybridization tests were undertaken for characterization. The isolates were identified as *Steinernema feltiae*. ITS sequences confirmed this. 16S rRNA sequences and phenotypic characters of its symbiotic bacterium were determined and used for identification. In the phylogenetic analysis the relationship among different isolates of native *Steinernema* were indicated. Laboratory test on *Spodoptera exigua* showed high pathogenicity of these entomopathogens.

Key words: Entomopathogenic nematodes, *Steinernem*a *feltiae*, *Xenorhabdus bovienii*, Iran

Introduction

Entomopathogenic nematodes (EPNs) are suitable candidates for use in insect pest management. It has been demonstrated that for routine identification of EPNs, DNA based diagnostics are quicker than the traditional strategies using morphology and morphometrics (Poewer *et al.*, 1997). Sequences of the ITS region of *Steinernema* species have been used by different authors in taxonomic and phylogenetic studies (Stock *et al*., 2001; Nguyen & Duncan, 2002; Nguyen & Adams, 2003; Kuwata *et al*., 2006).

During the last 5 years, few studies have been conducted to characterize EPNs from Iran (e.g., Tanha Maafi *et al.*, 2003). In several surveys, *S. carpocapsae, S. biocornotum, Heterorhabditis bacteriophora* and recently *S. glaseri* were identified from different locations.

Material and methods

Entomopathogenic nematodes were isolated from soil taken from the Tehran province, Iran.The nematodes were maintained on last instar larvae of the greater wax moth *Galleria mellonella*. The third stage infective juveniles (IJs) emerging from host insect cadavers were washed and stored in distilled water at 15°C until use (Kaya and Stock, 1997).

Isolated IJs were prepared for light microscopy examination. EPNs DNA was extracted from a first generation adult. The nematode was crushed in 50 µl worm lysis buffer in a sterilized 0.5 ml microcentrifuge tube. After heat treatment and centrifugation, the supernatant containing nematode DNA was collected and stored at -30°C. In PCR, ITS regions were amplified in a 50 µl reaction using Takara ExTaq® as described in Kuwata *et al*. (2006). After amplification, PCR products were loaded by electrophoresis and purified with a Gel-M Gel Extraction system (Viogene). Sequencing reactions were performed by using an ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City ,CA ,USA).

Sequences of double-stranded PCR products were carried out by ABI Prism® 310 Genetic Analyzer.

Initial direct sequencing showed ambiguous positions and multiple peaks, so ITS product were cloned and resequenced. Pure PCR products were cloned into the pGEM-T Easy vector and transformed into XL-10 Competent Cells. Screening was conducted as described in Spiridonov *et al*. (2004). From each strain three clones were selected and sequenced in both directions. Sequences obtained during this study were deposited in GenBank (FJ657532). Multiple-sequence alignments were created using CLUSTAL X version 1.81 (Thompson *et al*., 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007).

Crossbreeding tests with *S. feltiae* (Belgium strain) were carried out on *G. mellonella* hemolymph according to the method described by Nguyen & Duncan (2002).

Symbiotic bacteria were isolated from surface-sterilized IJs using 0.1% merthiolate solution. After crashing IJs, 0.5 ml of LB broth was added to the suspension and the suspension was spread on an NBTA plate (Akhurst, 1980). Single colonies were successively extracted. The 16S rDNA fragment was amplified by PCR from bacterial cultures and from total DNA isolated from adult and juvenile stages of nematodes. PCR and purification conditions were those described by Kuwata *et al* (2006) using Fischer-Le Saux *et al*. (1999) primers. Purified PCR products were sequenced directly as described earlier.

Pathogenicity of *S. feltiae* THR strain was studied in the laboratory. The concentrations were 10, 50 and 100 IJs/larva. Fourth larvae of *Spodoptera exigua* were used as test host. Thrity larvae were used in each treatment in 9 cm Petri dishes with filter paper Whatman n. 1. 0.5 ml of nematode suspensions were applied. Two hours later host larvae were individually placed into a container. The larval mortality was assessed after 24 h. Statistical analysis was performed using the test of least significant difference (LSD) at the 5% level (SAS, 1989).

For comparing, another isolate of *S. feltiae* (Belgian isolate kindly provided by M.Moens and M.A.Ansari, Ghent University) was used. This isolate was cultured in *G. mellonella* larvae at 25° Ċ (Kaya & Stock, 1997). Experiments were conducted at room temperature. In control treatments only DW was applied. Percentage mortality after 48 h were analysed using ANOVA and Tukey ‘s test for separation of means. Differences among mean mortality considered significant at $p<0.05$.

Results and discussion

The isolated nematode showed specific characters of *Steinernema*. Morphological examination indicated that *Steinernema* sp. (THR) resembles most of *S. feltiae* characters. Key diagnostic traits of the IJs and males were identical to *S. feltiae*. Phylogenetic analysis of the ITS rDNA sequence data placed this species in a clade with other isolates of *S. feltiae*. The partial 18S, 5.8S gene sequence and 28S portion show little variation among different isolates. The ITS1 and ITS2 regions are much more variable and provide most of the base differences for species diagnosis (Adams *et al*., 1998; Nguyen *et al.,* 2001). In the phylogenetic analysis of the ITS sequences, all Iranian isolates of EPNs were categorized tentatively into four different clades. These clusters were supported by high bootstrap numbers. The isolate aligned clearly with those of the other isolates of *S. feltiae*. Phylogenetic cladogram of the symbiont bacterium of this nematode made a clade with other strains of *X. bovienii*. Bootstrap values (1000 re-samplings) of up to 83% each confirm the statistical significance of the branching point of the *Xenorhabdus* strain.

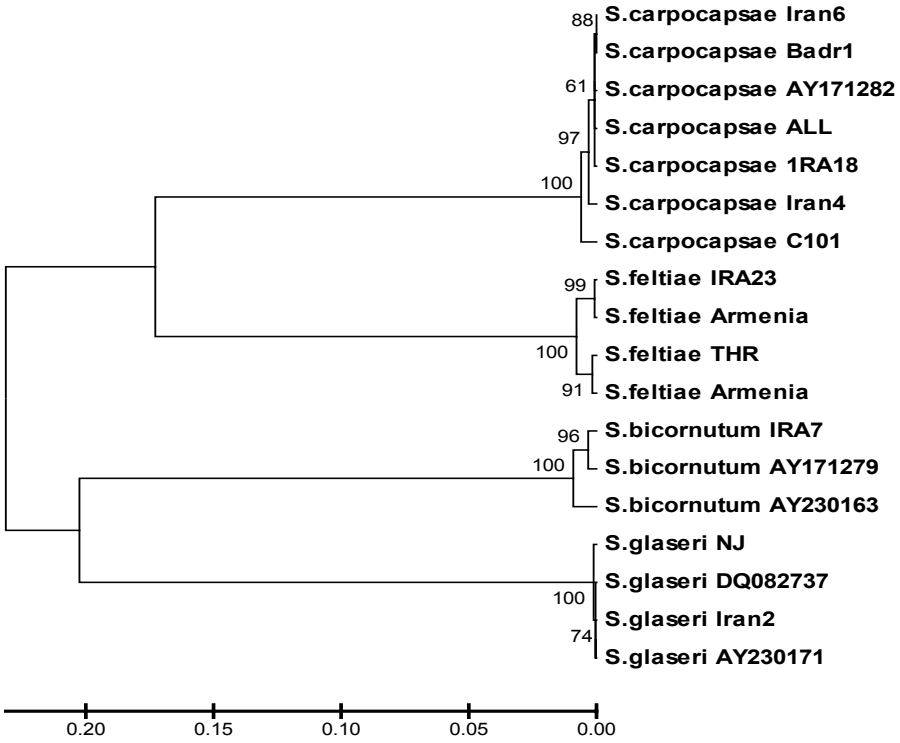

Figure1. Phylogenetic analysis of steinernematid species/isolates based on ITS sequences. The dendrogram was constructed by the maximum parsimony method and Kimura-2 parameter with 1000 resamplings values of bootstrap

Males and females of *Steinernema* sp. THR did interbreed with *S. feltiae* Belgium strain. In the control treatments, males and females of isolated nematode mated and produced offspring.

This study is the first record of the bacto-helminthic complex *S. feltiae – X. bovienii* from Iran. The sequences analysis of the ITS and 16S regions confirmed this.

Colonies of the symbiotic bacteria associated with *Steinernema* sp. THR on NBTA were similar to those found for *Xenorhabdus* spp. The almost complete 16S rDNA sequence was generated from *X. bovienii,* 1502 bp in lenght. This strain is sharing high sequence similarity with *X. bovienii*, symbiont of *S. feltiae*. nBLAST search showed that the 16S sequence of the bacterium has high identity with sequences of other *X. bovienii* strains. Homology matrix analysis showed 97-99 % similarity with other strains of *X. bovienii*.

The host mortality was 74.2–100%. Ranges of LT50 values were from 23.6 to 37.1 h. Pathogenicity determination of this isolate showed that it is highly pathogenic to *S. exigua*. It was indicated that *S. feltiae* could cause 100% mortality at a concentration of 50 IJs/larva of *S. exigua*. Compared with the Belgian isolate, the Iranian isolate caused higher mortality.

The present investigation clearly demonstrated the presence of *S. feltiae* in Iran. An important step towards achieving an effective EPN for pest control is to seek naturally occurring endemic EPNs isolates. In addition to *S. feltiae* isolate THR, some other steinernematid and heterorhabditid isolates have been isolated from white grubs. Among them, the THR isolate of *S. feltiae* had the moderate pathogenecity.

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Pristionchus sp. (Rhabditida: Diplogastridae) from Italian populations of *Anoplophora chinensis* Forster (Coleoptera: Cerambycidae)

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Abstract: *Priostonchus* sp. n. (Rhabditida: Diplogastridae) is described from a population of Citrus Longhorn Beetles. Alive adults of this Cerambycid were collected from *Acer saccharinum* trees at Assago and Parabiago (Villastanza), two localities near Milan (Italy). The natural occurrence of the entomogenous nematode in the beetle was not frequent and widespread in the study area. Preliminary laboratory infectivity tests highlight the potential parasitic nature of the nematode. However, it was not successfully maintained in storage and culture. The diplogasterid *Pristionchus* sp. has rather complicate systematic thus a biomolecular analysis based on DNA is necessary for more specific identification and to understand whether the nematode is autotochtone or was brought with this new invasive Coleopteran. Further studies are necessary before this nematode can be considered as a biological control agent of the *A. chinensis*.

Key words: Pathogen, Citrus Longhorn Beetle , invasive species

Introduction

The Citrus Longhorn Beetles (CLB), *Anoplophora chinensis* Forster (Coleoptera: Cerambycidae) has become a serious pest in the northern part of Italy (Hérard *et al*., 2005; Maspero *et al*., 2007; Jucker *et al.*, 2007). *A. chinensis* is native from Asia, particularly spread in China, Korea and Japan, where it is a serious pest of *Citrus* and many other ornamental and forestal plants. Recorded in the A2 list of quarantine pests of EPPO and regulated according to the EU Directive 2000/29/CE, the species was first detected in Europe in 1980 in the Netherlands, then in 1998 in Austria, in 2000 in the UK, in 2003 in France, in 2007 in Croatia and Germany. In these countries, the pest has been eradicated after the detection. In Northern Italy *A. chinensis* was observed in 2000 (Colombo & Limonta, 2001) and the species could settle and spread. Extremely polyphagous, it attacks different species of broadleaf plants (e. g. *Acer, Aesculus, Platanus, Betula, Carpinus, Fagus, Malus, Pyrus, Rosa*). Damages are caused by the xylophagous larvae, which bore tunnels into the trunk and roots. Hardly attacked plants can easily die.

Despite to the serious problems of the attack with *A. chinesis*, little is known about its natural enemies and pathogens both in the origin areas and in the regions where *A. chinensis* was introduced. In China, predation by the weaver/red ants, *Oecophylla smaragdina* (Fab.) are effective predators of CLB larva (Yang, 1984). The fungus *Beauveria brongniartii* (Sacc.) has also been shown to cause high adult mortality (CABI, 2004). In Italy research has shown the presence of some indigenous non-specific parasitoids and a new species of specific parasitoid of *A. chinensis*, *Aprostocetus anoplophorae* Delvare (very likely native from the Far East) (Delvare *et al.*, 2004; Hérard *et al*., 2005).

Rhabditoid nematodes have been used successfully in control programs for other cryptic pests also in the case of the genus *Anoplophora* especially *A. glabripennis* (Katio, 1986; Li 1988; Liu *et al*, 1992; Solter *et al*, 2001; Gomez Vives *et al*., 2008). However, to our knowdlege there are no records on natural entomopathogenic nematodes infesting *A. chinensis*. This paper reports the first record of a naturally occurring entomopathogenic nematode found in *A. chinensis* recently introduced in Italy.

Material and methods

Insect sampling and nematodes collection

Nematode populations were isolated from adult specimens of *A. chinensis* Forster. These coleopterans were collected from *Acer saccharinum* trees at Assago and Parabiago (MI), localities of the Lombardia region, Italy, on June 2008. Nematodes were maintained on nutrient agar plates containing larvae of wax moth (*Galleria mellonella* L.) to perform preliminary bioassay on the pathogenicity.

Nematode identification

The collected and reared nematodes from *A. chinensis* were killed at high temperature and then mounted in permanent collection slides. Observations and photographs were made with an Inverted Phase Contrast Microscope (Leitz Diavert) with magnifications of up to x 1,000 with a digital imaging system (Nikon). All measurements are in micrometers unless otherwise stated.

Results and discussion

A diplogastrid nematode of the genus *Pristionchus* was found parasitizing specimens of the Citrus Longhorn Beetle collected on trees of *Acer saccharimun* in the Lombardia region (Italy). Considerations regarding the taxonomy and the biology of the *Pristionchus* genus are reported.

Pristionchus* Kreis, *1932 (Rhabditida: Diplogastridae)

The total length of nematodes was in average 1446 µm for females and 995 µm for males (Fig. 1a). In both of the sexes the tail shape is long and tapering to filamentous but the female tail is generally longer. They are characterized by a stoma prominent with metarhabdion with a tooth in the stoma and with very muscular metacorpus and glandular postcorpus (Fig. 1b). The median bulb is not elongate. The specimens of the diplogastrid found are gonochoristic, the gonads are paired, the uterus is not kidney-shaped. The length of the spicules is in average 55.5 µm and the gubernaculum is 19.0 µm (Fig. 1c). No hermaphroditic individuals were found.

The genus *Pristionchus* is closely associated with scarab beetles, 23 species have the association with various species of beetles. Sommer et al (2008) separate species based on one nucleotide difference. Hong et al. (2008) report that the nematode–insect association, in which nematodes infest the surface of insects and wait for their hosts to die before resuming development on the cadaver, is known as necromeny. We also notice that *Pristionchus* infest live insects. In fact, the initial bioassay infesting *Galleria melonella* gave contrasting results on the mortality and the nematode was not to replicate.

Developmental stages such as dauer larva and different forms of the mouth can be influenced by changes in temperature and food availability in the host insect (Sommer et al.,

1996; Hong et al., 2008), thus a further analysis is necessary to investigate the pathogenicity of this nematode.

It was found that each *Pristionchus* species is able to detect its corresponding beetle by olfactory signals and the nematodes are attracted to the sex pheromones of the beetles. For this reason the specific determination of species is essential. In fact, recent studies indicate a close association of these nematodes to various scarab beetles and the Colorado potato beetle, thus extending the knowledge of *Pristionchus* ecology (Hong et al., 2008). For example, *P. pacificus* is found on the Oriental beetle *Exomala orientalis* in Japan and the United States, whereas two common European species *P. maupasi* and *P. entomophagus* are predominantly found on cockchafers (*Melolontha* ssp.) and dung beetle (*Geotrupes* ssp.), respectively (Sommer et al., 1996; Hong et al., 2008). Moreover it is important to understand if the diplogasterid was autochtone or imported with A. *chinensis.*

However, nematode species of the genus *Pristionchus* have little diagnostic morphological characters. Results indicate that the specimens may belong to the species *P. entomophagus* but it usually shows overlapping ranges of morphometric values (Srinivasan et al., 2008) and thus a clear identification was not possible. We therefore will continue the research using molecular tools to distinguish the species. The role of this entomogenous nematode in the reduction of the *A. chinensis* should be investigated.

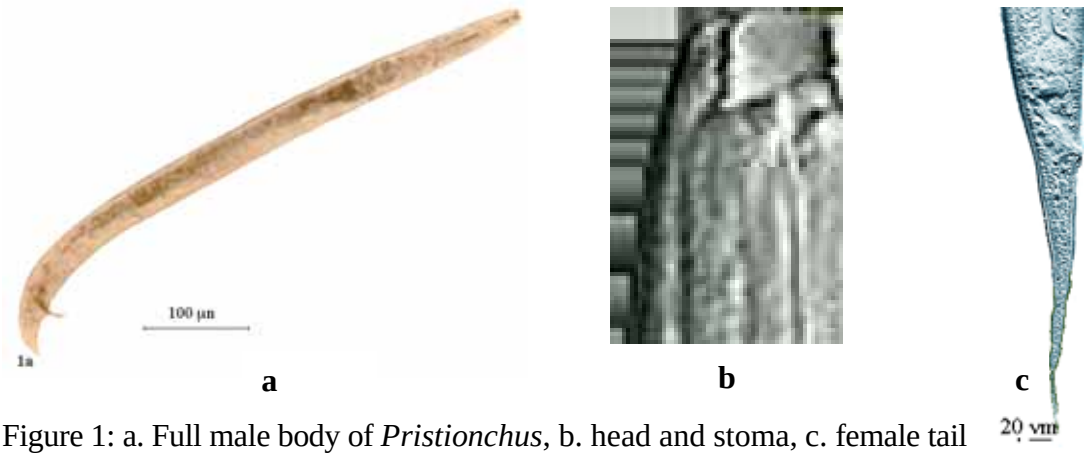

Figure 1: a. Full male body of *Pristionchus*, b. head and stoma, c. female tail

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Susceptibility of the tomato leaf miner, *Tuta absoluta* to entomopathogenic nematodes

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Abstract: The tomato leaf miner, *Tuta absoluta,* is a new imported pest of tomato crops in Europe. Although chemical pesticides are quite effective, factors such as insect resistance or environmental pollution limit their use for management of *T. absoluta.* Biological control with entomopathogenic nematodes can provide a good control of this pest. The efficacy of different entomopathogenic nematode strains of the families Steinernematidae and Heterorhabditidae was tested against last instar larvae and pupae of *Tuta absoluta.*

Key words: *Tuta absoluta,* entomopathogenic nematodes, *Steinernema feltiae*, *S. carpocapsae*, *Heterorhabditis bacteriophora*, biological control.

Introduction

The tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most important insect pests on tomato crops in South America although it also attacks other solanaceous crops as aubergine, potato or pepper plants (Biurrun, 2008). The first report of introduction in Europe was at the end of 2006 in Spain where it spread throughout some regions of the Mediterranean coast (EPPO, 2008). Recently it has been detected in France and in the Netherlands (NPPO, 2009) and can become a great problem in greenhouses or in open crops in other European countries.

Females of the tomato leaf miner lay their eggs on leaves either individually or in little groups. After hatch, larvae feed on leaves and fruits during their development. They are quite active and can move to different plants. Pupation can occur on the plant or in soil (Fernández and Montagne, 1990). The damages are caused by the larvae during all growth stages, producing large galleries in the leaves and burrowing apical buds, and green or ripe fruits (Cáceres, 1992). Both yield and fruit quality can be significantly reduced and losses of up to 100% have been observed on infested tomato crops (Lourençao *et al.,* 1985; Giustolin *et al.,* 2001).

Principally, control strategies for *T. absoluta* consist of insecticide applications against larvae (Collavino and Giménez, 2008; Ortega *et al.,* 2008), but effective control is difficult because the larvae are mining and insects develop resistance to some chemical pesticides. Alternative control methods are being investigated including field trapping with pheromone and light traps (Oliveira *et al.*, 2008; Robredo and Cardeñoso, 2008), selection of insect-resistant plant varieties and the use of biological control agents: parasitic insects like *Trichogramma* sp.and *Macrolophus* sp. (Faria *et al.,* 2008), mites as *Pyemotes* sp. (Romero *et al.,* 2007), bacterias as *Bacillus thuringiensis* (Giustolin *et al.,* 2001; Niedmann and Meza-Basso, 2006) and entomopathogenic fungi as *Metarhizium anisopliae* and *Beauveria bassiana* (Rodríguez *et al.,* 2007; Pires *et al.,* 2009). However, the potential use of entomopathogenic nematodes against the tomato leaf miner has not been investigated yet.

The objective of this study was to evaluate the susceptibility of larvae and pupae of the tomato leaf miner to three native strains of entomopathogenic nematodes.

Material and methods

Nematodes

Three native strains of different nematode species were used: *Steinernema feltiae* (Bpa), *S. carpocapsae* (B14) and *Heterorhabditis bacteriophora* (DG46). The nematodes were reared at 25ºC in last instar *Galleria mellonella* (Lepidoptera: Galleridae) according to the method of Woodring and Kaya (1998). The infective juveniles that emerged from cadavers were recovered using modified White traps (Kaya and Stock, 1997). After storage at 7ºC for 7-14 days, they were allowed to acclimate before use at room temperature for 12h.

Insects

Last instar larvae and pupae of *Tuta absoluta* were obtained from the Department of Plant Protection, IRTA (Cabrils, Barcelona), from insect laboratory cultures in tomato plants.

Bioassays

The experiments with larvae were done in Petri dishes (5.5 cm diam.) filled with 23 g of sterile sand and moistened with sterile tap water (10% w/w). Pupae assays were lined with three moistened filter paper discs. A single insect was exposed to infective juveniles (IJs) of each strain. Two nematode doses were used: 50 IJs/cm^2 and 25 IJs/cm^2. The dishes were sealed with parafilm to avoid dehydration and maintained in a climate chamber at 23 ± 2ºC in the dark. Controls were identical to the treatments except that no IJs were added. Insect mortality was checked every 72h and presence of nematodes inside dead insects was observed to confirm nematode infection. For each insect stage, larva and pupa, there were 15 replications per nematode strain and the experiment was repeated twice.

Results and discussion

This work is the first report of the susceptibility of the tomato leaf miner, *Tuta absoluta* to entomopathogenic nematodes. The results revealed that the last instar larvae of the tomato leaf miner were highly susceptible to all three entomopathogenic nematodes tested. When the dose of 25 IJs/cm^2 was applied, mortality of larvae reached 78.6% with *H. bacteriophora*, 85.7% with *S. carpocapsae* and 100% with *S. feltiae*. For the dose of 50 IJs/cm^2 mortality reached 86.6% with *S. carpocapsae* and 100% with *H. bacteriophora* and *S. feltiae*.

Pupae were less susceptible than larvae to entomopathogenic nematodes. Percentage of pupae parasited by nematodes varied from 0% (*H. bacteriophora*) to 13% (*S. feltiae* and *S. carpocapsae*). During the experiments some pupae became adults and some of them were infected by entomopathogenic nematodes (6.7% infected by *H. bacteriophora* and 40% by steinernematids), confirming that adults of *Tuta absoluta* are also susceptible to entomopathogenic nematodes.

The results obtained in the present study offer interesting starting points for further evaluation of entomopathogenic nematodes as biocontrol agents against *T. absoluta*.

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Natural occurrence of entomopathogenic fungi and nematodes in hazel orchard soils from Catalonia (N.E. Spain)

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Abstract: The occurrence of entomopathogenic fungi and nematodes has been studied sampling 295 hazel fields in Catalonia (N.E. Spain). Fungi were recovered from 133 of 295 fields, which represent 45.1% of the samples. *Beauveria bassiana* and *Metarhizium anisopliae* were the most often collected fungi, and both were recovered in all types of crops analyzed (integrated, conventional, ecological, abandoned, and wild hazels). Nematodes were recovered from 15 (5.1%) of the sites sampled. The most common family of entomopathogenic nematodes isolated was *Steinernematidae*, which made up 93.3% versus 6.6% of *Heterorhabditidae.* The nematodes were present in four of the five types of crops sampled, lacking only from conventional hazel orchards.

Key words: biological control, *Beauveria*, *Metarhizium*, *Steinernematidae*, *Heterorhabditidae*, soil survey.

Introduction

Hazelnut culture in Spain is mainly located in Catalonia with a production ranging 15,000 and 25,000 t of hazelnuts. The Tarragona province in Catalonia concentrates 90% of the national production with about 20,000 ha of orchards.

The hazelnut weevil (*Curculio nucum* L., Coleoptera, Curculionidae) is the most destructive pest in hazel orchards (Maeso *et al.*, 1988). Its distribution is partially determined by different factors including variety of hazel and altitude cultivation. Thus, orchards in high altitudes like those in the geographic area, which are the object of our research, are more sensitive in suffer attacks from hazelnut weevil according to Tuncer and Ecevit (1997). The weevil female lays single eggs into the hazelnut. Larvae feed inside the fruits and at the end of its larval period fall down with the fruits, emerged from hazelnuts and entering the soil where they overwinter as a full grown diapausing larvae until the following spring. During this period larvae can be exposed to infestation by different entomopathogenic agents. The objective of this study was to screen the natural occurrence of entomopathogenic fungi and nematodes in hazel fields. These findings could be used to estimate the role of these biocontrol agents in the natural control of this pest. Isolation of new fungi and nematode strains adapted to the environmental conditions of the hazel crop could allow us to select the best entomopathogenic agents to control the hazelnut weevil pest.

Material and methods

We collected samples in 295 hazel fields selected from orchards that were attacked by *C. nucum*. The sample method involved collecting one sample of soil per site. Each sampling site was characterized by the type of crop (conventional, integrated, ecological, abandoned or wild hazels) (Table 1). Latitude, longitude and altitude were all recorded with a GPS (Garmin®).

Each soil sample was a composite of four subsamples each one collected at a depth of 2-20 cm around one hazel. The four subsamples were pooled in a bucket and mixed gently, but thoroughly, by hand. Approximately 1 kg of soil was placed in a polyethylene bag to prevent water loss and transported under cool conditions to the laboratory. Each soil sample was sifted and any stones and plant residues were removed. Sterile tap water was used to moisten dry soil if necessary. All soil samples were analyzed for pH and the samples containing nematodes were analyzed for soil type (USDA textural analysis), electrical conductivity (EC, as a proxy for salinity), organic matter and pH.

Samples were tested for entomopathogenic nematodes by baiting with wax moth (*Galleria mellonella* L., Lepidoptera: Galleridae) larvae (Bedding and Akhurst, 1975), and for fungi according to Zimmermann (1986). For each sample six Petri dishes (90 mm diameter) were filled up with soil and six *G. mellonella* larvae were placed on the soil surface. Petri dishes were inverted, sealed and incubated at 23ºC ± 2ºC in the dark. Larvae were removed from the Petri dishes after seven days. When the result was negative for entomopathogenic nematodes the assay was repeated once as above. Dead larvae from the baiting showing signs of infection with nematodes were placed individually on modified White traps (Kaya and Stock, 1997) and held at 23ºC ± 2ºC. Nematodes were collected and stored at 7ºC. Some of the collected nematodes were exposed to fresh *G. mellonella* larvae to confirm pathogenicity and to establish new cultures. Dead larvae with signs of infection with fungi (i.e. with mummified or hardened body) were isolated in a moist chamber and incubated at 23ºC ± 2ºC waiting 7-10 days for sporulation. Later, the fungi were cultured in SDAY media to be finally stored at -75ºC in CRIOTECA® vials. Parasitized larvae were kept dry for further reference.

Table 1. Number of soil samples collected in hazel orchards with different methods of production and presence of entomopathogenic fungi and nematodes (*B.b*: *Beauveria bassiana*; *M.a*: *Metarhizium anisopliae*).

Type of production / orchard	**Samples**									
	no.	**%**	**no. with Fungi**	**% with Fungi**	**no.with *B.b***	**% with *B.b***	**no. with *M.a***	**% with *M.a***	**no. with nematodes**	**% with nematodes**
Integrated	142	48.1	69	48.6	37	26	39	27.5	4	2.8
Conventional	63	21.4	27	42.8	19	30.1	14	22.2	0	0
Ecological	10	3.4	4	40	4	40	1	10	1	10
Abandoned	50	16.9	25	50	18	36	9	18	2	4
Wild	30	10.2	8	26.7	3	10	5	16.7	8	26.7
TOTAL	**295**	**100**	**133**	**45.1**	**81**	**27.4**	**68**	**23**	**15**	**5.1**

Results and discussion

Fungi were isolated in 133 of the 295 hazel fields that represent 45.1% positive samples. The presence of fungi related to the type of crop is showed in Table 1. The percentage of fungi obtained in this study is less than those mentioned in similar studies. Quesada-Moraga *et al*. (2007) reported 71.7% of soils with fungi from different localities in Spain. Most fungi isolated in this study belong to *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Metschn.) Sorok. The few other fungi (including *Fusarium* spp. and *Paecilomyces* spp.) represented less than 1 % of the collections and have been neglected in these results.

Nematodes were recovered from 15 (5.1%) of the 295 sites sampled. The incidence of entomopathogenic nematodes in hazel orchard in Catalonia agree with data reported by Morton and Garcia-del-Pino (in press) who isolated nematodes from 5.2% of soils of stone fruit orchards in Catalonia and with Campos-Herrera *et al.* (2007), who recovered nematodes in 5.4% of the soil samples from La Rioja (North of Spain). In the present study, the most common family of entomopathogenic nematodes was *Steinernematidae*, which made up 93.3% of the nematode isolates versus 6.6% of *Heterorhabditidae*. These results are similar to percentages of steinernematids (94%) and heterorhabditids (6%) reported by García-del-Pino and Palomo (1996) in different habitats (cultivated fields, woodlands and pastures) in Catalonia. The nematodes were present in the four of the five types of crop sampled: integrated (2.8%), ecological (10%), abandoned (4%) and wild hazels (26.7%) (Table 1). No entomopathogenic nematodes were isolated from conventional hazel orchards, where the continuous use of pesticides could restrict its presence.

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Soil Insect Pests

The relationship between *Agriotes* click beetle (Coleoptera: Elateridae) wireworms and environmental factors

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Abstract: Wireworms, the soil inhabiting larvae of click beetles (Coleoptera: Elateridae), are world-wide pests causing loss of crop quality and/or yield. In the UK a pest complex of three species (*Agriotes obscurus*, *A. sputator* and *A. lineatus*) is thought to be responsible for the majority of damage. Knowledge of the distributions of individual adult click beetle and wireworm species, and the factors that affect this distribution, is important. However, there have been limited studies in this area, and where they have been carried out wireworms have been grouped together as a complex, mainly because wireworms are morphologically cryptic. Here we used a recently developed molecular tool, terminal restriction fragment length polymorphism (T-RFLP) (Ellis *et al.* 2009), to identify *Agriotes* wireworms collected as part of a previous study by Hicks (2008). Hicks (2008) collected wireworms, adult click beetles and data on a number of environmental variables to investigate possible relationships that may be of use in future control strategies. However, wireworms were grouped as a complex in the analysis as the study preceded the development of the T-RFLP tool. We have re-analysed these data with the wireworms identified to species to establish whether knowledge of the wireworm species present affects the results of an analysis looking at the relationship between adults and wireworms.

Wireworms were collected from 99 organic fields in the South Hams in Devon in 2004, using soil cores (10cm deep 10cm wide) and bait traps (1:1 wheat-Barley seed mixture). Adult males were collected from the same fields using sex pheromone traps, and a range of environmental, chemical, physical and cultural data were taken for each field. The T-RFLP protocol was used to identify *A. obscurus*, *A. sputator* and *A. lineatus* wireworms. An initial Principal Components Analysis (PCA) was carried out using Hicks' (2008) wireworm complex and adult species data, and then a further PCA was performed combining the data on wireworm species identified in this study with the adult data collected by Hicks (2008).

The T-RFLP informed results suggest that the pest complex of three *Agriotes* species may need to be reconsidered. Comparison of wireworm and adult data in the PCA suggested that spatial relationships between counts of adults and wireworms are complicated, and that therefore pheromone trapping of adults might not be appropriate for assessing the proportions and abundances of wireworm species present. These results imply that a greater understanding of species ecology is needed to gain the full potential of using sex pheromone traps as monitoring tools.

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Predicting the size of leatherjacket populations in grassland: a pilot study using water traps

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Abstract: Leatherjackets, *Tipula paludosa* larvae, are sporadic pests of a range of crops. There are indications that they may potentially be controllable with biocides, but these need to be applied in the early autumn when estimating populations is all but impossible. This paper reports an investigation into the use of water traps to catch adults as predictors of subsequent larval populations. It is concluded that female trap counts will be a better predictor than male or total cranefly counts. It is also noted that there appears to be a spatial dimension to correlations suggesting that predictions may be specific to the immediate vicinity of the traps.

Key words: Leatherjackets, *Tipula paludosa*, monitoring, forecasting, water traps

Introduction

Leatherjackets, the larvae of the cranefly *Tipula paludosa*, are noted pests of grassland (Blackshaw 1984; 1985), which provides their main agricultural habitat. However, the majority of the economic damage that is noticed occurs in crops that immediately follow grass (Blackshaw 1988; French 1969). These include spring cereals and several vegetable crops with maize (sweetcorn), summer brassicas and lettuce being the most vulnerable (Blackshaw 2009).

Leatherjackets are susceptible to biological control agents such as entomophilic nematodes and *Bacillus thuringiensis* (e.g. Oestergaard et al. 2006). The optimal time for their application appears to be early in their larval development (Oestergaard et al. 2006), and this is confirmed by simulation modelling (Blackshaw 2009). At the favoured October application time, the larvae are small and not easily detected, even by specialists. Furthermore the variance:mean ratio of population estimates at this time is much greater than in later development stages. Thus direct leatherjacket sampling to aid decision making is unlikely to be successful.

Since *T. paludosa* has an annual cycle and intergenerational dispersal is limited by female morphology and behaviour (Blackshaw & Coll 1999), and there is evidence for density-dependent feedback (Blackshaw & Petrovskii 2007), it is possible that adult numbers could be related to larval numbers. This paper presents data from a pilot study and addresses the question of identifying which components of cranefly catches best map onto leatherjacket numbers.

Materials and methods

The study was made at the University of Plymouth's Seale-Hayne farm in south Devon, England. At 26 well-separated locations in six grass fields a green, circular water trap (Blackshaw 1983) 28 cm diameter x 15 cm deep was positioned on 22 August 2007. The traps were filled with water to which a little detergent had been added. Each week thereafter, for

nine weeks, the traps were emptied, and any craneflies recovered and taken to the laboratory for identification and sexing.

Over the period January to March 2008 5 cm diameter (x 10 cm deep) soil cores were collected from a systematic sampling array centred on each water trap. The array was established by collecting cores from the intersects of concentric circles (5, 10, 20 and 40 m radius) and 24 radii separated by 15°, giving 96 cores per location. The radius at 0° was set at magnetic north. Leatherjackets were extracted from the soil cores in a dry heat extractor within three days of sample collection.

From the cranefly catches, counts were obtained for numbers of total catch, total males, total females, peak total catch, peak males and peak females for each of the 26 locations. These were then correlated with total numbers of leatherjackets and the numbers recovered from each of the concentric circles.

Cumulative male, female and total cranefly catch over the sampling period was correlated with the cumulative leatherjacket count at increasing distances from the trap (i.e. at 5 m, up to 10 m, up to 20 m and up to 40 m).

Results

More males than females were recovered over the nine week sampling period (Fig 1). Males were caught in the first week of trapping but no females until the second week. Generally the peak catches occurred in week ending 26 September 2007, with some being the following week.

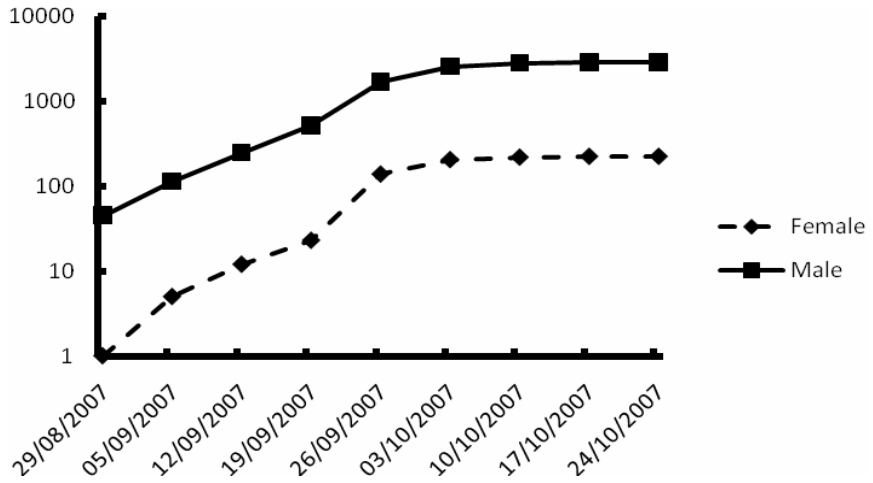

Figure 1. Cumulative total catch of female and male *T. paludosa* adults in 26 water traps

The R^2 values from the simple correlations of trap counts and leatherjacket numbers are shown in Table 1. These values show that counts of females (either as total or peak) were substantially superior to either male or total craneflies in accounting for the variability in the leatherjacket data. Furthermore there was a noticeable spatial effect with the maximum R^2 values occurring with the 10 m leatherjacket counts for all potential explanatory variables. There was little to choose between total counts in each category and peak counts, with the two variables almost certainly collinear.

Table 1. Explained variance (R^2) in relationships between cranefly catches and leatherjacket numbers at 5, 10, 20 and 40 m from water traps, and total leatherjacket numbers up to 40 m from the traps. Peak refers to the maximum number caught in any one week period

	5 m	10 m	20 m	40 m	Total
Total craneflies	0.02	0.18	0.00	0.01	0.07
Peak craneflies	0.06	0.25	0.00	0.01	0.13
Total males	0.01	0.15	0.01	0.01	0.05
Peak Males	0.05	0.20	0.00	0.01	0.11
Total females	0.14	0.58	0.04	0.00	0.41
Peak females	0.16	0.66	0.02	0.02	0.42

Discussion

This was a pilot study and the data analysis preliminary in nature. Any conclusions will need to be verified by a complete analysis. Nevertheless there do appear to be two noticeable patterns in the data. Firstly, it appears that simply counting craneflies caught in water traps does not look to be adequate for predicting subsequent leatherjacket populations. The data support the contention that females are a better predictor, with over 60% of the variation in leatherjacket populations explained with the best correlations (Table 1).

The reason for this probably lies in female oviposition and dispersal behaviour; most eggs are laid within 12 hr of emergence from the pupa and females are unable to fly well until they have shed the weight. In contrast males are much better fliers – which explains why they dominate water trap catches (Fig 1). The consequence of this is that females are likely to be more spatially related to the distribution of the subsequent generation of leatherjackets.

The second pattern is seen in Table 1 where the R^2 values are at their highest when cranefly counts are correlated with leatherjacket counts at 10 m away from the water trap locations. Whilst such a spatial pattern may be an artefact and needs verifying, it is at least consistent with what is known of female behaviour.

These results are encouraging and it looks as if it may be possible to develop a predictive monitoring system for leatherjackets. The caveat is that it will necessitate sorting and sexing craneflies. Irrespective of the resource issues, the available evidence is that non-experts are prone to mistakes when doing this (Blackshaw 1987).

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Isolation of bacteria pathogenic on wireworm larvae *Agriotes* spp. (Coleoptera: Elateridae)

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Abstract: Wireworms, *Agriotes* spp. (Coleoptera: Elateridae) larvae are susceptible to several pathogens. In order to develop a potential microbial control agent against *Agriotes* spp. we have isolated 20 bacterial strains from living *Agriotes* spp. larvae, collected from various fields of Eastern Black Sea Region. All isolates have been identified based on morphological, physiological and biochemical properties and 16S rRNA gene sequence analysis. They were identified as *Paenibacillus* sp. (Ag1), *Cellulomonas* sp. (Ag2), *Bacillus subtilis* (Ag3), *Staphylococcus* sp. (Ag4), *Enterococcus mundtii* (Ag5), *Arthobacter aurescens* (Ag6), *Staphylococcus* sp. (Ag7), *Sphingobacterium* sp. (Ag8), *Staphylococcus pasteuri* (Ag 9), *Arthobacter gandensis* (Ag10), *Bacillus muralis* (Ag11), *Chryseobacterium* sp. (Ag12), *Streptomyces* sp. (Ag13), *Oerskovia turbata* (Ag14), *Bacillus thuringiensis* (Ag15), *Pseudomonas fluorescens* (Ag16), *Oerskovia jenensis* (Ag17), *Arthobacter gandavensis* (Ag18), *Bacillus thuringiensis* (Ag19) and *Pseudomonas plecoglossicida* (Ag20). Virulence of all isolates was evaluated on *Agriotes* spp. larvae. Highest killing efficiency of 100% within ten days using a dose of 3×10^8 bac./larvae was determined for *Arthobacter gandavensis*, *Bacillus thuringiensis* and *Pseudomonas plecoglossicida*. It was possible to further reduce killing time by increasing bacterial doses. This is the first report on isolation of *A. gandavensis* from an insect and its pathogenicity on insects. Consequently, *A. gandavensis*, *B. thuringiensis* and *P. plecoglossicida* have a good potential for use in an augmentative biological control program of wireworm.

Key words: *Agriotes* spp., bacterial flora, microbial control, wireworm

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Biological control of the European cockchafer: Does *Beauveria brongniartii* have effects on fungal community structures in soil microcosms?

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Abstract: Investigation of non-target effects is an important aspect for risk assessment of biological control agents (BCA). *Beauveria brongniartii*, which is commercially applied to control the European cockchafer, *Melolontha melolontha*, was used to assess effects of a fungal BCA on soil fungal communities. The experimental system consisted of six soil microcosm treatments with and without *M. melolontha* larvae and included BCA- and carbofuran-based chemical control agent (CCA) treatments. Quantitative real-time PCR analysis of a specific microsatellite marker was used to quantify *B. brongniartii* in soil and fungal ribosomal internal spacer analysis (RISA) was applied to assess changes in soil fungal communities over a period of 91 days. Strongest and most significant changes in soil fungal communities were detected for treatments containing larvae that had died from either control agent. The BCA alone revealed much smaller and transient effects, while CCA effects were also small but significantly increased at the end of the experiment. The study has shown that both control strategies induced relatively small effects on soil fungal communities and it demonstrated that molecular genetic techniques are efficient tools for monitoring and effect assessment of fungal BCAs.

Key words: Risk assessment, monitoring, community profiling

Introduction

Risk assessment constitutes an integral part of the development chain of biological control agents and knowledge on potential effects of biological control agents on non-target organisms represent the basic data needed for such analyses (Hokkanen & Hajek, 2003). In the past investigations of non-target effects have focused on macroorganisms. Assessment of potential effects on microorganisms did not receive much attention, primarily because appropriate methods were missing. During the last decade various molecular tools have been developed that now allow to address this issue (Schwarzenbach et al., 2007; Widmer, 2007). Ribosomal internal spacer analysis (RISA) constitutes one of the methods with great potential for such applications (Hartmann et al., 2006; Ranjard et al., 2001). This approach is based on specific amplification of the internal transcribed spacer (ITS) region of the ribosomal RNA gene cluster from bulk soil DNA extracts and subsequent analysis of size variation among the PCR fragments. Amplifications are performed with PCR primers that are specific for certain taxonomic groups or kingdoms for instance fungi or bacteria. Size separation of the PCR fragments for instance with a capillary sequencer allows to generate profiles, which represent relative images of the community structure of the targeted organisms in the sample. Comparison of the community profiles from samples exposed to different treatments like “BCA applied” and “BCA not applied” allows to asses the effects caused by a particular treatment.

The entomopathogenic fungus *Beauveria brongniartii* is a well established biological control agent to control the European cockchafer, *Melolontha melolontha*, a serious pest in

orchards and permanent grassland throughout Central Europe (Keller, 2000). Effects of the *B. brongniartii* control agent on non-target macroorganisms like epigeous and soil dwelling arthropods, or earthworms have been investigate in great detail, however studies on potential effects on microorganisms are largely missing (Zimmermann, 2007).

The goal of this study was to assess and compare potential effects of the biological control agent (BCA) *B. brongniartii* and the chemical control agent (CCA) Curaterr® on soil fungal community structures in soil microcosms. In a first step a real-time based cultivation-independent quantification of the BCA in soil was developed and applied to determine exposure of soil organisms to the BCA. In a second step, effects of the control agents on soil fungal community structures were assessed based on RISA profiling followed by multivariate statistical analyses.

Material and methods

The commercially available product 'Beauveria-Schweizer' (E. Schweizer Seeds Ltd., Thun, Switzerland) was used for the *B. brongniartii* treatments. This product consisted of fungus colonized barley kernels (FCBK) overgrown with *B. brongniartii* strain 996. For the chemical treatments the insecticide Curaterr® (containing 5% carbofuran; Bayer, Leverkusen, Germany) was used. *M. melolontha* larvae were collected from an infested hay-meadow in Central Switzerland, in June 2005 and kept in quarantine for 11 weeks prior to use in the experiments.

Microcosms were set up in transparent plastic pots of 9.5 cm diameter and 13 cm height, filled with 200 g equilibrated, *B. brongniartii*-free soil collected from a pasture. A total of 36 microcosms was established, representing six treatments with six replicates each consisting of soil supplemented with either: no addition (Ctrl), 8 FCBK (BCA), 350mg Curaterr® (CCA), one *M. melolontha* larva (Larva), one *M. melolontha* larva and 8 FCBK (L+BCA), or one *M. melolontha* larva and 350mg Curaterr® (L+BCA). BCA and CCA quantities applied were ten times higher than the recommended 40 kg ha-1 for *B. brongniartii* FCBKs and 50 kg ha-1 for Curaterr®. All microcosms were maintained in the dark at 20°C and 85% relative humidity for the entire duration of the experiment (91 days). Living larvae were fed every ten days with one carrot slice per microcosm and leftovers were removed three days after feeding. Larvae were analyzed daily for treatment-characteristic symptoms, i.e. desiccation and dark brown coloration of the insect body for the CCA treatment or red coloration of the insect body followed by emergence of white mycelium for the BCA treatment. Each microcosm was sampled three times throughout the experiment, i.e. at days 7, 42, and 91.

Nucleic acids were extracted from 600 mg fresh soil using a bead beating procedure and quantified according to Schwarzenbach et al. (2007). Pooled soil DNA extracts, referred to as "pools", were prepared by mixing equivalent volumes from each of the six replicates. Cultivation-dependent quantification of *B. brongniartii*, was performed according to Enkerli et al. (2004) with three replicates per sample. For cultivation-independent quantification of *B. brongniartii* in soil DNA, copy numbers of *B. brongniartii*-specific SSR marker Bb5F4 were determined using real-time PCR with two replicates per sample (Schwarzenbach et al., 2009). RISA profiling was accomplished using forward primer fRISAfor (5'-GTTTCCGTAGGTGAACCTGC-3'; HEX-labeled) and reverse primer fRISArev (5'-ATATGCTTAAGTTCAGCGGGT-3' to PCR amplify fungal ITS fragments (Schwarzenbach et al., 2009). Amplified fragments were analyzed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) and scored according to Schwarzenbach *et al.* (2009). Average profiles were determined as arithmetic means of the six replicate profiles from each treatment and will be referred to as "means".

Data from cultivation-dependent and cultivation-independent quantification of *B. brongniartii* were analyzed with Statistica version 6.1 (StatSoft, Tulsa, OK) using Pearson product-moment correlation and Student's t-test. Hierarchical cluster analyses of the RISA data were performed as outlined in Schwarzenbach et al. (2009) applying the Ward method based on pairwise squared Euclidean distances of normalized profile data using Statistica version 6.1. Statistically significant differences among fungal RISA profiles were tested for all treatments with Monte Carlo permutation testing using Canoco for Windows 4.5 (Microcomputer Power, Ithaca, NY).

Results and discussion

All the larvae of the untreated control microcosms survived until the end of the experiment, whereas all CCA treated larvae died between day 8 and day 14, and all BCA treated larvae died between day 18 and day 28.

Quantification of B. brongniartii *and exposure analysis*

Cultivation-dependent as well as cultivation-independent quantification of *B. brongniartii* revealed a significant increase in BCA density during the course of the experiment in the microcosms that received the BCA treatment (Fig. 1; BCA, L+BCA). *B. brongniartii* was not detected in any of the other microcosms. (data not shown). The cultivation-dependent and -independent quantifications significantly correlated ($p < 0.05$) with correlation coefficients of $r = 0.82$ for treatment "BCA" and $r = 0.89$ for treatment "L+BCA".

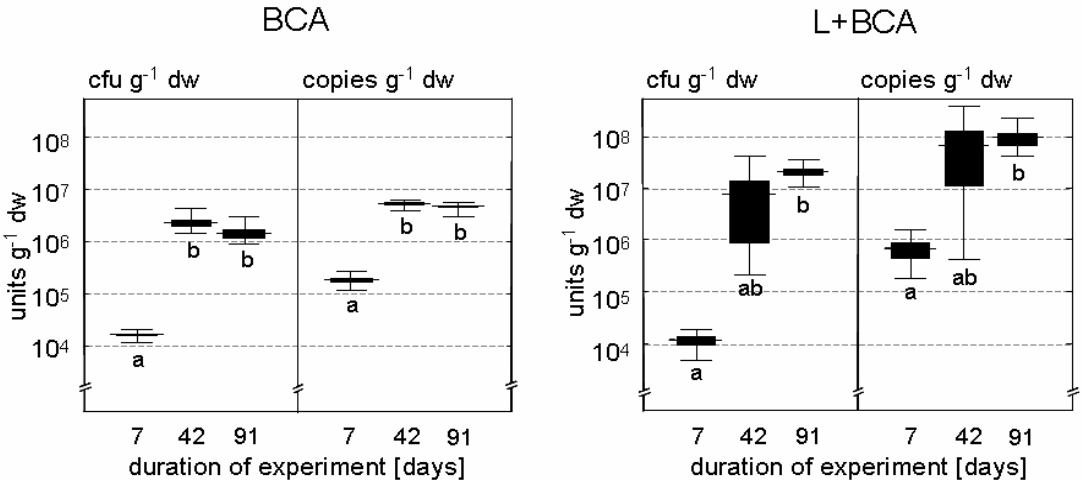

Figure 1. Means (middle-bar), standard errors (box) as well as min and max (bottom- and top-bar) of *B. brongniartii* densities from six replicated soil microcosms amended with eight fungus colonized barley kernels ("BCA"), or eight fungus colonized barley kernels and one *M. melolontha* larva ("L+BCA"). *B. brongniartii* densities determined as colony forming units per gram soil dry weight (cfu g-1 dw) from cultivation on selective medium and copy numbers of SSR-marker Bb5F4 (copies g-1 dw) determined with quantitative real-time PCR

The new cultivation-independent method proved to be reliable and of high analytical precision. In addition, it allowed for reduction of the time required for quantification to about half if compared to the cultivation-dependent approach. This new real-time PCR based tool provides an efficient approach for monitoring of *B. brongniartii* in the future.

Effects of* B. brongniartii *on fungal community structures

Analyses of the RISA community profiles from corresponding pooled soil DNA extracts (pools; p in Fig. 2) and calculated averages of RISA profiles (means; m in Fig. 2) clustered together for all six treatments and all three time points in the Ward dendrograms, which indicated the robustness of the method. No significant clustering of the fungal RISA profiles among the six different treatments was detected seven days after the start of the experiment (Fig. 2). However, at day 42, the treatment group containing dead larvae, i.e. "L+BCA" and "L+CCA" were significantly separated, from the other treatments. This separation became even more pronounced after 91 days. In addition, the difference between the two control agents (BCA, CCA) also became significant, both if applied in treatments with larvae ($p<0.01$) or just to soil alone ($p<0.05$; Fig. 2). Analyses detected no significant differences among the treatments "Ctrl", "Larva", and "BCA" (Fig. 2.).

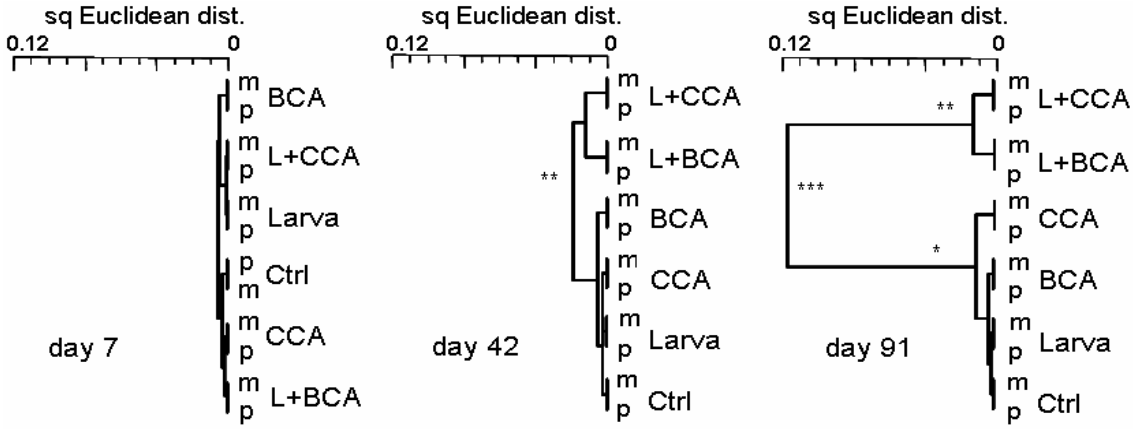

Figure 2. Cluster analyses of fungal community structures derived from pools (p) and means (m) of RISA profiles for all six treatments after 7 days, 42 days, and 91 days in the experiment. Significance levels are *$p<0.05$, **$p<0.01$, ***$p<0.001$. Treatments are control (Ctrl), biological control agent (BCA), chemical control agent (CCA), *M. melolontha* larva (Larva or L)

The results revealed that the BCA as well as the CCA control strategy induced relatively small effects on soil fungal communities. In natural ecosystems stochastic variation of data can be high and effects may quickly be compensated. Therefore, it may be difficult to detect the here reported effects in a field study. Furthermore, effects in the field may be even smaller because ten times lower quantities of the BCA and the CCA are applied in the field and because maximum densities of the *B. brongniartii* BCA in field studies have been reported hundred times lower than detected in this study (Keller et al., 2002). Results obtained have demonstrated that the applied molecular genetic and statistical methods are powerful tools for assessing and ranking effects of BCAs on non-target fungal community structures.

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Observations on natural mortality factors in wireworm populations and evaluation of management options

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Abstract: Wireworms (*Agriotes sordidus, Agriotes ustulatus*) have been reared in rearing cages placed into fields over a five year period (2004 – 2008) in order to evaluate the effect of rotation on larval development and the potential role of biocidal plants (*Brassica juncea* var. ISCI 99) to reduce wireworm populations in comparison with other mortality factors. Wireworm populations in cages planted with different rotations (**continuous rotation**, very short interruption between different crops, **discontinuous rotation**, long periods with bare soil between crops, meadow) were estimated by using bait traps at monthly intervals. Soil with continuous vegetation cover allowed more larvae to survive. Biocidal plant root systems did not cause significant larval mortality. The incorporation of the above ground material of *Brassica juncea* sel. ISCI 99 at a dosage of 55 t ha^{-1} of fresh matter corresponding to about 290 µmoles of GLs l^{-1} of soil, significantly reduced wireworm populations. In the end of the rotation period the larvae were placed in vials inspected twice per week in order to establish larval mortality. According the cause of dead, dead larvae were divided into the following groups: *Metarhizium* spp., *Beauveria* spp., Nematodes, Uncertain. The percentage of larvae found dead because of insect pathogen infection was low and did not differ between larvae coming from different rotations.

Key words: Wireworms, *Agriotes*, mortality factors, rotation, biocidal plants, pathogens

Introduction

Wireworms, the soil-dwelling larvae of click beetles (Elateridae), are widely distributed throughout the world and are important pests of several crops. In Europe, the species of economic importance predominantly belong to the genus *Agriotes.* Traditionally, control of wireworms has relied on conventional pesticide chemistry. However, these treatments are not always fully effective and their use poses potential environmental risks. Therefore, studies on the alternative, sustainable strategies for managing wireworms were performed over the last years. The effects of different rotations and particularly of biocidal plants on wireworm population dynamics were studied. The potential role of wireworm pathogens was also investigated.

Material and methods

Facilities and experimental layout

The rearing facility cages were placed at the border of an open field. They were made of concrete 1 m^2 x 1 m deep, open at the bottom to allow drainage. The cages were closed on the top with netted cages (1-2 mm mesh) 1m^2 x 1m high that allowed rain to penetrate. The cages were filled with a sandy soil, which had been dried in the open air for two months. During dry

(low rainfall) periods, the cages were irrigated at least once a week to maintain a suitable soil moisture level in the upper part of the soil.

The following rotations were compared with 4 replications in cages, which were divided into two parts (each half cage was a replication): 1) **Continuous rotation** (very short interruption between different crops, continuous soil cover with plants): Year 1 (04-05): pea seeds (cv. Corallo) – hemp; Year 2 (2006) spring barley, biocidal plant (*Brassica juncea* sel. ISCI 99, after barley harvest), which subsequently was incorporated into the soil at flowering, Year 3 (2007): sugar beet; 2) **discontinuous rotation** (long periods with bare soil between crops and more tillage disturbance); maize (2005), winter wheat (2005-2006), sugar beet (2007); 3) **meadow** (continuous soil cover with plants, no soil disturbance, optimal conditions for wireworms): rye grass - *Festuca* spp. 2004 – 2006; sugar beet 2007.

In order to evaluate the effect of individual factors on wireworm populations eight additional cages were planted with continuous rotation and split into two groups after barley harvest: A) four cages were half kept with bare soil and half planted with *B. juncea*; B) four cages were planted with *B. juncea.* In one half of each of the four cages, plants were incorporated as described below and in the other half the above ground plant parts were cut at flowering and removed from the cages (untreated - no incorporation). Removed plant material was used to arrange the required dosage (described below) to be incorporated in any other cage sectors where needed.

In cage sectors with plant incorporation the *B. juncea* plants were cut and chopped into 2-5 mm pieces and were immediately homogeneously mixed and incorporated (55,5 t ha^{-1}) into the upper 17 cm of soil; sinigrin glucosinolate content of the fresh plants was about 9 µmoles/g; the average sinigrin dosage was about 290 µmoles of GLs l^{-1} of soil;

The biological activity of *Brassica juncea* is linked to the cellular presence of the glucosinolate (GLs) - myrosinase system which, in the presence of water, produces a number of biologically active compounds including isothiocyanates, nitriles, epithionitriles and thiocyanates (Fahey *et al*., 2001).

Groups of 50-100 adults of *A. sordidus* Illiger (late spring) and of *A. ustulatus* Schäller (summer) were put into each cage every 7-15 days from early May to mid June. A total of about 200 adult females per cage was used.

Study of larval development and mortality factors

From July onwards, two bait traps (Chabert and Blot, 1992) per cage were placed in the centre of each cage once per month. These were assessed by hand after 7-10 days. In order to recover all the larvae, all material from the trap was first manually sorted and then put into Tullgren funnels and allowed to dry for 15-20 days. Larvae were collected in vials containing moist soil as described above. The head width of all the larvae collected was measured and recorded before returning them to the cage. The larvae were assigned to the respective instar based on the head measurements found in laboratory studies taking into account the standard deviation ranges (Furlan, 1998 and 2004).

The larvae were returned to the cages until the beginning of the last year; from this point on all the larvae were placed individually in plastic vials (2,8 cm in diameter and 8,7 cm high and closed by an airtight plastic lid), filled with moistened sandy soil to half-height and inspected twice per week. The vials were put in small chambers maintained at 25°C.

Statistical analysis

Data were subjected to a $\sqrt{(n+0.5)}$ transformation and analyzed by analysis of variance (ANOVA). Tukey test was used to determine differences between means.

Results and discussion

Rotation effect

Population levels were significantly affected by the rotation (Table 1). Soil with continuous vegetation cover allowed more larvae to survive as observed for the same species *Agriotes sordidus* Illiger in field conditions (Furlan and Talon, 1997). In the first years meadow competition reduced the number of larvae caught by bait traps; in the last year after the removal of previous crops the highest populations were found in cage sectors planted with meadow where there was no disturbance during the first two growing seasons.

Table 1. Effects of rotation on wireworm populations (number of larvae/cage sector/ inspection). Means with different letters are significantly different at P = 0,05

	Before biocidal incorporation (3/3/2006 - 12/10/2006)			**After biocidal incorporation** (27/10/2006 - 7/10/2007)		
Continuous rotation	0,60		b	0,79	a	b
Discontinuous rotation	0,28	a		0,38	a	
Meadow	0,22	a		1,11		b

Effect of the growing biocidal crop

Biocidal plant root systems allowed wireworm development and did not cause significant larval mortality (Table 2). The rotation including the biocidal crops without incorporation of the above ground part of the plants allowed the establishment of a population comparable to that found in meadow, but significantly higher than in discontinuous rotation.

Table 2. Effect of cultivation of biocidal plants on wireworm populations (number of larvae/ cage sector/inspection). Means with different letters are significantly different at P = 0,05

	Before biocidal plants 3/03/2006 - 17/08/2006		**During biocidal plants** 5/09/2006 - 9/10/2006		**After biocidal plants** 27/10/2006 - 7/10/2007	
Biocidal plants in rotation	0,47	a	0,44	a	1,17	b
Untreated (bare soil)	0,24	a	0,44	a	0,38	A

Effect of the incorporation of the above ground part of biocidal plants

Broadcast incorporation of chopped plants of *B. juncea* sel. ISCI 99 at a dosage of 55 t ha^{-1} of fresh matter corresponding to about 290 µmoles of GLs l^{-1} of soil caused a high larval mortality (Table 3). This confirmed the preliminary results obtained in laboratory conditions (Furlan *et al*., 2004)

Larval mortality

The number of larvae affected by pathogens did not differ between different rotations (Table 4). Since the observations were limited to developed larvae (6^{th} to 8^{th} instars for *A. sordidus* and 9^{th} to 11^{th} instars for *A. ustulatus*) it was not possible to evaluate the effect of pathogens on the first instars.

Rotation was the primary factor influencing larval populations and pathogens were not capable to decrease wireworm populations.

Table 3. Effect of biocidal plant incorporation on wireworm populations (number of larvae /cage sector/inspection). Means with different letters are significantly different at P = 0,05

	Before biocidal incorporation (3/3/2006 - 12/10/2006)		**After biocidal incorporation** (27/10/2006 - 7/10/2007)	
Incorporation of chopped biocidal plants	0,43	a	0,65	b
Untreated (no incorporation)	0,30	a	1,90	b

Table 4. Mortality factors: number of larvae killed by pathogens within 90 days from collection from cages planted with the different rotations under study

	Caught larvae	**Metarhizium spp.**	**Beauveria spp.**	**Nematodes**	**Uncertain**
Continuous rotation	41	1	0	0	2
Discontinuous rotation	30	1	0	0	1
Meadow	58	2	1	1	3
Total	**129**	**4 (3,1%)**	**1 (0,7%)**	**1 (0,7%)**	**6 (4,5%)**

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Bioprotection for management of soil dwelling pests

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Abstract: Soil dwelling pests are a major challenge for farmers and pest managers. In New Zealand, a bioprotection strategy has been developed to reduce damage from the endemic grass grub *Costelytra zealandica*. The strategy makes maximum use of knowledge of the insect's biology for prediction of outbreaks of the target pest and uses a natural pathogen for control. A bacterium (*Serratia entomophila)* isolated from diseased larvae has been incorporated into the commercial product Bioshield™, which is marketed in New Zealand for control of the pest. The strategy utilises knowledge of the insect's biology to predict application timing for best effect to minimise damage. Control of the grass grub could be improved by better predictive systems and formulations to increase persistence in the soil. Extension of bioprotection strategies to other soil-dwelling pest species will depend on the discovery of suitable microbial control agents.

Key words: bioprotection, Scarabaeidae, New Zealand

Introduction

Soil insect pests remain a major challenge to agricultural production in many parts of the world. They are hard to anticipate, it is not easy to estimate their damage potential and they are difficult to control. While many species have been pests for decades, agricultural intensification and changes in land use create new opportunities for these old pests and provide a niche for previously unrecognised insects to emerge as new pests. Both old and new pest species are present among the Scarabaeidae, which are a particularly resilient group and have been difficult to control since the prohibition of toxic, persistent chemical pesticides that have adverse non-target effects on the environment. An integrated system based on a thorough knowledge of pest biology is needed to minimise damage from these pests and this approach was encapsulated in integrated pest management (IPM), which generally reduced pesticide inputs. With the withdrawal of many toxic pesticides a new approach is needed. Bioprotection (Jackson *et al.* 2002) moves beyond integrated pest management by aiming to maximise biological and non-chemical controls and meet the consumer demands for pesticide-free produce.

Targeting the grass grub

In New Zealand, a bioprotection system has been developed for control of the endemic grass grub, *Costelytra zealandica*. The insect infests pastures throughout the country but pest outbreaks can be predicted from regional characteristics, weather patterns and pasture history (Jackson *et al.* 1999). The insect tends to occur at highest densities on volcanic and loam soils. On light soils, prone to drought, grass grub populations will build up in synchronous outbreaks that can lead to widespread damage. Outbreaks are usually brought to an end by severe drought, which will cause populations to collapse. In regions less prone to drought stress, the pattern of attack is determined by the pasture history. Cultivation usually kills most

of the insects leaving only a small residual population. From this initial low density the population will build up in a predictable manner until it reaches a high density, damaging population 3–4 years after sowing of the pasture. Peak numbers are maintained for no more than 1–2 years before population collapse. The damage pattern is a reflection of the biology of the insect. Female beetles have low fecundity but lay most eggs close to their initial point of emergence leading to damage patches that increase in size between generations until populations reach densities that can be ten-fold that of densities in natural habitats. At these population densities, disease can spread and pathogens thrive. More than 20 pathogenic microbe and nematode species have been isolated from natural populations of *C. zealandia* (Glare *et al*. 1993b).

A disease condition, found to be widespread among collapsing populations, was found to be caused by strains of two bacteria, *Serratia entomophila* and *S. proteamaculans*, which carry a specific plasmid (Glare *et al*. 1993a) encoding toxin genes (Hurst *et al*. 2000). A pathogenic strain of *S. entomophila* has been produced by fermentation and was initially commercialised as the liquid product Invade™. This has been superseded by the dry granule Bioshield™. The bacteria produced by fermentation are incorporated into a biopolymer prior to coating onto a zeolite granule. The granules are packaged and can be stored in ambient conditions for several months without loss of viability. Once applied to the soil, the bacteria become separated from the granule and integrate into the soil microflora.

In the soil, bacteria are ingested by larvae and rapidly cause cessation of feeding followed by clearance of gut contents and digestive enzymes (Jackson *et al.* 1993, Jackson *et al.* 2001). Remarkably, the disease is specific to *C. zealandica* despite the similarity of digestive enzymes to those of other insect species (Marshall *et al*. 2008). The midgut contents are voided into the hind gut, which in the Scarabaeidae is modified as a “fermentation chamber” containing a wide diversity of micro-organisms (Zhang & Jackson 2008) thought to be necessary symbionts for digestion of recalcitrant plant material. The complexity of the gut and its microbial associations, offers a potential target for manipulation by endogenous agents to disrupt the insect digestive processes. As a specific control, *S. entomophila* poses no danger to non-target organisms (Jackson 2003).

Implementing bioprotection for grass grub control

The bioprotection system for grass grub management involves prediction of pastures at risk through consideration of pasture history and weather conditions that will influence population increase. Adult trapping using pheromone traps can also assist the timing of biocontrol applications (Unelius *et al*. 2008). Adult female grass grubs produce phenol as a microbial byproduct in the reproductive tract. Commercial phenol can be used to bait traps to catch large numbers of male beetles. While quantification of field populations from trap catches has not been calibrated , the absence of beetle catches in the flight season indicates no threat from the beetles in the following season.

Predictions are validated by field sampling of larvae in the second and early third instar stage prior to occurrence of pasture damage. Bioshield™ application for best effect is recommended when the population is between 75 and 250 larvae/m^2 (http://www.ballance.co.nz). If the population is too low, there will be insufficient hosts for the bacteria to recycle. If the population is too high, there will be too much damage caused by healthy larvae before the disease can spread through the population. Another important issue is seasonal timing of application. The bacteria must be applied while the larvae are feeding in the soil, both to prevent damage and allow infection and multiplication within the insects. The key to success of Bioshield™ is bacterial recycling. Once established, pathogenic bacteria will

persist until the grass grub population declines to low levels or are killed by adverse soil conditions. This means that one application of bacteria is usually sufficient to provide pasture protection for 5 years, making the product economical for use by pastoral farmers.

Future bioprotection

Thus, the bioprotection strategy uses an understanding of biology to maximise the benefits of natural controls. Pest populations are monitored to identify when they are at the optimum stage of susceptibility for uptake of disease and prevention of damage. A natural controlling agent is applied that can recycle and keep the population at low levels, preventing damage. The management action is to provide the pathogenic bacterial inoculum earlier in the population cycle than it would naturally occur and by doing so prevent a major outbreak and extensive damage. The bioprotection strategy, however, is not a simple panacea, as it requires attention from farmers and advisors to pest prediction and application. The biocontrol system for grass grub in New Zealand could be improved by better prediction systems that would provide information to farmers without the high costs of physical sampling. The narrow window of application, during peak larval feeding, could be widened by developing formulations for long term survival in the soil or baits to increase uptake.

The greatest limitation for application of bioprotection for soil dwelling insect pests is the lack of suitable biocontrol agents. Although many pathogens are reported from soil dwelling insects, it appears that soil insects show intrinsic resistance to many generalist pathogens. This has probably arisen as a result of frequent challenge of larvae by microbes during their long evolution in the soil. This means that to use a pathogen effectively against scarab pests we must select strains that have evolved to overcome the host resistance. These will be relatively rare and are likely to be host specific (Jackson 1999). The challenge for researchers is to find them.

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Efficacy evaluation of the entomopathogenic fungus *Beauveria bassiana* strain ATCC 74040 against wireworms (*Agriotes* spp.) on potato

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Abstract: In 2008, the efficacy of the entomopathogenic fungus *Beauveria bassiana* strain ATCC 74040 was evaluated in three field trials, one conducted in Germany and two in Italy. The *B. bassiana* strain ATCC 74040-based product, when applied both alone and in an IPM strategy, always significantly reduced potato tuber damage compared to the untreated control. Mean efficacy values ranged from 54 to 94% and were comparable to those of the clothianidin- and fipronil-based reference strategies (77-84%), and higher than those of the chlorpyriphos-based strategy (<35%). The fungus strain can therefore be considered a valuable tool for the control of *Agriotes* spp. in both organic farming and integrated pest management.

Key words: Elateridae, biocontrol agent, integrated pest management, organic farming

Introduction

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota, Hypocreales) is an entomopathogenic fungus affecting a wide range of arthropod pests, such as coleopterans, mites, and hemipterans, and all their developmental stages, but different *B. bassiana* strains differ in their host range (Talaei-Hassanloui *et al*., 2007). The entomopathogenic *B. bassiana* strain ATCC 74040 was isolated from *Anthonomus grandis* (Boheman), the cotton boll weevil, in the Lower Rio Grande valley, Texas, USA (BCPC, 2004). It affects white flies, two-spotted spider mites, thrips, fruit flies, and nut weevils (Mayoral *et al.,* 2006; Duso *et al*., 2008), and acts primarily by contact. Once attached to the insect's cuticle, the conidiospores germinate producing penetration hyphae, which enter and proliferate inside the insect's body. The fungus feeds on its host, causing its death due to dehydration and/or depletion of nutrients. Infection can take between 24 and 48 hours, depending on the temperature (BCPC, 2004). The proliferation of the fungus inside the insect's body leads to the insect's death within 3-5 days.

Wireworms, the larvae of click beetles (Family Elateridae), are destructive to a wide range of plants, but damage can be especially severe on potato (Furlan, 1999). They feed on the seeds, roots and stems of their food plants. Potato tubers may be attacked directly. Damage caused in early stages of tuber growth may result in deformed tubers, while tubers attacked at maturity reveal narrow, shallow galleries, which greatly reduce their value. Wireworm control is difficult because of the long life cycle of the pest (larvae may take from two to six years to reach full maturity and pupate), and it is becoming increasingly challenging due to the withdrawal of many chemical insecticides and due to ecotoxicological issues. Vacante *et al.* (2001) showed that *B. bassiana* strain ATCC 74040 can be a valuable tool for wireworm control in carrot production. We therefore decided to investigate whether this strain would provide satisfactory wireworm control also in potato production.

Material and methods

The entomopathogenic fungus

B. bassiana strain ATCC 74040, formulated in Naturalis® (Intrachem Bio Italia S.p.A., Grassobbio, Italy) is a concentrated suspension of viable conidiospores containing, at least, $2.3x10^7$ viable spores/ml. In 2005, Intrachem Bio International S.A. (Geneva, Switzerland) acquired the intellectual property of Naturalis from Troy Biosciences and the commercial product is now manufactured in Europe.

Table 1. Study site location, potato cultivars, applied spray volume and field rate, and timing of the applications in the different treatments and trials.

No.	Active substance (conc. a.s.)	Formulated product	Spray volume	Applied rate	Timing*
Trial 1 – Sanitz (Germany), potato cv Fasan					
1	*B. bassiana* (7.16%)	Naturalis	300 l/ha	3 l/ha	AB
2	Clothianidin (50%)	Dantop	300 l/ha	0.3 kg/ha	A
3	Untreated control	-	-	-	-
Trial 2 – Lavezzola (Italy), potato cv Vivaldi					
1	*B. bassiana* (7.16%)	Naturalis	60 l/ha	3 l/ha	AB
2	*B. bassiana* (7.16%) + Thiamethoxam (29.9%)	Naturalis + Cruiser FS	60 l/ha	3 l/ha 0.4 l/ha	A
	Tefluthrin	Force	300 l/ha	15 kg/ha	B
3	Fipronil (2%) + Thiamethoxam (29.9%)	Regent G + Cruiser FS	60 l/ha	7.5 kg/ha 0.4 l/ha	A
	Tefluthrin	Force	300 l/ha	15 kg/ha	B
4	Untreated control	-	-	-	-
Trial 3 – Vedrana (BO), potato cvs Melba and Primura					
1	*B. bassiana* (7.16%) + Thiamethoxam (29.9%)	Naturalis + Cruiser FS	100 l/ha	3 l/ha 0.4 l/ha	A
2	*B. bassiana* (7.16%) + Thiamethoxam (29.9%)	Naturalis + Cruiser FS	100 l/ha	3 l/ha 0.4 l/ha	A
	B. bassiana (7.16%)	Naturalis	400 l/ha	2 l/ha	AB
3	Fipronil (2%) + Thiamethoxam (29.9%)	Regent G + Cruiser FS	100 l/ha	7.5 kg/ha 0.4 l/ha	A
4	Chlorpyriphos (7.5%) + Thiamethoxam (29.9%)	Cyren 7.5 G+ Cruiser FS	100 l/ha	15 kg/ha 0.4 l/ha	A
5	Untreated control	-	-	-	-

* A = at transplanting (BBCH 01), B = just prior to earthing-up (BBCH 08)

Field trials

The efficacy of the *B. bassiana* to reduce wireworm damage was tested in 2008 in three field trials. The product was tested using different application strategies (1 application of the fungus at transplanting; 2 applications, 3 at transplanting and 4 at earthing-up) and in IPM strategies in comparison to chemical strategies and untreated controls. Information on study site location, potato cultivars, applied field rates and spray volumes and timing of applications are summarized in Table 1. A randomized complete block design with four replicates per treatment was used in Trial 1 and 2 (plot size: 24 and 180 m², respectively), while a large-plot

design with 4 repeated subplots per treatment was used in Trial 3 (plot size: approx. 300 m²). In all trials, treatments were applied along the rows immediately round the tubers into the furrow at transplanting, and along the rows just prior to earthing-up. In order to evaluate the efficacy of the different treatments in reducing tuber damage, at harvest (Trial 1: 30 Sept.; Trial 2: 11 Aug.; Prova 3: 16 July), the number of tubers damaged by wireworms was counted on 100 randomly selected tubers per plot/subplot. The percentage of tuber damage was then calculated and the efficacy according to Abbott of the different treatments in reducing tuber damage was determined.

Statistical analysis

For trials and cultivar the percentage of tuber damage was compared across treatments using one-way ANOVA, followed by Tukey's test for post-hoc comparison of means in Trial 1 and 2 and by Duncan's multiple range test in Trial 3. In Trial 1, data were arcsin(radq(x/100))-transformed. Levene's test was used to test for homogeneity of variances.

Table 2. Percentage of tuber damage (mean ± s.e.) in the different treatments and trials and efficacy (mean ± s.e.) of the treatments in reducing tuber damage at harvest.*

No.	Treatment	Tuber damage (%)		Efficacy (%)	
Trial 1 – Sanitz (Germany), potato cv Fasan					
1	*B. bassiana* (7.16%)	1.0 ± 0.4 a		92.7 ± 2.7	
2	Clothianidin (50%)	2.5 ± 0.6 a		79.4 ± 6.8	
3	Untreated control	12.8 ± 1.6 b		-	
Trial 2 – Lavezzola (Italy), potato cv Vivaldi					
1	*B. bassiana* (7.16%)	0.4 ± 0.2 a		93.8 ± 3.8	
2	*B. bassiana* (7.16%) + Thiamethoxam (29.9%) + Tefluthrin	1.6 ± 1.1 a		70.6 ± 22.9	
3	Fipronil (2%) + Thiamethoxam (29.9%) + Tefluthrin	0.8 ± 0.4 a		83.8 ± 9.1 a	
4	Untreated control	6.0 ± 0.7 b			
Trial 3 – Vedrana (BO), potato cvs Melba and Primura					
		Melba	**Primura**	**Melba**	**Primura**
1	*B. bassiana* (7.16%) + Thiamethoxam (29.9%)	11.5 ± 4.2 ab	10.0 ± 0.7 a	64.9 ± 2.5	54.4 ± 16.7
2	*B. bassiana* (7.16%) + Thiamethoxam (29.9%) + *B. bassiana* (7.16%)	5.3 ± 1.5 a	8.0 ± 1.4 a	71.9 ± 4.8	79.2 ± 5.9
3	Fipronil (2%) + Thiamethoxam (29.9%)	5.8 ± 3.1 a	6.0 ± 2.5 a	78.9 ± 8.7	77.2 ± 12.3
4	Chlorpyriphos (7.5%) + Thiamethoxam (29.9%)	17.3 ± 1.8 bc	19.0 ± 0.9 b	33.3 ± 3.2	31.6 ± 6.9
5	Untreated control	25.2 ± 4.1 c	28.5 ± 2.4 c		

*Different letters within the same column/trial indicate statistically significant differences ($P<0.05$)

Results and discussion

In all trials, significant differences among treatments were observed (Table 2). Under these trial conditions of low (Trial 2: 6% tuber damage in the untreated control) to medium-high pest pressure (Trial 1 and 3: 13, 25 and 29% tuber damage in the untreated control), the *B. bassiana* strain ATCC 74040-based product, when applied both alone (Trial 1 and 2) and in an IPM strategy (Trial 2 and 3), always significantly reduced potato tuber damage compared to the untreated control. Mean efficacy values ranged from 54 to 94% and were comparable to those of the clothianidin-based (mean efficacy: 79%) and fipronil-based reference strategies (mean efficacy range: 77 - 84%), and higher than those of the chlorpyriphos-based strategy (mean efficacy: below 35%). The addition of thiamethoxam, a common practice in Italian potato growing areas, did not seem to affect the activity of the fungus (Trial 2 and 3; Table 2).

Our trial results indicate that *B. bassiana* strain ATCC 74040 can be considered a valuable tool for the control of *Agriotes* spp. in both organic farming, where no insecticides for wireworm control are allowed, and in integrated pest management strategies.

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Field trials on the use of *Beauveria brongniartii* against *Melolontha* spp. white grubs in forest plantations in Poland – a case study of Kozienice

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Abstract: *Melolontha hippocastani* Fabr. and *Melolontha melolontha* L. (Coleoptera: Scarabeidae) larvae are the most noxious soil insect pests in forest plantations and nurseries in Poland. In 2007-2008 several field trials were carried out to estimate *Beauveria brongniartii* wheat colonized kernels efficacy in the reduction of damages and control of *Melolontha* spp. larvae in forest plantations and nurseries in Poland. Two of the trials described in this paper were projected as a part of a dossier to be submitted for the registration of the used product. They were located in Kozienice Forest District (a coniferous forest growing on sideric soil, pH of the soil 3.5-5.0) in pine *Pinus silvestris* L. and oak *Quercus robur* L. plantations. A defined portion of colonized kernels have been put into the planting hole and mixed with a soil. Three doses were tested: 60, 120 and 240 kg/ha. The survival rate of seedlings was calculated (after 6 months for pine and after 13 months for oak), the white grub population density was checked, white grubs collected in experimental plots were reared in the laboratory and soil samples were taken to recover *Beauveria* spp. isolates by insect baiting using *Galleria mellonella* larvae. Results suggest that depending on seedling species and white grub population density the time of *B. brongniartii* application to the forest soil should proceed one or two years the forest planting and the application should be repeated in the following years. The *B. brongniartii* strain used to the colonizing of grains is probably not well adapted to the low pH values, typical for most of the forest soils in Poland.

Key words: *Beauveria brongniartii, Melolontha melolontha, Melolontha hippocastani,* microbial control

Introduction

Melolontha hippocastani Fabr. and *Melolontha melolontha* L. (Coleoptera: Scarabeidae) larvae are the most noxious soil insect pests in forest plantations and nurseries in Poland. White grubs are able to destroy root systems of young seedlings totally and they make reforestation and afforestation impossible in some regions of the country (Stocki & Malinowski, 2000). Before 2008 five chemicals were registered for the control of white grubs in forest plantations and nurseries in Poland. Since 2008 three of them are not on the Annex 1 of Directive 91/414 list anymore, therefore their use is not allowed any longer. Additionally, in forests certified according to FSC principles and criteria (around 50% of forests in Poland) the use of any kind of chemical for soil insect control is not approved. In this situation a biological control agents represents a valuable option.

In 2007-2008 several field trials were carried out to estimate *Beauveria brongniartii* wheat colonized kernels efficacy in the reduction of damages and the control of *Melolontha* spp. larvae in forest plantations and nurseries in Poland. Two of the trials, described in this paper, were projected as a part of a dossier to be submitted for the registration of the used product.

Material and methods

Trials were carried out in Kozienice Forest District, in plantations of pine (*Pinus silvestris* L.) and oak (*Quercus robur* L.) in 2007. Plantations were established in a habitat of mixed coniferous forest growing on sideric soil, pH of the soil 3.5-5.0 (Konecka-Betley et al. 1999).

The fungus was applicated in March, when 2 years old pine seedlings and one year old oak seedlings were planted. The measured portion of colonized wheat kernels (10^9 *B. brongniartii* conidia/ g of product) has been placed into the planting hole and mixed with the soil surrounding roots. Four treatments (0, 60, 120 and 240 kg of *B. brongniartii* product/ ha equal 0, 6.5, 13 and 26 g/ seedling respectively) were done in pine and oak plantations (completely randomised block designs). One plot measured 12 x 12 m.

The mean survival rate of seedlings in pine (September 2007) and oak (May 2008) plantations was measured – all dead and all vigorous seedlings were counted on every plot (1 m belt of border ground was left). The mean percentage of alive seedlings and standard deviation were calculated for every treatment. The data were statistically analysed with an analysis of variance (ANOVA).

The white grub population density was checked: in March and September 2007 in plots with pine and in March 2007 and May 2008 in plots with oak seedlings. Before an application 8 survey pits (0.5 m^2 x 0.4 m depth) were checked in the experimental area for the white grub density. In September 2007 and May 2008 2 survey pits per plot were checked. For estimation of the population density, *Melolontha* spp. larvae were collected and reared 40 days in the laboratory (in a sterile sand with carrot slices) to estimate the level of white grub infection after 6 months due to fungus application. Mortality data for white grubs taken from treated plots were corrected with mortality data of white grubs taken from untreated plots according to the Abbott formula (Abbott, 1925).

At the same time soil samples (10 samples per variant) were taken from treated and untreated plots. *Beauveria* spp. isolates were recovered from soil samples by insect baiting using *Galleria mellonella* larvae (Zimmermann, 1986).

Results and discussion

Six months after application of *B. brongniartii* the mean survival rate of pine seedlings was very low and similar for all untreated and treated variants of the experiment (7.6% ± 6.6% to 13.0% ± 3.8%). In May 2008, there were no significant differences ($p = 0.630$) between the survival rate of oak seedlings in treated and untreated variants. However, the mean survival rates calculated for oak seedlings treated with doses of 120 and 240 kg/ha were around 15% higher than the mean survival rate for seedlings treated with a dose of 60 kg/ha or for untreated seedlings (Table 1).

Table 1. The mean survival rate of seedlings in the oak plantation Kozienice, May 2008

Dose [kg/ha]	Mean survival rate [%]	Standard deviation	Maximum [%]	Minimum [%]
240	47.5	15.3	55.9	24.8
120	46.0	31.8	86.4	10.0
60	33.1	29.4	76.9	14.0
0	30.5	28.3	30.1	2.8

The mean survival rate of oak seedlings treated with doses of 120 and 240 kg/ha were similar (46.0 and 47.5% respectively), suggesting that the lower of the two doses reached a threshold value under these specific conditions. STD values suggest higher consistence of survival rate results of oak seedlings, when 240 kg/ha had been applicated.

Just before planting and fungus application, in March 2007, a high population density of white grubs was observed in the experimental area (an average 3.4 L_2 *Melolontha* spp. larvae per survey pit). Six months after the application, the number of larvae found in plots with pine seedlings was almost twice as high in the control (6.0) as the number of larvae found in treated plots (3.8). In September 2007 only 3 larvae out of 47 found in treated plots succumbed to *B. brongniartii*. After 40 days laboratory rearing, 27.7% of the collected white grub larvae died because of *B. brongniartii* infection. In May 2008, when the white grub population density was estimated in plots with oak seedlings, a mean of 1.9 L_2 *Melolontha* spp. larvae per survey pit were found treatments with 60 kg/ha, a mean of 0.5 larvae in treatments with 120 kg/ha or 240 kg/ha and a mean of 1.8 in untreated plots. In May 2008 none of the 24 white grub larvae collected in treated plots had signs of *B. brongniartii* infection. Only 5.1% of collected white grubs died because of *B. brongniartii* infection after 40 days of laboratory rearing.

Very low level of white grubs succumbed to the *B. brongniartii* found in treated plots and despite of the 27.7% mortality of larvae observed after 40 days of laboratory rearing, the experiment suggested that the strain used in the fungus colonized wheat kernel product is not very efficient, possibly due to insufficient adaptation to the low pH conditions typical for most forest soils in Poland. The around 6 times lower mortality of laboratory reared white grubs collected in May 2008 in comparison to mortality of white grubs collected in September 2007, suggests that the fungus survival rate decreased after winter. The above observation was confirmed with the *Galleria* baiting experiments (Table 2). In September 2007 levels of *Beauveria* spp. infections ranged from 56.6% to 66.7% depending on the dose used. In May 2008 infection levels of *G. mellonella* larvae decreased to a value comparable to the one observed in untreated plots.

Table. 2. Results of *G. mellonella* baiting experiments (samples taken in oak plantation)

Date	Dose [kg/ha]	No. of *G. mellonella* larvae	% of larvae with *Beauveria* ssp. infections
03.2007.	0	389	7.5
09.2007.	0	100	8.0
09.2007.	60	99	56.6
09.2007.	120	92	64.1
09.2007.	240	99	66.7
05.2008.	0	42	6.3
05.2008.	60	60	1.6
05.2008.	120	48	12.4
05.2008.	240	57	10.0

Results suggested that depending on the seedling species and the white grub population density, *B. brongniartii* application to the forest soil should continue with repeated treatments for one or two years following plantation of the seedlings. The *B. brongniartii* strain used to the colonizing of kernels is probably not well adapted to the low pH value, typical of the most forest soils in Poland.

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Movement of the Western corn rootworm (*Diabrotica virgifera virgifera*) adults in a trial with Bt and non-Bt maize plots

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Abstract: In spring 2008 a trial with 32 plots was initiated at Borovce (near Piestany in district Trnava) in Slovakia. The trial included 8 maize hybrids randomly arranged in 4 repetitions. Three repetitions and half of the forth repetition were sown in plots with a preceding crop different than maize and the other half of the forth repletion was sown in plots with maize as preceding crop. The results indicate that high numbers of the Western Corn Rootworm (WCR) adults were found at damaged plots on July 8. After their emergence, the adults seem to not move from their site of emergence. On September 16, there were no differences between the plots damaged or not damaged by larvae. This suggested that in August and in September the WCR adults may have moved between plots. WCR-resistant maize genotypes strongly influenced the level of the WCR damage on the maize plants (there were no lodged plants on the plots with MON 88017).

Key words: *Diabrotica virgifera*, Bt-maize, movement of adults

Introduction

Despite the substantial amount of research already performed (e.g., Al-Deeb and Wilde, 2005; Nowatzki et al., 2006; Marquardt and Krupke, 2009) there are still many open questions on how Bt-maize hybrids influence the behavior of the Western Corn Rootworm (*Diabrotica virgifera virgifera*) (WCR). It seems clear that Bt-maize hybrids minimally decrease the larval development (Oyediran et al., 2007) or damage by larvae (Al-Deeb and Wilde, 2005). The aim of this study was to investigate whether Bt maize influences the movement of WCR adults during the season.

Material and methods

A field trial was initiated at Borovce, Slovakia (N 48°34.831´ E 17° 43.302´) in 2008. The trial included 8 maize hybrids (MON 89034 × MON 88017, MON 89034, MON 88017, Conventional Parental - DKC 5143, Reference 1 – CISKO, Reference 2 – FORTIUS, Reference 3 - PR36D79, Reference 4 - MERIDIEN) randomly arranged in 4 repetitions. Plot size was 33.6 m (48 rows, distance between rows 70 cm) x 30 m (Table 1). Three repetitions and half of the forth repetition were sown in plots with a preceding crop different than maize and the other half of the forth repletion was sown in plots with maize as preceding crop.

WCR density was monitored nine times between June and September 2008 using yellow sticky traps (Pherocon AM). For each monitoring time point three traps were placed per plot and the number of trapped individuals determined after 7 days. Collection dates were: June 10, June 24, July 8, July 22, July 29, August 5, August 19, September 2, and September 16.

Table 1. Plot organization, plot numbers, and maize genotypes of the field trial at Borovce, Slovakia in 2008. Grey plots show WCR-resistant maize genotypes (MON 88017). Boxes in grey indicate plots with maize as preceding crop heavily infested with WCR larvae

MON 89034 x MON 88017	**101**	**Reference 2**	**201**	**Reference 3**	**301**	**Reference 1**	**401**
Reference 3	**102**	**MON 89034**	**202**	**MON 89034 x MON 88017**	**302**	**Control**	**402**
Reference 2	**103**	**Reference 4**	**203**	**Control**	**303**	**Reference 2**	**403**
MON 88017	**104**	**MON 88017**	**204**	**MON 89034**	**304**	**MON 89034 x MON 88017**	**404**
MON 89034	**105**	**Reference 3**	**205**	**Reference 4**	**305**	**Reference 3**	**405**
Reference 4	**106**	**Control**	**206**	**Reference 2**	**306**	**MON 88017**	**406**
Control	**107**	**MON 89034 x MON 88017**	**207**	**MON 88017**	**307**	**Reference 4**	**407**
Reference 1	**108**	**Reference 1**	**208**	**Reference 1**	**308**	**MON 89034**	**408**

Results and discussion

Results indicate that high numbers of the WCR adults were found at plots where preceding crop was maize. Damage in these plots was reduced in plots cultivated with genotype MON 88017 (plots 404 and 406). In plots 404 and 406 no plants were lodged, whereas in plots 405, 407 and 408 there were 25.32 %, 57.66 %, and 68.77 % of the plants lodged, respectively.

Emergence of adult WCR was observed between June 10 and July 22. There were significant differences in adult densities between plots 405, 407 and 408 and the other plots (ANOVA, Tukey test) on July 22 (Table 2). This suggested that adults remained in the plots in which they emerged. On September 16 there were no significant differences detectable between the plots damaged or not damaged by larvae (Table 3), which indicated that during August and September adult WCR may have moved between the plots. Furthermore, this suggested that the MON 88017 genotype did not influence the distribution of the WCR adults within the trial. The results of the trial support the opinion that adult rootworms are not significantly deterred by the presence of Cry3Bb1 and ingestion of toxin does not adversely affect adult longevity (Nowatzki et al., 2006).

Table 2. Mean and standard deviation of number of WCR adults in each plots (field at Borovce, Slovakia) on July 22, 2008 caught with 3 yellow sticky traps per plot. For plot organization refer to Table 1

2,3 ± 2,1	**5,3 ± 2,5**	**5,0 ± 1,0**	**4,3 ± 4,2**
5,7 ± 4,6	**5,0 ± 3,6**	**5,0 ± 4,6**	**1,7 ± 1,5**
2,7 ± 2,3	**5,0 ± 3,5**	**3,0 ± 3,6**	**4,3 ± 4,0**
2,3 ± 0,6	**1,3 ± 1,2**	**3,0 ± 1,0**	**3,0 ± 1,0**
3,7 ± 4,6	**7,7 ± 1,5**	**6,7 ± 4,0**	**101,3 ± 61,5**
5,3 ± 0,6	**4,3 ± 2,5**	**7,3 ± 4,5**	**15,0 ± 3,5**
4,7 ± 2,3	**5,3 ± 3,5**	**8,0 ± 3,0**	**177,0 ± 63,5**
10,7 ± 2,1	**16,0 ± 7,0**	**18,7 ± 10,6**	**132,7 ± 27,7**

Table 3. Means and standard deviations of number of WCR adults detected in each plots (field at Borovce, Slovakia) on September 16, 2008 caught with 3 yellow sticky traps per plot. For the plot organization refer to Table 1

9,3 ± 5,0	**6,0 ± 1,7**	**5,0 ± 1,0**	**6,3 ± 1,5**
3,7 ± 2,5	**5,7 ± 1,2**	**6,7 ± 1,5**	**9,0 ± 3,0**
5,7 ± 3,8	**7,3 ± 3,5**	**7,3 ± 1,5**	**3,3 ± 2,1**
3,7 ± 1,5	**6,3 ± 1,5**	**15,7 ± 16,9**	**6,7 ± 2,1**
4,7 ± 0,6	**2,7 ± 1,2**	**6,0 ± 2,0**	**6,0 ± 5,2**
7,0 ± 3,5	**4,0 ± 1,0**	**7,7 ± 2,1**	**7,3 ± 4,7**
6,0 ± 1,7	**4,0 ± 2,0**	**6,7 ± 0,6**	**1,7 ± 1,5**
5,0 ± 1,0	**3,3 ± 2,3**	**4,0 ± 2,0**	**1,0 ± 1,7**

WCR-resistant maize genotypes strongly influenced the level of the WCR damage on the maize plants. There were no lodged plants at plots with MON 88017 genotype (plots 404 and 406) and there were lower numbers of WCR adults at these plots (Table 4).

Table 4. Means and standard deviation of number of WCR adults observed (3 yellow sticky trap Pherocon AM per plot) in plots with maize as preceding crop at five monitoring time points in 2008

Monitoring time point	**Growth[b] stage**	**Plot[a]**				
		404	**405**	**406**	**407**	**408**
June 24	**V10**	**3,3 ± 0,6**	**12, 7 ± 11,6**	**6,3 ± 3,2**	**16,0 ± 7,0**	**10,0 ± 2,7**
July 8	**V14-16**	**4,7 ± 2,1**	**84, 7 ± 33,3**	**12,3 ± 1,2**	**161,7 ± 54,0**	**124,3 ± 29,0**
July 22	**VT-R1**	**3,0 ± 1,0**	**101, 3 ± 61,5**	**15,0 ± 3,5**	**177,0 ± 63,5**	**132,7 ± 27,7**
July 29	**R2**	**20,0 ± 5,2**	**110, 7 ± 11,9**	**65,0 ± 13,0**	**154,0 ± 35,1**	**85,0 ± 38,4**
August 8	**R3**	**12,7 ± 4,2**	**118, 3 ± 9.0**	**36,3 ± 4,7**	**93,7 ± 22,1**	**52,7 ± 20,0**

[a] plot numbers and maize genotypes according to Table 1
[b] Ritchie et al., 2005

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Optimised protocol for wireworm rearing

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Abstract: Wireworms for bioassays are usually collected in the field. This can be quite tedious since wireworm infested fields may be difficult to be found and wireworm infestations can be difficult to predict. An optimised protocol has been developed to rear wireworms of the species *Agriotes obscurus*, *A. lineatus* and *A. sputator* in the glasshouse. Adults are collected in the field in May and transferred to plant pots in the glasshouse for egg laying at 20 to 25ºC. In January, 20 to 200 larvae large enough to be used in bioassays may be retrieved per pot. Each May the rearing is restarted by collecting adults in the field. Using this method, a sufficient number of larvae can be produced for bioassays with limited effort and without having to rely on field collections.

Key words: Wireworms, click beetles, *Agriotes*, culture, rearing

Introduction

When performing bioassays with wireworms of *Agriotes* spp., one of the basic questions is where to get these insects from. Usually the larvae are collected in the field. Collecting wireworms in the field for use in bioassays can be quite tedious, cumbersome, tricky and frustrating. Wireworms live in the soil and infestations are difficult to predict. One year there may be a heavy infestation and the next hardly any wireworms can be found in the same field. Even in infested fields, it may still be difficult to catch sufficient numbers of wireworms as their presence may be very patchy within the field. Since it takes the larvae several years to develop into adults, field populations consist of larvae at different developmental stages, while for bioassays larvae of similar age have to be used. In addition, larvae of the different *Agriotes* species are very difficult to distinguish and larvae from field collections may belong to different species. Also field collected wireworms may be infected by pathogens.

Rearing wireworms from adults may offer an alternative to wireworm collection in the field. Adults are collected in spring, brought to the glasshouse and put in plant pots where they lay eggs. Then the hatching larvae are reared up to the developmental stage required for bioassays. Rearing wireworms in the glasshouse overcomes the problems encountered when collecting wireworms in the field.

Optimised protocol

Wireworms of the species *Agriotes obscurus*, *A. lineatus* and *A. sputator* are cultured in the glasshouse at 20 to 25ºC in plant pots with a diameter of 30 cm filled with 10-15 L sterile soil rich in humus. The holes at the bottom of the pots are sealed with mesh screen so that water can go through but the wireworms stay inside the pot. Wireworms are fed by sowing a mixture of *Festuca rubra*, *F. pratensis*, *Poa pratensis* and *Lolium perenne* into the pots. Then the pots are covered with a mesh screen bag.

Collecting adult click beetles in spring represents the main work load. At the end of April plastic sheets (40 x 40 cm) are placed on bare soil on agriculture farmland (eg. between rows

of maize or beets). Adults can also be collected from natural meadows where wireworm infestations are expected. There the sheets are placed on the freshly cut grass. The sheets are then covered with fresh grass. On the following morning beetles aggregated between the grass and the plastic sheet are collected (Fig. 1).

Figure 1. Adults collected from plastic sheets in a meadow

Most click beetles are found when the weather is warm and humid. After collecting click beetles from the field, they are kept in plastic containers for 24 hours and fed with a honey/yeast (9:1) mixture (Fig. 2).

Figure 2. After collecting adults in the field, they are kept in plastic containers for 24 hours and fed with a honey/yeast mixture

Then the click beetles are transferred to the glasshouse. Per plant pot, 20-30 adults are placed into the mesh screen bag for egglaying (Fig. 3). A funnel is used to insert the adults into the mesh screen bag. Adults in the mesh screen bag are fed using the honey/yeast mixture. The mixture is smired onto the mesh screen bag and renewed once to twice weekly.

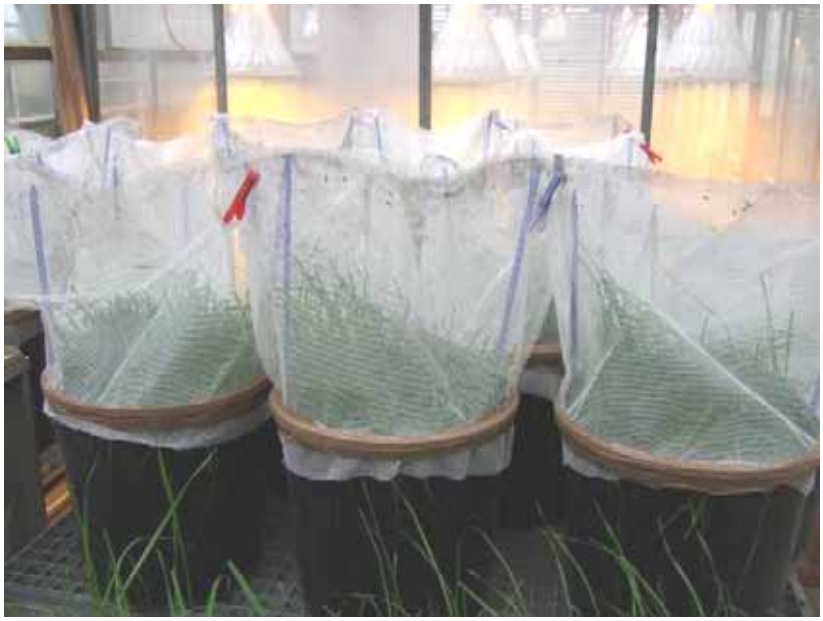

Figure 3. Plant pots covered with a mesh screen bag into which the adult click beetles are released

At the end of June the egg laying period is finished and the mesh screen bag is removed. The grassmixture is immediately resown, providing food for the hatched larvae. The young larvae like to feed on germinating seeds. As the soil needs to stay moist the plant pots are watered three times per week. By observing how well the grass grows allows to predict the development of larvae in the plant pots. If there is hardly any grass growing in the pot, there will be a large number of larvae feeding on the roots of the grasses in the pot. On the other hand if the grass is growing well, then there won't be many larvae in the pot. From the middle of August onwards, wheat or oat is used to resow the pots twice per month (Fig. 4).

Figure 4. Wireworms reared in plant pots in the glasshouse. Bare pots indicate the presence of larvae that feed on the germinating seeds. Pots covered with grass won't contain many larvae

In January the wireworms will be large enough for use in bioassays. *A. sputator* is developing faster than the other two species. The size of the larvae may need to be checked towards the end of the year and if necessary the larvae are transferred to a cool room (10ºC) to slow down the development. Some pots may have to be discarded due to wireworms infected with entomopathogenic fungi, especially when rearing susceptible species like *A. obscurus*. The number of wireworms developing per pot varies a lot. At the beginning of the year 20 to 200 individuals may be retrieved per pot.

Conclusion

By using this method, a sufficient number of larvae of a certain species and a similar age can be produced for bioassays with limited effort and without having to rely on field collections.

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New challenges for European cockchafer control in Swiss alpine valleys

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Abstract: The European cockchafer (*Melolontha melolontha* L.) is a major insect pest of alpine grasslands in several regions of Switzerland. The larvae extensively feed on roots of almost all grassy and herbaceous plants in meadows and pastures where they do repeatedly cause increased or total yield loss combined with soil erosion and infrastructural damages. In 2009, more than700 hectares of infested alpine grasslands will be treated against this pest. Since the 1980ties area wide control of cockchafers was achieved in many alpine valleys throughout Switzerland by sowing barley seed inoculated with the entomopathogenic fungus *Beauveria brongniartii* into grassland soils. In most cases, a single application has been successful to achieve a long-term control of the cockchafer population in treated areas. In recent years, increasing pest populations infested grassland areas previously not attacked by the pest and infestations repeatedly reached up to higher altitudes. Moreover, increasing numbers of larvae tend to complete their common three-year life cycle already within two-years, causing new challenges for the established biological pest control practice.

Key words: Cockchafer, entomopathogenic fungi, area wide control

Introduction

The use of the entomopathogenic fungus *Beauveria brongniartii* for the biological control of the European cockchafer, *Melolontha melolontha* L., has a long tradition in Switzerland. Since 1985 the Agroscope research station ART (formarly Swiss federal research station for agroecology and agricluture) accompagnied the treatment of about 1500 ha of grassland with Swiss isolates of *B. brongniartii* in seven cantons (regions). Fungal treatments were generally carried out in the year following the pests flight and targeted the most damaging second pest instar. Treatments were restricted to areas with high infestation levels of more than 20 larvae per m^2 (economic threshold). At lower infestation levels economic damage was usually not important and fungal treatments not necessary. Experiences of more than 20 years of *B. brongniartii* use against cockchafer in Switzerland often confirmed that one single treatment can be sufficient to establish the fungus and to control the pest for many generations up to 15 years and more in infested grasslands.

In recent years, however, strongly increasing pest populations and expanded infestation areas as well as the tendency of parts of the pest population to fullfill a shortened life cycle, initiated a re-evaluation of the current biological cockchafer control practice. With regard to planned fungal treatments in spring 2009 an infestation monitoring was carried through in two affected alpine regions in late summer 2008. These monitoring data are summarized in the present study and proposals to optimise the established control strategy are lined out.

Material and methods

Infestation monitoring

Infestation of *M. melolontha* larvae in grasslands of two Swiss alpine regions was monitored in late summer 2008 by sampling pest larvae on 0,25m^2 plots in meadows and pastures. A total of 144 samples were taken in 22 municipalities between 20 August and 4 September. The aim of the monitoring was to identify the areas with infestation levels above the economic threshold of 20 larvae per m^2 . These data were subsequently used to define the total surface to be treated with *B. brongniartii* in 2009 to achieve an area-wide pest control within the infested regions.

Results and discussion

In the canton of Grisons 21 municipalities were included and 126 sample plots were analysed. A total of 12'740 larvae were collected. The mean number of larvae per 0.25m^2 plot was 101±111. Only 19% of sample plots showed infestation levels below the economic threshold, whereas 42% of the sample plots showed increased numbers (20-99 l/m2) and 39% showed extremely high infestation levels of more than 100 larvae/m^2 and up to a maximum of 728 larvae/m2. Altogether about 600 ha were selected for *B. brongniartii* treatments in 2009.

In the canton of Glarus only one big municipality was considered and a total of 2656 larvae were collected from 18 sample plots. The mean number of larvae per 0.25m^2 plot was 148±73, which was again extremely high. In fact, all of the sampled meadows and pastures showed infestations above the threshold (up to a maximum of 320 larvae/ha) and a continuous area of 113 ha was selected for fungal treatments in 2009 in the canton of Glarus.

The additional observation of shortened larval development time and the expansion of infestation areas up to 1250 meters above sea level and higher indicated clearly that the established biological control strategy has to be re-evaluated. Both application time and technique have to be optimised for future treatments. In particular, the potential advantages of early treatments during the year of adult flight have to be investigated and compared to the common control strategy. The successful use of *B. brongniartii* treatments in grassland soils at high altitudes has still to be proven.

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Slugs and Snails

The effects of slugs on the plant communities in upland hay meadows

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Abstract: Species rich upland hay meadows are of high biodiversity importance and are internationally rare. There is increasing interest in restoring botanically diverse meadows in the uplands, but little is known about the role invertebrates may play in this process. The role of slugs, as seedling herbivores, in affecting the success of individual plants and community structure is being examined. The effect on the hemi-parasitic herb, *Rhinanthus minor* L. (Hay rattle) is of particular interest. *R. minor* is a keystone species that plays an important role in species rich grasslands by suppressing the growth of competitive grasses. A glasshouse experiment is currently underway to investigate how slug grazing affects this process in terms of parasite performance, host performance and the outcome of competition between grass, legume and forb hosts. Preliminary results will be presented and discussed.

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Slug control in iceberg lettuce heads using slug parasitic nematodes

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Abstract: Young iceberg lettuce plants (*Lactuca sativa var. capitata*) are very susceptible to slug damage, which may cause yield loss as seedlings are destroyed. During the vegetation period, slugs have a preference to hide inside the lettuce head, resulting in quality reduction due to feeding damage and faecal contamination. Just before or immediately after planting iceberg lettuce, bait pellets are frequently used to prevent this problem by controlling slugs. The use of bait pellets during the growth of iceberg lettuce is not allowed, because of potential product residue in the head. In the Netherlands in many cases the grey field slug *Deroceras reticulatum* is the responsible slug species causing these problems in iceberg lettuce. *Phasmarhabditis hermaphrodita* is a slug parasitic nematode and is already used to control the grey field slug in Brussels sprouts and green asparagus. This biological control agent was thought to be a good alternative for bait pellets in iceberg lettuce production. The nematodes can be applied during the growing season, without risk of residuals in the final product. In 2002 and 2003 several semi field trials were conducted in Lelystad, using one square meter bordered plots. The aim of the first trial was primarily to determine the application method and the potential of different dosages, assessing the capability of *P. hermaphrodita* to control *D. reticulatum*. The second trial focussed on the number of slugs to be used per plot. Based on these semi field trials it was concluded that the slug parasitic nematode, *P. hermaphrodita*, has good potential in controlling slugs.

Key words: Grey field slug, *Deroceras reticulatum*, slug parasitic nematodes, *Phasmarhabditis hermaphrodita*, iceberg lettuce (*Lactuca sativa* var. *capitata*), dose response

Introduction

Iceberg lettuce is susceptible to slugs, particular agrolimacids like *D. reticulatum* (Port and Ester, 2002). In cultivation of iceberg lettuce slug feeding can reduce yield as seedlings disappear by feeding. During crop development, iceberg lettuce heads provide suitable conditions to slugs and slug reproduction as it is moist and cool, which may result in inside damage and faecal contamination affecting product quality. Presence of slugs in the saleable heads is not accepted by the whole-sale buyer. The activity of *D. reticulatum* with corresponding plant damage is mainly restricted to heavier soil types. Due to a surplus of shelter places and well humid conditions, clay soils provide slugs a pleasant habitat throughout the field. Before planting seedlings of iceberg lettuce, bait pellets containing a molluscicide are widely used to control the slug population. In contrast, after planting bait pellets should be avoided. One slug may be good for a complaint, but the observation of a bait pellet is sufficient to reject a complete lettuce production part for sale at the auction. Tackling the slug problem during culture, Dutch iceberg lettuce growers request acceptable and effective methods controlling slug populations.

Phasmarhabditis hermaphrodita, a slug parasitic nematode (Wilson *et al*., 1993) with practical potential controlling slugs in agriculture crops (Ester and Wilson, 2005), was tested to control the grey field slug *D. reticulatum* in lettuce production. Firstly, a methodological trial was conducted primarily to determine the application method and to get an indication of

the dosages of *P. hermaphrodita* to be applied to control *D. reticulatum*. Secondly, a trial was conducted to assess the number of slugs/m². After the first data results of this trial, a density of 20 slugs per m^2 was nominated as being quite representative to regular slug presence in iceberg lettuce crops. The moments of treatment were chosen at quite practical stages of plant development, which may consequently result in a practical recommendation.

Materials and methods

General trial conditions

All trials were conducted in iron fenced boxes of one m^2 plots, on top surrounded with a copper gaze against escaping of slugs, on sandy clay soils (16 % lutum). Bottom and top were open; the boxes of 0.31 m height were pushed approximately 0.14 m into the soil. Inside each plot one wet board of 10 x 20 cm was placed as an artificial shelter for slugs. Iceberg lettuce seedlings were planted as 64 cm^3 root bolls at crop stage of four leaves. *D. reticulatum* in the adult stage was the target organism. *P. hermaphrodita* used in these trials is commercially available as Nemaslug®. Nematodes were applied with a Spraymatic 10 S knapsack sprayer, with one nozzle (TeeJet XR 8006). Plots were evenly sprayed in ten seconds with 300 ml water per m^2 (0.3 mm), except for the methodological trial (200 ml per m^2). Applications were mainly conducted late in the evening, occasionally on dark and cloudy days, to protect nematodes against detrimental sunshine. During rather dry conditions the trials were broad-cast irrigated before and after nematode treatment. Standard chemical reference bait pellets were 6.4 percent a.i. metaldehyde, broadly applied by hand in a dose of 0.0488 g a.i. m^{-2} (Luxan bait pellets Super 6.4 % pellets at 7 kg per ha formulated product). Bait pellets were always applied after irrigation. Assessments consisted of counting the number of leaves damaged by slugs and number of slugs in the heads and totals per plant (inclusively outer leaves). Heads as well as the outer leaves were soaked separately in water and leaves were disrupted and washed. Leaves were removed and the water with content was sieved. The number of slugs was counted. Random block designs consisting of four replicates were used. Data were analysed using analysis of variance (ANOVA) in GenStat. Treatments without corresponding letters differ significantly with a reliability of 95 percent.

Methodological research (trial 1, summer 2002)

The first trial using a single treatment of slug parasitic nematodes in iceberg lettuce aimed to give general information about the application method and doses in comparison to a single treatment of molluscicides used in practice in The Netherland. On the fifth of July, after introducing 40 adult slugs per plot, nematode treatments were carried out. After treatment, the lettuce was planted the same day. A treatment with 300,000 nematodes per m^2 in 200 ml water was conducted six days after planting, on 11 July. Due to large feeding damage, new plants were planted on empty places on 9 July. On 19 July all plants were removed and new plants were planted in all plots. The nematode applications were conducted with a watering can, except for one spraying treatment. In this trial next to standard bait pellet treatments with metaldehyde, a treatment with bait pellets with 1 percent a.i. ferric phosphate at the maximum dose legislated in The Netherlands of 0.05 g a.i. m^{-2} was added.

Population density (trial 2, spring 2003)

Nematodes and bait pellets were tested in the dose recommended on the label of the products. Four densities of adult slugs were introduced to the plots; 5, 10, 20 and 40 per m^2 plot. Nematodes were applied four days before planting on 25 April. Immediately after planting on 29 April bait pellets were applied. Assessments were conducted on 13 May and 17 June.

Table 1. Characteristics of the semi-field trials, 2002 – 2003

	Methodological research	Population density
Trial no.	1	2
Year	2002	2003
Period	Summer	Spring
Slug no./plot	40	5-10-20-40
Slug introduction	3-4 July	25 April
Treatment 1 nematodes	5 or 11 July	25 April
Treatment baits	5 July	29 April
Planting date	5 and 19 July	29 April

Results and discussion

Methodological research(trial 1, summer 2002)

Using 40 slugs/m² resulted in heavy plant attack on 8 July, just 3 days after planting and treatment in the summer period (Table 2). Nematode treatments were not sufficient to protect iceberg lettuce when applied at planting (moment 1). Both bait pellet treatments showed a significant decline of the percentage of consumed plants. The ferric phosphate treatment did not differ significantly from the metaldehyde treatment. On 9 July, due to 100 percent consumed plants in some plots, empty places were replanted with new plants. On 15 July, ten days after treatment, all treatments including the nematode treatments showed reliable lower percentages of plants consumed by slugs than the untreated plots. No dose response effect was observed. On 30 July, eleven days after a complete transplant on 19 July and 25 days after the applications of most treatments except one (300,000 nematodes per plot on 11 July), nearly any plant was consumed completely in all the nematode and bait treated plots. No significant difference was found in either the spraying method or the watering can method of nematode application, regarding the percentage of consumed plants by slugs. On 22 August, forty-eight days after treatment and the first planting, the iceberg lettuce plants had an average 17 leaves per plant. Although all treatments showed lower numbers of slugs per leaf compared to the untreated plots, only significant effects are shown by the two lowest doses of nematode treatments and the bait pellet treatments (Table 3). All slugs observed in the heads and plants were newly hatched offspring, with a body weight of approximately 0.04 g per individual. None of the original slugs introduced on 5 July, with an average individual weight of 0.4 g, were observed on the 22nd of August. On 12 September at harvest time, ten weeks after the first transplanting, the average slug weight was 0.2 g, some introduced slugs may have been present as some extreme bodyweights were present. Application of 300,000 nematodes per m^2, applied both by sprayer and with the watering can, and the lower doses of 150,000 and 37,500 nematodes per m^2 and also the bait pellet applications resulted in significantly lower numbers of slugs per head compared to the untreated plots. The application of 300,000 nematodes per m^2 six days after the first transplanting did not differ significantly in numbers of slugs per head from the untreated plots, neither did the lower dose of 75,000 nematodes per m^2. Counting's on 15 and 30 July showed no significant differences between the different control measures but they differed all significantly from untreated.

Population density (trial 2, spring 2003)

With increasing slug numbers from 5 to 40 slugs per plot, an increased leaf damage occurred 14 days after planting in the untreated plots (Table 4). The initial density of 40 slugs resulted

in significant more slugs in the heads than 5 or 10 slugs introduced per plot, while in the total plant 40 slugs resulted in significantly more slugs in the harvested product than the lower densities.

Table 2. Trial 1: Average percentage of completely consumed plants per plot, summer 2002. First planting date July 5. Application date at planting with exception of **

Treatment	**Dose a.i. per m^2**	**countings 8 July**		**countings 15 July*** **		**countings 30 July**** **	
Untreated	0	53.1	bc	45.3	b	21.9	b
Nematodes	300,000*	43.7	b	6.3	a	0.0	a
	300,000	64.1	c	4.7	a	0.0	a
	300,000**	-	-	-	-	0.0	a
	150,000	57.8	bc	12.5	a	0.0	a
	75,000	65.6	c	9.4	a	1.6	a
	37,500	62.5	bc	15.6	a	0.0	a
Ferric phosphate	0.05 g	4.7	a	3.1	a	0.0	a
Metaldehyde	0.0488 g	6.2	a	12.5	a	1.6	a
F-probability		< 0.001		< 0.001		< 0.001	
LSD (α = 0.05)		19.3		13.9		5.3	

* Treatment with spraying equipment, remaining treatments with watering can.
** Treatment date 11 July, 6 days after the other treatments and the first planting date on 5 July.
*** Six days after replanting the completely consumed plants on 9 July.
**** Assessment 11 days after the second planting date on 19 July.

Table 3. Trial 1: Number of slugs per leaf (22 August) and number of slugs per head (12 September), summer 2002

Treatment	**Dose a.i. per m^2**	**Number of slugs per leaf**		**Number of slugs per head**	
Untreated	0	0.33	b	3.8	b
Nematodes	300,000*	0.21	ab	1.5	a
	300,000	0.20	ab	0.6	a
	300,000**	0.27	ab	1.8	ab
	150,000	0.23	ab	1.4	a
	75,000	0.18	a	2.2	ab
	37,500	0.18	a	1.4	a
Ferric phosphate	0.05 g	0.13	a	0.4	a
Metaldehyde	0.0488 g	0.17	a	0.7	a
F-probability		0.013		0.033	
LSD (α = 0.05)		0.14		2.0	

* Treatment with spraying equipment, remaining treatments with watering can.
** Treatment date 11 July, 6 days after the other treatments and the first planting date on 5 July.

Treatment with metaldehyde 0.0488 g a.i. per m^2 bait pellets, 20 and 40 slugs per plot showed significant more leaf damage compared to the plots with 5 and 10 slugs. No differences were found between the three lower slug numbers, but 40 slugs showed significant more slugs in

the heads than the lowest density. Also in the treatment with 300,000 nematodes per plot, significant more leaf damage was observed in the plots with 40 slugs compared to the numbers of 5 and 10 slugs per plot. Nematode treatment did not show any significant difference between slug densities in numbers of slugs found per head or per plant. At all densities, nematode as well as bait pellet treatment gave significant fewer leaves damaged than untreated plots. Treatments with nematodes or bait pellets decreased numbers of slugs per head and per plant observed, only at a density of 40 slugs per plot, except the numbers of slugs inside the heads treated with bait pellets.

Table 4. Trial 2:Average percentage of damaged leaves by slugs 14 days after planting and average numbers of slugs inside the heads and totals per plant 49 days after planting, spring 2003

Treatment	**No. of introduced slugs**	**Percentage of Leaf damage**		**Number of Slugs inside head**		**Number of Slugs per plant**	
Untreated	5	34.7	d	0.6	Ab	1.2	abcd
	10	53.3	e	1.3	Abc	2.5	bcd
	20	69.7	f	1.8	Bcd	3.1	cd
	40	83.8	g	3.0	D	7.6	e
Bait pellets*	5	9.0	abc	0.4	A	0.7	ab
	10	15.9	c	0.8	Abc	1.0	abc
	20	35.5	d	0.7	Abc	1.2	abcd
	40	38.7	d	2.0	Cd	3.3	d
Nematodes*	5	2.7	a	0.1	A	0.2	a
	10	4.6	ab	0.1	A	0.3	a
	20	10.6	abc	0.5	Ab	0.9	ab
	40	15.3	c	0.4	Ab	0.8	ab
F-probability		< 0.001		0.002		< 0.001	
LSD (α = 0.05)		11.1		1.3		2.2	

* Bait pellets of metaldehyde were applied in a dose of 0.0488 g a.i. per m^2, nematodes were applied in a dose of 300,000 individuals per m^2.

The results of the first trial (Table 1) indicate the perspective of the currently recommended rate of 300,000 nematodes per square meter in iceberg lettuce. The *P. hermaphrodita* protective effect of crops against *D. reticulatum* has been shown before in sugar beet (Ester and Geelen, 1996). Additionally, the possibilities of using lower numbers of nematodes per square meter have been tested in Brussels sprouts (Ester et al., 2003). Assessment of numbers of healthy leaves was conducted 4 and 11 days after the second transplanting, these results have been published, indicating the possibility of using lower doses of nematodes per square meter (Ester et al., 2005). Applying nematodes by watering can or the applied spraying method did not differ, the latter indicating nematode tolerance to water pressure and passing small openings. Just prior to heading of the iceberg lettuce, treatments with 300,000 nematodes per m^2 and lower doses showed potential reducing slug numbers in the saleable iceberg lettuce heads. Treatment six days after planting was not sufficient. Nematodes are probably too late to prevent egg production by the slugs.The population density trial (2) shows a strong correlation between slug numbers and damage. None of the treatments can prevent damage at higher slug

numbers. In this trial nematodes perform better at higher slug numbers than the bait pellets. Both applications of nematodes and bait pellets in doses of 300,000 respectively 0.0488 g a.i. per m^2 may control slugs in a sufficient way when slug pressure is not too high. Assessment of leaf damage by slugs proved to be a more accurate parameter than counting the numbers of slugs per plant, as more significant differences are observed. Higher population densities of slugs may be better controlled by *P. hermaphrodita* then with metaldehyde baits. Slug population densities are of major importance in the choice of control measures (Wilson et al. 2004). Following growers experience, 20 to 30 slugs per m^2 seem to be quite representative for a regular slug pressure on iceberg lettuce fields in The Netherlands.In practice, many growers decide to control slugs in iceberg lettuce, after observation of slug damage. Based on these experiments it is concluded that nematodes should be applied one week before planting. This makes it important to know whether slugs are present in de chosen production field. Nematodes should be applied before planting, as the nematodes need sufficient time to parasitize the slugs.Application of *P. hermaphrodita* can be done using regular spraying techniques. Implementation of slug-parasitic nematodes within Integrated Pest Management systems in iceberg lettuce crops should be considered. *P. hermaphrodita*, commercially available as Nemaslug®, may also be used in organic farming systems. Nematode products are generally more expensive than the standard chemical pesticides (Grewal *et al*., 2005). The product costs are still one of the major thresholds using nematodes, especially in the case of large scale outdoor vegetables as iceberg lettuce. In The Netherlands costs are successfully reduced by collective orders of nematodes by farmer organisations representing a certain crop. This is e.g. done by Brussels sprouts growers . It can be concluded that the slug parasitic nematode *Phasmarhabditis hermaphrodita* is a reliable tool in the control of *Deroceras reticulatum* in iceberg lettuce production.

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Investigation into effects of sewage sludge application on populations of terrestrial molluscs

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Abstract: Sewage sludge (biosolids) is routinely applied to agricultural land as a fertilizer. It is known to contain a mixture of endocrine disrupting compounds (EDCs) and potentially toxic metals (PTM) which can perturb many different parts of an organism, including the reproductive system, the immune system, the thyroid gland, bones and behaviour. The value of molluscs as sentinel species in the marine environment is well known, and they have also been used to assess the effects of toxic metals in the terrestrial environment. However, the effects of EDCs on terrestrial molluscs are not known and they are not routinely used as bioindicator species for EDCs. A field study comprising six, randomly assigned, replicate plots (three control and three treated) was used. Sewage sludge was applied twice at year at a dose of 2.25 tonnes dry matter per hectare for two years. Application of sewage sludge reduced mollusc populations and numbers of slug eggs found. Histological and gene expression studies were done to investigate how sewage sludge influences slug biology.

Key words: Sewage sludge, *Deroceras reticulatum*, *Endocrine disruptors*, potentially toxic metals.

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Phylogeny of nematodes associated with terrestrial slugs inferred from 18S rRNA sequences

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Abstract: The present paper proposes a phylogeny of nematodes associated with terrestrial slugs using 18S rDNA sequences from nematodes isolated from slugs in the Belgium, Chile, Norway, Slovenia, UK and USA. Interpretation of these data will elucidate the relationship between these distinct taxonomic groups of nematodes and attempt to solve several unanswered questions including the number of times the acquisition of parasitism in terrestrial molluscs has occurred throughout history and the phylogenetic relationship between known groups of mollusc-parasitic nematodes.

Key words: *Agfa, Alloionema, Angiostoma, Cosmocercoides,* molluscs, nematodes, *Phasmarhabditis,* phylogeny.

Introduction

The phylum Nematoda is one of the most abundant and diverse invertebrate groups in the world (Blaxter *et al.,* 1998). Classification within this phylum can be troublesome due to lack of reliable morphological characters; however this has recently been overcome with the development of molecular phylogenetics. Advances in such molecular based techniques have allowed the increased understanding of the evolutionary history of the Nematoda. Blaxter *et al.* (1998) were the first to produce the molecular phylogeny of the phylum using nuclear ribosomal small subunit (SSU) sequences of important parasitic nematodes. Although the classification of the phylum continues to develop, there remains to be a lack of data on the phylogenetic positions of nematodes associated with terrestrial slugs. Current understanding of mollusc/nematode associations is based on reviews by Mengert (1953), Morand (1988) and Grewal *et al.* (2003) describing main taxa and biological peculiarities of nematode associated with terrestrial slugs and snails. Surveys of Germany (Mengert, 1953), France (Morand, 1988), Maine, USA (Gleich et al., 1877) and Australia (Charwat and Davies, 1999) reveal there are a total of seven families of nematodes associated with terrestrial slugs including, Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae (particularly the genus *Phasmarhabditis*). The biological and taxonomic diversity of these nematodes remain understudied. The objective of this study was to investigate the relationships between taxonomic groups of slug-parasitic nematodes, to investigate the number of times the acquisition of parasitism in terrestrial slugs has occurred and to determine the phylogenetic relationship between known groups of slug-parasitic nematodes

Material and method

Slugs were collected from sites in Belgium, Chile, Norway, Slovenia, UK and USA. All slugs were rinsed to remove surface-dwelling nematodes then dissected and examined for the presence of internal nematodes. Extracted nematodes were conserved in 70% ethanol. DNA was then extracted and PCR was conducted using a combination of 18s universal primers. Sequencing was conducted using ABI3730 sequencer.

Table 1. Species used in the analysis

Species	Host	Locality	Number of Isolates
Agfidae			
Agfa flexilis	*Limax flavus* *Deroceras reticulatum* *Arion hortensis*	UK	4
Agfa flexilis	*Limax flavus* *Limax maximus* *Limas marginatus*	USA	3
Alloionematidae			
Alloionema appendiculatum	*Arion lusitanicus*	Belgium	1
Alloionema appendiculatum	*Arion lusitanicus*	Slovenia	1
Alloionema appendiculatum	*Arion ater*	USA	6
Alloionema appendiculatum	*Arion lusitanicus*	Norway	1
Alloionema appendiculatum	*Arion flagellus*	UK	2
Angiostomatidae			
Angiostoma limacis	*Arion distinctus*	UK	5
Angiostoma dentifera	*Limax flavus* *Limax maximus* *Limas marginatus*	USA	5
Cosmocercidae			
Cosmocercoides dukae	*Deroceras panormitanum* *Arion subfuscus*	USA	2
Diplogasteridae			
Diplogaster sp.	*Deroceras reticulatum*	USA	1
Mermithidae			
Mermithid sp.	*Deroceras caruanae*	UK	1
Rhabditidae			
Curviditis sp.	*Arion ater*	USA	1
Caenorhabditis briggsae	*Laevicaulis alte* *Deroceras reticulatum*	USA	2
Phasmarhabditis hermaphrodita	*Deroceras reticulatum* *Arion ater*	UK	13
Phasmarhabditis hermaphrodita	*Arion lusitanicus*	Norway	5
Phasmarhabditis hermaphrodita	*Deroceras reticulatum*	Chile	1
Phasmarhabditis neopapillosa	*Deroceras reticulatum* *Deroceras panormitanum* *Arion ater* *Arion dsitinctus*	UK	6

Results and discussion

A total of 60 18s rRNA sequences were generated from nematodes isolated from slugs in Belgium, Chile, Norway, Slovenia, UK and USA (Table 1). Of these 60 isolates, eleven species were identified, including; *Agfa flexilis, Alloionema appendiculatum, Angiostoma dentiferan, Angiostoma limacis, Cosmocercoides dukae*, *Diplogaster* sp., *Mermithid* sp., *Curviditis* sp., *Caenorhabditis briggsae*, *Phasmarahdbitis hermaphrodita* and *Phasmarhabditis neopapillosa*. These 11 species can be classified into seven families known as Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae and Rhabditidae. These families represent four out of the five clades of Nematoda proposed by Blaxter (1998), implying multiple origins of mollusc parasitism.

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Prospects for microbial molluscicides to control slugs

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Abstract: Current agronomic practices in commercial agriculture and climatic conditions favour the growth of slug populations and there is the need for better management of these pests. Sustainable production requires greater use of biological approaches to pest control which could be used alone in protected crops or included in an integrated system to complement existing options in a broadacre setting. This paper discusses prospects for a microbial approach to slug control, focusing on the niche such a product could fill in the molluscicide market and the characteristics it would require. The need for an economic appraisal of the damage caused by slugs is highlighted along with the role of education in promoting uptake of biocontrol technology.

Key words: molluscicides**,** slugs, pests, microbial control, crop protection, economics

Introduction

Slugs cause considerable damage in all sectors of the plant growing industry. In agriculture, for example, widespread adoption of low-tillage cultivation and increases in the area of susceptible crops grown, have contributed to a swell in slug populations. Commercial nurseries and field crops have always had a low tolerance for slug damage and recent trends towards warmer winters and wetter summers have compounded the problem; indeed, following a wet 2007 summer in the UK slug numbers grew by over 50% to unprecedented levels, with estimates of 1000 slugs per square metre in certain areas (BBC, 2007). Sales of molluscicides rose by up to 40% (R. Meredith, *pers. comm.*), and some growers more than doubled bait applications.

Control on a field scale is currently dominated by chemical baits. Formulations have improved over time; however they are centred around a limited number of active ingredients and one main method of delivery (Meredith, 2003). Furthermore, baits are only effective against the surface active segment of the slug population, which may be proportionally small and hence the challenge of getting the active ingredient to its target persists (Port, GR in Redbond, 2003). Cultural modifications such as repeat cultivations with soil compaction can help, but are not universally applicable, e.g. they exclude low-tillage systems, and may not be sustainable. Biological control using the nematode *Phasmarhabditis hermaphrodita* (Schneider) is effective (Wilson *et al.*, 1994), but at the present time cost prohibits its use on a field scale. In addition, it can only be used in countries where it is known to be endemic.

There is no 'magic bullet' to keep slug populations below economic damage thresholds; an integrated approach is required and it is clear that a new strategy is needed to augment existing measures to achieve better control of slug pests as populations grow. This paper will discuss the prospects for an approach using microbes.

A market opportunity

A microbial approach to slug control is novel and would therefore represent a new entry into the biocontrol market, which is approximately 1–1.5% of the total crop protection market worldwide (Lacey *et al.*, 2001). This amounts to around $US 244 million across all sectors at 2002 prices (based on Thacker, 2002) with 2008 estimates of $US 215 million in agriculture alone (S. Lisansky, *pers. comm.*). Furthermore, the microbial pest control market is growing much more rapidly than the agrochemical market; for the 10 years to 2002 the growth rate of the latter was 1–2% per annum, in contrast to 10% per annum for microbials, with some estimates as high as 25% (Thacker, 2002).

An effective biological solution to slug pests, especially if applicable to broad-acre crops, has the potential to gain a strong foothold in a fast-expanding area of plant protection technology. It will be positively affected by pesticide use-reduction programmes and re-registration requirements applicable to certain chemicals, including metaldehyde, leading to a gradual reduction or withdrawal of their use in some crops and countries. This will open opportunities for alternatives (Lisansky, 1997; Straus and Knight, 1997).

It is worth noting that there is no single all-encompassing industry perspective as small businesses pursue different markets to those targeted by bigger companies (Harris, 1997), however increased patent protection and the emergence of leading suppliers recognised for their expertise in the biological control of pests will build stability into the market.

There is increasing consumer awareness concerning how food is produced (Tatchell, 1997) and ‘green credentials’ positively add to the reputation of both product suppliers and growers (Lacey *et al.*, 2001). Benefits to agriculture could include fewer losses at establishment by decreasing the below ground slug population prior to sowing. This in turn would reduce the need for mitigation strategies such as higher planting densities. Home-gardeners and the retail sector, whose concerns may be more lifestyle or customer driven would benefit from a greater choice of control options, less safety risks for human health and domestic animals (Lacey *et al.*, 2001) and competition acting to regulate purchase prices.

Current status of microbial mollusc control

Over 40 years ago Mead (1961) stated that “the most neglected aspect…in the entire field of malacological biology, is the study of the role of micro-organisms in molluscan symbiosis and pathology”, and this may still be true today. There has been very little work on the microbes pathogenic to, or associated with slugs.

Of the few observations reported for terrestrial molluscs, bacterial infections tend to be implicated in adult mortality, whereas fungi are cited more in the failure of eggs to hatch (e.g. Mead, 1961; Arias and Crowell, 1963; Dundee, 1977). A study by Kienlen *et al* (1996) investigated the toxicity of *Bacillus thuringiensis* Berliner against three pest species under controlled conditions, but found that none of the strains tested had any toxic effects. A research programme in New Zealand is currently underway to explore further the potential for microbes in slug control.

Ideal characteristics of a biomolluscicide

In defining the ‘ideal’ characteristics of a biomolluscicide, it is essential to acknowledge perceived and real limitations of biological control so that they can be addressed during the discovery and development process. Hence, a microbial control should be effective on more than one species. Due to its pest status worldwide, controlling *Deroceras reticulatum* (Müller)

is crucial but ideally the bait would also impact *D. panormitanum* (Lessona & Pollonera) and the *Arion hortensis* aggregate. A predictable level of control under a range of conditions will be necessary for the product to have commercial credibility and therefore promising laboratory results should be trialled in the field.

To maximise uptake, the biocontrol agent will need to be formulated such that it can be applied using existing equipment in a range of situations from home gardens to broad-acre plantings, e.g. watering cans, boom sprayers or pellet spreaders, and it should have a shelf-life of at least a year so that growers are able to buy on an annual basis, and supply and demand can be more easily anticipated.

An ideal biomolluscicide will need to work at the relatively low temperatures at which slugs are active and, preferably, be readily transmissible between individuals; consequently the propensity for some slug species to aggregate in crowded or adverse conditions could be exploited to increase the product efficacy. The primary outcome needs to be the cessation of feeding in hatched slugs, even if mortality is more protracted. It is also very important to thoroughly assess the non-target effects. Costs of manufacture clearly need to be kept to a minimum with the aim of the final retail product being price competitive with chemical control alternatives.

Challenges

In addition to the technical challenges of developing a microbial control for slugs, an area requiring concerted effort from researchers and industry alike is a sound economic appraisal of the impact of slug damage. Quantification of the scale of the problem in terms of the amount of damage and its financial impact is inherently difficult due to factors such as mistaken symptom identification, the reliance of slug activity on weather and lack of consistency in reporting schemes between countries. It is essential, however, to work towards a united estimate of the economic burden of these pests in order to highlight more tangibly the need for, and benefits of, research in this area.

Educating consumers and growers so that expectations of microbials are realistic will also be an important component in promoting the uptake of new biocontrol technology. Despite the increasing public demand for food which is sustainably produced, consumers often still place high value on blemish free groceries, which are easier to produce with chemical control (Lisansky, 1997). Similarly, many growers perceive biocontrol to be more expensive, difficult to use and less reliable than conventional methods (Straus and Knight, 1997) and a greater understanding of the differences between the two approaches in terms of both input and output needs to be communicated.

Summary

The damage caused by slugs affects all sectors of the growing industry. Current agronomic practices and climatic conditions favour the growth of slug populations and while existing controls have their strengths, they are not applicable in all situations. This, together with the drive for more sustainable production, highlights the need for a new biological approach for improved control, particularly in broad-acre crops. A microbial molluscicide could fill this niche and the advantages, and challenges, of such an option are discussed. In addition, the need for an industry wide economic appraisal is emphasised.

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The role of parasite release in invasion of the USA by European slugs

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Abstract: Previous surveys have shown that European slug species are invasive in the USA, threatening native species and damaging agricultural and horticultural crops. A possible explanation for the success of these invasive slug species is parasite release. This is a process where invading species are released from their co-evolved natural enemies during invasion into their new habitat. To test this hypothesis we collected European slugs in part of their native range (United Kingdom) and in the USA. We then compared the prevalence, distribution and species richness of their nematode parasites. All slugs were dissected and examined for the presence of nematodes. Nematodes were then fixed directly into hot ~60°C 5% formaldehyde and mounted on slides for morphological identification. Results showed that in the UK, nematodes were present at 93% of study sites and 16.4% of all slugs examined were associated with nematodes. In the USA the respective figures were 34% of sites and 5.4% of slugs. Twelve species of nematode were found in the UK, whereas only nine were found in the USA, indicating our data supports a role for parasite release during the invasion of the USA by European slugs.

Key words: *Agfa, Alloionema, Angiostoma,* Molluscs, Nematodes, Parasites, *Phasmarhabditis* pests, diseases, integrated control

Introduction

The terrestrial mollusc fauna of the USA was reviewed by Pilsbry (1948) and found that many slug species present in the USA were invasive and most were of European origin. There are many factors involved with the success of biological invasions including the enemy release hypothesis (Mack et al., 2000). This is a process where invading species are released from their co-evolved natural enemies during invasion into their new habitat, resulting in a competitive advantage over native species (Marr et al., 2008; Mitchell and Power, 2003; Torchin et al., 2003).

A well studied group of parasites associated with terrestrial molluscs are nematodes. Current knowledge of mollusc/nematode associations is based on surveys in Germany (Mengert, 1953), France (Morand, 1988), Maine, USA (Gleich et al., 1977) and Australia (Charwat and Davies, 1999). Nematodes are found to parasitize both slugs and snails, however, slugs are parasitized more frequently and by a greater diversity of nematodes than snails (Mengert ,1953). This is due to slugs inhabiting the soil thus increasing their exposure to nematodes (Mengert, 1953). There are seven nematodes families known to be associated with terrestrial slugs including; Agfidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogastridae, Mermithidae and Rhabditidae (particularly the genus *Phasmarhabditis*). These families represent four out of the five clades of Nematoda proposed by Blaxter (1998), indicating there are multiple origins of mollusc parasitism.

Hypotheses

Based on the enemy release hypothesis, we hypothesized that:

1. Nematode infection in European slug species will be more prevalent in the UK than in the USA.
2. Nematode diversity will be greater in the UK than in the USA.

Material and method

Slugs were collected from 30 sites in the UK and 70 sites in the USA. All slugs were dissected and examined for the presence of nematodes. Nematodes were fixed directly into hot ~60°C 5% formaldehyde, processed into glycerol (Seinhorst, 1959) and mounted on slides for morphological identification.

Results and discussion

Nematodes were found to be associated with slugs at 93% of UK study sites and 16.4% of all UK slugs contained nematodes. The comparable figures were much lower in the USA with nematodes present at 34% of sites and only present in 5.4% of individual slugs. In both cases chi^2 tests showed a significant association between samples site and prevalence of nematode parasites (P<0.001) (Fig. 1).

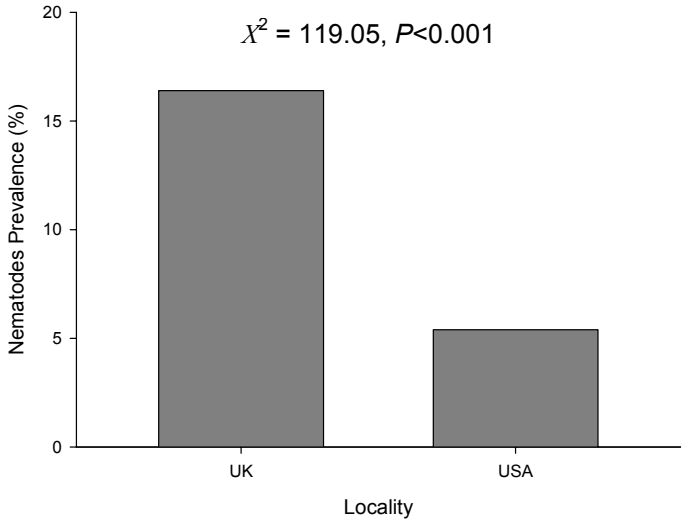

Figure 1. Nematode prevalence in terrestrial slugs collected in UK and USA.

Twelve species of nematodes were found associating with slugs in the UK compared with only nine in the USA, indicating that species richness is lower in the USA than in the UK (Table 1). If non-parasitic nematodes are excluded from these results, the difference is proportionately greater with seven parasitic species in the UK compared with four in the USA (Table 1). Furthermore, three out of the ten European slug species examined in the USA were completely free from nematodes whereas all were infected by nematodes in at least some sites in the UK (Fig 2).

The data in this paper clearly supports a role for parasite release in the success of European slugs invading the USA based on both prevalence and species richness of nematode parasites.

Table 1. Species of nematode associated with terrestrial slugs in the UK and USA

UK Nematodes	**USA Nematodes**
*Agfa flexilis**	*Agfa flexilis**
*Alloionema appendiculatum**	*Alloionema appendiculatum**
*Angiostoma limacis**	*Angiostoma dentifera**
Angiostoma sp.*	*Caenorhabditis briggsae*
Diplogaster sp.	*Cosmocercoides dukae**
mermithid sp.	*Curviditis sp.*
Pelodera sp.	*Diplogaster* sp.
*Phasmarahdbitis hermaphrodita**	*Panagrolaimis* sp.
*Phasmarhabditis neopapillosa**	Unidentified rhabditid juvenile
*Phasmarhabditis papillosa**	
Rhabditids gracilicauda	
Unidentified rhabditid juvenile	

*Thought to be true parasites rather than necromenic or phoretic associates.

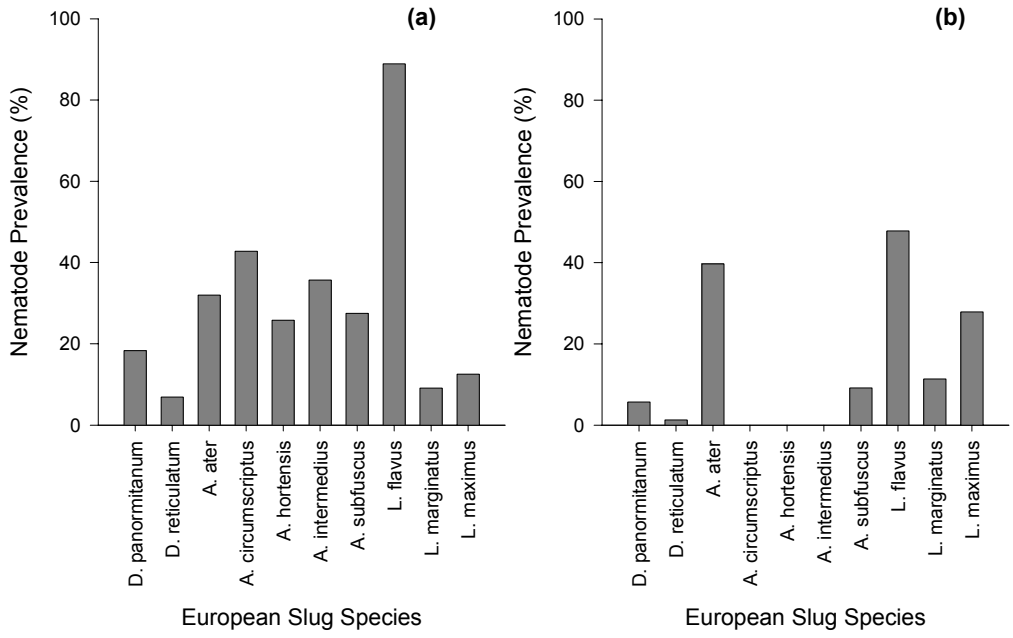

Figure 2. Nematode prevalence in ten European slug species collected in the UK (a) and the USA (b)

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Miscellaneous

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Biocontrol of *Ostrinia nubilalis* and *Sesamia nonagrioides* by *Bt* maize in South Western France: Search of biological indicators by a model-based approach for managing mycotoxin risks

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Abstract: The aim of this study was to assess the consequences of the biocontrol of two majors maize insect pests, *Ostrinia nubilalis* Hüb. and *Sesamia nonagrioides* Lef. by *Bt* maize (MON 810 event) on *Fusarium* spp. mycoflora and mycotoxins trichothecenes (DON), fumonisins B1 and B2 and zearalenone (ZEA). Field trials were conducted during summer 2005 in South Western France. *Bt* maize induced a significant reduction for both Lepidoptera, *Fusarium* spp. mycoflora and fumonisins. But difference was noted following mycotoxin families. The characterization of biological indicators by model-based approach showed that Lepidoptera and *Fusarium* spp. biocontrol could be considered to be biological indicators for the model fumonisins B_1 and B_2, although the model trichothecenes appeared to be linked only to *Fusarium* spp. Both results suggest the hypothesis of a competition of mycoflora between *Fusarium* spp. producing fumonisins and *Fusarium* spp. producing trichothecenes.

Key words: *Bt* maize**,** *Ostrinia nubilalis, Sesamia nonagrioides, Fusarium*, mycotoxins, model-based biological indicators

Introduction

Ostrinia nubilalis (Hübner) (ECB: European Corn Borer)) and *Sesamia nonagrioides* (Lefebvre) (CSB: Corn Stalk Borer) are considered to be a worsening factor for ear root infection, because their occurrences were correlated with mycotoxins in ears (Sobek and Munkvold, 1999). A previous work highlighted a link between the agrochemical control of these Lepidoptera and *Fusarium* spp. and mycotoxins (Folcher *et al*., 2009).

Because the sanitary quality is not guaranteed and crops are not commercialized when mycotoxin levels are above European Commission Regulation 1126/2007 thresholds, it is important to explore if an early detection of Lepidoptera in the field could be considered as a risk indicator for the later occurrence of mycotoxins in the crop. To verify this hypothesis, the links between the different factors, involving lepidopteran infestation and the consequences for the plant, fungi and mycotoxins should be well understood. The present work explores, with mathematical and statistical models, which of these parameters have to be taken into account as risk factors. The aim of this study was to assess, in a modeling approach, the consequences of the biocontrol of these two majors maize insect pests (ECB and CSB) by planting *Bt* maize (Mon 810) on the *Fusarium* spp. mycoflora and their associated mycotoxins, trichothecenes (DON), fumonisins B1 and B2 and zearalenone (ZEA).

Materials and methods

Field Trial

Experiments involved two varieties of corn (*Zea mays* L.): Isogenic PR33P67 (control) and its genetically modified (GM) counterpart PR33P66 (event MON 810). Experiments were conducted in 21 twin plots (>1 ha) located in the "Région Midi-Pyrénées" during the year 2005. Trials were done by the French Ministry of Agriculture through the "Services de la Protection des Végétaux (SPV)" assisted by the "Fédérations Régionales de Défense contre les Organismes Nuisibles" (FREDON). Fields were seeded over a period of 20 days beginning April 15th. The meteorological data (temperature, relative humidity and rainfalls) were recorded by local meteorological stations belonging to the National Network of the French Ministry of Agriculture. We verified that climatic data were favorable for fungi infestation. These trials were located in an area where corn was currently infested by multivoltin *O. nubilalis* and *S. nonagrioides*. During the cropping and at harvest, the number of caterpillars of the two borers was checked by dissection of 20 stalks and ears from each plot. Two 1kg samples of kernels were taken from 20 mixed corn ears collected randomly in each plot and then analyzed for *Fusarium* spp. and mycotoxins.

Fungal identification and mycotoxin analyses

Fungal species were identified by the "Laboratoire National de Protection des Végétaux – Unité de Mycologie Agricole et Forestière" (Nancy zip F-54000), according to the Official Method of French Agriculture Ministry (Ioos *et al*., 2004). Mycotoxins were analyzed by LC-MS-MS by the "Laboratoire de développement et d'analyses" (Ploufragan 22, France). The following mycotoxins were evaluated: Trichothecene (DON); fumonisins B1 and B2; zearalenone and metabolites. The protocol was according to AFNOR V03-110.

Statistical analyses

The following variables were subjected to statistical analyses:

- Sites: Twin plots location,
- TTRav: Infestation by *O. nubilalis* (ECB) and *S. nonagrioides* (CSB),
- FuB1B2 and Ln(FuB1B2+1): Fumonisin $B_1$and B_2 levels,
- TriABD and Ln(TriABD+1): DON and trichothecenes levels,
- ZEA and Ln(ZEA+1): Zearalenone and metabolites levels,
- FuProd: Percentage of infected grains by fumonisin producer *Fusarium* spp.,
- TriProd: Percentage of infected grains by trichothecene producer *Fusarium* spp.,
- Ptige and I(Ptige^2): Average number ECB per stalk and quadratic factor,
- Stige and I(Stige^2): Average number of CSB per stalk and quadratic factor,
- GALepi: Lepidoptera borers damages

The statistical unit was the couple of plots that involved analysis of paired comparison. Student *t* test (paired data) was used for normally distributed variables and non-parametric bilateral Wilcoxon signed-rank tests (paired data) for non Gaussian distribution. Differences are considered significant if $P < 0.05$. Model developments used Generalized Linear Models (GLM) (Nelder and Wedderburn, 1972) associated with Akaike information criterion (AIC) (Venable and Ripley, 2002). Wilcoxon and *t* test analysis were conducted using the software StatBox (Version 6.1) and model constructions used free R software (version 2.5.1 on-line http://www.rproject.org/, June 2007).

Results

Lepidoptera control
The control of ECB and CSB by the event MON 810 on stalks and ears was effective. No insect colonized the plant. The statistical analyses showed that all variables expressing the infestation of insects discriminated clearly the GM maize from the isogenic counterpart (Folcher *et al*., 2006).

Fusarium spp. contamination
Identification of *Fusarium* spp. were: *F. culmorum, F. graminearum, F. crookwellense, F. sambucinum, F. avenaceum, F. poae, F. sporotrichioides, F. tricinctum, F. equiseti, F. verticillioides, F. proliferatum* and still *Furarium subglutinans.* Fungi were affected by *Bt* variety: Reduction of contaminations of *F. verticillioides* and *F. proliferatum* synthetizing fumonisins ($t = 5.868$; $df = 21$; $P < 10^{-4}$) and an increase of *F. graminearum* and *F. culmorum* synthesizing trichothecenes ($t = 2.744$; $df = 21$; $P = 0.013$).

Effect on mycotoxins
We observed a significant ($Tw = 231$; $N = 21$; $P < 10^{-4}$) positive effect of MON 810 event on fumonisin levels. DON levels were significantly lower within isogenic than Bt cultivars ($Tw = 65$; $N = 21$; $P = 0.04$). For zearalenone no significant difference was observed.

Links between mycotoxin levels, Lepidopteran and Fusarium spp.
Generalized Linear Models associated with Akaike information criterion allowed to hold significant variables for the models (Table 3).

Table 3. Biological indicators according the models for fumonisins, DON and Zearalenone

Model Fumonisins	
Ln (FuB1B2+1) ~	Sites+TriProd+Ptige+I(Ptige^2)+Stige+I(Stige^2)+I(GALepi^2)
Model Trichothecenes	Ln (TriABD+1) ~ FuProd+TriProd
Model Zearalenone	Ln (ZEA+1) ~ Sites+FuProd+TriProd+I(Ptige^2)

The model fumonisin [Ln(FuB1B2+1)] involved sites, percentage of fungi producing trichothecenes, average number of *O. nubilalis* and *S. nonagrioides* larvae in stalks and associated quadratic term and also insect damages (wounds and trails). The model trichothecenes [Ln(TriABD+1)] involved the percentage of fungi that produced respectively fumonisins and trichothecenes, i.e., it is independent of corn borers density. The model zearalenone involved sites and both fungi, as well as the quadratic term associated with *O. nubilalis* in stalk.

Discussion and conclusion

Results highlighted that the *Bt* event affected significantly *Fusarium* spp. contamination in the same way as the mycotoxin levels they synthesize. The main fumonisin fungal producers *F. proliferatum* and *F. verticillioides* were reduced by the transgenic event and consequently fumonisin levels were strongly reduced in *Bt* plots. However, trichothecenes fungal producers

F. graminearum and *F culmorum* were significantly more present in these plots and DON levels were higher. Trials were all located in a polyvoltin area for ECB and CSB. The warm climate of "Region Midi-Pyrénées" exposed crops to higher pressure of lepidopteran pests and also to contamination by *Fusarium* spp. that need warmer temperature to develop (i.e., *F. proliferatum* and *F. verticillioides).* Fumonisins vs DON levels could consequently be explained by a competition involving fungal species. A similar phenomenon between *F. graminearum* and *Michrodochium nivale* was observed on wheat crop by Ioos et *al*. (2005).

As a result, we hypothesize that we are dealing with "an inversion of the mycoflora". This hypothesis takes into account the equation for the trichotechene model that only involves the fungi producing mycotoxins and not the insects. However, lepidopteran damage appeared to be implicated in the fumonisins model and consequently the frequency of wounds of a plant might give a clue for a mycotoxin risk. This model-based approach is preliminary and must be replicated to validate the explanatory variables.

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Entomopathogenic *Rickettsiella* bacteria: From phylogenomics to biology of infection

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Abstract: The application of molecular techniques to the phylogenetics of "rickettsias of insects" has triggered a recent controversy on the most appropriate taxonomic organization of the bacterial genus *Rickettsiella*. We have made use of the first whole genome sequence available from this taxon – that of the pathotype `*Rickettsiella armadillidii*´ – to establish its phylogeny beyond the 16S rRNA gene level. Employing a combination of phylogenetic reconstruction and likelihood-based significance testing over a basic core set of 211 single-copy orthologous gene (SCOG) families we demonstrate that `*R. armadillidii*´ is correctly assigned to the gamma-proteobacterial order *Legionellales*, but that its 16S rRNA-based current assignment to the family *Coxiellaceae* is not positively supported by genomic data. Consequences of these phylogenetic findings for an experimental approach to the biology of infection by *Rickettsiella* bacteria are discussed.

Key words: *Rickettsiella armadillidii,* Legionellales, Coxiellaceae, single-copy orthologous genes (SCOG), type-IV secretion system (T4SS)

Introduction

Rickettsiella bacteria are intracellular pathogens of a wide range of arthropods that typically multiply in vacuole structures within fat body cells and are frequently associated with protein crystals. As such, they are of principal interest as a possible biocontrol agent.

Due to early descriptions, "rickettsias of insects" were comprised under the genus *Rickettsiella* of the alpha-proteobacterial order *Rickettsiales* (Weiss *et al.,* 1984). At the infrageneric level, the taxon is primarily subdivided into bacterial pathotypes designated after a strain's original host. However, determination of a 16S rRNA-encoding sequence from *R. grylli* (Roux *et al.,* 1997) revealed highest homology to orthologous genes from the gamma-proteobacterial genera *Coxiella* and *Legionella.* In the sequel of this finding, the entire genus *Rickettsiella* has recently been assigned to the family Coxiellaceae within the order Legionellales (Garrity *et al.,* 2005).

At the order level, this taxonomic reorganization receives support from the determination of 16S rRNA-encoding sequences from further *Rickettsiella* pathotypes, e.g. from ticks (Kurtti *et al.*, 2002), collembola (Czarnetzki & Tebbe, 2004), aquatic isopods (Cordaux *et al.*, 2007), scarabaeids (Leclerque & Kleespies, 2008b), and dipteran insects (Leclerque & Kleespies, 2008d). However, further arthropod-associated bacteria originally described as *Rickettsiella* pathotypes (Drobne *et al.*, 1999; Radek, 2000) were removed from this taxon and reorganized instead in the candidate genus `*Rhabdochlamydia*´ of the order Chlamydiales after the respective 16S rRNA-encoding sequences had been determined (Kostanjsek *et al.,* 2004; Corsaro *et al.,* 2007). Nevertheless, monophyly of the genus *Rickettsiella* has been claimed (Cordaux *et al.*, 2007) and critically discussed (Leclerque, 2008b).

Moreover, the genome sequence of the pathotype `*Rickettsiella armadillidii*´, a pathogen of the pill-bug, *Armadillidium vulgare*, has meanwhile been established and found to contain two identical copies of a 16S rRNA gene with highest homology to orthologs from

Rickettsiella-like bacteria assigned to the order Legionellales (Leclerque & Kleespies, 2008a). In particular, the existence of multiple spatially dispersed 16S rRNA operons in the *Rickettsiella* genome makes interoperonic heterogeneity a possible explanation of the observed diverging 16S rRNA-based phylogenies of different *Rickettsiella* pathotypes. We have, therefore, extensively mined the `*R. armadillidii*´ genome data in order to create a sound basis to establish the phylogeny of *Rickettsiella* bacteria beyond the 16S rRNA level.

Material and methods

A detailed description of the relevant methodology of bioinformatic analysis can be found in Leclerque (2008a).

Results and discussion

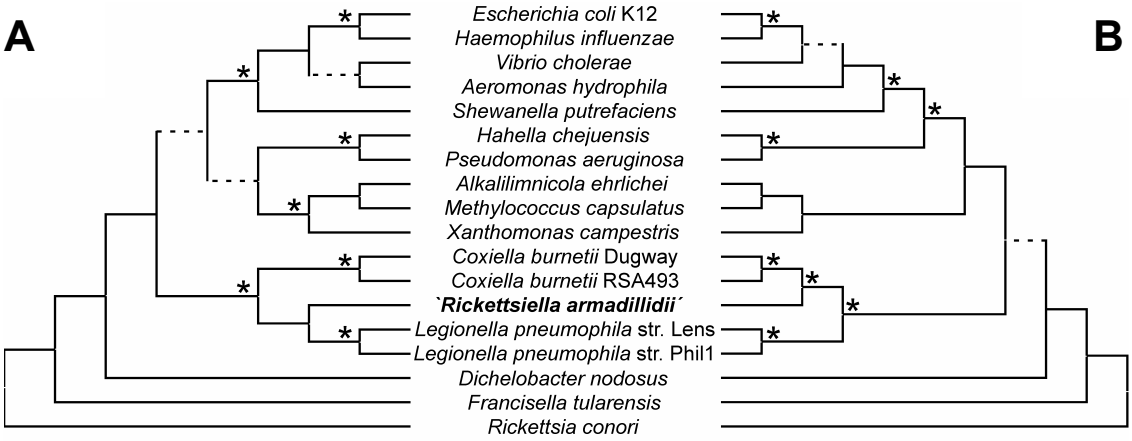

Figure 1. Extended majority rule consensus trees generated from nine hypothetical organismal phylogenies. Original phylograms were grouped according to the alternative *Legionellales* clade topologies. Both groups are represented by consensus trees A (6 phylogenies) and B (3 phylogenies). Dashed lines denote branches that are not present in all underlying topologies; the asterisk indicates optimal bootstrap support in all phylograms.

Order level classification of `Rickettsiella armadillidii´

Bioinformatic analysis of the `*R. armadillidii*´ genome sequence revealed the presence of single copies for 194 out of 211 gene families that had been identified earlier as a core set of single-copy orthologs (SCOGs) across the Gamma-proteobacteria (Ciccarelli *et al.*, 2006; Lerat *et al.*, 2003). In order to re-assess the taxonomic position of `*R. armadillidii*´, its genome sequence was compared to 16 gamma-proteobacterial and one rickettsial, i.e. alpha-proteobacterial, genomes, and 132 genes from the SCOG core set were identified across this genome space. These were further grouped into three subsets of gene families made up of 88 informational, 44 operational, and 35 universally panorthologous families, respectively. For each SCOG subset, metaprotein data sets comprising 35,772, 19,685, and 11,831 sites, respectively, were created by sequence concatenation and alignment. Subsequent phylogenetic reconstruction by the ML, ME, and NJ methods gave rise to nine different hypothetical organismal phylogenies represented in Figure 1. These nine phylogenies firstly coincide in placing the metaproteins generated from genes of `*R. armadillidii*´ in a single branch together with the sequences representing the genera *Legionella* and *Coxiella.* Optimal bootstrap support at the root of this branch makes it a consistent representation of the

presumed Legionellales clade within the Gamma-proteobacteria and thereby unambiguously corroborates the current order level classification of `*Rickettsiella armadillidii´*.

Family level classification of `Rickettsiella armadillidii´

Secondly, the nine metaprotein trees can be subdivided into two groups of topologies (Figure 1) according to the internal organization of the Legionellales clade, with the `*R. armadillidii´* sequence being located in a sister clade position either relative to the *Coxiella* (Figure 1A) or with respect to the *Legionella* clades (Figure 1B). To solve this apparent contradiction, we have used likelihood-based significance testing in a model system comprising 6 bacterial genomes: two sequences each from the genera *Coxiella* and *Legionella*, the `*R. armadillidii´* sequence, and the *E. coli* genome as an exogenously fixed outgroup. Exhaustive permutation of these genomes over two topological backbone structures generated a total of 105 possible candidate tree topologies that were evaluated for the 181 SCOG families from the basic core set identified across this genome space using an SH-test (Shimodaira and Hasegawa, 1999) at the 5% significance level. In line with expectations from the above analysis, all but candidate topologies A through C (Figure 2) were positively rejected as significantly worse descriptions of each of the 181 SCOG family-specific alignment data. Of the 181 SCOG family-specific best trees, 87 had topology 2A, 78 topology 2B, and 23 topology 2C; second best trees of topology 2A, 2B or 2C were in no case rejected. Despite the availability of sufficient information in the alignments there is, therefore, no statistically sound basis to exclude two of the three possible bifurcative topologies to the benefit of the third.

In summary, a phylogenomic approach based on both phylogentic reconstruction and significance testing corroborates the current assignment of `*Rickettsiella armadillidii´* to the order Legionellales, but at the same time contradicts its classification in the family Coxiellaceae. Further support for a hierarchically neutral taxonomic assigment of the genus *Rickettsiella* in the order Legionellales comes from existence and analysis of a set of genes encoding a type-IV secretion system in the genome of `*R. armadillidii´* and other *Rickettsiella* pathotypes (Leclerque and Kleespies, 2008c). Implications of these phylogenetic findings for the study of the biology of infection of *Rickettsiella* bacteria will be discussed.

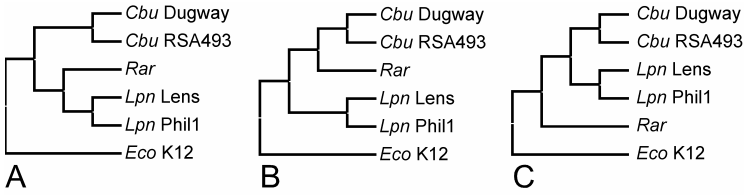

Figure 2. Legionellales clade topologies (A, B, C) that are not rejected by the SH-test. *Cbu, Coxiella burnetii; Lpn, Legionella pneumophila; Rar, Rickettsiella armadillidii; Eco, Escherichia coli.*

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Infection of *Lymantria dispar* (Lep.: Lymantriidae) larvae with the midgut pathogen *Endoreticulatus schubergi* (Microsporidia) has little influence on growth and development of the host

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Abstract: We studied the effects of infection with the microsporidium *Endoreticulatus schubergi* on growth, development and consumption of *Lymantria dispar* larvae in the laboratory. While rearing temperature (21° or 26°C) significantly affected most parameters at least in female insects, infection with *E. schubergi* had very little effects. Neither fresh mass of pupae, nor weight gained during larval stage (four and five) and food consumption was significantly altered by infection.

Key words: Development, growth, microsporidia, *Lymantria dispar*, pathogens

Introduction

Entomopathogenic microsporidia are regularly detected in *Lymantria dispar* populations in Central and Eastern Europe. These pathogens belong to the genera *Endoreticulatus*, *Nosema*, and *Vairimorpha*; they differ in site of infection and effects on the host (McManus and Solter, 2003). Microsporidia are intracellular parasites that can cause severe disease in their hosts leading to death during the larval stage or resulting in reduced larval development, growth and fecundity of adults. Some of these, such as *Vairimorpha disparis* and *Nosema lymantriae* that infect the fat body have severe effects on the host causing high larval mortality and markedly affect host physiology (Hoch and Schopf, 2001, Hoch et al., 2002, Goertz et al., 2004). Others, like *Endoreticulatus schubergi* that infects the midgut cause little larval mortality (Goertz and Hoch 2008). In this study, we measured growth, development and food consumption of larvae infected with *E. schubergi* under laboratory conditions at two temperatures to contribute to a better understanding of this host-parasite interaction at a physiological level.

Material and methods

Infection

Freshly molted 3rd instar *L. dispar* larvae were inoculated with 1 µl of *E. schubergi* spore suspension (concentration: 5000 spores/µl; isolate: Asenovgrad, Bulgaria) applied to 2-mm^3 diet cubes. Larvae were individually reared in plastic cups and fed with meridic diet at 21°C and 26°C, respectively, and 16:8 L:D photoperiod.

Growth of the larvae and food consumption

To evaluate growth, larvae were weighed starting on the 1st day post infection (dpi 1), the last day of the 4th instar (L4), the first day of 5th instar (L5), every 2nd to 3rd day in L5. Pupae and

fresh cadavers were also weighed. Growth of larvae in L4 and L5 was calculated based on fresh mass of the larva. Fresh diet was weighed and given to larvae every 3rd day. Food that was not consumed was dried for 3 days at 70°C and weighed. Food consumption was calculated based on dry mass. Therefore, the fresh mass of diet blocks provided to larvae was converted into dry mass based on test blocks weighed before and after drying. The majority of tested insects developed through five instars. Only data from these were used in the analysis. All adults were dissected at the end of the experiment and inspected for infection with *E. schubergi*. All insects that were inoculated with microsporidia but were diagnosed "not infected" were excluded from the analysis.

Data were analyzed using SPSS 15.0 for Windows. To test the effects of the treatments on larval growth and food consumption, ANCOVA (GLM procedure) was computed using infection and rearing temperature as factors and larval fresh mass on dpi 1 as covariate. Total developmental time was compared by non-parametric procedures. Sex had a dominant effect on all evaluated parameters already in the 4th instar, Therefore, we analyzed data for male and female insects separately.

Results and discussion

Infection with *E. schubergi* had only little effect on development of *L. dispar* larvae; it induced no mortality during the larval stage. Moreover, total developmental time from start of the experiment to pupation was not affected by infection. While temperature had a highly significant effect on the time of pupation – female insects at 26°C pupated on average 5.9 days earlier than at 21°C – the infection did not lead to prolonged development within each temperature regime (Kruskal-Wallis H test followed up by pairwise Mann-Whithey U tests) (Tab. 1).

Table 1. Mean (±SE) time of pupation, fresh mass of pupae, growth and consumption of uninfected (control) and *E. schubergi* infected (Endo.) *L. dispar* larvae reared at 21° and 26°C

				Fresh mass	Day of	Growth [mg FM]		Consumption [mg DM]	
			n	Pupa [mg]	Pupation [dpi]	in L4	in L5	in L4	in L5
Males	21°C	Control	7	626.2 ± 46.4	25.1 ± 0.6	187.6 ± 10.1	358.4 ± 41.5	93.6 ± 8.3	337.6 ± 46.7
		Endo.	3	654.4 ± 49.6	24.7 ± 0.3	162.0 ± 15.1	405.4 ± 36.9	76.9 ± 10.3	410.9 ± 37.1
	26°C	Control	6	757.9 ± 24.4	20.7 ± 0.2	188.1 ± 7.1	448.2 ± 20.0	82.9 ± 6.7	420.8 ± 21.8
		Endo.	3	700.4 ± 68.2	20.3 ± 0.7	188.2 ± 24.4	401.7 ± 57.5	98.1 ± 9.3	463.9 ± 9.5
Females	21°C	Control	17	1805.5 ± 57.1	29.1 ± 0.3	320.7 ± 11.5	1378.2 ± 45.4	165.7 ± 7.5	1109.5 ± 40.5
		Endo.	7	1757.4 ± 104.6	28.7 ± 0.3	305.3 ± 19.1	1343.2 ± 83.8	163.2 ± 10.4	1151.1 ± 58.3
	26°C	Control	13	2192.3 ± 57.4	22.9 ± 0.2	344.4 ± 10.9	1697.0 ± 45.5	156.2 ± 6.2	1426.8 ± 36.1
		Endo.	13	1921.1 ± 112.7	23.2 ± 0.2	327.4 ± 20.2	1447.7 ± 88.1	149.7 ± 11.3	1250.9 ± 96.4

Our analysis showed very little effects of infection by *Endoreticulatus schubergi* on growth and food consumption parameters of *L. dispar* larvae (Tab. 1). Temperature had a more pronounced effect. Fresh mass of pupae was higher at 26°C. The effects of temperature and infection were not significant in males (ANOVA: model F=1.479, P=0.258); however, numbers of male insects in our experiment were too low to allow definitive conclusions. Fresh mass of female pupae was significantly affected by temperature (F=16.312, P<0.001, η^2=0.258) but not by infection (F=3.144, P=0.083, η^2=0.063); there was no significant interaction of temperature × infection (Fig. 1a).

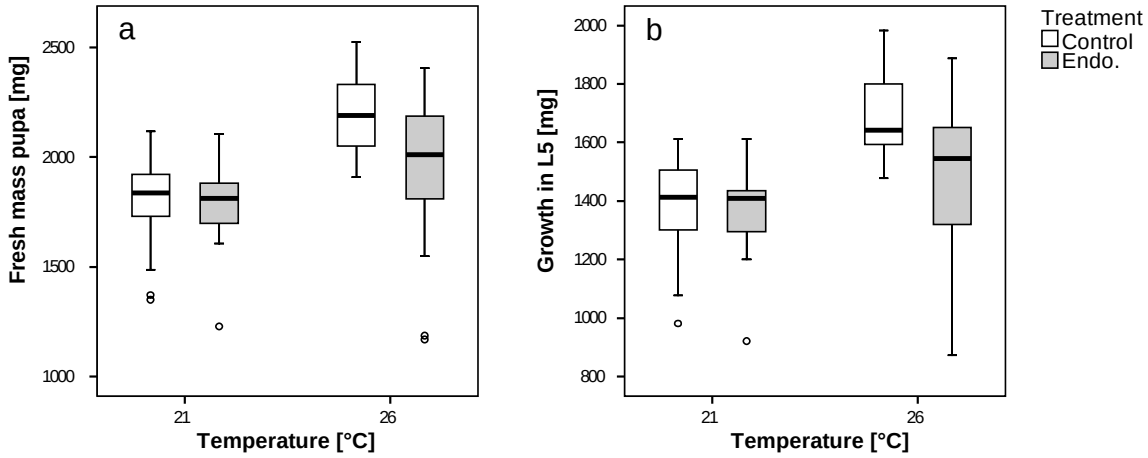

Figure 1. (a) Fresh mass of infected (Endo.) and uninfected (control) female *L. dispar* pupae and (b) growth of female fifth instars reared at 21°C and 26°C, respectively.

Growth (gain in fresh mass) in the 4th instar was not significantly affected by temperature or infection. In the 5th instar, the effects were still not significant in males (F=0.833, P=0.525). Female 5th instars, on the other hand, showed higher growth at higher temperatures (F=14.830, P<0.001, η^2=0.240). *E. schubergi* infection led to noticeable but not significant reduction in weight gained in this stadium (F=3.954, P=0.053, η^2=0.078); no significant interaction of temperature × infection occurred (Tab. 1, Fig. 1b). Food consumption in the 4th instar was neither significantly affected by infection nor by temperature. In the 5th instar, higher temperature led to increased consumption by the larvae; the effect was significant only in females (F=13.422, P=0.001, η^2=0.226). Infection did not induce significant alterations in food consumption (F=0.863, P=0.358, η^2=0.018). Again, there was no significant interaction of temperature × infection (Tab. 1).

Overall, this experiment shows that infection of the larval midgut by *E. schubergi* does not only cause little mortality in larval *L. dispar* but moreover hardly produces any effects on growth, development and food consumption of its host. Given microscopic findings show that the midgut tissue becomes completely infected with this microsporidium and the epithelium cells become filled with spores at advanced stages of the disease, it is quite remarkable that host nutrition continues to function without measurable reductions. The infection, however, is not totally without pathological effects. At a dosage of 1000 spores per larva applied to 3rd instars, 40% of the infected insects die during the pupal stage (Goertz and Hoch, 2008). Nutrient analysis in *E. schubergi* infected *L. dispar* larvae showed only slightly reduced levels of trehalose in hemolymph as well as glycogen and lipids in whole body samples. Only at the final days prior to pupation, amounts of the storage carbohydrate glycogen were significantly

lower in infected than uninfected larvae (Pilarska et al., 2007). *L. dispar* larvae are apparently able to compensate for the loss of resources to this midgut parasite. The dataset of this experiment does not suggest that increased food consumption is the primary mechanism. Infected insects may respond with enhanced conversion of food. However, before definitive conclusions can be drawn more detailed data are needed. Future experiments should bring more clarity.

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Potential of mites, fungi and microsporidia for biological control of *Pityokteines spinidens*

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Abstract: *Pityokteines spinidens* is the most abundant and most important pest species on Silver fir in Croatia. Due to a lack of knowledge about the potentials of their natural enemies, emerging beetles from incubated stem sections were examined for phoretic mites and pathogenes. A total of 8 mite species was documented as associates of *P. spinidens* of which *Ereynetes scutulis* could have some potential for biological control. The fungus *Beauveria bassiana* is a common pathogen of bark beetles and was often found in breeding galleries of *P. spinidens* (13% mortality). Furthermore, three microsporidia and one gregarinan species were observed: *Canningia spinidentis*, *Menzbieria* sp., *Chytridiopsis* cf. *typographi* and *Gregarina* sp. Data presented in this study show possible candidates for further tests to utilize pathogens as microbial control agents against fir bark beetles in the future.

Key words: Integrated control, bark beetles, silver fir

Introduction

Increased local populations of bark beetles of the genus *Pityokteines* (Coleoptera, Curculionidae, Scolytinae) have been recently noted in conifer forests in various parts of Croatia, resulting in high damage levels on Silver fir (Pernek, 2005). Among three occuring species in Croatia the most abundant and most important is *Pityokteines spinidens* Reitter. The increase in importance of *P. spinidens* as forest pest on *Abies alba* Mill. prompted research on the biology, natural enemies and on mite associates (Pernek, 2005).

Use of trap logs (Postner, 1974) is the only non-chemical pest control practice for bark beetle control. Little is known about the potential of insect pathogens as environmentally safe and effective biological control agents against bark beetles. Only a few reports have been yet published about naturally occurring pathogens in field populations of *P. spinidens*.

The ecological roles of most of the phoretic mites associated with *P. spinidens* spp. are poorly known (Pernek et al., 2008). However, based on the knowledge of other bark beetle-mite-fungal systems and previous records of the biology of these mites in the scientific literature, it is justified to assume that their biology and ecology is diverse (Moser et al., 1995; Klepzig et al., 2001) and some species could have potential for biological control of bark beetles. The purpose of this study was to investigate the species spectrum and abundance of phoretic mites and pathogenes associated with *P. spinidens*.

Material and methods

Bark beetle specimens of *P. spinidens* originated from autochthonous Silver fir stands (at two locations in Croatia: Trakoscan and Litoric). Stem sections were cut from the trunk of Silver fir trees infested by *Pityokteines* spp. The sampled material was incubated in separate rearing cages at 23°C (±2°C) and under a 16L:8D photoperiod. All bark beetles emerging from the stem sections were collected daily from the cage screens during the entire emergence period.

All emerging bark beetles were removed daily during their emergence periods, stored in separate Petri dishes with some bark pieces at 6°C until they were examined microscopically. Beetles for mite examination were randomly chosen and placed into vials with 70% ethanol. Specimens were placed in separate vials and transferred to lactophenol for clearing. Their accompanying mites were counted, plucked from their bodies and determined. Living beetles were dissected and inspected under the light microscope according to the method described in Wegensteiner et al. (1996). Beetles were decapitated and dissected in a drop of water on a microscope slide. The entire gut, part of the adipose tissue, gonads, and the abdominal segments were removed from the carcass. After all beetles had emerged, the bark was removed from three the stem sections (average height = 36cm and d = 38cm) in order to count the remaining beetles and to find beetles infected with entomopathogenic fungi.

Results and discussion

Mites associated with Pityokteines spinidens

Eight mite species were detected among the 582 mite individuals from *P. spinidens*: *Dendrolaelaps quadrisetus* Berlese, *Uroobovella ipidis* Vitzthum, *Tarsonemus minimax* Vitzthum, *Histiostoma piceae* Scheucher, *Paraleius leontonychus* Berlese, *Proctolaelaps hystricoides* Lindquist and Hunter, *Trichouropoda lamellosa* Hirschmanna and *Ereynetes scutulis* Hunter. None of the phoretic mites found, except *E. scutulis*, which may feed on small mites, eggs and immature stages of larger arthropods, have potentials to be used for biological control of *P. spinidens*. Further field and laboratory studies are required to precisely assess the feeding habits of *E. scutulis*.

Occurance of Beauveria bassiana in Pityokteines spinidens

The average mortality rate caused by the fungi *Beauveria bassiana* (Bals.) Vuill. in three stem sections *P. spinidens* was 13%. Due to the high infection rates compared with other pathogens found in this study, it can be assessed as the most important reduction factor of *P. spinidens* populations. Additional field studies need to be conducted to confirm the exact impact of *B. bassiana* on this bark beetle.

Occurrence of microsporidia and gregarines in Pityokteines spinidens

Four pathogens were diagnosed in 4298 *P. spinidens*. Trophozoits and two different types of gamonts of gregarines occurred in the midgut lumen. Pansporoblasts of the microsporidium *C.* cf. *typographi* were found in the midgut epithelium. Spores of the microsporidium *C. spinidentis* were found in the cells of the midgut epithelium, fat body, ovarioles and testes. Cysts and spores of *Menzbieria* sp. were found in the midgut of *P. spinidens.*

Future prospects

Data presented in this study show that the fungi *B. bassiana* and the microsporidia *C. spinidentis* can be considered as microbial control agents against fir bark beetles. Further testing will need to be thoroughly researched for potential use. One possibility could be the combination of pheromone traps with microbial control agents as proposed with *Ips typographus* and *B. bassiana* (Kreutz et al., 2000). Another model for *C. spinidentis* could be the pathogen *C. tomici* which has been tested for possibilities in microbial control against the pine bark beetles *Tomicus piniperda* (Linnaeus) and *Tomicus minor* (Hartig) (Wegensteiner et al., in prep.).

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Use of insect pathogens in Ukraine: Currant status and future perspectives

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Abstract: Commercial use of microbial biocontrol agents has a long and rich history in Ukraine, which is closely linked with other countries of the Former Soviet Union. The use of insect pathogens against Ukrainian pests, particularly *Cydia pomonella L.* has been practiced. Entomopathogenic nematodes have generated some interest for future use. We consider here the potentials and pitfalls to commercial production of insect Finally, we identify steps that should be taken for growing of microbiological pesticides market.

Key words: Codling moth, microbiological pesticides, entomopathogens, entomopathogenic nematodes

Introduction

Historically, insect pathogens have played a significant role in biological control in the Ukraine (i.e., Metchnikoff's experiments). Various pathogens have been used widely in biological control of pests in Ukraine until the beginning of the 1990s. Biocontrol represented 4% of the total methods used in crop protection in 1991(Stefanovska et *al*, 2005). Insect pathogens are used inundatively in many cropping systems with bacterial pathogens being the most important in Ukraine for insect suppression. At least five varieties of *Bacillus thuringiensis* are being used against lepidopteran and coleopteran species in various cropping systems and one variety is being applied against mosquito species. Entomopathogenic fungal-based products, such as *Beauveria bassiana*, *Metarhizium anispoliae*, and *Lecanicillium lecanii* have also been used in greenhouses, field crops and vegetable crops to control pests. Baculoviruses (nucleopolyhedroviruses and granuloviruses) are not extensively used because of their narrow host range and relatively slow speed of kill and the technical and economic difficulties in producing them *in vitro*. One exception is the granulovirus of the codling moth, which has been produced *in vivo* and is economical to use. After the collapse of the Soviet Union most of the research on insect pathogens was cancelled and the market of microbial biocontrol agents has declined significantly. Regardless that insect pathogens continue to be an attractive alternative to chemical pesticides, the market for microbiological control products is not growing.

Results and discussion

Codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a major pest of apple, pear, apricot pear and walnuts in Ukraine (Vasiliev *et al.*, 1998). When the use of broad-spectrum pesticides is minimized or eliminated, natural enemy populations in apple orchards increase (Shelestova *et al.*, 2001). Codling moth is commonly infected by naturally occurring entomo-pathogens. The use of microbial control in apple orchards have been intensely studied. In

particular, the granulovirus of the codling moth (CpGV) is commonly found in populations and can reduce the fecundity and longevity of the adults.

The CpGV was isolated from dead overwintering codling moth larvae collected in Russia in the 1940s. Since that time, its biology, virulence, and diversity have been studied and it has been evaluated as a biocontrol agent in apple orchards. Furthermore, the use of microbial products in apple orchards against the codling moth and other lepidopteran species, separately or in combination, with an inundate release of *Trichogramma* spp., has shown high efficacy and IPM programs have been successfully established in apple orchards (Matvievskiy, 1979).

Another insect pathogen that has received a lot of attention for codling moth control is the fungus *Beauveria bassiana*. It is one of the most common fungal species infecting codling moth in nature (Drozda, 1978). Attempts to develop *B. bassiana* as a microbial control agent of several insects have been successful. *B. bassiana* is most effective when combined with chemical pesticides (Goral and Lapa, 1979). However, its use as a microbial insecticide has been limited because of the lack of humid, warm conditions required for infection. In spite of some limitations of microbial agents, commercially products based on CpGV, *Bacillus thuringiensis* Berliner and *Beauveria bassiana* have been developed and these pathogens are registered for use in apple orchards (Fedorinchik, 1972; Goral and Lapa,1979; Tkachev,1982 and1987; Stefanovska *et al.*,1998 and 2000).

A fourth pathogen that may have use as a bioinsecticide is the entomopathogenic nematode *Steinernema carpocapsae* (Weiser). It has been recorded from natural populations of the codling moth in Ukraine (Dyadechko,1990). Data about the natural occurrence of nematodes and their biology, ecology, host range and the effectiveness of *S. carpocapsae* Agriotes strain against lepidopteran and coleopteran pests are available (Stefanovska and Shelestova, 2001).

The codling moth is used here as an example of how a fungus, a virus and a bacterium can be used for pest suppression. To further foster use of microbial control in Ukraine, commercially produced natural enemies (insect pathogens) must be readily available to farmers at reasonable costs. Previously, the majority of commercial producers of microbial control agents at affordable prices were located outside Ukraine, when it was part of the Soviet Union. Following the collapse of the Soviet Union, production of biocontrol products in the countries of the Former Soviet Union declined significantly, and it was not feasible for Ukrainian farmers to buy these products and remain profitable. In some cases, farmers are not aware of biological control agents because they are not commercially available and companies that sell chemical pesticides often are not interested in introducing biocontrol products. The number of companies interested in registration of new biocontrol agents is not increasing also because of the absence of harmonized data requirements; high registration costs and delay in registration.

Ukraine has the potential to be a major player in organic farming and export of organically grown products to Europe, where the demand for such products is high. Without biocontrol products, the organic market will not be growing.

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Pathogen and nematode occurrence in the spruce bark beetle *Ips typographus* (Col., Scolytidae) in two different region of Georgia

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Abstract: Occurrence of pathogens and nematodes in the spruce bark beetle *Ips typographus* was investigated. Beetles were collected in 2008 from two different regions of Georgia: The Shovi resort (1370 m a.s.l) of the Caucasian Mountains (Northern region) and Daba (950 m a.s.l) at the Borjomi–Bakuriani gorge (Southern region). Various pathogens and nematodes were recorded: *Gregarina typographi* (18.9-49.5%) and *Chytridiopsis typographi* (6.2% and 15%) were found in adults from both regions. *Nosema* sp. (like *Nosema typographi*) was observed in 5.3% of the beetles from the Daba district. The entomopathogenic fungus *Beauveria bassiana* (0.9%) occurred at the same place. Two species of parasitic nematodes, *Contortylenchus diplogaster* and *Bursaphelenchus* sp. were found in bark beetle populations of both regions (40.7–50%).

Key words: *Ips typographus, Gregarina typographi*; *Chytridiopsis typographi, Nosema* sp., *Beauveria bassiana, Contortylenchus diplogaster*

Introduction

The spruce bark beetle, *Ips typographus* L (Coleoptera: Scolytidae*)* is a very important pest insect of Oriental spruce (*Picea orientalis* L) in coniferous forests of Georgia. At present, it is very active and massive outbreaks are recorded in Georgia. The occurrence of epizootics of pathogens in bark beetles is one of the least studied aspects influencing their population dynamics. Recent studies brought evidence of several new pathogen species in bark beetles (Balazy, 1996; Haidler et al., 2003; Händel and Wegensteiner 2005; Wegensteiner et al., 2005; Burjanadze, 2006; Burjanadze et al., 2008; Tonka et al., 2007). The aim of our study was to investigate the occurrence of the pathogen complex in *I. typographus* in two regions of Georgia.

Material and methods

The survey was carried out in 2008 at two different regions of Georgia: The Shovi resort (1370 m a.s.l) in the Caucasian mountains (CM) (Northern region) and Daba (950 m a.s.l) at the Borjomi–Bakuriani gorge (BBG) (Southern region). Adult bark beetle were collected by cutting infested logs of spruce trees into sections and by peeling off infested bark or by directly taking them out of their galleries in the phloem. Log sections and bark with beetles were brought to the laboratory and kept at 15 ±1°C in a refrigerator**.** Beetles were dissected just after collection. Only living or slowly moving beetles were removed from their galleries every day and were examined, first for macro-organisms. Subsequently, beetles were dissected and the whole gut, gonads, fat body and other organs were removed. Diagnosis and search for pathogens was conducted on wet smears, with a light microscope (Wegensteiner and Weiser, 1996). After fixation with methanol, smears were stained with Giemsa's dye (Weiser, 1977) and re-inspected in normal light microscope. The presence of pathogens in

different organs or free in the haemolymph was recorded and the presence of nematodes was noticed. Identification of entomopathogenic fungi was according to Humber (1997). Fungi were cultivated on the PDA for 10-14 days at 25°C. Isolation of parasitic nematodes was done according to Kaya and Stock (1997) and Pavlovski (1957). Beetles were dissected in Petri dishes filled with Ringer's solution under the stereomicroscope.

Results and discussion

In spring *Picea orientalis* in the district Daba were colonized by *Ips typographus*. Reproductive adults bored holes through the outer bark, construct oviposition galleries within the inner bark and phloem of host trees. Initial entrance tunnels were surrounded by red-to grey-brown boring dust. Average number of galleries with chamber was 4-5 on 1 dcm^2. In each of the chamber we observed 3 or 4 beetles. Eggs, larvae and pupa were not observed. The pathogen complex in *Ips typographus* from different regions of Georgia is presented in Tab.1.

Table 1. Occurrence of pathogens and nematodes in *Ips typographus* and infection rates (in %) from beetles collected in Northern and Southern Georgia in 2008. (D.b= Dissected beetles; *G.t = Gregarina typographi*; *Ch.t = Chytridiopsis typographi*; N.sp = *Nosema* sp.; F=Fungus; Nem=Nematodes; n=number)

#	Sites	D.b (n)	Infected		*G.t*		*Ch.t*		N.sp		F.		Nem	
			n	%	n	%	n	%	n	%	n	%	n	%
1	Shovi (CM)	196	116	59.2	37	18.9	12	6.2	-	-	-	-	98	50
2	Daba (BBG)	113	82	72.5	56	49.5	17	15	6	5.3	1	0.9	46	40.7

In total, 309 beetles were dissected individually, from two sampling plots (Shovi = 196, Daba = 113). Different microorganisms and nematodes were found in *I. typographus*. The protozoan species *Gregarina typographi* (Sporozoa, Gregarinidae) was described by Fuchs (1915) as a parasite of *Ips typographus* and it was found in the mid-gut lumen (size of gamontes: 50–75µm x 70–130 µm) of adult beetles from both regions. Infection was detected in 18.9% (CM) and 49.5% (BBG). The pathogen was present in both genders. *G. typographi* was very common in *I. typographus* populations at high prevalence (Wegensteiner, 1994 and 2004; Händel et al., 2005; Kereselidze and Wegensteiner., 2007; Takov et al., 2007; Tonka, 2007). According to Wegensteiner & Weiser (2004), the pathogen was present in both genders. However, gregarines are presumed to be less virulent pathogens (Tanada and Kaya, 1993), which do evidently not interrupt migration of beetles.

In light microscopical observations of fresh smears of *I. typographus*, the microsporidium *Chytridiopsis typographi* Weiser (1954) could be identified in the cells of the midgut epithelium (Spores 1.5–2.0 x 2.0–2.5 µm; Pansporoblasts (thick walled): 10–20 µm in diameter). This pathogen was found in beetles from both regions. Infection rates were lower at both sites (6.2% and 15%). The occurrence of *Ch. typographi* in population of *I. typographus* is known from former investigations (Burjanadze et al., 2008; Händel et al., 2005;Wegensteiner 1994; Takov et al., 2007).

Nosema sp. (similar to *Nosema typographi*) could be observed in some few cases in beetles from Daba (BBG). The pathogen with single binucleate spores in the fat body was found. The infection rate was 5.3%. Weiser (1955) described *Nosema typographi* by means of light microscopy from the adipose tissue of *Ips typograhus* with low infection rates.

In the district of Daba (BBG) a single record (0.9%) of the entomopathogenic fungus *Beauveria bassiana* occurred. Fungal pathogens associated with *Ips typographus* populations are well-known at present (Balazy, 1996; Wegensteiner et al., 1996).

I. typographus was associated with two species of nematodes: *Contortylenchus diplogaster* and *Bursaphelenchus* sp. They were found in the gut, on the surface of the body and under the elytra of *I. typographus*. Infection with nematodes was 50% in Shovi (CM) and 40.7% in Daba (BBG). Nematodes were also found free in the haemolymph and in the gut lumen. In case of presence of nematodes it was possible to identify *C. diplogaster* in the haemolymph, 2-3 individuals in one bark beetle. This species was already described from *I. typographus* from Georgia (Kakuliya, 1989, Burjanadze et al., 2006 and 2008). *Bursaphelenchus* sp. is known as a nematode parasite found under elytra. Under each elytra >50 individuals were sampled and 70-90% of the bark beetle were infested. This is a facultative ectoparasite (Kakulia, 1989).

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Microbial antagonists of the codling moth, *Cydia pomonella* L., diagnosed from 1955 to 2008

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Abstract: At the Institute for Biological Control in Darmstadt, Germany, more than 20,000 specimens of the codling moth, *Cydia pomonella* L., were diagnosed for insect pathogens from 1955 to 2008. Altogether, 87 accessions were received for diagnosis, mostly from Germany (79), but also from Switzerland (4), Austria (2), Italy (1), and Israel (1). Fifty accessions (57.5%) with larvae and with adults of field catches by light and pheromone traps revealed high infection rates up to 70% by the microsporidium *Nosema carpocapsae*, causing a chronic disease process. In 21 accessions (24.1%), the fungus *Beauveria bassiana* was identified most frequently on old and diapausing larvae and on pupae, but also *Isaria farinosa* (syn. *Paecilomyces farinosus*), *Hirsutella* spp., *Lecanicillium* spp., *Isaria fumosorosea* (syn. *Paecilomyces fumosoroseus*), *Metarhizium anisopliae*, as well as different fungi assigned to saprophytes were found. The *C. pomonella* granulovirus (CpGV) was identified in eight accessions (9.2%) of larvae and pupae of rearings or pertinent field experiments. Several bacteria, mostly undetermined, were also diagnosed. The results and possible contributions of the most important pathogens to natural control of codling moth populations are briefly discussed.

Key words: Codling moth, *Cydia pomonella*, insect pathogens, granulovirus, fungi, *Beauveria bassiana*, microsporidia, *Nosema carpocapsae*, diagnosis, natural antagonists

Introduction

The codling moth, *Cydia pomonella* L., is a key pest of apple that occurs worldwide and needs regular control. Generally, chemical insecticides are applied, but also the codling moth granulovirus (CpGV) is used as a biological control agent. Regular applications of chemical insecticides often cause resistance problems, yet recently also reduced susceptibility of *C. pomonella* against CpGV was recognized (e.g., Asser-Kaiser *et al.*, 2007). This stimulated discussion on the use of further insect pathogens and some of them were already tested. So far, only few ecological investigations on natural microbial antagonists and their impact on codling moth populations have been published (e.g. Huger, 1976; Subinprasert, 1987; Zimmermann & Weiser, 1991; Cross *et al*., 1999). This presentation gives an overview on the results of our diagnostic studies of more than 20,000 specimens of *C. pomonella* examined from 1955 to 2008.

Material and methods

For diagnostic investigations of the codling moth, different microbial and histopathological methods and techniques have been used together with light and electron microscopy as described in many papers and books, e.g., Huger (1970; 1974); Romeis (1989); Lacey (1997). For determination of fungi and adaptation of their nomenclature, recent publications, as for instance by Humber (1997, 2007), proved useful.

Results and discussion

In 87 accessions of larvae, pupae, and adults of the codling moth received between 1955 and 2008 (Table 1), the most frequently found pathogens were: 1. The microsporidium *Nosema carpocapsae*; 2. the fungus *Beauveria bassiana*, but also *Isaria farinosa* (syn. *Paecilomyces farinosus*), *Hirsutella* spp., *Lecanicillium* spp., *Isaria fumosorosea* (syn. *Paecilomyces fumosoroseus*), *Metarhizium anisopliae*, as well as different fungi assigned to saprophytes; 3. the *C. pomonella* granulovirus (CpGV); and 4. several mostly undetermined bacteria.

Microsporidia (now assigned to Fungi)
In 57.5% (n=50) of all accessions, the microsporidium *N. carpocapsae* was the most frequently detected pathogen of adults caught in light and pheromone traps. Infection rates up to 70% (mean 20 – 50%) were diagnosed in adult catches from different states of Germany (Hessen, Baden-Württemberg, Bayern) (Huger, 1978). This microsporidium causes a chronic disease and is transovarially transmitted to the offspring. In studies with infected adults, a reduction of fecundity and fertility up to 56% was observed (Huger, 1978).

Fungi
The fungus *B. bassiana* was mostly found in 21 accessions (24.1%) from field collected old larvae in autumn and diapausing larvae or pupae in spring. Infection rates were between 0.9% and 100% (mean 32%). Also mixed infections with *I. farinosa*, *Hirsutella* sp. and other fungi occurred (0.3% – 25%). In few cases, *B. bassiana* and *I. farinosa* were hyperparasitized by the fungus *Syspastospora parasitica*.

Viruses
The CpGV was diagnosed in eight accessions (9.2%) of larvae and pupae from rearings or field experiments with CpGV. The infection rates were between 3.7% – 100%. Natural occurrence of the virus without relation to CpGV field applications could not be ascertained.

Bacteria
In many cases vegetative and spore-forming, undetermined bacteria were diagnosed, while members of the genus *Serratia* were identified a few times. As bacteria are belonging to the gut flora, their role as antagonists is often unclear.

Conclusions
Natural infections by the microsporidium *N. carpocapsae* in adults of *C. pomonella* are widely spread in different states of Germany. Mean infection rates ranged from 20% – 50%. As laboratory studies with infected adults disclosed that fecundity and fertility is reduced up to 56% (Huger, 1978), it is concluded that the diagnosed natural infection rates by *N. carpocapsae* may nicely contribute in decreasing codling moth populations. Therefore, it is suggested to introduce *N. carpocapsae* as an additional microbial antagonist into non-infected codling moth populations in orchards.

Insect pathogenic fungi, especially *B. bassiana* and *I. farinosa*, were the most important pathogens of old and diapausing larvae and of pupae of *C. pomonella*. These fungi were the most important limiting factors in hibernating populations, and primarily *B. bassiana* seems to be permanently existent in orchards. Systematic investigations on the effect of fungi as natural mortality factors are missing in Germany. Applications of *B. bassiana* against old larvae in autumn could also contribute to decrease codling moth populations. CpGV is the only pathogen registered and successfully used as biocontrol agent against *C. pomonella* (e.g., Kienzle *et al.*, 2006). But recent findings of reduced susceptibility or even resistance of the

codling moth against this virus point out the necessity to search for new effective strains of CpGV and to develop further alternative control measures.

The results demonstrate that our knowledge on the ecology and efficacy of microbial antagonists in codling moth populations still is rather poor.

Table 1. Overview on pathogens/micro-organisms of the codling moth, *Cydia pomonella*, diagnosed at the Institute for Biological Control, Darmstadt, from 1955 to 2008 (adapted from Kleespies *et al*., 2008); DE – Germany, AT – Austria, CH – Switzerland.

Pathogens/Microorganisms	**Countries of Origin**
Viruses	
Granulovirus	CH, DE
Granulovirus + *Nosema carpocapsae*	CH, DE
Bacteria	
Bacteria, indet.	DE
Bacteria (spore-formers), indet.	DE
Serratia liquefaciens	DE
Serratia sp.	DE
Hafnia alvei + *Serratia* sp. (?) + *Pseudomonas* sp. (?)	DE
Serratia sp. (?) + *Nosema carpocapsae*	DE
Bacillus cereus (?) + nematodes, indet.	DE
Fungi	
Alternaria sp.	DE
Aspergillus flavus	DE
Aspergillus sp.	AT, DE
Beauveria bassiana	AT, DE
Beauveria bassiana, hyperparasitized by *Syspastospora parasitica* (*Melanospora parasitica*)	DE
Beauveria sp.	AT
Cephalosporium sp.	AT, DE
Cladosporium sp.	AT
Fungi, indet.	AT, DE
Fusarium avenaceum	AT
Fusarium sp.	AT, DE
Hirsutella gigantea	AT
Hirsutella sp.	AT, CH, DE
Hirsutella subulata	AT
Isaria farinosa (*Paecilomyces farinosus*)	AT, DE
Isaria farinosa (*Paecilomyces farinosus*) hyperparasitized by *Syspastospora parasitica* (*Melanospora parasitica*)	AT, DE
Isaria fumosorosea (*Paecilomyces fumosoroseus*)	DE
Lecanicillium sp. (*Verticillium lecanii*)	DE
Metarhizium anisopliae	AT
Mucor sp.	AT, DE
Penicillium sp.	AT, DE
Verticillium sp.	DE
Beauveria bassiana + bacteria, indet.	AT
Alternaria sp. + *Cephalosporium* sp.	DE
Alternaria sp. + *Fusarium* sp.	DE
Aspergillus sp. + *Fusarium* sp.	AT, DE
Aspergillus sp. + *Mucor* sp.	AT

Pathogens/Microorganisms	**Countries of Origin**
Fungi (continued)	
Beauveria bassiana + *Aspergillus* sp.	AT
Beauveria bassiana + *Cephalosporium* sp.	DE
Beauveria bassiana + *Cladosporium* sp.	AT
Beauveria bassiana + *Hirsutella* sp.	DE
Beauveria bassiana + *Mucor* sp.	AT
Beauveria bassiana + *Isaria farinosa* (*Paecilomyces farinosus*)	DE
Beauveria bassiana + *Penicillium* sp.	DE
Hirsutella sp. + *Aspergillus* sp.	AT
Hirsutella sp. + *Mucor* sp.	AT
Isaria farinosa (*Paecilomyces farinosus*) + *Alternaria* sp.	DE
Isaria farinosa (*Paecilomyces farinosus*) + *Fusarium* sp.	DE
Isaria farinosa (*Paecilomyces farinosus*) + *Penicillium* sp.	AT
Paecilomyces sp. + *Alternaria* sp.	DE
Paecilomyces sp. + *Beauveria* sp.	DE
Paecilomyces sp. + *Mucor* sp.	DE
Paecilomyces sp. + *Verticillium* sp.	DE
Penicillium sp. + *Alternaria* sp.	DE
Penicillium sp. + *Aspergillus* sp.	DE
Penicillium sp. + *Cephalosporium* sp.	DE
Penicillium sp. + *Mucor* sp.	AT, DE
Verticillium sp. + *Alternaria* sp.	DE
Verticillium sp. + *Penicillium* sp.	DE
Fungi, indet. + *Nosema carpocapsae*	DE
Aspergillus sp. + *Fusarium* sp. + *Penicillium* sp.	AT
Beauveria bassiana + *Aspergillus* sp. + *Hirsutella* sp.	AT
Beauveria bassiana + *Mucor* sp. + *Penicillium* sp.	AT
Beauveria bassiana + *Penicillium* sp. + *Aspergillus* sp.	AT
Isaria farinosa (*Paecilomyces farinosus*) + *Beauveria bassiana* + *Penicillium* sp.	AT
Isaria farinosa (*Paecilomyces farinosus*) + nematodes, indet.	DE
Microsporidia (now assigned to Fungi)	
Nosema carpocapsae	CH, DE
Microsporidia, indet. + bacteria, indet.	CH
Nosema carpocapsae + nematodes, indet.	DE
Nematodes	
Nematodes, indet.	DE

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Fungi, microsporidia and nematodes in *Gastrophysa viridula* (Col., Chrysomelidae) from Eastern and Central Europe

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Abstract: As a result of a comparative study on the occurrence of pathogens and parasites on the beetle *Gastrophysa viridula* in Austria and Poland in 2007 and 2008, an entomophthoralean fungus from the genus *Zoophthora* was found on adults and larvae as well, but only from locations in Poland. Microsporidia (*Nosema* sp.) were found, in a relatively high proportion of beetles from all Polish sites, but only in some few individuals from one Austrian site. Nematode larvae (Mermithidae) were found in beetles from some Austrian sites only.

Key words: Chrysomelidae, entomopathogenic fungi, microsporidia, nematodes, natural occurrence

Introduction

The Green Dock Leaf Beetle, *Gastrophysa viridula* DeGeer is an oligophagous insect on plants of the genus *Rumex* or *Rheum*. It feeds mainly on broad-leaved dock, *Rumex obtusifolius* L. These plants are weeds in meadows in Austria and Poland and *G. gastrophysa* has therefore been considered as a possible biological control agent (Hann and Kromp, 2003; Piesik, 2000). In contrast, garden dock (*Rumex rugosus* Campd.) is used in cooking in Poland and in therefore *G. viridula* is rated mainly as a pest.

Whatever the status of the beetle, few pathogens have been found on *G. viridula*, mainly adults: One eugregarinid species, *Gregarina* sp. (Wegensteiner *et al*., 2008), two microsporidian species, *Nosema equestris* and *Nosema gastroidea* (Hostounsky and Weiser, 1980, Toguebaye *et al*., 1988), and two entomophthoralean species, *Entomophthora sphaerosperma* Fres. (Judenko, 1943), and one species of the genus *Zoophthora* (Tkaczuk *et al*., 2005). As preliminary data from collections made in 2006 in Austria and in Poland brought evidence of differences in pathogen or parasitic species of *G. viridula* populations (Wegensteiner *et al*., 2008), occurrence of pathogens and parasites was studied at several localities in these two countries the two following years.

Material and methods

Observations were made in several localities of Austria (Lower Austria, Styria and Upper Austria) and Western and Eastern Poland in 2007 and 2008 (Figure 1). First of all, larvae and adult beetles (starting from the ones of the second generation) were observed *in situ* for the presence of possible fungus-infected individuals. *G. viridula* specimens were counted on 20 infested leaves randomly chosen throughout the *Rumex* stand. The number of living and fungus-killed larvae and beetles was recorded and infection rate due to the fungal infection was calculated. A sample of the infected specimens was taken to the laboratory to determine

the fungal species. Further, living adult beetles were collected in both years and dissected on microscopical slides. Fresh smears were inspected, dry and methanol fixed smears were re-inspected after staining with Giemsa's dye

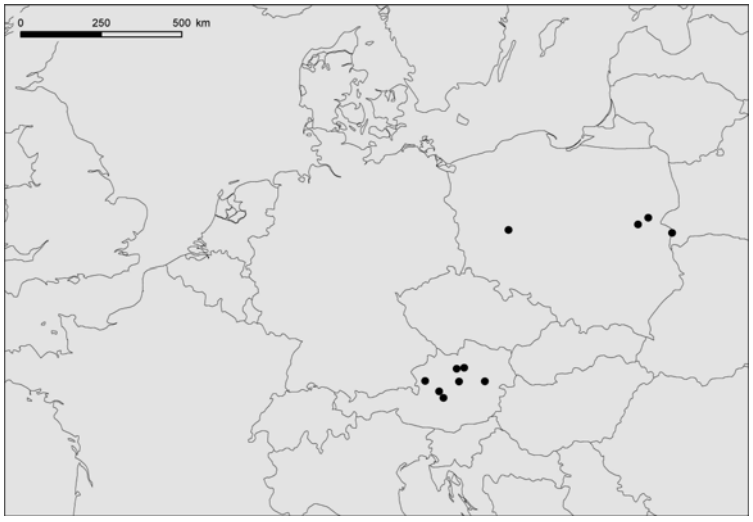

Figure 1: *G. viridula* sampling sites (black dots) in Austria and Poland, 2007 and 2008

Results and discussion

In our study an Entomophthoralean fungus species from the genus *Zoophthora* was found on adults and larvae of *G. viridula* both in 2007 and 2008, but only in Poland. At the beginning of June and July 2007 and 2008 numerous larvae and adults of *G. viridula* infected by *Zoophthora* sp. were found on leaves of many *Rumex* plants growing among the meadows near Bug river (Drohiczyn and Kostomłoty localities) and Liwiec river in Chodów locality. The fungus caused a mortality of 12.4-37.8% in the second generation of host populations (Table 1), but continuous development of the pathogen was observed on successive host generations during the vegetative season. The first infected specimens were usually found at the beginning of June, but in 2008 the first case of mycosis of a single beetle in Chodów locality exceptionally occurred at 28th of April. The last cases of mycosis caused by *Zoophthora* sp. occurred in the end of September. These data confirmed previous information about occurrence of a similar (most probably the same) species of the genus *Zoophthora* in *G. viridula* populations in Poland in the years 2004 and 2006 (Tkaczuk *et al*., 2005; Wegensteiner *et al*., 2008). The fungal disease was observed in populations of *G. viridula* in Eastern Poland only. In the two inspected localities of Western Poland (Turew and Winna Góra) and in all Austria localities no specimens infected by *Zoophthora* sp. were found, in 2007 and in 2008. Dissection of living beetles led to observe the presence of a pathogen and a parasite (Table 2). In some cases (e.g. Schönau and Klostertal in 2007 and Donnersbachwald, Bad Mitterndorf and Mondsee in 2008) only very low numbers of beetles have been dissected, which could give just an indication about the possible presence of natural enemies. Microsporidia (*Nosema* sp.) were recorded, in relatively high numbers of beetles from all Polish sites and only in some few individuals from one Austrian site in 2007. For the Drohiczyn locality, results are consistent with the observations made in 2006 (Wegensteiner *et al*., 2008). In 2008, *Nosema* sp. was found only in beetles from Poland (one location), in a similar proportion as in 2007 (Table 2).

Table 1: Mortality (in %) caused by *Zoophthora* sp. in the second generation of *G. viridula* in different locations in Poland in 2007 and 2008. N = Number of inspected beetles.

Location	Year	N	% of infected specimens
Drohiczyn	2007	164	25.4
	2008	129	18,0
Chodów	2007	210	37.8
	2008	182	12.4
Kostomłoty	2008	124	20,7

Table 2: Infection with *Nosema* sp. and parasitisation by Mermithidae (in %) in *G. viridula* from different localities (1 and 2 = different places at the location) in Austria and Poland in 2007 (a and b = different sampling dates) and 2008. N = numbers of dissected beetles.

Location	Country	Year	N	*Nosema* sp.	Mermithidae
Drohiczyn a	PL	2007	102	44.1	-
Drohiczyn b	PL		109	43.1	-
Chodów	PL		187	29.9	-
Turew	PL		19	5.3	-
Hohenlehen 1	A		80	-	1.3
Hohenlehen 2a	A		79	-	7.6
Hohenlehen 2b	A		87	2.3	33.3
Nöchling	A		72	-	1.4
Schönau	A		9	-	-
Klostertal	A		8	-	-
Chodów	PL	2008	92	21.7	-
Hohenlehen 1	A		25	-	-
Donnersbachwald	A		2	-	-
Bad Mitterndorf	A		3	-	-
Mondsee	A		5	-	20.0

In 2007 and 2008, larvae of Mermithidae were found in several *G. viridula* specimens from Austrian sites, where these parasites have been found in 2006 already. No Mermithidae were observed in Polish localities in 2007 and 2008, but in 2006 (Wegensteiner *et al*., 2008).

It should be noticed that no G*regarina* sp. (Apicomplexa, Eugregarinida) were found in beetles from any of the Austrian and Polish sampling sites both in 2007 and 2008, in contrast to the record 2006 in one of the Austrian locations (Hohenlehen) (Wegensteiner *et al*., 2008).

Different pathogens and a parasite occurred in *G. viridula* populations in Austria and Poland but situations differed according to locations. An Entomophthorale of the genus *Zoophthora* was found from Polish locations in relatively high abundance, with some variation within the year and between years however, but the pathogen was constantly present. In contrast, the fungus was not recorded in Austria yet. The microsporidium, *Nosema* sp., is constantly present in populations in Poland, even in relatively high prevalence, but only exceptionally in beetles from Austrian locations. In addition, Mermithidae are more abundant

in beetles from some Austrian sites compared to the situations observed from Poland. Further ecological studies are needed to understand such differences, given climatic conditions are likely to play a role.

In practice, when establishing a *G. viridula* mass production it will be necessary to select healthy beetles from a location without any infection with *Zoophthora* sp. or *Nosema* sp. and also to avoid Mermithidae. On the other hand, augmentation of *Zoophthora* sp. and *Nosema* sp. infections may be an effective tool to reduce *G. viridula* populations in the field.

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Genetic engineering of baculoviruses for biocontrol: Dead end or bright future

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Abstract: Baculoviruses are insect pathogens that have long been considered as ideal biological control agents. They are natural agents and they often play a role in the ecology of the host insect by controlling the size of insect populations. Baculoviruses often cause fatal disease and produce large amounts of progeny virus. They can be used to control insect pests by inoculative and inundative (spraying) technologies and in a variety of agro-ecological settings, such in arable and fiber crops and in forestry. Baculoviruses are mainly isolated from lepidopteran insects, although they do occur in hymenopteran and dipteran insects. These viruses are specific for one or a few related insect species and are therefore considered environmentally safe. They are compatible with chemical and integrated control strategies. A further advantage of these viruses is that the infectivity is retained in large cuboidal to granular proteinaceaous structures, called occlusion bodies, which give the virus protection against decay and allow them to be stored for years until use. A bonus is that baculoviruses can easily be produced in insect larvae and therefore manufactured on site, on the farm. Quite a few baculoviruses are available on the market, either commercially or provided through governmental agencies. The most successful ones are baculoviruses against cotton bollworm in Asia (China), against soybean looper in South America (Brazil) and against codling moth in Europe.

The major downside in the use of baculoviruses has been the relatively slow speed of action. It takes a few days before the insect dies and that may be too long to protect against damage. In addition to finding faster acting isolates, the engineering of baculoviruses has become a real alternative, since these viruses proved amenable to genetic engineering. More than 25 years ago the first successful attempt to modify the baculovirus genome was reported with the replacement of the polyhedrin gene (the gene responsible for the formation of occlusion bodies) and for interleukin-2. This treatment eliminated the production of occlusion bodies, but opened up a whole new venue for the application of baculoviruses as protein expression and gene therapy vector. It also provided the technology for improving baculoviruses as biocontrol agent. In the early 1990's the first engineered baculoviruses for biocontrol became available. The first type was a baculovirus lacking the *egt* or ecdysteroid UDP-glucosyl transferase gene. This virus proved to be considerably faster than the wild-type virus in stopping feeding and inducing mortality without affecting the required dose. The second type was a baculovirus expressing insect-specific toxins. These viruses were extremely effective with a quick speed of action, and resulted in one or two days in drop-off from the plant through paralysis. However, they were difficult to produce in large amounts as the yield was smaller as larvae died earlier. A suite of baculoviruses have now been successfully engineered for improved insecticidal activity.

So, in principle the armory of baculoviruses to control insects has been extended with quick-acting variants, but the application and commercialization never took off. After an initial surge in the 1990's industrial interest tapered off and did not regain interest. This is not because of lack of potential, but as a consequence of the public concern on the biosafety of these genetically engineered baculoviruses in the environment and for animal and human health. A considerable amount of research has gone into the assessment of the biosafety of these genetically engineered baculoviruses in the laboratory and in the field. None of these showed a major effect on any non-target insects or provided unexpected results. Recent experiments showed that the competitive fitness of such genetically-engineered viruses in an agro-ecological setting is at best equal but mostly inferior to the wild-type baculovirus variants. Nevertheless, it is difficult to conclude that genetically-engineered baculoviruses are absolutely safe as long-term fitness and persistence experiments have not been done. Unless public

acceptance of genetically-engineered baculoviruses – and genetically-engineered organisms (crops) in general – does not change, the future of such viruses for implementation in biocontrol programs is not bright.

Despite the above the real and immediate benefit of genetically-engineered baculoviruses is their use in insect pathology to tailor insect biocontrol strategies. Using engineering technology it is now possible to knock-out any gene of interest of the baculovirus to study its function, to tag baculoviruses to obtain a more detailed understanding of their pathology and to study the baculoviruses for which no suitable cell culture systems exist. Such information on baculoviruses and their pathology should ultimately give a wider scope to strain selection in agro-ecological settings and provide further insight in understanding and avoiding baculovirus resistance in insects, which has recently been reported.

Key words: Baculovirus, genetic engineering, speed of action, persistence, safety

Gut gene expression profiles of *Heliothis virescens* larvae fed *Bacillus thuringiensis* toxins

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Abstract: The mode of action of Cry toxins produced by *Bacillus thuringiensis* has been extensively studied. In comparison, there is only limited information on how target midgut cells respond to the intoxication challenge. Our work was aimed at identifying genes involved in response to Cry1Ac intoxication in *Heliothis virescens*. Developmentally synchronized *H. virescens* 4th instar larvae were randomly assigned to experimental groups and starved for 6 hours. Larvae were then fed on a commercial diet containing 1 µg/ml of Cry1Ac, Cry3Aa, or buffer and dissected after 0, 0.5, 2, 6, or 24 hours. Total RNA was isolated from larval gut tissue and used to produce dye-labeled complementary RNA (cRNA) for hybridization to an oligonucleotide microarray representing over 15,000 transcripts from *H. virescens*. Expression levels of *H. virescens* gut-specific transcripts after toxin feeding were compared with those of control larvae. Genes that responded to toxin feeding by up- or down-regulation were identified and the expression profiles of these genes during the experiment time course established.

Key words: *Bacillus thuringiensis, Heliothis virescens,* Cry1Ac, Bt toxin, microarray, gene expression