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Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status

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REVIEW ARTICLE

Production, Formulation and Application of the Entomopathogenic Fungus *Beauveria bassiana* for Insect Control: Current Status

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This review summarizes the progress and achievements made in the last decade in mass production formulation and application technology of the entomopathogenic fungus, Beauveria bassiana. *Reports published on relevant research from Belgium, Canada, China, Cuba, Czechoslovakia (former), France, Germany, Great Britain, Philippines, Poland, Switzerland, USA and USSR (former) regarding this topic have been covered. Much of the non-English language literature, particularly that from Eastern European and Chinese sources, has not been translated and is inaccessible to most English or other western language readers. We have done this translation and through this review provide technological details about mass production of* B. bassiana *in China. Various aspects of* B. bassiana *growth, substrate use, production of mycelia, conidiospore and blastospores, process technologies associated with separation, drying and milling, formulation, storage and 'shelf-life', and field efficacy are reviewed. Data are presented on: a modified diphasic production technology developed in China during the 1980s; comparisons between submerged fermentation, which usually produces blastospores, and those producing conidia; the use of mycelial preparations pelletized with alginate or gelatinized with cornstarch or cornstarch-oil; and data on low or ultra-low volume sprays of emulsifiable or oil conidial suspensions and dust formulations.* B. bassiana *has proved to be competitive with chemical insecticides for the annual protection of 0.8–1.3 million hectares against forest and farm insect pests in China. It is hoped that this review will help to bridge the language gap between eastern and western scientists in microbial control using* B. bassiana.

Keywords: Beauveria bassiana, *entomopathogenic fungi, fungal mass production, biocontrol formulations, fungal storage and stability, microbial control*

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INTRODUCTION

Concerns about the negative effects of chemical insecticides have led to emphasis on alternative strategies for pest control. There is a world-wide resurgence of interest in the use of entomopathogenic fungi as biological control agents (BCA), and a significant advance in development and manufacturing of these agents in the future is expected with recent biotechnological innovations (Khachatourians, 1986). There have been several major reviews on entomopathogenic fungi in general (Ferron, 1978a; Roberts & Humber, 1981; Hall & Papierok, 1982; Zimmermann, 1986; McCoy *et al.*, 1988; Gillespie & Moorhouse, 1989; Roberts, 1989; Ferron *et al.*, 1991; Roberts *et al.*,1991; Goettel, 1992; Roberts & Hajek, 1992; Leathers *et al.*, 1993), their epizootiology and ecology (Feng, 1985; Carruthers & Soper, 1987; Fuxa, 1987; Carruthers & Hural, 1990; Onstad & Carruthers, 1990; Glare & Milner, 1991), their physiology and genetics (Khachatourians, 1991), the insecticidal activity of their toxic metabolites to insects (Roberts, 1981; Wright & Chandler, 1982; Charnley, 1989; Gillespie & Claydon, 1989), their stability in response to environmental factors (Roberts & Campbell, 1977) their safety to non-target invertebrates (Goettel *et al.*, 1990) and vertebrates (Austwick, 1980; Siegel & Shadduck, 1990; Semalulu *et al.*, 1992), and the commercial approaches to their use (Bartlett & Jaronski, 1988; Powell & Faull, 1989; Bradley *et al.*, 1992). Also, there have been several reviews addressing specific fungal agents such as *Beauveria* and *Metarhizium* (Ferron, 1981), *Verticillium lecanii* (Zimmerman) Viegas (Hall, 1981), *Hirsutella thompsonii* Fisher (McCoy, 1981), *Nomuraea rileyi* (Farlow) Samson (Ignoffo, 1981) and entomophthoralean fungi (Wilding, 1981; Wilding *et al.*, 1986; Wolf, 1988; Wilding, 1990). Only two reviews discussed in some detail the mass production and formulation of entomopathogenic fungi (Soper & Ward, 1981) and other microbial agents (Couch & Ignoffo, 1981) although this was mentioned in some other reviews (e.g. Ferron, 1971). A brief discussion on production and formulation was recently given by Goettel and Roberts (1992). We felt that a consolidated review, on mass production and formulation technology, was needed to facilitate the development and application of *Beauveria bassiana* (Balsamo) Vuillemin.

There are specific requirements for successful, commercial production and use of fungal insect control agents (Roberts *et al.*, 1991; Samsináková *et al.*, 1981). Firstly, a fungal isolate for mass production must be selected with rapid growth, abundant sporulation and sufficiently high pathogenicity to the target pests. Secondly, production costs must be minimal; this may be achieved by developing a medium that is simple in composition, cheap in price and available in large quantities, and a production procedure that is easy to run and minimizes labour. Thirdly, microbial products must be formulated to control different target pests with distinct biological aspects. Finally, formulated products must be suitable for long-term storage under natural or nearly natural conditions without significantly losing their viability and infectivity. Shelf-life is considered a pivotal factor that determines the commercial success of a biocontrol agent as well as its field efficacy. An 18-month shelf-life is recommended for the agricultural market (Couch & Ignoffo, 1981).

The aim of research into the production, formulation and application of entomopathogenic fungi is to deliver, inexpensively, a sufficient number of resilient infective propagules at a suitable place and time to control the pest. In this review, we have restricted ourselves to *B. bassiana*, although, occasionally, reference will be made to *B. brongniartii* (Saccardo) Petch. There are over 700 species of entomopathogenic fungi (Roberts, 1989; Roberts *et al.*, 1991) and it would be difficult to include details of all fungi with potential as biological control agents in a single review. Of these, however, *B. bassiana* has been studied most extensively since it was first reported as a pathogen of the silkworm, *Bombyx mori* L., by Agostino Bassi in 1834 (see Glare & Milner, 1991), and experiences gained with *B. bassiana* reflect the current status of the microbial control of pest insects by fungi. There is also a need to present the numerous reports regarding the production, formulation and application of *B. bassiana* that are published in non-English language journals, particularly those from China, which are either untranslated or remain inaccessible to most.

BIOLOGICAL ASPECTS

Mode of Infection

B. bassiana has no known sexual cycle. Insects are infected by conidia (asexual propagules) which attach to the host cuticle. Conidia germinate in an environment with high humidity. The germ tubes developing from the conidia penetrate the host cuticle and invade the haemocoel. A successful infection by *B. bassiana* is dependent primarily on various enzymatic activities for degradation of proteins, chitin and lipids in the insect integument (see Ferron *et al.*, 1991; Khachatourians, 1991).

Despite the general mode of infection through the integument, there is evidence that *B. bassiana* may infect insects *per os*, particularly insects with chewing mouthparts, e.g. the Colorado potato beetle, *Lepitinotarsa decemlineata* (Say) (Allee *et al.*, 1990); the red imported fire ant, *Solenopsis invicta* Buren (Siebeneicher *et al.*, 1992); the pine caterpillar, *Dendrolimus punctata* Walker (Long & Du, 1988); the German cockroach, *Blattella germanica* (L.) (Cai & Liu, 1988); the lesser cornstalk borer, *Elasmopalpus lignosellus* (Zeller) (McDowell *et al.*, 1990); the cabbage looper, *Trichoplusia ni* (Hübner) (Ignoffo *et al.*, 1982); the mulberry silkworm, *B. mori* L. (Huang, 1988); and the migratory grasshopper, *Melanoplus sanguinipes* (F.) (Feng *et al.*, unpublished data).

On invading the haemocoel, the fungus proliferates. Mycelia from the elongated germ tubes are septate and release blastospores. Host insects are killed due to depletion of their haemolymph nutrients and/or due to toxaemia caused by fungal toxic metabolites (Khachatourians, 1991; Roberts, 1981). Under moist conditions, the fungus emerges and produces a layer of aerial conidia on the surface of host cadavers.

In addition to its lethal effect, infection by *B. bassiana* may have sub-lethal or secondary effects. For example, doses of *B. bassiana* near the LD_{50} reduced the reproductive potential of adult *Sitona lineatus* (L.) (Müller-Kögler & Stein, 1970), and the fertility (N'Doye, 1976) and fecundity (Faizi, 1978) of adult *Chilo suppressalis* (Walker) surviving infection. The fungus also influenced the fecundity and egg fertility in adult Colorado potato beetles surviving larval infection (Fargues *et al.*, 1991), and egg development of rice plant hoppers and leaf hoppers (Homoptera) (Zhang & Huang, 1988).

Natural Occurrence and Epizootics

Over 200 species of insects in nine orders, mainly Lepidoptera and Coleoptera, have been recorded as hosts of *B. bassiana* (Li, 1988). But the fungus has also been recorded on cereal aphids (Feng *et al.*, 1990a; Feng & Johnson, 1990), hop aphids (Dorschner *et al.*, 1991) and grasshoppers (see Khachatourians, 1992). Despite this wide range of hosts, epizootics caused by *B. bassiana* in natural insect populations occur infrequently. Feng *et al.* (1990b) reported that in 2930 aphid cadavers examined, ten species of fungal pathogens, including eight Entomophthorales and two Hyphomycetes, were recovered. Of the fungal infections $<1\%$ each were attributed to infection by *B. bassiana* or *V. lecanii*, and the remainder were from the Entomophthorales. However, a large swarm of the red locust, *Nomadacris semptemfasciata* (Serville), was killed by a *B. bassiana* epizootic (Schaefer, 1936), and several *B. bassiana* epizootics were observed to decrease the populations of pine caterpillars, *Dendrolimus* spp., in southern China (see Chen *et al.*, 1990). In 1954, a white muscardine (i.e. mycosis caused by *B. bassiana*) caused an epizootic in >2000 ha of pine forest in Hunan Province, causing 85–95% mortality of the caterpillar populations. In 1958, almost all pine caterpillars in a forest of Longxi County, Fujian Province died of the white muscardine. During the period from 1973 to 1976, *B. bassiana* epizootics effectively suppressed the caterpillar populations to low levels ($>90\%$ mortality recorded) in >2600 ha of forest in Jing County, Anhui Province. White muscardine epizootics also were relatively often reported in numerous counties of Hubei, Guangdong and Guangxi Provinces. These epizootics usually occurred in the overwintered generation of the caterpillars during spring; a warm, rainy season in southern China. Various degrees of natural suppression by *B. bassiana* were observed in rice leaf hoppers and the elm leaf beetle, *Pyrrhalta luteola*

(Müller), (>50% mortality) in Hubei and Hunan during the 1970s (Lü & Zhao, 1988). Based on a field survey in several counties of Shandong Province from 1985 to 1987, Tao *et al.* (1988) found that 4.8–13.19% of peach fruit moths, *Carposina sasakii* Matsumura, were killed by *B. bassiana*. Additionally, the fungus *B. brongniartii*, closely related to *B. bassiana*, caused 8.6–50% mortality in the population of white grubs, mainly *Holotrichia parallela* Motschulsky and *H. oblita* (Faldermann), in bean fields in southwestern Shandong in 1984 (Lin *et al.*, 1988).

Variations in Pathogenicity Among Isolates

To date, more than 200 insect species have been recorded as hosts of *B. bassiana* world-wide (see Li, 1988). The virulence of the fungus (Fargues, 1981) and pathogenicity of isolates towards any insect varies, and in the absence of a clear definition of virulence for entomopathogenic fungi (*vis-à-vis* specified gene-for-gene relationship between phytopathogenic fungi and their hosts) the situation is warranting 'improved' strain selection. The term virulence has been defined as the 'degree of pathogenicity within a group or species' of entomopathogenic fungi, in the context of a particular host bioassay (see Khachatourians (1991) for a discussion). Often, virulence is measured in terms of LC_{50}, but then bioassays can be performed under different relative humidities and temperatures because of the experimentalist's choice or particular requirements of an insect. In this review, we have adopted the terminology used in the publications and hoped to avoid further confusion.

Bioassay of a collection of over 50 *B. bassiana* isolates from around the world on the Colorado potato beetle produced LT_{50} values ranging from 2 to 10 days (see Soper & Ward, 1981). A similar range of LT_{50} values was obtained with the migratory grasshopper (Khachatourians, 1992). Generally, *B. bassiana* isolates tend to have higher virulence to their original hosts, or species closely related to the original hosts (Xu, 1988). However, exceptions exist; for instance, an isolate from the Colorado potato beetle was more virulent to the Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), than isolates derived from other homopteran hosts (Feng & Johnson, 1990). Interestingly, high concentrations of a suspension of conidia of an isolate highly virulent to the European corn borer, *Ostrinia nubilalis* (Hübner), caused only 4–10% mortality in the perny silkmoth, *Antheraea pernyi* Guérin-Méneville, even under laboratory conditions (Xu *et al.*, 1988); while repeated use of a formulation of this isolate yearly for control of the corn borer in the fields never resulted in white muscardine in the silkmoth populations located in nearby fields. Kononova (1978) classified 190 *B. bassiana* isolates into two groups: on solid media, one group was characteristic with suppressed vegetative growth and abundant sporulation, whereas the other group had fluffy, cottony colony (excessive vegetative growth) and suppressed sporulation. This author also found that the first group tended to be more virulent to insect hosts than the second group.

Variation in virulence of *B. bassiana* may also be related to enzyme production and activities during the course of the penetration of the host cuticle (Bidochka & Khachatourians, 1990; Hegedus & Khachatourians, 1988). Isoesterase analysis can be used to identify a virulent isolate because of variation in isoesterase profiles among isolates from different insect hosts and/or different geographic regions (Poprawski *et al.*, 1988; Riba *et al.*, 1986; Tigano, 1985; Wu *et al.*, 1988; Yin *et al.*, 1988a). In the laboratory, repeated subculture of an isolate sometimes leads to the attenuation of its original features such as growth, sporulation and virulence (for a discussion, see Khachatourians, 1991; Hayden *et al.*, 1992). It can be speculated that this problem may be a consequence of genetical change through a parasexual cycle in *B. bassiana* reported by Paccola-Meirelles and Azevedo (1991), and perhaps can be minimized by routine host passage and single spore isolation (Hayden *et al.*, 1992). Although the evidence for the genetic basis for variation is still to be researched, once discovered, through *in vitro* recombinant DNA-derived vectors and electroporation-mediated transformation (Pfeifer & Khachatourians, 1992) or the parasexual cycle, one could methodically approach the genetical stabilization or enhancement of *B. bassiana* isolates.

Culture

B. bassiana is easily cultured on solid and liquid media, e.g. Saubouraud or potato dextrose agar and broth. Aerial conidia are produced on solid media and, in morphology and infectivity, are

indistinguishable from those produced on the surface of insect cadavers. Development in submerged cultures has been classified into six stages (Bidochka *et al.*, 1987). Blastospores are usually formed, as in the insect haemocoel, by schizolytic separation at septa or by mechanical fragmentation of hyphae due to shearing forces in the liquid medium. Blastospores can also be produced by yeast-like budding from parent single cells. The formation of blastospores in submerged culture has been well studied (Samsináková, 1966, 1969; Goral, 1978; Bidochka *et al.*, 1987; Yin *et al.*, 1988b; Rombach, 1989; Trinci *et al.*, 1990). Because of the mode of formation, the blastospores of *B. bassiana* are considered functionally identical to short hyphal cells and thus called 'hyphal bodies' more properly (Rombach, 1989). Since the term 'blastospore' has been widely used in the literature, we still use it throughout this review unless mentioned otherwise.

The blastospores of *B. bassiana* are thin walled and unstable during drying after fermentation and when applied under field conditions (Yin *et al.*, 1988b). Conidia are much more resilient and attempts to produce them on a large scale have drawn much research attention. Mass production of aerial conidia by a diphasic fermentation, i.e. vegetative mycelia production by liquid batch culture followed by surface conidiation of the mycelia on a nutrient or inert carrier (Soper & Ward, 1981), is considered to be labour-intensive and unsuitable for conventional processing of fungal material in fermenters (Rombach, 1989). This has challenged researchers to investigate the possibility of producing submerged conidia that are similar to aerial conidia in environmental stability and virulence as well as in morphology. Submerged conidiation of *B. bassiana* has been illustrated by Kononova (1978), Thomas *et al.* (1987), Rombach (1989), Yin *et al.* (1988b) and Hegedus *et al.* (1992), and also discussed in some earlier publications (Kondrat'yev & Alyeshina, 1971; Goral, 1971, 1973, 1975; Belova, 1978). Various other publications on submerged *B. bassiana* cultures have used the terms blastospores, blastoconidia, spores, conidia and conidiospores indiscriminately and thus are difficult to evaluate.

According to Kononova (1978), submerged conidiation includes five developmental phases:

(1) swelling and germination of conidia;
(2) formation of mycelia and conidiophores;
(3) branching of the mycelia and initial conidiation;
(4) massive conidiation;
(5) detachment of conidia in liquid culture.

In addition to this mode of submerged conidiation, Thomas *et al.* (1987) observed conidiation without the usual vegetative phase of growth and development of a *B. bassiana* isolate. The latter mode of submerged conidiation includes the production of blastospores during the first 48 h of cultivation in a defined broth and the subsequent formation of conidia followed by germination of the blastospores and conidiation at the tip. Submerged conidation can also be completed by the budding of the conidia formed in the broth (Hegedus *et al.*, 1992). However, this micro-cycle conidiation was not observed by Rombach (1989) who studied a different strain. It is thus postulated that submerged conidiation is likely to depend on the nature of the isolate and the particular physiological conditions used.

Not all *B. bassiana* strains have the potential to produce conidia in submerged cultures (Kononova, 1978). Generally, however, isolates with suppressed vegetative growth and abundant sporulation on solid media are more likely to produce submerged conidia. The composition of the liquid medium can also determine production of conidia. According to Yin *et al.* (1988b), only one of three liquid media consisting of 2% corn syrup, 3% sucrose and varying basal salts produced submerged conidia with a globose (2.52 μm) or subglobose (2.28 $\times$ 3.24 μm) morphology. It was found that submerged conidiation was related to the nature of the carbon source and the presence of nitrate as a nitrogen source, e.g. KNO_3 (Thomas *et al.*, 1987) and $NaNO_3$ (Kononova, 1978). However, the use of NH_4NO_3 led to the production of blastospores rather than conidia in fermenters (see Chen *et al.*, 1990). Thomas *et al.* (1987) also found that limiting the vegetative growth of the fungus by reducing the amount of phosphate in the liquid medium

resulted in the production of submerged conidia which resembled aerial conidia more closely than any other preparation of submerged conidia. Replacing the quantity of glucose with the same amount of another carbon source (e.g. citrate, lactose, soribitol, starch, fructose, maltose or glycerol) decreased the yield of submerged conidia and/or increased the proportion of blastospores in the resultant cultures. According to Hegedus *et al.* (1990), growth media containing *N*-acetyl-D-glucosamine proved to be better for submerged conidiations of *B. bassiana* than yeast extract–peptone–glucose, or glucose plus ammonium salts or *N*-acetyl-D-galactosamine; however, the conidial yields ranged only from $2.1–15.8 \times 10^5$ conidia/ml despite the higher percentage (up to 86%) of submerged conidia formed in the liquid cultures.

Whether the aerial and submerged conidia differ in infectivity and environmental stability is a concern, when it comes to decisions of modes of production. Boucias *et al.* (1988) considered that hydrophobicity is important for the attachment of the spores of several entomopathogenic Hyphomycetes to the insect cuticle. Recently, Hegedus *et al.* (1992) has shown little difference in hydrophobic and lectin-binding activities between the two types of *B. bassiana* conidia. They found that the submerged blastospores were less hydrophobic than the two types of conidia and also differed in lectin-binding activity. The three types of spores had similar LT_{50} values for the migratory grasshopper: 6.50 days with the blastospores, 7.12 days with aerial conidia and 7.24 days with the submerged conidia (Hegedus *et al.*, 1992). However, for submerged and aerial conidia and the blastospores, respectively, the time for the loss of 50% viability in distilled water at $-70°C$ was 30.7, 15.2 and 5.2 weeks. Yin *et al.* (1988b) reported that during a 19-month storage at 4°C, dried submerged conidia of *B. bassiana* had significantly higher viability and infectivity than the submerged blastospores. The viability of the submerged conidia was 93.3, 87.3 and 35.3% for 8-, 12- and 19-month storage, respectively. In contrast, the viability of the submerged blastospores decreased to 32.0, 29.3 and 4.8% when stored at 4°C for 8, 12 and 19 months. The aqueous suspensions of submerged conidia and blastospores, at concentrations of 5.9×10^8 spores ml^{-1}, respectively, produced mortalities in the pine caterpillar of 69.7% and 28.3% when fresh, 46.6% and 17.2% after 2-week storage, and 40.6% and 1.6% after 8-month storage (Yin *et al.*, 1988b).

MASS PRODUCTION

At present, mass production of *B. bassiana* is generally based on diphasic and submerged fermentation techniques. As pointed out by Soper and Ward (1981), diphasic fermentation combines the advantages of both solid and liquid media. The fungus is allowed to grow in fermenters to the end of the log phase for maximal production of mycelial biomass, which is subsequently transferred on to nutritious or inert substrates for the production of aerial conidia in the form of natural inocula. However, the diphasic method is, though simple, considered to be the most expensive (Soper & Ward, 1981) and labour-intensive (Rombach, 1989). Submerged fermentation is designed to take advantage of existing industrial fermenters which typically can hold up to 200 m^3. However, with large-scale fermentation, precautions must be taken to control properly several factors (such as aeration, pH, temperature and foaming) which may significantly affect the quality and quantity of a fungal preparation. Furthermore, submerged fermentation often gives rise to blastospores or a mixture of blastospores and conidia (Belova, 1978; Thomas *et al.*, 1987; Yin *et al.*, 1988b; Rombach, 1989). For example, Goral (1971) reported a *B. bassiana* preparation containing 40% conidia and 60% blastospores.

Solid and Diphasic Production Technologies

Fundamentals of solid substrate fermentation of several entomogenous fungi including *B. bassiana* and *B. brongniartii* have been reviewed in a comprehensive article by Bartlett and Jaronski (1988). A solid culture process for *B. bassiana* production has recently been developed by Mycotech Corporation, USA (Bradley *et al.*, 1992). This process is based on a packed bed, solid culture system. A liquid phase is absorbed in a starch-based substrate. The fungus grows on the substrate particles between which the gaseous phase remains available for aeration. With

this method, yields of 10^{13} conidia can be obtained in fermenter volumes of less than 1 litre. Under pilot production, dry powder with an average of 2.6×10^{11} conidia g^{-1} were reported.

In Canada, a simple and inexpensive method for culturing entomopathogenic fungi using cookware roasting pans, wheat bran, cellophane and autoclavable bags was described by Goettel (1984). With this method, conidial yields of 10^{10} g^{-1} of dry biomass were obtained for *B. bassiana* and several other fungi, free from substrate contamination.

Alves and Pereira (1989) obtained a yield of 2×10^{11} *B. bassiana* conidia g^{-1} of powdery preparation using rice as a basic growth substrate. Rice (500 ml) was soaked in 200 ml of deionized water in autoclavable polypropylene bags (25×45 cm) for 1 h. Bags and contents were then autoclaved at 121°C for 45 min, and their tops folded and stapled when removed. Once cooled, the rice in each bag was inoculated with conidial powder introduced from one corner of the bag, the bag was restapled and the conidia were mixed uniformly throughout the rice. Then, the bags were incubated at 25°C in 16–24 h light for ≥ 3 days, allowing a profuse mycelial outgrowth on the rice kernels. The contents of the bags were then poured on to plastic trays (30×46 or 40×40 cm) with the rice layer being < 6 cm thick. The cultures were covered with the tray lids or plastic wrap to trap in moisture, and maintained for 12–15 days for conidial production. Thereafter, the cultures were allowed to dry by removing the wrap and transferring the trays to a refrigerated cold chamber at 3°C. Finally, conidia were harvested by means of a vibrating sieve apparatus enclosed in a container. Each 100 kg of rice culture generated 3 kg of conidial powder. It should be mentioned that the rice ready for inoculation has the firmness of hard rubber which ensures uniformity of the moisture content in the rice. The amount of rice and water in plastic bags can vary to some extent. For instance, the rice can be soaked for 1–2 h before autoclaving or cooked in a metal barrel with water heated directly by steam from a boiler for 4–5 min.

Rombach *et al.* (1988b) produced conidia of *B. bassiana* on wheat bran by a small mass production unit using a diphasic fermentation process. Over 95% of the conidia germinated in viability tests after more than 3-years storage at 4°C. In field tests, mortality of *Nilaparvata lugens* (Stål) in rice fields was in the range 65–95%, 3 weeks after application of an aqueous suspension of *B. bassiana* ($4–5 \times 10^{12}$ conidia ha^{-1}). There are some other simple media and methods used for the mass production of *B. bassiana*. A mixture of 70% wheat bran, 25% corn flour and 5% bean flour plus water (1:1.25) resulted in a preparation containing 2.9×10^{10} conidia g^{-1} of dry powder after 10–days surface cultivation at 28°C (Tao *et al.*, 1988). Three hundred kilograms of wheat bran and 20 kg wheat flour plus a small amount of corn flour (about 2%) produced 21 kg of conidial powder through surface cultivation (Shi, 1988). Applying the diphasic method to *B. brongniartii*, Lin *et al.* (1988) obtained a yield of 2.9×10^9 conidia g^{-1} of dry powder from a medium composed of 70% cotton-seed-shell powder, 25% wheat bran and 5% corn flour (plus water). With the same fungus grown in polyamide bags at 23°C, Aregger (1992) obtained a maximal yield 1×10^8–2×10^9 conidia g^{-1} after 24- or 42-day cultivation, respectively, on shelled barley, mixed with sunflower oil and water.

In the past decade, Chinese entomologists and microbiologists have successfully improved the traditional diphasic fermentation technology for mass production of pure *B. bassiana* conidial powder for large-scale control of pine caterpillars and other insect pests (Li & Yang, 1988; Lü,1988; Lü, & Zhao, 1988; Mou, 1988; Pan *et al.*, 1988; Shi, 1988; Xu, 1988; Yao *et al.*, 1988; Yin *et al.*, 1988b; Zhou, 1988; Chen *et al.*, 1990). The improvement involved the introduction of machinery equipment into the production process and the use of cheap, large-source materials for growth substrates. This modified diphasic technology made mass production of high-quality *B. bassiana* conidial powder simple, easy, cheap and safe.

Figure 1 is a flow diagram of the modified diphasic technology following the description of Chen *et al.* (1990). Production starts with a fungal isolate with characteristics of rapid growth, abundant sporulation and high infectivity. The isolate is incubated on slants of a solid medium for 12 days. Inocula from the slant culture containing spores and mycelia are used to start a liquid culture in flasks, which are incubated on a rotary shaker (94 rpm) at 25 ± 1°C for 3 days. The resulting 'first-level' inoculum culture is then transferred into a 20 l fermenter and incubation

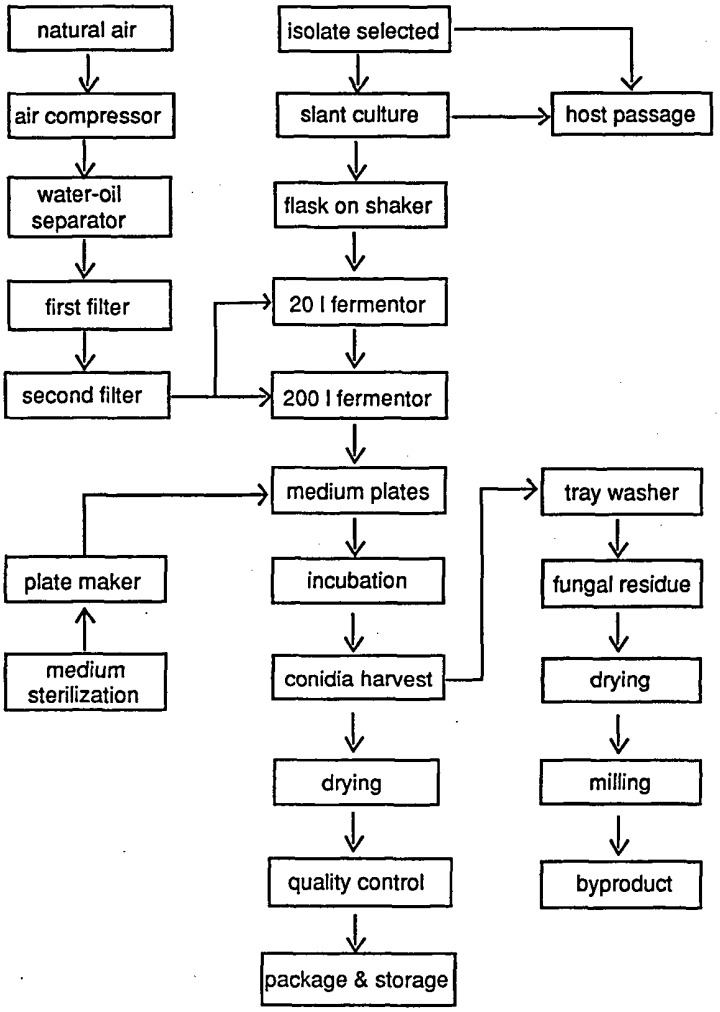

FIGURE 1. Flow diagram of the modified diphasic technology used in production of pure conidial powder of *B. bassiana* in China (adapted from Chen *et al.* (1990)).

proceeds for a further 40 h. The resultant culture is transferred into a 200-l fermenter and after another 48-h incubation period the 'third-level' inoculum culture is produced, which is eventually used to inoculate plates for conidia production. The three-level cultures for inoculation are produced using the same liquid medium: 6% wheat bran (infusion), 2% sucrose, 0.2% peptone, 0.05% KH_2PO_4, 0.02% $MgSO_4$ and 0.3% $CaCO_3$ with pH unadjusted. A desirable ratio of carbon to nitrogen (C/N) in this medium is determined as 3.75. At levels 2 and 3, 0.15% peanut oil is added as a defoamer. The liquid medium is sterilized by piping high pressure steam into the fermenter (1.1–1.4 kg cm^{-2}) for 30 min. When cooling to 27 ± 1°C, inoculation is completed using inoculum culture of the previous level. The ratio of inoculum to liquid culture medium (v/v) is 1/10. Environmental conditions for fermentation at levels 2 and 3 are as follows: (1) temperature, 25 ± 1°C; (2) aeration, 1:1 (v/v) per min; (3) fermenter pressure, 0.5–0.6 kg cm^{-2}.

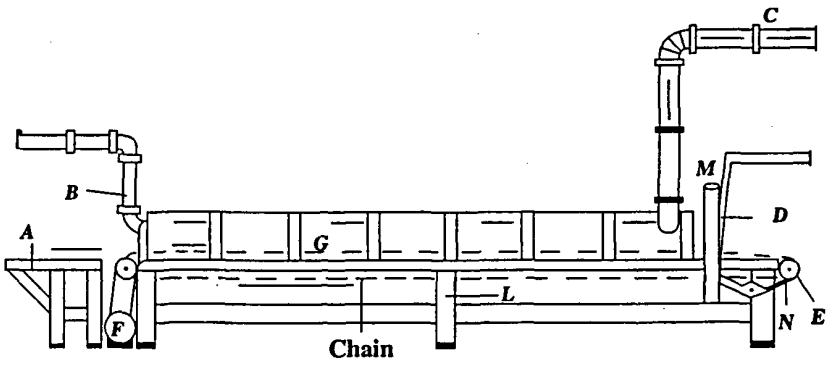

FIGURE 2. Diagram of the automatically mechanized system used for plate making and inoculating during production of pure conidial powder of *B. bassiana*: (A) support, (B) 100-mm air input pipe, (C) 200-mm exhaust pipe, (D) 32-mm inoculum input pipe, (E) cam, (F) motor, (G) wind cover, (L) principal support, (M) support of inoculum sprayer, (N) crankshaft (adapted from Chen *et al.* (1990)).

Plate making and inoculating are completed by specifically designed machinery which functions as follows.

Sterilization of trays. A steel cabinet ($2 \times 1.3 \times 1.05$ m) allows for sterilization of 200 trays (0.252 m^2 each) at one time. A pipe connects the cabinet to a steam-producing boiler. Sterilization is achieved by piping the steam into the cabinet from the boiler and keeping the temperature at 100°C for 1 h.

Medium preparation. The medium for production of conidia contains 3% wheat bran (infusion), 1% sucrose, 0.05% KH_2PO_4, 0.01% $MgSO_4$ and 3% agar. A tank for sterilization of the medium is modified from a 2-ton fermenter. Sterilization is completed by piping the steam under high pressure into the tank filled with the medium and kept at a pressure of 1.1 kg cm^{-2} for 30 min. Subsequently, tap water is piped into the double-layered walls of the tank to cool the medium. When the temperature has dropped to 70–80°C, the medium is piped out for plate making (Figure 2, D).

Conveying. When working, a conveyer chain moves at a speed of 89.4 cm min^{-1}, driven by a 2.2 kW motor using a 40:1 gearbox. As the chain moves, sterilized trays (63×40 cm) are sent to the plate-making system to receive melted medium, which is cooled off while moving on the conveyer. Next, the trays are exposed to the inoculation sprayer, where the tray plates are sprayed with the liquid inoculum culture from fermentation at level 3. After inoculation, the trays are moved to an exit, where they are collected and then sent to the incubation room.

Plate making. The amount of medium that a tray receives is controlled by a cam (Figure 2, E) which is connected to a cam shaft. As the cam rotates, an on–off switch opens and closes regularly, allowing a controlled amount of medium (1500 ml per tray) to be disposed into a tray at a given time.

Plate cooling. The temperature of the poured medium is about 70°C. In order to cool rapidly the medium in trays on the conveyer, a 1-kW blower (22 m^3 min^{-1}) is set at one end of the conveyer. Natural air is forced through an air-filtering system before entering into an air-cleaning pipe (100 mm in diameter) (Figure 2, B), which starts from one end of the wind cover (Figure 2, G) above the conveyer. At the other end of the wind cover is a 200-mm exhaust pipe (Figure

2, C) connected with a 0.14-kW fan. Heat in the medium is thus removed from the system as the trays are moved on the conveyer while the blower and the fan are working.

Plate inoculating. An inoculation sprayer (near M in Figure 2) controls the amount of liquid seed culture sprayed on to each tray plate (usually 70 ml per plate) on the conveyer.

Incubation. The inoculated tray plates are placed on shelves in an incubation room positioned at 8-cm intervals and are maintained at $25 \pm 1°C$ for 10 days. The incubation room is pre-sterilized using germicides such as 75% ethanol or 40% methanol. Relative humidity in the room is maintained at $>85\%$ during the first 3 or 4 days of incubation to facilitate germination and vegetative growth and can be reduced to $<75\%$ thereafter. Contamination can be avoided by increasing the amount of inoculum culture per plate and spraying the plate as evenly as possible.

Harvest of conidia. The machine that was developed for harvesting the conidia (Yu, 1983) is composed of a conveyer chain, roller brushes, a vacuum and a collector, as shown in Figure 3 (see Chen *et al.*, 1990). The conveyer chain driven by a 1.5-kW motor (Figure 3, A) via a 38:1 gearbox, moves the 10-day-old tray cultures through a wind cover (Figure 3, C) at a speed of 2.5 m min^{-1}. Here, conidia are removed from each tray by rolling brushes and are vacuumed into a pipe system by a working blower (Figure 3, D). The brushes set in function under the wind cover are five rollers with dense 46-mm bristles and are rotated at 48 rpm by a 1.5-kW motor (Figure 3, B) connected to a gearbox. After being brushed off, conidia in the wind cover are vacuumed up by the blower (0.75 kW, 18 m^3 min^{-1}) into a 100-mm pipe (Figure 3, E) which is connected with a 200-mm pipe (Figure 3, F). While coarse particles of mycelium–medium mixture gravitate down to a waste exit (Figure 3, K), conidial aggregates are piped into an upper collection bucket (Figure 3, G) via the 200-mm pipe. Below the upper collection bucket are located 15 flannelette bags (Figure 3, H) where air is removed, and conidia settle in the lower collection bucket (Figure 3, J) for packing. This conidia-harvesting machine can harvest $>95\%$ of the conidia from the tray cultures with an average of 3.3 trays being processed per min. Each gram of conidia powder harvested in this way contains $>1.8 \times 10^{11}$ conidia with an average viability of 94.6% and a water content of 14.5%. An average yield of 54.6 g of pure conidial powder per m^2 of the plate can be attained. A conspicuous advantage of the above technology is that the drying of conidia is achieved in the pipe system under completely natural conditions in the collection process.

Yao *et al.* (1988) have developed another technique for mass production of *B. bassiana* conidial powder in Fujian, China. Their medium is equally simple and cheap: wheat flour + water (1:2.4), wheat flour + bean flour + water (1:0.05:2.4) or wheat flour + agar + sucrose + water (1:0.02:0.02:5). The medium, a paste when well prepared, is sterilized in a tank with high pressure steam (1.5 kg cm^{-2}) for 2 h. Inoculation is completed by mixing the liquid inoculum culture of 15–25% (v/v), obtained at the second level of fermentation, with the sterilized paste that has been cooled to $<40°C$. The inoculated paste is then filled in a funnel on a mechanical device and smeared on to a copper roller. While the roller is being rotated, the inoculated paste is evenly smeared on to nylon mesh frame (85×50 cm), forming a paste membrane 1.0–1.5 mm in thickness. Then, the mesh frames with the paste membrane are maintained in an incubation room. During the first 72-h incubation, temperature and relative humidity are controlled to 22–26°C and $>90\%$, respectively. Thereafter, the temperature is increased to 26–28°C for rapid conidiation and the humidity is reduced. As the paste membrane is drying, conidia become mature. From the 7th to 10th (or 15th) day, the resulting cultures are transferred into an aerating room, where drying continues at 30–32°C with $<50\%$ relative humidity. Conidial powder, harvested with roller brushes, is sieved, further dried at 38°C for 5 h and finally packed. The powdery preparation contains $>1.5 \times 10^{11}$ conidia g^{-1} ($>85\%$ viable) and $<5\%$ moisture (a standard issued by the Forest Department of Fujian Province). The advantages of this technique

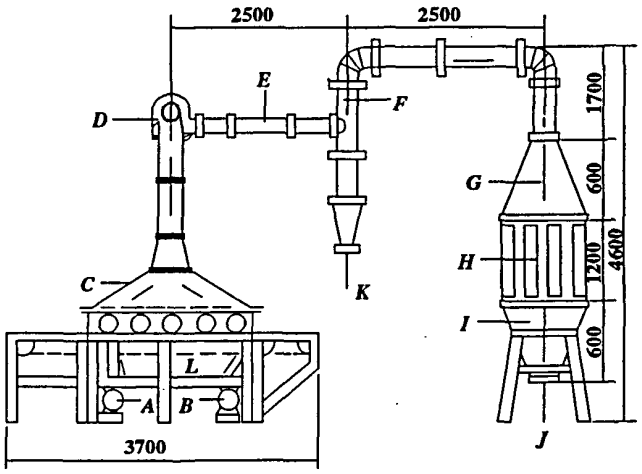

FIGURE 3. Diagram of the machinery equipment specifically developed for harvest of pure conidial powder of *B. bassiana* from plate cultures: (A) motor, (B) motor, (C) wind cover, (D) blower, (E) 100-mm pipe, (F) 200-mm pipe, (G) upper collection bucket of conidial powder, (H) flannelette bags, (I) lower collection bucket of conidial powder, (J) valve of exit to conidial powder, (K) valve of exit to by-product particles, (L) chain of conveyor (adapted from Chen *et al.* (1990)).

are the use of cheap materials which are available in large supply in China and the simple drying process.

SUBMERGED FERMENTATION

Boverin, a commercial product based on *B. bassiana*, was developed in the USSR during the 1970s (Kondrat'yev & Alyeshina, 1971; Lappa & Goral, 1973; Alyeshina *et al.*, 1976). It has been used extensively to control the Colorado potato beetle, the larvae of codling moth, *Cydia pomonella* (L.), and other local insect pests. Although almost all reports on Boverin are published in the Russian language, the Russian experiences and practices have been introduced to English readers through the publications of Alyeshina (1978), Belova (1978), Goral (1978), Kononova (1978), Lappa (1978), Sinitsyna (1978) and Yevlakhova (1978). Two strains, 113N-278 and 124-R, obtained by screening 190 strains from different hosts in various geographic regions were recommended for industrial production (Kononova, 1978). Submerged fermentation was considered to be the best technology which could satisfy the requirements of commercial production and practical application (Belova, 1978).

Using a mineral medium (0.9% $NaNO_3$, 0.225% KH_2PO_4, 0.075% $MgSO_4$, 1.25% $CaCl_2$) supplemented with 1% sucrose (Kononova, 1978), a yield of $3–5 \times 10^8$ conidia ml^{-1} was obtained after about 72-h fermentation in fermenters up to 63 m^3 capacity, at 26–28°C, with an aeration rate of 0.5 g O_2 l^{-1} h^{-1} (Belova, 1978). Inclusion of peptone in the medium resulted in an average yield of $2–3 \times 10^9$ spores ml^{-1}. However, as indicated by Goral (1971) this yield was a mixture of conidia and blastospores. Kononova (1978) also found that, under submerged conditions, conidia were formed in the so-called mineral medium mentioned above whereas a mixture of conidia and blastospores were produced when using a medium composed of 0.375% peptone, 1.87% sucrose, 0.56% $NaNO_3$, 0.046% KH_2PO_4, 0.046% $MgSO_4$, 2.34% $CaCl_2$. This finding is supported by recent reports regarding the conditions of *B. bassiana* conidiation in submerged culture (Thomas *et al.*, 1987; Yin *et al.*, 1988; Rombach, 1989). Using a medium with a glucose/nitrate ratio of 5:1 (50 g glucose, 10 g KNO_3, 5 g KH_2PO_4, 2 g $MgSO_4$, 50 mg $CaCl_2.2H_2O$, 12 mg $FeCl_3.6H_2O$, 2.5 mg $MnSO_4.H_2O$, 0.25 mg $Co(NO_3)_2.6H_2O$, 0.2 mg

$Na_2MoO_4.2H_2O$, 2.5 mg $ZnSO_4.7H_2O$ and 0.5 mg $CuSO_4.5H_2O$ per litre) and a pH of 5.0 adjusted with 0.1 N-NaOH, Thomas *et al.* (1987) obtained a maximum yield of 5×10^8 conidia ml^{-1}. In other studies, the maximal yields obtained were 5.9×10^8 (Yin *et al.*, 1988b) and 1.7×10^8 submerged conidia ml^{-1} (Rombach, 1989). Currently, a yield of submerged conidia in much lower quantities than blastospores remains an obstacle to the use of the submerged culture method for commercial production of conidia.

The production of Boverin was based on a multi-level fermentation technology (Belova, 1978). The first two levels of liquid cultivation on a shaker or in smaller fermenters were designed to provide for sufficient inocula for use in inoculation of medium in large fermenters 2–63 m^3). A yeast–polysaccharide medium (2% yeast, 1% corn meal and mineral salts) yielded an ideal inoculum culture containing 8.79×10^8 spores ml^{-1} after 42-h incubation at 25–28°C, with an aeration rate of 0.8 g O_2 l^{-1} h^{-1}. The optimal volume of inoculum in the production medium was >2%. Increasing the amount of inoculum to 10% did not significantly enhance the yield compared with that obtained when using the 2% inoculum culture. In large fermenters, each cubic metre of liquid medium produced 15–20 kg of conidia paste containing $1.0–1.5 \times 10^9$ viable spores g^{-1}.

Harvest of spores can be achieved either by sedimentation of conidia suspended in a solution of aluminium sulphate (Globe *et al.*, 1977) or by separating the spore paste from the liquid culture and subsequent drying (Belova, 1978). Separation of the spore paste from the liquid culture can be completed by means of suction filters, vacuum drum filters or an ASG 3M separator spun at 9000 rpm with a separation factor of 10^4. Drying the spore paste requires maximal preservation of spore viability and infectivity. Several drying techniques have been considered including vacuum drying, freeze drying, spray drying, drying by mixing the paste with an inert filler, and drying in a fluidized bed. Drying in a vacuum desiccator was not suitable for commercial production despite the high viability and infectivity of spores in the resulting dry samples. Freeze drying required a temperature of −40°C and resulted in a maximum of 3.2×10^{10} viable spores g^{-1} of the dry product (>70% spores viable after drying). Spray drying of the paste in the form of a suspension containing 2% molasses as a protective medium led to a preparation with a maximum of 1.75×10^{10} viable spores g^{-1}. However, spray drying without the use of the protective medium caused the spores to lose viability completely. Drying by mixing the paste with an inert filler such as kaolin, perlite, bentonite, chalk or starch resulted in a preparation with a spore viability of <20% (4×10^9 viable spores g^{-1}). To preserve a higher percentage of viable spores in the drying process, it was recommended that the zone of critical moisture content of 30–40% must be passed as rapidly as possible. This was achieved by the use of an apparatus for drying in a fluidized bed. Freeze drying which is superior to other drying techniques and is recommended in Boverin production is of more general use. Besides the multi-level fermentation technology. Yevlakhova (1978) reported a single-step fermentation technology for Boverin production. This method was said to depend on so-called 'self-sterilization' by antibiotic metabolites excreted by the fungus into a non-autoclaved liquid medium.

A similar multi-level, submerged fermentation technology developed for production of *B. bassiana* in China is described in detail by Chen *et al.* (1990). The medium used to initiate inoculum culture on a rotary shaker is composed of 12 g glucose, 5 g corn meal, 8 g NH_4NO_3, 0.7 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$ and 0.2 g $CaCl_2$ per litre of hydrolyzed bean curd by-product solution (which in Chinese is called Huang-Jiang-Shui, i.e. a by-product from the process of bean curd making). After 72-h incubation at 26–28°C, the first-level inoculum culture ($27.9 \pm 1.6 \times 10^8$ blastospores ml^{-1}) is transferred into a 50-l fermenter to increase the amount of inoculum culture. Again, the 72-h-old, second-level inoculum culture is used to inoculate the liquid medium in 5-ton fermenters. The component, $FeSO_4.7H_2O$, is not included in the medium used in the second-level fermentation whereas the other components, plus a defoamer, are used in the same quantities as in the first-level inoculum culture. The 5-ton fermenters are filled with a cheaper medium containing 2.7 g glucose or sucrose, 2 g corn meal, 4 g NH_4NO_3, 0.1 g K_2HPO_4 and 0.1 g $MgSO_4.7H_2O$ per litre of the bean curd by-product

solution, plus 2% vegetable oil residue as defoamer, with pH adjusted to 5. Aeration during the fermentation is based on an empirical estimate of soluble oxygen (K_d) in the liquid medium:

$$K_d = 7.32 \times 10^{-7}\ V_s^{0.713}\ P_g/V \qquad (\text{M } O_2\ \text{ml}^{-1}\ \text{min}^{-1})$$

where V is the volume of the fermentation liquid, V_s is the flow rate of O_2 (m s^{-1}) at the level of the liquid surface under the pressure, and P_g is the power (kW) of a stirrer when working. The third-level fermentation of 38–40 h gives rise to a culture of $20.8 \pm 2.0 \times 10^8$ blastospores ml^{-1}.

Blastospores are harvested by mixing a filler (talcum powder or $CaCO_3$) with the fermented cultures and then forcing the mixtures to pass through a filtering device. The material obtained is then formulated into 1.2–1.4 mm granules on a medicine formulator and dried in a blow desiccator, where moisture is removed by flowing air of <30°C while the granules are being blown up and down by a blower. Dry blastospores obtained are as viable as, or slightly less viable than, those dried under natural conditions. The spray drying method is also used to dry the blastospore material. To do so, the blastospore paste is sprayed at a pressure of 3 kg cm^{-2} in a spray-drying tower. Temperature is controlled to 35–45°C at the entrance of the tower, but 35–40°C at the exit. Moisture is removed by blowing. Dry powder obtained in this manner contains up to 10^{10} blastospores g^{-1} and a water content of only 4.66%.

In addition to the production of conidia or blastospore preparations, McCabe and Soper (1985) have developed a method for mass production of mycelial preparations of entomopathogenic fungi based on a submerged fermentation process. The fungi are grown in commercially available media and harvested while in the active growth phase before nutrient exhaustion begins. Harvesting of mycelia is completed by discarding the liquid medium and making a slurry of the resulting mycelia in a volume of water. The slurry is then filtered on a vacuum apparatus to obtain a thin, moist mycelial mat. The fungal mat is then treated with a chemical protectant, dried and finally ground for storage and use (McCabe & Soper, 1985). This method has been used to produce *B. bassiana* mycelia for field treatment of the rice black bug, *Scotinophara coarctata* (F.), based on a liquid medium containing 1.5% molasses and 1.5% yeast extract for fungal growth and a 10% maltose solution as a protectant (Rombach *et al.*, 1986). A maximum of 12.31 g mycelia biomass l^{-1} was obtained using a broth containing 3.5% sucrose as a carbon source and a 3.5% yeast extract as a nitrogen/vitamin source, but mycelia from a broth of 2% maltose and 0.75% yeast extract produced 4.62×10^9 conidia g^{-1} (Rombach *et al.*, 1988a). Recently, Pereira and Roberts (1990) produced mycelial preparations of *B. bassiana* and *Metarhizium anisopliae* var *anisopliae* (Metchnikoff sorokin) using a medium consisting of 1% dextrose, 1% yeast extract, 0.1% sunflower oil and 0.05% penicillin–streptomycin (200 000 units penicillin and 250 mg streptomycin ml^{-1}). Yields of 4 g l^{-1} dry mycelium of culture for *B. bassiana* and 6 g l^{-1} for *M. anisopliae* were obtained.

Pereira and Roberts (1990) harvested the mycelial biomass formed in the liquid culture on a novel extraction table. The extraction table consisted of a plastic tray with a perforated false bottom. A pipe connected the table to a vacuum flask attached to a vacuum source. To harvest the mycelial mats, a coarse filter paper was placed on the top of the perforated base and the liquid culture was simply poured on to the paper while vacuum was applied. The mycelial mats were easily peeled off the paper and transferred to wire racks for final processing.

Polyethylene Cushion Cultivation

A technique that was developed for stationary cultivation of microorganisms (Kybal & Vlcek, 1976) has been modified for mass production of *B. bassiana* conidia in Czechoslovakia (Samsináková *et al.*, 1981). The use of polyethylene cushions requires large, thin-walled polyethylene tubing. In the course of manufacture, the cushions are sealed into sections which are partially filled with liquid medium and inflated with air. Sterilization is unnecessary because the polyethylene tubing manufacturing process renders the system sterile. Air is introduced as needed during cultivation. In a simple medium containing 0.8% peptone and 1% sorbitol (the best carbon source among sources tested including glucose, sucrose, fructose, maltose and starch), the fungus grows as a floating fungal mat and is harvested by simply discarding the

liquid. A 12-day cultivation at 27°C results in a yield of 10^{11} conidia 100 cm^{-2}, equalling 3.5×10^{11} conidia g^{-1} of dry biomass. Since the fungal mat is floated on the liquid during cultivation, this method apparently takes advantages of both submerged fermentation and surface conidiation. In addition to the high yield of conidia and being technically undemanding, the method was said to be simple and cheap (Samsináková *et al.*, 1981).

FORMULATION, SHELF-LIFE AND APPLICATION

Commercial mycoinsecticides must be formulated with two objectives in mind:

(1) ease of field application to target insects within their habitats;
(2) enhancement of shelf-life and environmental persistence after application.

These reasons could equally serve as criteria to justify a fungal formulation.

The general considerations and criteria of fungal formulations given by Soper and Ward (1981) are still the primary source of guidance. A formulation attempting to provide the correct combination of the active and final components is crucial for agricultural application. The proper formulation of a fungal agent requires an understanding of the life cycle and, particularly, the biological aspects of not only the fungus but also the target insects concerned. The physical and chemical characteristics of the infective fungal propagules to be formulated affect the final qualities and properties of the resultant formulation. Formulation ingredients selected should at least not interfere with the infection process and at best should enhance fungal viability, virulence, disease transmission and field persistence. Incompatibility of formulation ingredients must be avoided to prevent potential biodegradation. In other words, the compatibility of ingredients used for a formulation means that the infective stages of the fungus formulated have longer shelf-life, higher viability and greater virulence than those unformulated or formulated otherwise. On the other hand, the control of target insects with distinct biological habitats (e.g. soil insects, surface dwellers, foliage eaters, and root, stem or fruit borers, etc.) requires different formulation and application techniques. To adapt field application conveniently, the formulation should be compatible with existing technology, i.e. the application methods of conventional chemical insecticides.

All three developmental stages of *B. bassiana*, i.e. conidia, blastospores and mycelia, have been successfully formulated for small-scale field trials or large-scale application. For convenience, we attempt to consider the formulations of the three forms separately in the following discussion and summarize the practices and achievements in the past decade. Soper and Ward (1981) can be referred to for general information on selection of formulation types, ingredients such as dispersants, surfactants, wetting agents, protectants and other additives, and their physical and chemical features.

Investigations on Conidial Formulation

Conidia of *B. bassiana* (whether aerial or submerged) are smooth-walled, globose and only 2–3 μm in diameter. They can be suspended in aqueous liquid or mixed with a powder carrier and sprayed as mist or dust with conventional equipment used for the application of synthetic chemical insecticides. Dry formulations (in which the active ingredient is formulated and stored until used; Soper & Ward, 1981) are the forms which so far have been considered for *B. bassiana* conidia although oil- and water-based formulations are being tried in experimental trials.

To study the persistence of uncoated, unformulated conidia of four entomopathogenic Hyphomycetes in soil, including *B. bassiana*, an experimental method using a trap technique at 19°C was developed by Fargues and Robert (1985). At various times of incubation, traps were collected to measure changes in biomass and inoculum potential. Inocula of *B. bassiana, Paecilomyces fumosoroseus, N. rileyi* and one isolate of *M. anisopliae* var. *anisopliae* were substantially degraded after 6-months incubation (e.g. 70–80% dry weight loss) whereas the activity of another isolate of *M. anisopliae* remained at the initial level after 21 months; the authors concluded that the persistence of conidia depends on the fungal isolate and that

micro-cyclic conidiation could be implicated in the *M. anisopliae* isolate with high survival of conidia.

Pure conidial powder, mechanically harvested, can be stored unformulated in airtight containers at 4°C and still retain a viability of 71% after 21 months if the water content is below 10% (see Chen *et al.*, 1990). The water content is considered a key factor influencing the shelf-life of conidia. According to Yin (1983), conidial viability of a preparation with 8% water content was 81% after 12-months storage at 4°C, but decreased to 4.5% after 46 months; however, if the water content is increased to 15%, the same preparation could be stored only for 6 months. Formulated with a filler (attaclay X-250) and 12-month storage at 26°C, no significant loss of virulence was observed (see Chen *et al.*, 1990). In a test using quick lime as a desiccant in the storage environment, Shi (1988) found that unformulated conidial powder could be stored under natural conditions for a considerably longer period of time. About 83% viability of the conidial powder was retained after 6-months storage.

For aerial spraying, conidial powder retrieved from storage is usually suspended in water supplemented with an emulsifier or in mineral oil for air spraying. Pan (1988) developed an emulsifier for ultra-low volume sprays of a *B. bassiana* conidial suspension by airplane against pine caterpillars, consisting of 38.9% oxidized resin, 22.2% diesel, 5.6% Na_2CO_3 and 33.3% water. It is processed as follows:

(1) a mixture of the resin and diesel oil is molten at 130°C;
(2) it is slowly added to a solution of Na_2CO_3 while gently stirring and then kept boiling for 'saponification' until foams disappear;
(3) a volume of water equivalent to 50% of the total volume is added and kept boiling (for 1.5–2.0 h) until the excess water evaporates and the desired volume is achieved. The resultant liquid is the desired emulsifier with a pH value of 8.65 and a specific gravity of 1.0; it is brown in colour but becomes milky when mixed with water.

This emulsifier has no negative effect on conidial viability and significantly enhances virulence. Furthermore in this report, the conidial suspension mixed with 12.5% of the above emulsifier (v/v) gave an ideal coverage of the forest canopy. An airplane fully loaded with 800 l of the emulsion can spray about 533 ha (125 g dry powder ($> 1.2 \times 10^{11}$ conidia g^{-1}) ha^{-1}). A mineral oil, referred to as Erxianyou (in Chinese), is also compatible with *B. bassiana* conidia (Pan & Zheng, 1988). Only 2–3 l of the oil conidial suspension ($5–10 \times 10^9$ ml^{-1}) are needed for ultra-low volume air spray of 1 ha (Pan & Zheng, 1988). Recently, ES-87, a wetting agent mainly composed of an emusifiable, neutrally saponified material, was developed in Hubei for use in normal or ultra-low volume sprays of conidial suspensions (see Chen *et al.*, 1990). This wetting agent is recommended for large-scale use because it is as effective as, but cheaper and more readily available than, the emulsifier mentioned above.

At present, the annual production of *B. bassiana* conidial powder in China (about 10 000 tons) has allowed for treatments of 0.8–1.3 million ha for protection against various forest and crop insects (Xu, 1988, 1992; Zhang, 1992; Ying, 1992). Dozens of locations have been established nation-wide for regular mass production. There are numerous records of successful applications. Target insects controlled include up to 30 genera, as listed in Table 1, but primarily are pine caterpillars, European corn borer and rice leaf hoppers. Pine caterpillar control provides good examples of large-scale applications. During the period from 1983 to 1987, emulsions or oil conidial suspensions were sprayed by airplane on to 367 000 ha of forest in 14 counties of Guangdong, causing mortalities of 43–93% (Pan & Zheng, 1988). In 1981, 72% control was achieved in 16 000 ha of forest in Yangjiang County, Guangdong (Pan *et al.*, 1988). During the mid-1980s, 70 tons of conidial powder were produced and applied to about 100 000 ha in Hunan, resulting in 80% control (Mou, 1988). There were 20 000 and 40 000 ha sprayed during 1984 and 1987, respectively, in several counties of Hubei, resulting in 80–90% control (Lü & Zhao, 1988). Zhou (1988) reported 85–90% control of the caterpillar populations by *B. bassiana* preparations in a 39 000-ha forest in Weichang County, Hebei from 1982 to 1987. Not only were the pine caterpillar populations suppressed in locations where *B. bassiana* conidial suspensions were

sprayed but also in nearby sprayed areas, following epizootics facilitated by suitable weather conditions (Xu, 1988). Furthermore, yearly repeated applications of *B. bassiana* preparations resulted in better control of the caterpillar populations in Hubei, where the acreage of annually infested forest has decreased to 200 000–260 000 ha from 530 000 ha (Lü & Zhao, 1988).

The cost for the control of pine caterpillars by *B. bassiana* is low. Based on data from Pan (1988) and Pan *et al.* (1988), ultra-low volume sprays of conidial emulsions by air costs 2.85 Chinese yuan (ca. US$0.7 per ha while aerial dusting costs 12.15 yuan (ca. US$3 per ha. Compared with the use of this emulsion, the cost of oil formulation is higher: 6 yuan (ca. US$1.5 per ha by ultra-low volume spray and 10.5 yuan (ca. US$2.63 per ha by normal low-volume spray.

In Canada, Fogal *et al.* (1986) provided a detailed description of a method for producing conidia of *B. bassiana*, on a bran medium. Procedures were outlined for determining and maintaining spore viability and ensuring a high degree of virulence against cone and seed insect pests of white spruce, *Picea glauca* (Moench). Possible modes of application for use against these insects were also suggested. Strobili of white spruce trees were dusted with conidia just after pollination when insects such as the spruce cone maggot, *Lasiomma anthracina* (Czerny), and spruce seed moth, *Cydia youngana* (Kearfott), are laying eggs. A 55% increase in the number of sound seed was obtained. Dusting the soil with a mixture of conidia (8.5×10^{10} g^{-1}) and wheat flour or talc, applied and mixed with litter and humus at a rate of 3.5–7.5 kg of conidia ha^{-1}, significantly decreased numbers of *L. anthracina* maggots in the soil.

In Cuba, Sanchez *et al.* (1992) reported a pilot trial for the production and formulation of *B. bassiana*. They described the production of a dry (dust) formulation that had a viability of 5.6×10^9 colony forming unit (CFU) ml^{-1}. During the separation of the product, by either filtration (25 l h^{-1}) or centrifugation (50 l h^{-1}), pastes containing 2.6×10^4 and 1.6×10^6 CFU ml^{-1}, respectively, were obtained. The relative humidity of the pastes was 74–77%, 29% after the secondary processing (prior to formulation) and 8% for the final product. The authors described the formulation process including the use of osmotic stabilizer, antioxidant, and granulating and shelf-life extending agents. Analysis of the particle size distribution for the final granulated product showed that 96.7% of the particles were between 0.10 and 0.65 mm in diameter, and the rest were between 1 and 1.6 mm. In assays with the larvae of *Diatraea saccharalis* (Fab) the LD_{50} was 1.13×10^6 CFU ml^{-1} and the LT_{50} was 3.07 days at a dose of 4.6×10^7 CFU ml^{-1}.

In Great Britain, *B. bassiana* has been studied for the control of a range of agricultural pests including *O. nubilalis* and *Hypothenemus* spp. (Jimenez & Gillespie, 1991). In a defined liquid medium the fungus produced a maximum, of 2.7×10^8 conidia ml^{-1} while in a complex medium, blastospores were formed (maximum 1.6×10^9 spores ml^{-1}). On a semi-solid medium (cereal grain), up to 1.9×10^{10} conidia g^{-1} were produced. Submerged and aerial conidia stored in water survived similarly at 5°C and more than 60% were still viable after 180 days. Blastospores survived less well and only 25% were viable after the same time. At 20°C, aerial conidia survived better than submerged conidia. All the stored spore types maintained pathogenicity for at least 180 days.

In the US, attempts were made by Nutrilite Products as early as 1962 to obtain registration of *B. bassiana* as Biotrol, but these were unsuccessful (Ignoffo *et al.*, 1979). More recently, a wettable formulation of *B. bassiana* conidia, based on isolate ARSEF 252 (Humber & Soper, 1986), has been developed by Abbott Laboratories and used in pilot tests against the Colorado potato beetle during the 1980s (Campbell *et al.*, 1985; Hajek *et al.*, 1987; Anderson *et al.*, 1988). Milled vermiculite was used at first to make the wettable conidial formulation but this resulted in serious clogging problems, both with or without nozzle filter screens (Anderson *et al.*, 1988). The problems were eventually overcome by replacing the milled vermiculite with whey solids in the *B. bassiana* preparation (Anderson *et al.*, 1988). No other details have been published about the Abbott formulation.

Little has been published about the shelf-life of the wettable formulation from Abbott Laboratories. However, a serious storage problem due to the adjuvant used in the formulation led

TABLE 1. Control of insect pests by *B. bassiana* preparations in China, adapted from Xu (1988)

Target insect	Plant[a]	Application reported	Control (%)
Dendrolimus spp.	fir, pine	Air spray (1.2×10^8 conidia ml^{-1}), ultra-low volume spray ($5–10 \times 10^9$ conidia ml^{-1}), dusting (3.75–22.5 kg ha^{-1}) or 'cannon of conidial powder' in 12 southern provinces	70–90
Ostrinia nubilalis	corn	Foliar application of granules to plants at whorl stage, spray of conidial suspensions to piled corn stalks post-harvest against overwintering larvae and pupae in Tianjing, Hebei, Shanxi and northeastern provinces	70–95
Nephotettix bipunctatus	rice	Spraying or dusting against first generation in Zhejiang, Hunan, Jiangxi and Hubei	58–84
Lymantria xylina	trees	Spraying, dusting or 'conidial cannon' on the coast of Fujian	70–90
Autexix cinnamoni	trees	Spray (2×10^8 conidia ml^{-1}) or 30–45 'conidial cannons' (10^{10} conidia g^{-1}) ha^{-1} (ca. 6 kg)	51–87
Empoasca formosana	tea	Air spray of conidial suspensions	95
Curculio sp.	tea	Air spray of conidial suspensions	54–79
Cylas formicarius	sweet potato	Application of conidial suspensions to furrows while being fertilized (6 kg powder ha^{-1})	>90
Leguminivors glyeinivorella	soybean	Dusting when larvae stay out of pods during the fall; field trials in Jinin and Shandong	50–80
Bothynoderes penetiventris	beet	Dusting of overwintering habitats (conidia: dry soil = 1:6)	75
Nilapavata lugens	rice	Dusting of 0.5% conidia powder	72
Cuculio nucum	NA	Caged trials in the field	>90
Curculio devidi	NA	Spray of 10% conidial suspension on to insects (A) or soil surface (B)	A: 72; B: 24
Parametriates theae	tea	Caged trials in the field	75
Aleidodes sp.	trees	Air spray (5×10^8 conidia ml^{-1})	60–81
Dasychira argentata	trees	Ultra-low volume spray of emulsifiable or oil conidial suspensions	60–85
Pyrrhalta aenescens	trees	Air spray ($0.5–2 \times 10^8$ conidia ml^{-1})	70–80
Ceratomia amyntor	trees	Air spray ($0.5–2 \times 10^8$ conidia ml^{-1})	67–100
Cryptorrhynchus lapathi	trees	Spray or injection of suspensions into tunnels from openings	73–89
Phassus excrescens	trees	Suspensions applied to tunnel openings	73–83
Agrotis sp.	Chinese cabbage	Dusting of conidial powder to plants at whorl stage	68–81
Holotrichia diomphalia and *H. morosa*	crops	Application of conidia–soil mixture (1:30) to furrows when seeding or weeding	60–70
Euxoa segetum	crops	Spray of 0.5% conidial suspension plus a small quantity of insecticide	74
clover moth	clover	Air spray (10^8 conidia ml^{-1})	68–96
Paranthreae tabaniformis	trees	Stopping of tunnel openings with cotton balls soaked with conidial suspensions	80–90
Adoxophyes privatana	tea	Air spray of conidial suspensions	80–85
Odontotermes fomosanus	trees	Spray of trunks inhabited	82–100
Epilachan vigintiec timasulata	potato	Foliar spray of conidial suspensions	>92
Agrotis sp.	crops	Spray of 2% conidial suspension	67–90
Tessaratoma papillosa	litchi	Spray of conidial suspensions	>80
Tryporyxa incertulas	rice	Treatment of residues with conidial suspensions (500–700× dilution) against overwintering larvae and pupae	78–80
Musca domestica	NA	Spray of 2% conidial suspension to habitats	60–100
Delia fleralis	cabbage, radish	Apply 2% conidial suspension to roots (or nearby) of plants	80–89

[a] NA: not available.

to rapid loss of >90% viability before application and affected the outcome of the field experiments during 1984 (Anderson *et al.*, 1988; Hajek *et al.*, 1987). The Abbott conidial preparation was usually maintained at 4°C and 0% relative humidity (Studdert & Kaya, 1990a, 1990b). There are, however, a few reports regarding the environmental persistence of the formulation. Sprayed through an overhead irrigation system, conidia suspended in peanut oil stayed in the upper 5-cm layer of soil more (95%) than those suspended in water (67%) 1 h post-application (Storey *et al.*, 1987); more water-suspended conidia were recovered in the 10- and 20-cm deep soil samples (23% and 10%, respectively). Seven days later, the number of CFUs recovered in the treatment with the oil-suspended conidia decreased 10–100 fold in the 5-, 10- and 20-cm deep soil samples, but no CFUs were recovered in the treatment with the water-suspended conidia (Storey *et al.*, 1987). According to Storey and Gardner (1988), the number of the CFUs recovered from depths below the soil surfaces 72 h after application of the formulated conidia in suspension was significantly correlated with water infiltration values of different soil types. The survival of the formulated conidia in the soil was found largely to depend upon soil water potential, temperature and soil type (Studdert & Kaya, 1990a). The longest half-life was 44.4 weeks for conidia mixed with the Yolo fine sandy loam soil at a water potential of −10 bars and a temperature of 10°C, and the shortest half-life was 0.3 week for conidia mixed with the Staten peaty muck soil at 0 bar and 28°C. Studdert and Kaya (1990a) also found that clay-coated conidia could survive longer than uncoated conidia regardless of combinations of soil water potential, temperature and type.

Results from field experiments using the Abbott's wettable formulation against the Colorado potato beetle were encouraging although they differed from year to year. Campbell *et al.* (1985) found no difference in egg mass size and defoliation rate by the first generation of the beetle between the *B. bassiana* (5×10^{13} CFU ha^{-1}) and fenvalerate (227 g active ingredient ha^{-1}) treatments in 1980. Although the larval populations of the first generation were larger in the *B. bassiana* treatment, oviposition by the first-generation adults and the second-generation beetle populations were lower than those in the fenvalerate treatment. As a result, the average yield of potatoes was 29.4 tons ha^{-1} for the fenvalerate treatment, 24.6 tons ha^{-1} (16% reduced) for the *B. bassiana* treatment and <7 tons ha^{-1} (>60% reduced) for the untreated control (non-spray). Anderson *et al.* (1988) reported that 98% and 64% of the beetle larvae collected from field plots sprayed at dosages of 5×10^{13} and 5×10^{12} CFU ha^{-1}, respectively, in 1983 developed *B. bassiana* mycoses when held at >90% relative humidity; in 1985, 65.8% and 39.5% of the larval populations were reduced by the same high and low dosages. In a pilot test including several states in northeastern USA (Hajek *et al.*, 1987), average potato yields from plots treated with *B. bassiana* (5×10^{13} and 5×10^{12} CFU ha^{-1}) were greater than yields from control plots in 8 of 24 trials. However, the potato yields in two plots treated with *B. bassiana* or with locally recommended insecticide did not differ. An altered plot design providing within-site replication in 1985 demonstrated increased yield with increased *B. bassiana* dosages when initial egg density was used as a covariate; a minimum dosage of $5.39–6.62 \times 10^{11}$ CFU ha^{-1} was required for the yield increase (Hajek *et al.*, 1987). A conclusion from these experiments was that foliage application of the wettable *B. bassiana* formulation from Abbott Laboratories could not compete with the use of the then existing chemical insecticides for potato yield increase although it may have significantly reduced the beetle population levels. In an experiment to evaluate the efficacy of the Abbott formulation against pupae of the beet armyworm, *Spodoptera exigua* (Hübner), under different soil conditions, adult emergence was reduced to 6% at the dosage of 1×10^{8} conidia cm^{-3} of soil (Studdert & Kaya, 1990b). Recently, Wright and Chandler (1992) reported that conidial formulations of ARSEF-252 and ARSEF-3097 applied with a feeding substrate and an attractant significantly reduced the population density of the boll weevil, *Anthonomus grandis grandis* Boheman, in field plots, resulting in increased lint yields of 74–113% compared with untreated controls.

During the mid-1980s, a new *B. bassiana* preparation, Boverol, was developed in Czechoslovakia (Dirlbecková *et al.*, 1987; Kybal & Kálalová, 1987). The formulation is a whitish powder, insoluble in water. It is applied as a water suspension alone or in combination with selected

pesticides and wetting agents. The formulation contains $>10^{10}$ conidia g^{-1} with >70% viability. Boverol can be stored at temperatures $\leq 10°C$ for 12 months (Kybal & Kálalová, 1987). In the laboratory and small plot field experiments, spraying Boverol against larvae of the Colorado potato beetle, at a dosage of 500–700 g ha^{-1} in 300 l water, resulted in satisfactory control of the first- and second-instar larval populations (Dirlbecková *et al.*, 1987). In another pilot field trial, this group used a dosage of 1 kg ha^{-1} during the peak of those larval stages and obtained 38–59% mortality several days after application. The population density was decreased by 36% after 7 days and by 50% after 15 days. An 80% control was achieved when a mixture of Boverol and an insecticide (Decis) was applied (Dirlbecková *et al.*, 1987). Recently, another *B. bassiana* preparation has been registered under the trade name Boverosil in Czechoslovakia and a yet-to-be named preparation has been mass produced in Poland (Lipa, 1991).

Conidia of *B. bassiana* have also been formulated as granules for application against the European corn borer. Bing and Lewis (1991) prepared a granular formulation by spraying conidial suspensions on to corn grits (30–40 mesh) in the rotating drum of a Gustafson minimixer. Hand-held inoculators were used to apply 0.4 g of the granules (containing 4.55×10^7 conidia) to each plant whorl at the whorl stage (56 000 plants ha^{-1}). As a consequence of the treatment, *B. bassiana* was able to colonize the corn plant at the whorl stage, move within the plant and persist within it to provide season-long suppression of the borer population. Recently, *B. bassiana* conidia produced using Mycotech's surface culture method has been formulated in bran bait and oil for grasshopper control (Goettel & Roberts, 1992) but results from field trials were inconsistent (Johnson *et al.*, 1992).

In the USSR (former), all *B. bassiana* preparations have been referred to as Boverin regardless of their infective forms. The preparations might be conidia if any surface conidiation was involved in the production process (see Goral, 1978); otherwise, they might primarily consist of blastospores or mixture of blastospores and conidia if produced only by submerged fermentation (see Belova, 1978; Lappa, 1978). The scale of Boverin production has been very large. Yevlakhova (1978) mentioned an annual Boverin output of 22 tons in an experimental production plant located in St Petersburg. The annual application of Boverin in the USSR (former) was said to be >10 000 ha (Deacon, 1983) mainly against Colorado potato beetles and codling moth (Lappa, 1978). In a field experiment in the US, spraying 5% Boverin preparation (7.4×10^{14} conidia ha^{-1}) in field plots of kale resulted in about 50% reduction of larval populations of the cabbage looper and 87% reduction of leaf damage; the half-life of the Boverin preparation on kale and soybean foliage was <24 h (Ignoffo *et al.*, 1979). Sikura and Sikura (1983) reported that over 90% mortality of Colorado potato beetle larvae was caused by a dosage of 3 kg of Boverin (2×10^9 conidia g^{-1}) ha^{-1} against the first instar, 6 kg ha^{-1} against the second instar and 11 kg ha^{-1} against the third instar. For practical application, it was recommended to use 2 kg ha^{-1} Boverin mixed with one-quarter of the dose of any insecticide registered against the pest. Two treatments during the growing season were suggested, the first during the mass occurrence of second-instar larvae and the second 10–14 days later. Full protection of potato fields was attained in the Ukraine, Byelorussia, the Baltic republics and in the eastern regions of the USSR (former). However, Sikura and Sikura (1983) did mention that Boverin had not yet been as widely used in the USSR (former) as one may expect.

In Belgium, under greenhouse conditions, Tillemans and Coremans-Pelseneer (1987) obtained up to 84% control of introduced larvae of the black vine weevil, *Otiorhychus sulcatus* F., after an application of 2×10^8 conidia of *B. brongniartii* per litre of peat.

Because of the incubation period of disease development, the control of insects with conidial formulations of *B. bassiana* alone is often slower than with chemical insecticides. Control is often expected to depend on combinations of both *B. bassiana* preparations and pesticides. Studies have shown the varying compatibility of *B. bassiana* preparations with several chemical insecticides, fungicides and herbicides. According to Anderson and Roberts (1983), four wettable powder insecticide formulations (carbaryl 50, azinphos methyl 50, diflubenzuron 25, endosulfan 50) and two flowable insecticides (carbofuran 4, azinphos methyl 2) were compatible with *B. bassiana* conidia whereas five emulsifiable concentrates (endosulfan 3, fenvalerate 2.4, perme-

thrin 2, piperonyl butoxide 8, oxamyl 2) and two others (piperonyl butoxide technical, permethrin 25WP) showed varying degrees of inhibition to conidial germination (4–99%). Recently, Anderson *et al.* (1989) reported that each combination of abamectin 0.15EC, treflumuron 4F, thuringiensin ABG-6162 (1.5% active ingredient) and carbaryl 50WP with *B. bassiana* did not significantly inhibit *B. bassiana* colony growth in *in vitro* tests. In bioassays with neonates of the Colorado potato beetle, combinations of *B. bassiana* with these insecticides were consistently more toxic than *B. bassiana* alone, with narrow limits of variation in toxicity among the combinations relative to the fungus. This indicated that the effect of each combination is additive (Anderson *et al.*, 1989). In a report from Loria *et al.* (1983), fungicides such as chlorothalonil and metalaxyl had no detrimental effect on the survival of *B. bassiana* conidia in both laboratory and field tests while mancozeb was most detrimental to the conidia; between the detrimental and non-detrimental fungicides was metiran, which was only slightly less inhibitory to the conidia than was mancozeb *in vitro* but was not different from the control treatment under field conditions. However, Aguda *et al.* (1988) demonstrated that the fungicides benomyl and edifenphos and the insecticide carbaryl inhibited germination of three Hyphomycetes, including *B. bassiana*, at concentrations of 0.1, 1, 10, 100 and 1000 ppm. In a laboratory bioassay with several entomogenous fungi on agar plates containing different herbicides, Poprawski and Majchrowicz (1993) found that the active commercial products Betanal, Dual and Pyramin either totally impaired or strongly inhibited the development of *B. bassiana* and other fungi when tested at 15 and 25°C at the manufacturer-recommended concentrations. The effect of the fourth herbicide, Venzar, on the development of *B. bassiana* was reversibly inhibitory at the recommended concentration or 10-fold of it but became temporarily stimulatory at one-tenth of the recommended concentration. Therefore, Poprawski and Majchrowicz (1993) recommended the use of Venzar in integrated pest control programmes where fungal biocontrol agents are involved.

Practical applications of *B. bassiana* preparations in combination with insecticides have often been reported. In addition to the tests with Boverin and Boverol mentioned above, Lü (1988) listed 15 cases in which ultra-low volume spray by airplane of a *B. bassiana* conidial suspension ($9–18 \times 10^{12}$ conidia ha^{-1}) containing NRDC 156 (6 g AI ha^{-1}) resulted in 85–99% control of pine caterpillar populations overwintered in Hubei. These results were better than those resulting from the use of *B. bassiana* alone (70–90%) (Xu, 1988). Presently, combinations of *B. bassiana* with carefully selected insecticides have been recommended for practical application against various crop and forest insect pests in China. In caged field experiments, the mortality of the Colorado potato beetle caused by combinations of *B. bassiana* (5.3×10^9 CFU l^{-1}) with the *Bacillus thuringiensis* toxin, thuringiensin, at 0.1 ppm, carbaryl at 70 ppm, abamectin at 0.004 ppm, triflumuron at 10 ppm or fenvelerate at 0.2 ppm was generally higher than that caused by the individual agents (Anderson *et al.*, 1989).

Investigations on Blastospore Formulation

Blastospores of *B. bassiana* produced in liquid culture are characterized by their smooth wall and ellipsoidal shape (6.5×2.5 μm). This morphology and their instability in the drying processes determine that blastospores can only be formulated and applied in limited ways. Clogging may occur if blastospores are formulated for air spray with conventional equipment.

In France, Fargues *et al.* (1980) evaluated the field efficacy of *B. bassiana* against larval populations of the Colorado potato beetle. Preparations used in the trials were either a suspension of blastospores in an aqueous solution of a wetting agent (0.05% Etaldyne 95) or an aqueous formulation of lyophilized (after washing and addition of 10% skimmed milk supplemented with 0.1% glycerin) clay-coated blastospores (the latter process is described in Reisinger *et al.* (1977)). Fargues *et al.* (1983) found that the action of the fungus (both preparations) on the insect populations *in situ* in the leaf canopy continued after insects burrowed into the soil; that one application of 10^{14} blastospores ha^{-1} significantly reduced larval populations (up to 100%) in 7 to 15 days; and that the fungus retained its infectivity on the foliage for over 1 week.

A granular formulation of blastospores of *B. bassiana* produced in submerged culture was used

for the control of the European corn borer in northern China during the 1970s. Despite low production cost and considerably high yield, the blastopore formulation had serious storage problems. The maximal shelf-life was 5–8 months under dark, dry and cold ($\leq 0°C$) conditions (Yin *et al.*, 1988b). As reviewed by Chen *et al.* (1990), the blastospores dried and formulated as granules (1.2–1.4 mm in diameter) with 1% water content, were relatively stable during the first 5-months storage at $\leq 0°C$ but dramatically lost their viability thereafter. The average viability was 50% after 8 months, 40% after 10–12 months and only 3–10% after 12–23 months. Spray-dried blastospores lost 83% and 91.5% of their viability when stored at 13–15°C for 30 and 60 days, respectively, and few remained viable when stored at ambient temperatures for 30 days during the summer. Therefore, the granular formulation of *B. bassiana* blastospores was used in the field only when fresh or stored for a short period of time (Xu, 1988), and its production was terminated in China when the mechanized production technology of pure conidial powder was developed.

According to Belova (1978), Boverin preparations produced in the USSR (former) were subject to similar storage problems. Freeze-dried blastospores could be stored at most for 3 months. In the soil environment, the survival of blastospores was reported varying between treatments (Fargues *et al.*, 1983). Naked blastospores were inactivated after 3 weeks whereas clay-coated blastospores remained active after 2 months at 20°C. Clay-coating was thus considered to prevent the blastospores from biodegradation by soil antagonists. Fargues *et al.* (1979) reported that spray drying at 150°C of *B. brongniartii* conidia (produced on solid medium) coated in bentonite gave a yield of 50–70% viable spores. In contrast, blastospores were too sensitive to this technique and were instead lyophilized on trays after mixing with powdered milk supplemented with glycerin; this resulted in 89% viable spores. The longevity of the two formulations was monitored at various temperatures by testing spore viability and infection potential. The viability of atomized conidia and that of lyophilized blastospores was not affected after 18- and 8-months storage, respectively, at 5°C. Bioassays against *L. decemlineata* and *Plutella maculipennis* Curtis larvae confirmed the need to store these biopreparations at 5°C.

Field trials in Switzerland and France demonstrated that blastospores of *B. brongniartii* introduced into populations of the cockchafer, *Melolontha melolontha* L., caused epizootics and the collapse of the pest population. When blastospores were incorporated in the soil at 2×10^{13} and 2×10^{14} spores ha^{-1}, they caused 14.4–56.6% and 38–80% mortality, respectively, in the larval stage of the chafer (Ferron, 1978b). In Switzerland, swarming adults were treated with $2–4 \times 10^{14}$ blastospores ha^{-1} (skimmed milk was added as a sticker and UV-protectant) at 14 feeding sites over an area of 89 ha and the females spread the disease to 85% of their progeny in a breeding area of 4000 ha; the disease became established and the effect persisted for two generations (Keller *et al.*, 1986; Keller & Zimmermann, 1989; Keller, 1991).

In the Paris (France) basin, field tests with a granular formulation of blastospores of a strain of *B. bassiana* caused 96% mortality of European corn borer larvae and a 14% increase of corn yield at dose of 10^{12} spores ha^{-1} (Riba, 1984). The results were superior to those obtained with 10^{12} spores ha^{-1} when an aqueous formulation of the same strain was applied, and equivalent to those obtained with an insecticide treatment.

Investigations on Mycelial Formulation

Incorporation of mycelium or spores into alginate pellets, with or without additional nutrient sources, was originally developed to formulate fungal biocontrol agents of weeds (Walker & Connik, 1983) and of soil-borne plant pathogens (Lewis & Papavizas, 1985, 1987; Lewis *et al.*, 1989; Knudsen *et al.*, 1990a, 1990b), and has been recently applied to formulate *B. bassiana* (Knudsen *et al.*, 1990c, 1991; Pereira & Roberts, 1991). One advantage of the alginate pellet formulation is enhanced shelf-life and environmental stability after application. Recently, Lewis *et al.* (1991) devised a new mycelial formulation for fungal biocontrol agents of soil-borne plant pathogens that does not require sterile conditions during preparation and is likely to be useful for *B. bassiana*. Additionally, methods have been developed to make cornstarch and cornstarch oil formulations of *B. bassiana* mycelia as well as pure dry mycelial preparations (Pereira &

Roberts, 1990, 1991). *B. bassiana* mycelial preparations pelletized with alginate and formulated with cornstarch or cornstarch oil are not only stable during storage at room temperature but also have an enhanced potential of conidial production after several months of storage. This advantage does not exist in any other *B. bassiana* formulation known to date. Also, the formulated mycelial preparations have better potential of conidial production than the pure dry mycelia after the same period of storage at the same temperature.

Formulation of *B. bassiana* mycelia in pellets starts from liquid cultivation. Commercially available media such as Sabouraud dextrose broth plus 1% yeast extract and Difco potato dextrose broth can be used to initiate the culture (Knudsen *et al.*, 1990c). Pereira and Roberts (1991) used a liquid medium consisting of 1% dextrose and 1% yeast extract. Antibiotics such as streptomycin sulphate and/or penicillin may be added to the liquid culture to prevent possible bacterial contamination (Knudsen *et al.*, 1990c; Pereira & Roberts, 1990). From fungal growth medium, the mycelia are harvested on a wire-mesh filter, rinsed with sterile water, then added to a 1% aqueous sodium alginate solution at a rate of 37 g wet mycelium per 100 ml alginate solution (Knudsen *et al.*, 1990c). The mixture is gently stirred until the mycelia appear evenly dispersed. Pellets are made by dropwise adding of the mycelium–alginate mixture into 0.25 M aqueous $CaCl_2$. Before the pellets are formed, 2 g of wheat bran can be added to each 100 ml of the mycelium–alginate mixture. Pellets formed in the $CaCl_2$ solution can be removed within 10 min, rinsed with sterile water and allowed to air dry on waxed paper for 24 h. The method used by Pereira and Roberts (1991) is somewhat different from that in Knudsen *et al.* (1990c). They prepared their pellet formulation by first adding 5 g of sodium alginate to 10 ml of 100% ethanol, and immediately pouring 500 ml of spent liquid culture to which the corresponding amount of mycelial paste had been added. The fungal culture was then mixed with an equal volume of a 4% (w/v) solution of calcium chloride ($CaCl_2.2H_2O$). An insoluble precipitate was formed, entrapping the mycelia. The resultant material was blended for 40 s to further break the alginate–mycelium particles and promote complete exposure of sodium alginate to the calcium chloride solution. After a 2–3 min reaction period, the liquid phase of this mixture was filtered out. Mycelia trapped in a matrix of calcium alginate were transferred to metal trays and allowed to dry in a fume hood.

The cornstarch formulation used by Pereira and Roberts (1991) was made by mixing mycelial paste from 500 ml of liquid culture with 25 g of gelatinized cornstarch. The gelatinized cornstarch was produced by cooking cornstarch in deionized water, autoclaving for 30 min and then mixing with 10 ml ethanol to precipitate the starch in the gelatinized form, which was allowed to dry to a powder. The gelatinized cornstarch absorbed the liquid culture and entrapped the mycelia in a rubbery material. The formulation was allowed to harden for 3–4 h and then was broken into small pieces and finally mixed with 50 g of ungelatinized cornstarch in a blender. The particles were allowed to dry overnight in a fume hood. The cornstarch oil formulation was prepared using the same procedure, but 15 ml of sunflower oil were added to the mycelial paste before the addition of gelatinized cornstarch.

On average, each 100 ml liquid culture yielded 362 ± 146 air dried pellets (4.6 ± 0.6 mg/pellet) without wheat bran and 254 ± 29 pellets (9.4 ± 0.6 mg/pellet) with the bran (Knudsen *et al.*, 1990c). Pereira and Roberts (1991) report that the yields for pure mycelium, alginate, cornstarch and cornstarch oil formulations were 8.1, 15.6, 90.1 and 166.8 g l^{-1} of culture medium respectively.

Mycelia in alginate pellets sporulate very well after rehydration. After 5-months storage at room temperature, pellets without the bran yielded $1.77 \pm 0.1 \times 10^8$ conidia each whereas pellets with the bran produced $2.54 \pm 0.17 \times 10^8$ conidia each (Knudsen *et al.*, 1990c). The inclusion of wheat bran in the alginate pellets appeared to increase the size and conidial production of individual pellets but did not enhance the total number of conidia produced by all pellets from a given amount of liquid culture. Yields of conidia were significantly different between the 'with-bran' and 'without-bran' pellets when based on conidial production per pellet; they were similar when based on conidial production per 100 ml of liquid culture (pellets with bran:

6.41×10^{10} conidia per 100 ml ($= 3.84 \times 10^{10}$ conidia g^{-1}); pellets without bran: 6.45×10^{10} conidia per 100 ml ($= 2.70 \times 10^{10}$ conidia g^{-1})).

Pereira and Roberts (1991) compared the recovery and stability of four *B. bassiana* mycelial preparations stored at different temperatures. Higher conidial production was reported after storage at 4 or 22°C and there were no significant differences between storage at room temperature and at 4°C. Each gram of alginate pellets produced $0.08 \pm 0.003 \times 10^{10}$ conidia when fresh, but $0.19 \pm 0.015 \times 10^{10}$ at 4°C and $0.19 \pm 0.075 \times 10^{10}$ at room temperature after 13 weeks. The corresponding conidial yields were 2.85 ± 0.637, 6.27 ± 0.188 and $4.60 \pm 2.514 \times 10^{10}$ conidia g^{-1} of cornstarch formulation; 0.63 ± 0.095, 2.60 ± 0.479 and $7.27 \pm 3.578 \times 10^{10}$ conidia g^{-1} of cornstarch oil formulation; and 0.83 ± 0.088, 2.33 ± 0.275 and $3.68 \pm 0.917 \times 10^{10}$ conidia g^{-1} of pure dry mycelia. Furthermore, the pelletized formulation was most resistant to simulated solar radiation. After 48 h constant exposure to simulated solar light (radiation level = 1.3 kW m^{-2} and temperatures as high as 57°C), about 80% of *B. bassiana* alginate pellets remained active and sporulated very well whereas $< 10\%$ of pure dry mycelia and cornstarch oil particles could produce conidia.

It has also been reported that pure dry mycelia treated with maltose or sucrose and stored at 4°C for 4.5 months produced more conidia, when rehydrated, than those stored at 4°C for 1.5 months followed by 3 months at room temperature; in contrast, dry mycelia unsprayed with sugar or sprayed with water lost almost all of their conidiation potential after the same period of storage at either of the temperature regimes (Pereira & Roberts, 1990). Rombach *et al.* (1988b) found that, stored at 5°C, *B. bassiana* dry mycelia without sugar treatment gradually reduced their potential for production of conidia during a period of up to 32 weeks; however, significantly fewer conidia were produced after 8 weeks at 25°C and no viable mycelia were left after 1 week at 35°C. Therefore, it was concluded that dry mycelia not treated with sugar could be stored only at < -15°C for long periods (Rombach *et al.*, 1988b).

In field experiments, caged populations of insect pests of rice such as the brown plant hopper, *Nilaparvata lugens* (Stal), and the black bug, *Scotinophara coarctata* (F.), were reduced significantly with application of suspensions of dry mycelial particles of *B. bassiana* (Rombach *et al.*, 1986; Aguda *et al.*, 1987). In a laboratory bioassay, a population of the greenbug, *Schizaphis graminum* (Rondani), was significantly reduced by application of rehydrated alginate pellets to potted wheat plants which had been artificially infested with the pest (Knudsen *et al.*, 1990c). According to Pereira and Roberts (1991), mortality of the southern corn rootworm, *Diabrolica undecimpunctata howardi* Barder, caused by equal dosages of mycelia were 92, 93, 100 and 68% for formulations of alginate pellets, cornstarch, cornstarch oil and pure dry mycelia, respectively, stored at 4°C for 13 weeks. The mortality of these insects decreased to 37, 84, 67 and 10%, respectively, for the four formulations stored at 22°C for 13 weeks. Knudsen *et al.* (1991) found that *B. bassiana* alginate pellets treated with a 40% aqueous solution of polyethylene glycol (PEG) 8000 for 24 h before storage at room temperature produced conidia far more rapidly than those not treated after rehydration.

Kinzel (1992) has reported a technology for the economical mass production of filamentous fungi for extraction of metabolites. The technology described as an attached-growth biological reactor (a US Department of Agriculture invention) compensates the problems certain fungi have trying to grow in standard fermentation tanks while it facilitates their successful harvest. A key to the invention is eliminating the need to ferment fungi in submerged tanks. Rather, a horizontal cylinder inside the reactor is submerged to half its depth in a nutrient solution. Sterile air is pumped into the reactor through a pipe. A shaft continuously rotates the cylinder, so the fungus gets an equal distribution of nutrients and oxygen. After 12 to 19 h, an inoculum is added to the nutrient liquid in the reactor, and the fungus begins attaching to the rotating cylinder. It continues to grow outwards until it eventually covers the entire cylinder. The attached material is periodically shaved off the cylinder and caught in a small pan. Once harvested, the fungal growth can be retrieved for extraction of desired compounds. The inventors have agreed to test their technology on the production of other entomopathogenic fungi including *B. bassiana*.

BIO 1020, a microbial preparation based on *M. anisopliae*, has been recently developed for use

in horticultural crops by Bayer AG in Germany (Bayer, 1990). The fungus is produced under controlled conditions in large fermentation tanks containing a nutrient-rich liquid. The conditions are such that the fungus grows in the form of small (0.5–1.0 mm in diameter), very compact pellets. After 3 days the pellets are harvested and dried. The finished product is a uniform granulate. The fungus remains viable in granulate form for more than 12 months, forming the conidia necessary for infection upon water uptake when mixed with soil, peat or other growing substrates.

SUMMARY

The body of information compiled in this review allows for a global overview of the status of basic and applied research that has been undertaken to use *B. bassiana* for biological control of insect pests. The lack of commercially available *B. bassiana* and other mycoinsecticides in North America and Western Europe indicates the difficulties encountered in the development of entomopathogenic fungi for insect pest control. It is clear that among the several methods of mass production of *B. bassiana* the automatically mechanized diphasic technology developed in China is most encouraging and promising for practical application. Not only is this technology easy and economical but it also yields large quantities of inoculum; the latter is a pure conidial powder of high quality ($>1.8\times10^{11}$ conidia g^{-1}, 94.6% viability) and can be stored for considerably long periods at 4°C, or in tanks over quick lime as a desiccant at natural temperatures. More important is that the conidial powder can be easily formulated as an aqueous emulsion or suspended in mineral oil, and sprayed at low or ultra-low volume.

Submerged fermentation technology remains some distance from practical application because of several factors. Firstly, submerged fermentation usually gives rise to blastospores which are often subject to considerable viability loss during harvest, formulation and storage even under controlled conditions. Secondly, although submerged conidia are known to be more stable during storage than the blastospores and can be produced by carefully controlling components of liquid media and the fermentation conditions, the yield is too low and the cost of production too high for commercialization. With a maximum yield of 5×10^{8} submerged conidia ml^{-1} of liquid culture, a field treatment at a rate of 5×10^{13} conidia ha^{-1} requires 100 l of the original culture if there is no loss of conidial viability during harvest and formulation (which is impossible in practice). Any attempt to use fermentation technology for mass production of submerged conidia of *B. bassiana* becomes impractical unless the conidial yield is increased to $>5\times10^{9}$. Thirdly, mycelial preparations of *B. bassiana* need more study to evaluate their field efficacy before consideration for practical use. The pelletized formulation of fungal materials was originally developed for use in the soil environment against weed roots and soil-borne plant pathogens. Likewise, alginate pellets of *B. bassiana* may be more promising against soil insect pests and those insects with at least one stage of their life cycle in soil.

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