Moroccan Journal of Biology 07-2008/N 4-5

Isolation and characterisation of yeast strains for the olive fly *Bactrocera oleae* biological control

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Abstract

Bactrocera oleae (Diptera: Tephritidae) is one of the major pests of the olive fruits all around the Mediterranean basin. Our laboratory is interested in developing microbial strategies to fight this insect. During *B. oleae's* breeding, certain pupas were unnable to accomplish their development cycle. These pupas were used to isolate pathogenic yeast strains against *B. oleae*. Two strains were shown to be particularly interesting. Their actions during different stages of *B. oleae*'s development were determined. The two strains were identified as *Pichia guilliermondii* and *Debaryomyces hansenii* according to both conventional and molecular methodologies.

Key words: *Bactrocera oleae* (Diptera: Tephritidae)*, Bio-control, Debaryomyces hansenii, Pichia guilliermondii, Yeast strains.*

Introduction

The olive fruit's fly *Bactrocera oleae (*Diptera: Tephritidae) is one of the major pests of the olive trees around the Mediterranean basin. In fact, the damages caused by this insect are very important (Arambourg, 1986): the infested olives become black and fall before maturation; and the quality of oil is degraded (high degree of acidity, low organoleptic quality and unpleasant flavour). This leads to the decrease of the commercial values of the olives and their oil. Furthermore, it is important to note that the international regulations tolerate less than 2 % of *B. oleae* infested olives.

The control of *B. oleae* population is vital. In this regard, the predominant method of control is based on the use of conventional pesticides. In fact, applications of some organophosphate compounds along with attractants are mainly used to kill *B. oleae* adults. However, such insecticidal treatments, usually spread by aircraft over vast areas, have ecological and toxicological side effects such as environmental contamination, action on untargeted organisms, severe outbreaks of other secondary pests, and persistence of insecticide residues in the olive oil mainly caused by high concentrations of lipophilic pesticides (Delrio, 1992).

Great efforts have been made to improve *B. oleae* control including the male sterilization technique (Tzanakakis, 1967) and the use of pheromone baits (Haniotakis *et al.* 1987, Broumas *et al.* 1990). Natural endoparasites have also been used (Arambourg, 1986).

Alternative methods based on yeasts's killing activity have been developed in bio-control of phytopathogenic micro-organisms and pathogens in agriculture and agro-alimentary industries (Bevan & Makover 1963, Young 1987, Golubev 1998, Buzzini & Martini 1999, 2001,

Janisiewcz & Korsten 2002). There exists a limited number of pathogenic yeast species and they generally belong to *Candida*, *Cryptococcus, Torulopsis*, *Trichospores*, *Rhodotorula*, *Saccharomyces* and *Malassezia* genera. These yeasts are known as "opportunists" i.e. that they are frequent in the environment but do not parasitize any host unless the latter presents intrinsic or extrinsic conditions supporting yeasts penetration, adhesion and multiplication *in vivo* (Drouhet & Dupont, 1979). The yeast killer toxins (mycocines) are suspected to play a significant ecological role like a form of amensalism implying various yeasts (Buzzini & Martini, 2000, 2001).

Species of Hyphomycetes cause occasional high infection levels in insect populations despite the fact that during the last years, several species have been and still are used and engineered as biocontrol agents (Tanada & Kaya 1993, Hajek & St. Leger 1994). Strains such as *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Metschnikoff), *Verticillium lecanii* (Zimmermen) and *Paecilomyces fumosoroseus* (Wize) are currently used for insect control (Lacey *et al.*, 2001).

Many higher fly species have been reported to suffer high infection levels caused by fungi. *Entomophthora muscae* infects several species (*Delia antigua*, *Delia coarctata, Chamaepsila rosae*) and flies associated with animal production (*Musca domestica*, *Fannia canicularis*) (Konstantopoulou and Mazomenos, 2005).

Recently, Konstantopoulou & Mazomenos (2005) have evaluated the virulence of some fungi against *B. oleae* adults, in particular *Beauveria bassiana*, *Beauveria brongniartii* and other species isolated from *B. oleae* pupae and *Sesamia nonagrioides* larvae. They have demonstrated that the *B. oleae* fly is sensitive to different tested isolates of toxic fungi. *Mucor hiemalis*, isolated from *Sesamia nonagrioides* was the most virulent to *B. oleae*. Its high toxicity was related to some metabolites production.

The objective of this study was to isolate pathogenic yeast strains from dead pupas and evaluate their toxicities against *B. oleae* adults in order to use them in different bio-control strategies.

Materials and methods

Yeast culture medium

YPG medium (1% yeast extract, 2% peptone, 2% glucose; pH 4.5; Kreger-Van Rij (1987) was used for yeast's isolation and culture. This medium was supplemented with three antibiotics (Ampicillin (100 µg/ml), Kanamycin (30 µg/ml) and Tetracyclin (30 µg/ml) to avoid any bacterial contamination.

Yeast isolated from dead pupas

We isolated the endogenous microflora of the dead pupas obtained during the breeding of *B. oleae* in our laboratory. To eliminate the micro-organisms contaminating the surfaces, the pupas were incubated for 2 seconds in ethanol 70%, 3 minutes in sodium hypochlorite 5% and five minutes in sodium thiosulfate 10%. The pupas were then washed three times in sterile distilled water (Cavados *et al.*, 2001). The treated pupas were crushed in 400 µl of YPG. A volume of 100 µl of various dilutions (10^{-1} to 10^{-12}) was then spread on YPG agar medium containing three antibiotics (see below). The yeast strains obtained were preserved at 4°C for frequent use and at -20°C for long storage.

Identification of yeast strains

Yeast identification was carried out by a molecular procedure based on PCR amplification of the ribosomal RNA 5.8 S using specific primers of the preserved areas. The ITS1 (ITS-1: 5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers allow the amplification of 5.8 S rRNA region located between the 18 S

rRNA and 28 S rRNA (Drik, 2000). The amplification reaction was performed in a final volume of 50 µl containing 50 pmol of each primer (ITS-1 and ITS-4), 200 µM each dNTP, 0.5 units Taq DNA polymerase and 3 µl of DNA sample in 1x Taq polymerase buffer. The mixture was first denatured at 94°C for 7 min. Then, thirty-five cycles of PCR were performed by denaturation at 94°C for 30 s, primers annealing at 55°C for 45 s, and primer extension at 72°C for 90 s. At the end of the last cycle, the mixture was incubated at 72°C for 7 min. For each reaction, a negative control missing DNA template, and a positive control were included. Efficient amplification was confirmed by gel electrophoresis on 1.5% agarose gel.

PCR products were purified using the Magnesil yellow solution (Promega) to eliminate the primers used for PCR reactions, then the sequence reaction for each purified product was done in a thermocycler. The sequencing reaction was performed in a total volume of 20 µl containing 20 pmol of each primer (ITS-1 or IST-4), 3 µl of Big Dye (version 1.1) and 2 µl of purified PCR product. Twenty five cycles were performed: denaturation at 96°C for 10 s, primer annealing at 55°C for 10 s, and extension at 60°C for 4 min. In order to eliminate the excess of labelled ddNTPs, sequencing reaction products were purified using the above mentioned Magnesil green solution.

Direct sequencing of amplified PCR products for the ITS fragment of the ribosomal RNA 5,8S gene was performed on an ABI PRISM sequencing apparatus (ABI Prism 310 Genetic Analyser, Applied Biosystem) and data analysis was done by sequencing analysis software. The sequencing was carried out for both strands.

To confirm the results obtained with the molecular procedure, the conventional method was used (Kreger Van Rij 1987, Barnett *et al.* 2000). The results analysis was done with the "Yeast identification PC program, version 5, May 2000" (Barnett *et al.*, 2000). Briefly, the physiological tests included fermentation of D-Glucose, assimilation of carbon compounds, assimilation of nitrogen compounds, determination of vitamin requirements, temperature tolerance tests, cyclohexemide resistance test, growth on osmophilic media and urea hydrolysis. The morphological testing included mode of vegetative reproduction and formation of pseudohyphae, true mycelium, and arthroconidia.

Test of yeast strains pathogenesis on *B. oleae* development

100 µl of an overnight culture of the yeast strain tested was spread over an YPG medium and incubated at 30°C for 48 h. A carpet of each strain was obtained in each dish. *B. oleae* L3 larvae recovered from olive fruits were placed in a synthetic medium (Tsitsipis, 1977) during 24 hours and then transferred in YPG agar medium containing the tested strain and incubated at 25°C. The development of these larvas was followed until their transformation to pupas or the emergence of adults. These tests were carried out four times.

Insects

B. oleae adults used in this work were obtained from our laboratory at 24 °C with 65-70% relative humidity and 12:12 (light:dark).

Control Strain

The control yeast strain used in this study was isolated in our laboratory from larvae of B. *oleae*. This strain identified as *Candida diddensiae* (Chakri *et al.*, 2007) was used as control so as to estimate the rate of pathogenic capacity of the tested strains.

Nucleotide sequences

The nucleotide sequences were registered in GenBank, the accession numbers are presented in table 1.

Table 1. Sequenced product and registered number in GenBank.

Yeast Strain	Specie	Sequenced product (DNA)	registered number in GenBank
YS1	*Debaryomyces hansenii*	581 pb	EU339932
YS2	*Pichia guilliermondii*	540 pb	EU339933

Results

Isolation of yeast strains

During *B. oleae* breeding, some dead pupas were observed. These pupas were used to isolate yeast strains that would be responsible for the observed lethality. Several strains were isolated on YPG agar containing three antibiotics (Ampicilline, tetracycline and kanamycine). Eight strains were randomly selected and retained for further investigations.

Test of yeast strains pathogenesis on *B. oleae* development

Larval development and pupation of *B. oleae* were followed up on a medium containing the tested yeast strain. In parallel, the number of dead larvae and pupae and the percentage of lethality were recorded. Among the eight tested strains, only two (YS1 and YS2) showed an important rate of lethality against *B. oleae*. The results obtained for these strains are summarised in table 2. SY2 showed a strong lethality of *B. oleae*. This mortality exceeded 95 % and acted mainly at the larval stage (85.2%), whereas the mortality at the pupa stage was only 10.1%. In the presence of SY1 strain, the larval viability was very weak (19.4), and the rate of emergence was more important than SY2.

Identification of yeast strains

The specific amplification of RNA 5,8S from the yeast strains YS1 and YS2 gave PCR products with 581bp and 540bp, respectively. The two PCR products were then sequenced on both strands. The sequences are reported in figure 1. These sequences were compared with available DNA sequence database using BLAST program (Table 3). For strain YS1, the 581 nucleotides sequenced show 98% of homology with the ITS DNA sequences found in the GeneBank database that correspond to two species *Candida psychrophila* and *Debaryomyces hansenii*. Furthermore, the 540 nucleotides sequence of strain YS2 show 98% of homology with the ITS DNA sequences found in the GeneBank database that correspond to *Pichia guilliermondii* (Table 3 and Figure 1).

To confirm these results, the strains were subject to conventional identification according to Barnett *et al*. (2000). This approach enabled us to differentiate between *Debaryomyces hansenii* and *Candida psychrophila*. Thus, strains YS1 and YS2 were identified as *Debaryomyces hansenii* and *Pichia guilliermondii* with a 0.994 score for both.

Table 2. Test of yeast strains pathogenesis on *B. oleae* development (Page 9; lane 15).
Larval development and pupation of *B. oleae* were followed up on a medium containing the tested yeast strain. In parallel, the number of dead larvae and pupae and the percentage of lethality were montred. SY2 showed a strong lethality of *B. Oleae* (95 %) and acted mainly at the larval stage (85.2%), whereas the mortality at the pupa stage was only 10.1%. For SY1 strain, the larval viability was very weak (19.4).

Strain	Total larvae	Dead larvae		Dead pupae		total lethality (larvae and pupas)		Emerged adults (viability)	
		Number	%	Number	%	Number	%	Number	%
SY1	134	26	19.4	45	33.6	71	53	63	47
SY2	149	127	85.2	15	10.1	142	95.3	7	4.7
***Candida diddensiae* (control)**	140	4	2.9	2	1.4	6	4.3	134	95.7

Table 3. Sequence analysis of RNA 5,8S PCR products amplified from YS1 and YS2 yeast strains (Page 10, lane 1 and 6).
The sequences of YS1 and YS2 strains are reported. These sequences were compared with available DNA sequence database using BLAST program. For strain YS1, the 581 nucleotides sequenced show 98% of homology with the ITS DNA sequences found in the GeneBank database that correspond to two species *Candida psychrophila* and *Debaryomyces hansenii*. The 540 nucleotides sequence of strain YS2 show 98% of homology with the ITS DNA sequences found in the GeneBank database that correspond to *Pichia guilliermondii*.

Yeast Strain	Specie	PCR product	Sequenced product	Percentage of homology	Reference
YS1	*Debaryomyces hansenii*	581	581	98	gb|EF197950.1
	Candida psychrophila	581	581	98	gb|AY040667.1
YS2	*Pichia guilliermondii*	540	540	98	gb|EF197816.1

1 TTGTTTGTTATATTGTAAGGCCGAGCCTAGAATACCGAGAAATATACCATTAAACTATTC 60
61 AACGAGTTGGATAAACCTAATACATTGAGAAGTGCATATAGCACTATCCAGTACCACTCA 120
121 TGCGCAATACATTTCAAGCAAACGCCTAGTTCGACTAAGAGTATCACTCAATACCAAACC 180
181 CGAAGGTTTGAGAGAGAAATGACGCTCAAACAGGCATGCCCTTTGGAATACCAAAGGGCG 240
241 CAATGTGCGTTCAAAGATTCGATGATTCACGAAAATCTGCAATTCATATTACTTATCGCA 300
301 TTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGAAGA 360
361 TTTTTTGAATTTAATCAACAAATTGACAATTAAAATAAATAACAATTCAATATAAATATT 420
421 GAAGTTTAGTTTAGTAAACCTCTGGCCCAAACTATTTCTAGTCCAGACCAAAGCAAGAGT 480
481 TCTTGTAATAACAAAAAACACTGTGTGTAAGGGTTTTTCGCCGCGCAATTAAGCGCTGGC 540
541 AAAAAGAATACTGGAATGATCCTTCCGCAGGTTCCCCTACG 581

Figure 1a.

1 TTGTTTGGTTGTTGTAAGGCCGGGCCAACAATACCAGAAGATATCCCGCCACACCATCTC 60
61 AACGAGTGTGGATAAACCTAATACATCTGAGAGGTCGACAGCACTATCCAGTACTACCCA 120
121 TGCGCAATACTCTCTTCAAGCAAACGCCTAGTCCGACTAAGAGTATCACTCAATACCAAA 180
181 CCCGGGGGTTTGAGAGAGAAATGACGCTCAAACAGGCATGCCCTCTGGAATACCAGAGGG 240
241 CGCAATGTGCGTTCAAAGATTCGATGATTCACGAAAATCTGCAATTCATATTACTTATCG 300
301 CATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGAA 360
361 GATTAATTCAAAATTTGACTAACTGTAAAAATAATTAAATTGTGTTTTGTTAAACCTCTG 420
421 GCCCAACCTATCTCTAGGCCAAACCAAAGCAAGAGTTCTGTATCAAAAAGACACTGTGTG 480
481 TAAGGTTTTTCGCCGCGCAGTTAAGCGCTGGCAAAAGAATACTGTAATGATCCTTCCGCA 540

Figure1b.

Figure 1 (Page 9, lane 12 and 18). Nucleotide sequence of the ITS amplified fragments of the two isolated yeast strains.
The two PCR products from the yeast strains YS1 and YS2 were sequenced on both strands.
Figure 1a: Sequence of the 5.8S ITS region of strain YS1
Figure 1b: Sequence of the 5.8S ITS region of strain YS2

Discussion

Development of alternative control strategies such as biocontrol can provide additional management tools as supplements to other control measures for plant diseases, as rotation with other control measures, or as back-up when favoured control measures are withdrawn from the market or fail due to new strains or races of pathogens (Fravel, 2005).

In our laboratory, we were interested in isolating pathogenic yeast strains from dead *B. oleae* pupas. Indeed, several investigations were made to evaluate the opportunity to use yeast strains in the biocontrol of *B. oleae* fly. Konstantopoulou & Mazmenos (2005) showed that several yeast strains, especially *M. hiemalis,* have a toxic effect against *B. oleae* adults. Two yeast strains were isolated from death *B. oleae* pupas. Conventional and molecular identifications of these strains showed that they belong to *Pichia guilliermondii* and *Debaryomyces hansenii,* respectively. The virulence of *Pichia guilliermondii* strain

was the highest - 95.3 % of lethality - when compared to that of *D. hansenii* strain with 53 % lethality only. On the other hand, *P. guilliermondii* strain seems to act mainly at the larval stage since only few larvae had undergone the pupation (10.1 %). In contrast, *D. hansenii* seems to act preferentially at the pupal stage (33.6 % of lethality).

To our knowledge, there are no reports up to date on the susceptibility of *B. oleae* fly to *P. guilliermondii* and *D. hansenii* yeast strains. However, *Pichia guilliermondii* was reported in several works to be used for bio-control essays. Wisniewski *et al.* (1991) have isolated a *P. guilliermondii* strain that protects apples from postharvest fruit rotting fungi *Botrytis cinerea* and *Penicillium expansum*. Other studies have demonstrated the ability of *P. guilliermondii* to inhibit the growth of grain with microflora using naturally contaminated soya beans and sterilized soya beans artificially inoculated with *Aspergillus flavus* (Paster *et al*., 1993). Arras *et al*. (1998) reported the use of a strain of *P. guillermondii* in bio-control of blue mould of citrus fruits. The abilities of *Pichia anomala*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae* to inhibit the growth of the mould *Penicillium roqueforti* in nonsterile high-moisture wheat were demonstrated (Petersson & Schnurer, 1995). Furthermore, yeast's application as biocontrol agents to prevent mold decay of fruits and vegetables has been described Richards *et al.* (2004) have shown that *Debaryomyces hansenii* and *Pichia guilliermondii* were antagonists to *Salmonella Poona* in cantaloupe juice.

Association between *Pichia guilliermondii* and insects has already been documented. Different strains in the *Pichia guilliermondii* clade were isolated from the digestive tract of basidiocarp-feeding members of seven families of Coleoptera (Frants & Mertvetsova 1986, Suh & Blackwell 2004).

D. hansenii is one of the most frequent yeast species to be associated with chilled food and is normally considered non-pathogenic. It has been reported that *D. hansenii* grew mainly on the cheese surface, where its oxygen demand was satisfied (Leclercq-Perlat *et al*., 1999; Bonaïti *et al.,* 2004). The single case of infection associated to *D. hansenii* was reported with bone infection (Yamamoto *et al*., 2002).

Even if the number of biocontrol products is increasing, they only represent about 1% of agricultural chemical sales. Yeast based biocontrol strategies offer an important alternative to conventional products. In this paper, we have demonstrated, for the first time, that *P. guilliermondii* and *D. hansenii* have toxicity against insects, and more specifically, the olive fruit's fly *B. Oleae*. The results obtained in this paper are of great importance and open a way for an application of these strains for bio control against the devastating insect *B. oleae*. Generally, the toxicity of yeast species resides in their produced metabolites. The high toxicity of *P. guilliermondii* and *D. hansenii* may be due to some metabolites produced by the two species. This makes it necessary for further research to identify and eventually isolate these potent active molecules.

Actually, trends in research are basically axed on increasing the use of biorational screening processes to identify microorganisms with biocontrol potential. Furthermore, increased testing under semicommercial and commercial production conditions, increased emphasis on combining biocontrol strains with each other and with other control methods and integrating biocontrol into an overall system will be of great interest for the control of the olive fruit fly *B. oleae* and the safeguard of the olive groves.

Acknowledgments

We thank Drs Sendid Khalid, Fikri Benbrahim Kawtar, Ziane Hamid and El Barji Abdelhak for their critical reading of the manuscript and help in improving English.

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