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Effect of *Beauveria bassiana* strains on the *Ceratitis capitata - Psyttalia concolor* system

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Abstract

The effects of a previously selected entomopathogenic fungus, *Beauveria bassiana* AL1 strain, and the *B. bassiana* based myco-insecticide Naturalis (ATCC 74040 *Beauveria bassiana* strain) were evaluated on the system *Ceratitis capitata - Psyttalia concolor* in laboratory assays. First, the entomopathogenic fungal strains were tested for their virulence against 2, 4, and 6-days old puparia of *C. capitata*. Subsequently, *P. concolor* emergence from *C. capitata* puparia treated or not with the fungal strains was evaluated at three different time points (2, 4 and 6 days) from the parasitization and the following pupation. Results showed that the entomopathogenic fungal applications affected the medfly survival. The effect of fungal treatments was higher on 2-day puparia (49.16 and 51.33% of mycosed puparia for ATCC 74040 and AL1 strain respectively) while the rate of mycoses was lower and ranged between 39 and 27.16% when fungal treatments were performed on 4 and 6-day puparia. Furthermore, fungal treatments affected the *P. concolor* emergence (c.a. 80% in the untreated control) particularly when applied 2 days after the parasitization and the *C. capitata* pupation (43.16 and 47.83% for the ATCC 74040 and the AL1 strains respectively), while when treatments were performed on older puparia, the *P.concolor* emergence ranged from 63.33 to 68.66%. Results suggest that the entomopathogenic *B. bassiana* strains are effective against *C. capitata* puparia but they may be detrimental against its endoparasitoid *P. concolor*, particularly when applied in the earlier stages of the parasitization process.

Key words: entomopathogenic fungi, insect-parasitoid-entomopathogens interactions, microbial control.

Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera Tephritidae), is one of the most destructive pests in the Mediterranean area. This is a multivoltine, widespread and highly polyphagous pest which has been recorded in more than 400 host plant species (Liquido *et al*., 1991; Aluja and Mangan, 2008). Females oviposit into the hosts fruits, which are directly damaged by the trophic activity of medfly larvae (Tremblay, 1994). The prepupating larvae drop from the fruits and pupate within the first 2-4 cm of the soil, forming puparia (Tremblay, 1994).

Psyttalia concolor (Szepligeti) (Hymenoptera Braconidae), the endoparasitoid associated to medfly, is considered an important factor in regulating natural populations of *C. capitata* and other Tephritidae as *Bactrocera oleae* (Rossi). In Italy, *P. concolor* can be found spontaneously on *B. oleae* in Sicily, southern Sardinia and in Tuscany (Raspi, 1995; Raspi *et al*., 1996; Loni *et al*., 2005). This species was used in Italy and in the Mediterranean area for biological control programs against the olive fruit-fly *B. oleae* by inundative and inoculative methods (Raspi and Loni, 1994).

The control of *C. capitata* can be carried out against adults (aerial treatments with chemical insecticides, sterile insect technique, chemosterilant, mass trapping, deterrents of oviposition), or prepupating larvae and puparia in the soil (terrestrial treatments with chemical insecticides) (Ros *et al*., 2002; CDFA, 1993; Mellado *et al.*, 1970; Rendon *et al*., 2006; Navarro-Llopis *et al*., 2004; Ekesi *et al.*, 2007). Chemical control of this pest has induced the selection of resistant medflies populations, and its negative environmental impact (especially on beneficial entomofauna) has encouraged the development of alternative pest management strategies.

Entomopathogenic fungi, which are the only insect pathogens infecting their hosts by direct penetration of the cuticle, show promising perspectives for the medfly control, both against adults (cover spray, fungus contamination devices) and puparia in the soil (soil inoculation) (Primo-Yúfera *et al*., 2002; Ekesi *et al*., 2007; Ortu *et al*., 2009; Daniel and Wyss, 2010; Garrido-Iurado *et al*., 2011). The effectiveness of entomopathogenic fungi such as *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin on *C. capitata* adults and puparia have been reported by several authors (Garcia *et al*., 1989; Castillo *et al*., 2000; Ekesi *et al*., 2002; 2003; 2005; Konstantopoulou and Mazomenos, 2005; Quesada-Moraga *et al*., 2006; 2008; Dimbi *et al*., 2009). Furthermore, the physical and biochemical properties of aerial *B. bassiana* conidia, in particular the rodlet layers of hydrophobins covering conidia surface, seems to determine a significant reduction in the number of visits and oviposition by *C. capitata* on treated fruits, such us to hypothesize a possible use of *B. bassiana* as deterrent of oviposition (Falchi *et al*., 2015).

Entomopathogenic fungi are commonly considered non-detrimental against parasitoids, although few experimental data are available, particularly about the effect on the parasitoids development, the parasitization mechanisms and interaction "host × parasitoid × pathogen". Several authors demonstrated that entomopathogenic fungal treatments did not affect significantly the survival and the activity of predators, parasitoids and

non target arthropod fauna (Parker *et al*., 1997; Goettel and Hajeck, 2000; Ludwing and Oetting, 2001; Thungrabeab and Tongma, 2007; Potrich *et al*., 2015) and the interactions between fungal pathogens and natural enemies are usually considered as positive (Roy and Pell, 2000).

Our study aimed to investigate the interaction among *C. capitata*, *P. concolor* and *B. bassiana* strains in laboratory assays so to evaluate the following: (1) the virulence of fungal strains against *C. capitata* puparia; (2) the effect of these strains on the emergence of *P. concolor* adults.

Materials and methods

Insects rearing

The rearing of the parasitoid *P. concolor* on the substitution host, *C. capitata,* was carried out at the insect growing facility of Mediterranean Agronomic Institute of Bari (C.I.H.E.A.M.) at the following controlled conditions: 24-26 °C temperature (Loni, 1997), 64% relative humidity, and 12 h light/12 h dark photoperiod (Carey, 1984). The parasitoids were initially provided by the "Centro Regionale Agrario Sperimentale (CRAS)", in Cagliari (Italy). Periodically, fresh populations of the parasitoid collected directly from fields were added to the breeding population to avoid the problems associated with multi-annual breeding. The target of the parasitoid, *C. capitata*, had been reared since 2000 according to the commonly adopted procedures, as described in Raspi and Loni (1994), Loni (1997) and Canale and Benelli (2012). The dipteran population of *C. capitata*, on which the breeding of the parasitoid was started, came from infested fruits collected from trees growing in local biological orchards; also in this case, the target population was periodically renewed by adding fresh individuals from outside.

C. capitata adults were reared into Plexiglas cages (40 × 50 × 45 cm) provided with a medium consisting in protein bait (30 g), dry yeast (8.4 g), sugar (40 g) and water (40 ml), and wet paper to supply water to the adults in the cages. Parasitoid adults were reared into Plexiglas cages (40 × 50 × 45 cm) provided with 20 g of a mixture of honey/sugar in a Petri dish and wet paper for water supply. *C. capitata* adults (about 8000 individuals) were placed into cages with one side covered by a net of tulle on which females laid their eggs. Eggs were allowed to fall into a water reservoir located at the base of the cage to avoid desiccation. The eggs were collected daily from the water reservoir and added to an artificial food, known as pabulum. Pabulum was prepared as described in Cavallaro and Girolomi (1969) and consisted in: bran (96.8 g), sugar (64.8 g), dry yeast (9.07 g), citric acid monohydrate (2.4 g) and sodium benzoate (2 g). These components were suspended in water (200 ml). Some cups were filled with 375 g mixture on which 0.25 ml insect eggs were distributed. We kept this mixture containing *C. capitata* eggs in the insectarium growth chambers until larval hatching. Mature larvae (III stage) were collected in trays filled with water to block them in the larval stage.

Afterwards, one group of larvae (about 10,000) was transferred into rectangular cages and exposed for 15-20 minutes to the parasitism of 10-25-day-living adult parasitoids (12,000, 50% females) (Loni, 1997), whilst a second group was let grow under the same conditions but with no exposition to parasitoids, in order to establish a new generation of *C. capitata* adults.

Fungal strains

The strain AL1 of *B. bassiana* was used in the experiments. This strain was previously selected from the fungal collection of the Department of Soil, Plant and Food Science - Section of Zoology and Entomology (Bari, Italy) upon its *in vitro* thermal regime and virulence against several insects target as *Galleria mellonella* L., *Tenebrio molitor* L., *Trialeurodes vaporariorum* (Westwood) (Oreste *et al*., 2011; 2012; 2015). The commercial *B. bassiana* strain ATCC 74040 (Naturalis; $2.3 \cdot 10^7$ conidia ml^{-1}; Biogard, division of CBC, Italy) was included in the assays. Conidia from 15 day-old sporulated colonies, grown in Petri plates containing 2% malt extract-agar medium and incubated at 25 °C in the dark, were harvested in 25 ml of sterile water added with 0.002% Tween 80 (Sigma Aldrich). The fungal inoculum was filtered by a cheesecloth, and conidial concentration was estimated using a Malassez chamber and adjusted to $2 \cdot 10^7$ conidia ml^1. The same conidial concentration was used for the commercial ATCC 74040 *B. bassiana* strain.

Virulence assays of the entomopathogenic fungal strains against *C. capitata*

The experiments were carried out in plastic boxes (20 × 20 × 10 cm) lined on the bottom with a filter paper, and closed on the top with a plastic net (1 mm mesh). Three assays were conducted: (1) on 2 days-old *C. capitata* puparia (2 days from pupation), (2) on 4 days-old *C. capitata* puparia (4 days from pupation), (3) on 6 days-old *C. capitata* puparia (6 days from pupation). 200 puparia of *C. capitata* were treated with a single fungal strain, by spraying 10 ml conidial suspension of each strain. This conidial suspension volume ensured homogeneous wetting of puparia. Inoculated insects were placed on filter paper for 20-30 minutes in order to remove the inoculum excess and then were put in each box. Sterile distilled water added with 0.002% Tween 80 was used as control. Boxes were incubated in a climatic chamber at 25 °C and 75% relative humidity. A complete randomized block design with three replicates (boxes) was used. The emergence of *C.capitata* adults and the individuals killed by the fungal strains were counted daily for 15 days and then removed. Individuals killed by the fungal strains were easily recognizable by discoloration, desiccation and/or fungal outgrowth. Mortality caused by the entomopathogenic strains was confirmed by re-isolation of the fungi on agar from random samples at each day. It was easily discriminated from that caused by other causes (e.g. bacteria).

Data were analyzed performing a logistic regression, considering the following independent blocks "Age of puparia" and "Fungal strain". Means were then com-

pared with the least-squares means statistics ($P < 0.05$). The correlation between the dependent variables was analyzed with the Spearman's correlation. Data were analyzed using the SAS/STAT 9.0 software (SAS Institute, Cary, NC).

Effect of the entomopathogenic fungal strains on *P. concolor* emergence

The selected fungal strains were tested: (1) on 2 days-old *C. capitata* puparia parasitized by *P. concolor* (2 days from the parasitization by *P. concolor* and the following *C. capitata* pupation); (2) on 4 days-old *C. capitata* puparia parasitized by *P. concolor* (4 days from the parasitization by *P. concolor* and the following *C. capitata* pupation); (3) on 6 days-old *C. capitata* puparia parasitized by *P. concolor* (6 days from the parasitization by *P. concolor* and the following *C. capitata* pupation). The assays were conducted inoculating 200 parasitized puparia with each fungal strains as previously described and incubating them in the same conditions. Sterile distilled water added with 0.002% Tween 80 was used as control. Insects were checked daily for 25 days to record the following data: the number of mycosed individuals (mortality caused by the entomopathogenic strains was confirmed by re-isolation of the fungi); the number of individuals dead due to other causes; the number of alive *C. capitata* adults; the number of *P. concolor* adults emerged. Data were submitted to the logistic regression, considering the following independent variables: "Block", "Age of puparia", "Fungal strain". Means were then compared with the least-squares means statistics ($P < 0.05$) and the Spearman's correlation was used to analyze the correlation between variables.

Results

Virulence assays of the entomopathogenic fungal strains on *C. capitata* puparia

Results of logistic regression (table 1) revealed that the interaction "Age of puparia × Fungal strains" influenced both the emergence of *C. capitata* adults and the *C. capitata* mortality (mycosed puparia), while the other variables resulted not significant. Results of the Spearman's correlation statistics showed that the dependent variables "Emerged *C. capitata* adults" and "Mycosed *C. capitata* puparia" were negatively correlated ($\rho = -0.99$, $P < 0.0001$).

Results showed that the entomopathogenic fungal applications affected the medfly survival in each experiment but the effect of fungal treatments was higher on 2-day puparia (49.16 and 51.33% of mycosed puparia for ATCC 74040 and AL1 strain respectively) (figure 1). When fungal treatments were performed on 4 and 6-day puparia, the rate of mycoses was lower and ranged between 39 and 27.16%.

Table 1. Logistic regression statistics for the virulence of AL1 and ATCC 74040 *B. bassiana* strains on *C. capitata* puparia with different age.

Source of variability	DF	Emerged *C. capitata* adults		Mycosed *C. capitata* puparia	
		χ^2	P	χ^2	P
Block	2	0.2	0.65	0.79	0.67
Age of puparia (AP)	2	0.02	0.99	0.02	0.99
Fungal strain (FS)	2	5.67	0.05	5.68	0.05
AP × FS	4	12.06	0.01	12.06	0.01

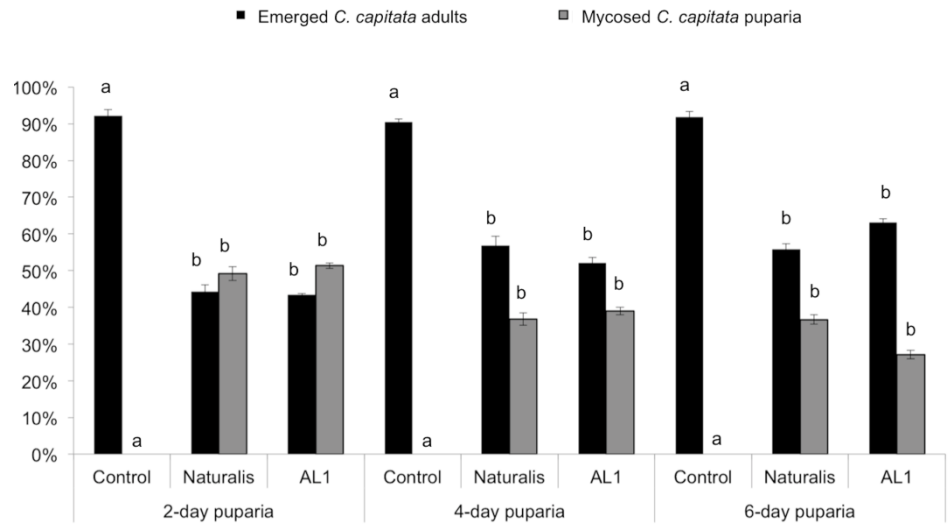

Figure 1. Virulence of AL1 and ATCC 74040 *B. bassiana* strains on *C. capitata* puparia with different age. Within each variable, means with different letters are significantly different according to the least squares means statistics ($P < 0.05$). Vertical bars indicate the standard errors (n = 3).

Also the rate of *C. capitata* emergence was influenced by the fungal treatments. The rate of emerged medflies was higher when fungal strains were applied on 4 and 6-day puparia (from 52 to 63.16%), while it decreased when treatments were performed on younger puparia (44.33 and 43.33% for ATCC 74040 and AL1 strain, respectively) (figure 1). In the untreated control, the rate of *C. capitata* emergence varied between 90.5 and 92.16% while the natural mortality ranged between 10.66 to 19.33% in all the assays.

Considering the performance of the AL1 strain, it was statistically not different from the commercial myco-insecticide.

Effect of the entomopathogenic fungal strains on *P. concolor* emergence

The logistic regression analysis (table 2) showed that the variables "Age of puparia", the "Fungal strains" and the interaction "Age of puparia" × "Fungal strains" influenced the emergence of *P. concolor* adults. Furthermore, the variables "Age of puparia" and "Fungal strains" influenced significantly the rate of mycosed puparia and the emergence of *C. capitata* adults, respectively. The Spearman's correlation statistics revealed a negative correlation existing between the variables "Emerged *C. capitata* adults" − "Mycosed *C. capitata* puparia" ($\rho = -0.75$, $P < 0.0001$) and "Emerged *P. concolor* adults" − "Mycosed *C. capitata* puparia" ($\rho = -0.94$, $P < 0.0001$), while the other variables seems to be not correlated ($\rho = 0.59$, $P = 0.001$).

Results showed that entomopathogenic fungal treatments affected the *P. concolor* emergence (figure 2), particularly when strains were applied on 2-day parasitized puparia, reducing the parasitoids emergence to 43.16% (ATCC 74040) and 47.83% (AL1), while it was 79.16% in the control. When treatments were performed on older puparia, the *P. concolor* emergence ranged from 63.33 to 68.66%. Considering the effect on *C. capitata* adults emergence, the fungal treatments reduced the rate of emergence (between 6.15 and 7.83%) in respect to the untreated control (on average 13.66%) but without significant differences in relation to the age of puparia. The age of puparia influenced the fungal treatments effectiveness. The rate of mycosed individuals was higher when fungal strains were applied on younger puparia (respectively 45.33 and 39.5% for ATCC 74040 and AL1), while it decreased when treatments were performed on 4 and 6-day puparia (ranging from 24.33 to 19.5%) (figure 2). Natural mortality values varied from 5.5 to 8.83% in the entire experiment. Also in this experiment, the two strains ATCC 74040 and AL1 were statistically not different.

Table 2. Logistic regression statistics for the effect of AL1 and ATCC 74040 *B. bassiana* strains on *P. concolor* parasitizing *C. capitata* puparia with different age.

Source of variability	DF	Emerged *C. capitata* adults		Mycosed *C. capitata* puparia		Emerged *P. concolor* adults	
		χ^2	P	χ^2	P	χ^2	P
Block	1	0.01	0.91	0.48	0.48	0.44	0.55
Age of puparia (AP)	2	0.82	0.66	171.05	<0.0001	65.81	<0.0001
Fungal strain (FS)	2	78.12	<0.0001	0.88	0.64	237.42	<0.0001
AP×FS	4	1.66	0.89	2.64	0.61	46	<0.0001

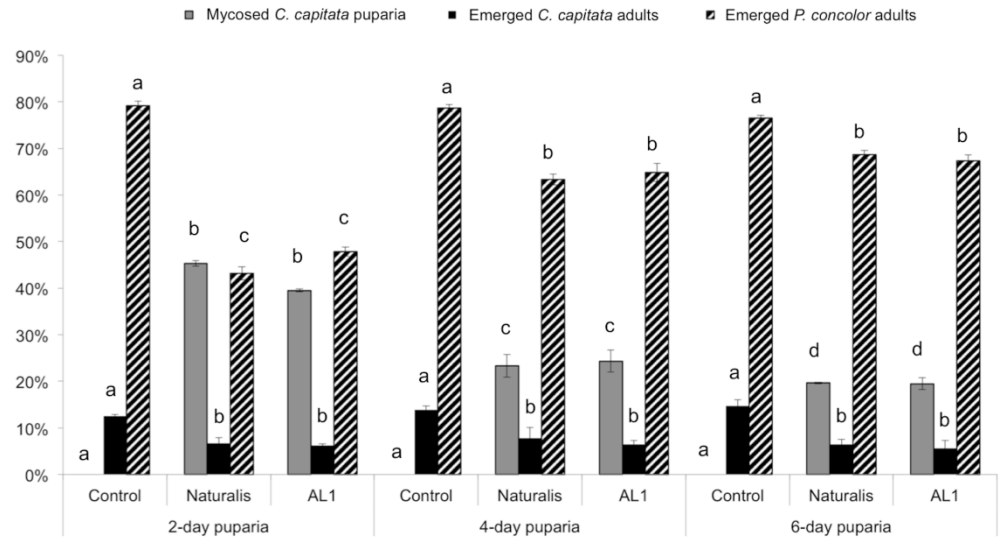

Figure 2. Virulence of AL1 and ATCC 74040 *B. bassiana* strains on *P. concolor* parasitizing *C. capitata* puparia with different age. Within each variable, means with different letters are significantly different according to the least squares means statistics ($P < 0.05$). Vertical bars indicate the standard errors (n = 3).

Discussion and conclusions

Results from our experiments revealed that the AL1 *B. bassiana* strain is effective against *C. capitata* puparia, without significant differences compared to the commercial ATCC 74040 strain, although the average mortality found was low and ranged between 27 and 51%. These results agree with previous researches. Lozano Tovar *et al.* (2013) found significant differences among strains of *Beauveria* spp. and *Metarhizium* spp. in the total percentage of non-viable puparia of *C. capitata* and non-viable puparia showing fungal outgrowth, with percentages ranging from 5.0 to 45.0%. Eldesouki-Arafat *et al.* (2007), obtained growth of *B. bassiana* on *C. capitata* puparia with percentages from 3.3 to 43.0% and a mortality range from 16.7 to 73.3% for a *M. anisopliae* strain. Quesada Moraga *et al.* (2006) tested the pathogenicity of 10 strains of *B. bassiana* and five of *M. anisopliae* against puparia and adults of *C. capitata*, finding that only 2 strains of *B. bassiana* and one of *M. anisopliae* caused mortality higher than 50% when puparia were immersed in the conidial suspensions. Beris *et al*. (2013) verified the pathogenicity of several strains of *B. bassiana*, *Isaria fumosorosea* Wize - formerly *Paecilomyces fumosoroseus* (Wize) Brown et Smith - and *M. anisopliae* under laboratory conditions against pupae and adults of Mediterranean fruit fly via different routes of exposure. They obtained that the average mortality of pupae after immersion into spore suspensions was in general low and ranged from 18.7 to 23.9% depending on fungal species and dose applied. Imoulan *et al.* (2011) tested several *B. bassiana* strains against medfly pupae, finding that when insects were exposed to 10^8 conidia/ml, the adult emergence ranged from 0 to 23.33% after 10 days post-treatment. Goble *et al.* (2011) evaluated the pathogenicity of 15 strains of *B. bassiana*, five strains of *M. anisopliae* and one strain of *Metarhizium flavoviride* (Gams et Rozsypal) against the subterranean life stages of *Ceratitis rosa* Karsch, *C. capitata* and *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera Tortricidae) in laboratory assays. In their work they showed a higher effect on the adults of *C. rosa* and *C. capitata* than on the puparia of these two fruit fly species.

Several author demonstrated that puparia of tephritids are less susceptible to entomopathogenic fungi than the adults. This finding may be imputable to two reasons: the hard barrier of puparia formed by the cuticle of the third larval stage, which may inhibit the fungal penetration and evasion (Kaaya and Munyinyi, 1995; Ekesi *et al*., 2007); the high levels of resistance to infection developed by the terrestrial stages of insects, due to a co-evolution with entomopathogenic fungi which are widespread in the soil (Vanninen *et al*., 1999; Cossentine *et al*., 2010).

Furthermore, our results agree with those of Ekesi *et al*. (2002), who found that pupal susceptibility of tephritids to *M. anisopliae* decreased with increasing pupal age. According to Ekesi *et al*. (2002), the higher susceptibility of younger compared to older puparia, may be explained by considering that younger puparia have a softer cuticle. In fact, although the logistic regression revealed that the "Age of puparia" variable did not influence the rate of mycosed puparia, we found that 2-day puparia were more susceptible than the older ones (4 and 6-days old), with mortality values which reached 49.16 and 51.33% for the ATCC 74040 and the AL1 strain respectively, while in the other experiments mortality ranged from 27 to 39%.

Considering the effect of entomopathogenic fungal strains on *P. concolor* emergence, we obtained that all fungal treatments affected the emergence of *P. concolor*, although the detrimental effect on the parasitoid was higher when treatments were performed in the earlier stages of the parasitization (2-day after the parasitization). These results are in contrast with those by other authors. Medina *et al.* (2008) found that the biopesticide *B. bassiana* did not cause *P. concolor* mortality when applied *via* treated host larvae, but the emerged adults originated a lower size progeny, so they concluded that the fungal treatments may reduce the parasitoids beneficial capacity in laboratory conditions. Ekesi *et al.* (2005) evaluated the effect of two *M. anisopliae* formulations against pupariating larvae of *C. capitata*, *Ceratitis fasciventris* (Bezzi) and *Ceratitis cosyrae* (Walker) and their associated endoparasitoids *P. concolor* and *Psyttalia cosyrae* (Wilkinson) in field cage experiments. Their results showed that the combined use of *M. anisopliae* and the two *Psyttalia* species for the control of *C. capitata* and *C. cosyrae* may be successful for the target pest control, due to both parasitoid species emerged from host puparia exposed to fungal treated soil, indicating that *M. anisopliae* had no adverse effect on the development of the parasitoids. Similar results were obtained by several authors investigating the effect of microbial control agents on parasitoids development (Ruiu *et al*., 2007; Labbé *et al*., 2009; Hamdi *et al*., 2011; Potrich *et al*., 2015). They found that parasitoids are able to develop on infected hosts without damaging or to detect and avoid infected hosts during their oviposition activity (Fransen and van Lenteren, 1994), favouring the combined use of both the control agents in organic or integrated pest management.

Our results agree with those of previous researches, which suggested that time between fungal application and exposure to parasitoids is crucial for the parasitoids survival (Askary and Brodeur, 1999; Avery, 2008; Oreste *et al*., 2015). Some authors demonstrated that most of the "host × parasitoid × pathogen" combinations are detrimental mainly due to premature death of the host caused by the pathogen (Los and Allen, 1983; Goh *et al*., 1989). According to this hypothesis, when fungal treatments are applied several days after the parasitization, parasitoids may "escape" to fungal infection and complete their development before the host death induced by fungi. On the other hand, the melanization reactions induced by the parasitization process may inhibit the entomopathogenic fungi in penetrating the host cuticle (Avery *et al*., 2008), as well as some fungistatic metabolytes produced by parasitoids may prevent the fungus development (Blackburn *et al*., 2002).

In conclusion, our results suggest that entomopathogenic fungi are effective against *C. capitata* puparia but they may be detrimental against its endoparasitoid *P. concolor*, particularly when applied in the earlier stages of the parasitization process.

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