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Pathogenic potential of *Beauveria pseudobassiana* as bioinsecticide in protein baits for the control of the medfly *Ceratitis capitata*

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Abstract

The medfly, *Ceratitis capitata* (Wiedemann) (Diptera Tephritidae), is a major insect pest affecting fruit production worldwide whose control is mainly based on the use of protein baits laced with chemical insecticides. Entomopathogenic fungi are well-known to be effective against a wide spectrum of insect pests and are commonly utilized in integrated pest management and biological control programs. Here, we assess the feasibility of using the recently described entomopathogenic species *Beauveria pseudobassiana* Rehner et Humber (Hypocreales Cordycipitaceae) as a biological insecticide in protein bait sprays for the control of the medfly. Firstly, we evaluated the pathogenicity of *B. pseudobassiana* against eggs, larvae, pupae and adults of the medfly. Secondly, we tested its efficacy as bioinsecticide in protein bait sprays. The results of the pathogenicity tests showed that *B. pseudobassiana* is able to infect, and lead to the death, all instar of the medfly. The efficacy of *B. pseudobassiana* was confirmed also when used as bioinsecticide in protein baits. *In planta* tests, the survival probability (Kaplan-Meier estimates) of flies in contact with the *B. pseudobassiana*-laced protein bait was significantly lower respect to control. Median survival time of flies treated with *B. pseudobassiana*-laced protein (6 ± 1.422 d) was at least three times shorter than in control (> 20 d). Based on our results, we confirmed the potential of *B. pseudobassiana* as bioinsecticide in entomopathogenic fungi-laced protein baits for the control of tephritid fruit flies.

Key words: *Beauveria pseudobassiana*, biological control, crop protection, entomopathogenic fungi, Mediterranean fruit fly, protein bait sprays.

Introduction

The Mediterranean fruit fly or medfly, *Ceratitis capitata* (Wiedemann) (Diptera Tephritidae), is an invasive, highly polyphagous, and multivoltine fruit pest native to the Mediterranean area that has spread to many parts of the world, including Australasia and North and South America (Papadopoulos, 2008). Because of its wide distribution, its ability to tolerate cool climates, and its wide host range, the medfly is regarded as one of the world's most destructive fruit pests (Stewart and Johanson, 1999) and it is ranked first among the economically important fruit fly species (Liquido *et al*., 1990). Damage to crops is the result of the medfly oviposition in plant soft tissues and fruits, of the larval trophic activity, and of the consequent infection of plant tissues by microorganisms.

Currently, a multiple-technique approach is recommended for the control of *C. capitata*. Area-wide integrated pest management (IPM) programs have been proven to reduce medfly population throughout a defined area (Vargas *et al*., 2010; Boulahia-Kheder *et al*., 2012). In IPM, several suppression techniques are implemented to reduce or eradicate fruit flies from an area. Those techniques include, the destruction of fallen fruits, the releases of parasitoids, application of larvicidals to fruit, release of sterile fly male (SIT), and the male annihilation technique (MAT) where insecticides are bounded to a male lure, (i.e trimedlure, for the medlfy), and the use of chemical insecticides, which are routinely bounded to protein baits (protein bait application technique, BAT) (Roessler, 1989; Allwood *et al*., 2002). BAT exploits the use of the nutritional need (protein) of fruit fly females for the maturity of the gonads. Adding a toxicant to the protein and applying the mixture in spots to the foliage of the trees is a very effective method of controlling fruit flies by killing overall female flies before they reach the egg-laying stage (Allwood *et al*., 2002).

Although effective, the intensive and continuous use of organophosphates, especially malathion and pyrethroids has, however, caused the rising of resistant strains of tephritid flies (Vontas *et al*., 2011). Moreover, synthetic insecticides residues have negative effects on human health and on the environment (Desneux *et al*., 2007), and their public acceptance is low. In this scenario, biological control methods, based on the use of specific predators, parasitoids, and pathogenic microorganisms could provide a promising, environmentally safe, alternative to synthetic insecticides (Higuchi *et al*., 1997; Lacey and Shapiro-Ilan, 2008). In fact, accounting for approximately 1-2% of the global pesticide market, microbial pesticides have shown a constant long term growth over the past decade in contrast to chemical pesticides, which have consistently declined in the global market (Thakore, 2006; Bailey *et al*., 2010).

Several attempts have been made in substituting chemical insecticide with microbial pesticides for the control of medfly. Among bacteria-based pesticides, promising results have been obtained with spinosad, a bacteria-derived toxin (Peck and McQuate, 2000), and the actinobacteria *Streptomyces phaeochromogenes* (Samri *et al*., 2017). However, since entomopathogenic fungi infect their host via the outer cuticle, they may be

preferable as biocontrol agents for adult fruit flies than bacteria or viruses that must be ingested to be effective (Goettel *et al*., 2005; Quesada-Moraga *et al*., 2006; Lacey and Shapiro-Ilan, 2008). Moreover, entomopathogenic fungi are effective against a wide spectrum of insect pests (see review by Lacey *et al*., 2015).

The entomopathogenic fungal genus *Beauveria* includes twelve polyphyletic fungal species among which *Beauveria bassiana* (Bals.) Vuill. and *Beauveria brongniartii* (Sacc.) Petch (Hypocreales Cordycipitaceae) are well-known species largely employed for the control of harmful insects (Rehner *et al*., 2011). Recently, the genus *Beauveria* has been revised by molecular phylogenetic analysis (Rehner *et al*., 2011) and the new species *Beauveria pseudobassiana* Rehner and Humber, has been described as a cryptic species morphologically similar, but phylogenetically distant from *B. bassiana* (Rehner *et al*., 2011).

Medfly was shown to be highly susceptible to entomopathogenic fungi, mainly *B. bassiana*, *B. brongniartii* and *Metarhizium anisopliae* (Metschnikoff) (Castillo *et al*., 2000; Ekesi *et al*., 2002; Dimbi *et al*., 2003; Konstantopoulou and Mazomenos, 2005; Quesada-Moraga *et al*., 2006; Beris *et al*., 2013) and the use of entomopathogenic fungi against fly populations by cover sprays (Ortu *et al*., 2009; Daniel and Wyss, 2010), infected sterile males (Ekesi *et al*., 2007; Flores *et al*., 2013) and soil inoculation (Ekesi *et al*., 2007; Garrido-Jurado *et al*., 2011; Lozano-Tovar *et al*., 2013) has been proposed. Here, we evaluated the infectiveness and the insecticidal ability of the recently described species *B. pseudobassiana* to *C. capitata* stages and assessed the feasibility of using *B. pseudobassiana* conidia as an active ingredient in protein bait sprays by *in vitro* and *in planta* experiments.

Materials and methods

Insect rearing

C. capitata eggs, larvae, pupae and adults were sourced from a colony maintained at 25 ± 2 °C and 60-65% RH and L12:D12 photoperiod at the entomological laboratory of the Department of Agriculture, Food and Environment of the University of Pisa since 1970. Adults of the colony were provided with water and a solid diet consisting of 80% sugar, 20% hydrolysed yeast. Larvae were reared on artificial diet (alfalfa meal, sucrose, beer yeast, nipagin, and water) (Raspi and Loni, 1994).

Molecular identification of *Beauveria* sp. strain

The entomopathogenic fungal strain was supplied by the culture collection of the Leibniz-Institut DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) as *B. brongniartii* DSM6651 (isolate E 1246/91). This strain was chosen because it is easily available and because of its proven compatibility with biocontrol bacteria against soil-borne phytopathogenic fungi and with non-target species of aquatic and soil macrofauna (Sicuia *et al*., 2014). For DNA extraction, mycelium of the fungal strain was scraped from seven-day-old colonies developed at 25 °C in the dark on a cellophane membrane overlying PDA (Potato Dextrose Agar) plates. Genomic DNA was extracted according to the Chelex 100 protocol with some modifications (Baroncelli, 2012). Sequences 1 and 2, including the 5.8S gene, of the nuclear ribosomal DNA were amplified and sequenced with primers ITS5 and ITS4 (White *et al*., 1990). ITS region was formally proposed for adoption as the primary fungal barcode marker to the Consortium for the Barcode of Life (Schoch *et al*., 2012). Since the obtained dataset was clearly suitable to characterize the isolate used in the study at the species level, we decided to not use other loci. Amplification and sequencing were performed according to Doveri *et al*. (2013). The sequence data have been submitted to the GenBank databases under accession number KY609506.

The forward and reverse sequences generated were assembled using the software Geneious® 10.0.6. Sequences of ex-type and other reference strains of species belonging to the genus *Beauveria* (Rehner *et al*., 2011), all available on GenBank, were added to the dataset and the sequences aligned with MAFFT v.6.7 (Katoh and Toh, 2008). Using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003), the Markov chain Monte Carlo (MCMC) algorithm was performed to generate phylogenetic trees with Bayesian posterior probabilities for sequence datasets using the nucleotide substitution models (K2+G) determined by MEGA7. Four MCMC chains were run simultaneously for random trees for 1000000 generations. Samples were taken every 1000 generations. The first 25% of trees were discarded as burn-in phase of each analysis and posterior probabilities were determined from the remaining trees.

Entomopathogenic fungal formulations

The fungal strain was grown on YpSs medium (yeast extract 4 g, K_2HPO_4 1 g, $MgSO_4$ 0.5 g, starch 15 g, agar 20 g, distilled water 1000 mL) for 14 days at 23 °C in darkness. Freshly collected conidia from 15 days old cultures were used for the experiments. The viability of conidia was checked by germination tests in YpSs broth (yeast extract 4 g, K_2HPO_4 1 g, $MgSO_4$ 0.5 g, starch 15 g, distilled water 1000 mL) according to Imoulan and Elmeziane (2014). In all experiments, germination rates were higher than 90%. Two fungal formulations were used in the experiments: i) conidial water suspension; ii) *Beauveria*-laced protein bait (Bpb). The conidial water suspension was prepared by scraping conidia from well-sporulated cultures in Petri plates into 0.02% Tween 80 sterile aqueous solution. The suspension was then filtered through several layers of sterile cheesecloth to remove mycelial mats and the conidia concentration was estimated using a Thoma cell counting chamber. Conidia concentration was then adjusted by sterile 0.02% Tween 80 aqueous solution to a final concentration of 1.0×10^8 conidia mL^{-1}. Bpb was prepared by adding 1% (v/v) of protein bait (Amadene, Chimiberg) to the conidial water suspension. Tween 80 sterile aqueous solution (0.02%) was used as control solution.

Pathogenicity to *C. capitata*

Eggs and larvae of *C. capitata* are endophytic and pupae develop in the ground, therefore these instars are scarcely susceptible to the treatment proposed. How-

ever, we decided to test the susceptibility of all *C. capitata* stages since this information can be useful for a complete description of the insect-pathogen interaction.

Pathogenicity to eggs

One hundred newly laid eggs (< 12 h) were surface-sterilized with 1% sodium hypochlorite aqueous solution for 3 min, rinsed twice with sterile distilled water and then placed in a Petri dish (5 cm ⌀) where the lower surface was covered with a black filter paper (Hahnemüehle black filter paper, grade 551) and soaked with 0.4 mL of conidial suspension corresponding approximately to 2×10^6 conidia cm^{-2}. Control dishes were arranged with 100 newly laid eggs placed on black filter papers treated with the same volume of control solution. Eggs hatching was recorded every 24 hours × 5 days. Each treatment was replicated five times.

Pathogenicity to larvae and pupae

The effect of the fungal conidia on larval mortality and subsequent pupal ecdysis were evaluated by adding the conidial suspension to the larval diet. One hundred first instar larvae of *C. capitata* (< 24 h) were placed in groups of 10 in plastic containers (15 × 10 × 5 cm) and fed on artificial diet (Raspi and Loni, 1994) in which water was substituted by the conidial suspension (final conidial density about 2.5×10^7 conidia g^{-1}). The same number of larvae was fed on artificial diet without the conidial suspension, as a control. Each treatment was replicated three times. Larval mortality was calculated on the basis of the percentage of pupation. Subsequent pupal mortality was calculated on the basis of the percentage of adult emergence.

Pathogenicity to adults

Ten newly emerged adults (5 females and 5 males), previously cold anesthetized, were placed in a Petri dish (9 cm ⌀) where the two surfaces were covered with a filter paper soaked with 500 µL of the conidial suspension (1000 µL each Petri dish) corresponding approximatively to 8×10^5 conidia cm^{-2}. As a control, the same number of adults was placed in a Petri dish with filter paper soaked with the control solution. After 24 h, treated adults were transferred to plastic jars (8 cm in diameter, 10 cm in height) covered by a net cloth; adult diet and water were provided *ad libitum*. Each treatment was replicated five times. Mortality was monitored daily for 20 d. To assess the fungal infection (mycosis), dead flies were removed daily and immediately sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water. Cadavers were then placed on sterile wet filter paper in sterile Petri dishes, sealed with Parafilm and kept at room temperature to be inspected for development of fungal structures.

All the pathogenicity experiments were conducted under laboratory controlled conditions (24 ± 2 °C, 60 ± 10 % RH, and L16:D8 photoperiod).

Insecticidal activity of *Beauveria*-laced bait

In vitro experiments

Ten newly emerged (24-48 h) adults (5 males and 5 females) of *C. capitata* were placed in a 500 mL glass jar containing a lemon (*Citrus* × *limon* (L.) Burm. f.) leaf (surface area about 40 cm^2) with the petiole inserted in a 1.5 mL Eppendorf tube containing water. The leaf was sprayed with 500 µL of Bpb (corresponding approximatively to 1.25×10^6 conidia cm^{-2}). Flies were freely provided with solid diet and water. The jar opening was covered by cheesecloth. Flies' mortality was checked daily until the end of the experiment (20 d). Control and protein bait only (pb) treatments were also performed. In the control, the leaf was treated with 500 µL of the control solution; in the pb, the leaf was treated with 500 µL of 1% protein bait solution. Each treatment was replicated three times. Mycosis of dead flies was established as described above and the bioassay conducted under the same laboratory conditions.

In planta experiments

Thirty newly emerged (24-48 h) adults (15 males and 15 females) of *C. capitata* were placed in a 75 × 75 × 115 cm net cage (Bugdorm-2400 rearing cages, MegaView Science Education Services Co., Taichung, Taiwan) containing a potted lemon plant about 50 cm high. One leaf of the plant (surface area about 40 cm^2) was treated with 500 µL of Bpb (corresponding approximatively to 1.25×10^6 conidia cm^{-2}). Flies were freely provided with solid diet and water. Flies' mortality was checked daily until the end of the experiment (30 d). Control and pb treatments were also performed. Each treatment was replicated three times. Mycosis of dead flies was established as above described. The experiments were conducted in a heated glasshouse (240 m^2) under artificial light during May 2014. Daily mean values of T, RH, and global radiation were 21.2 °C, 70% and 9.4 MJ m^{-2}, respectively.

Data analysis

Mortality data were processed by one-way ANOVA with the inoculum treatment as factor. When needed to fulfil the analyses assumptions, data were normalized by arcsine transformation. Averages were separated by Tukey's post-hoc test. $P < 0.05$ was used as significant level of differences between means. Means in the text and tables are reported as untransformed data.

Survival probability was calculated by Kaplan-Meier analysis by repeated-measures modelling, with time as a categorical variable. Differences between the survival probabilities of flies under different treatments were analysed by Mantel-Cox log-rank test. All analyses were performed by SPSS 22.0 software (SPSS Inc., Chicago, IL, USA).

Results

Molecular identification of *Beauveria* sp. strain

The nuclear ribosomal internal transcribed spacer (ITS) region was amplified and sequenced to characterize the fungal strain DSM6651. The resulting sequence was 100% identical to those of *B. pseudobassiana* strain ARSEF 3405 (NR_111598.1) obtained by a BLAST search in GenBank. The phylogenetic analysis carried out with the obtained sequence and reference sequences

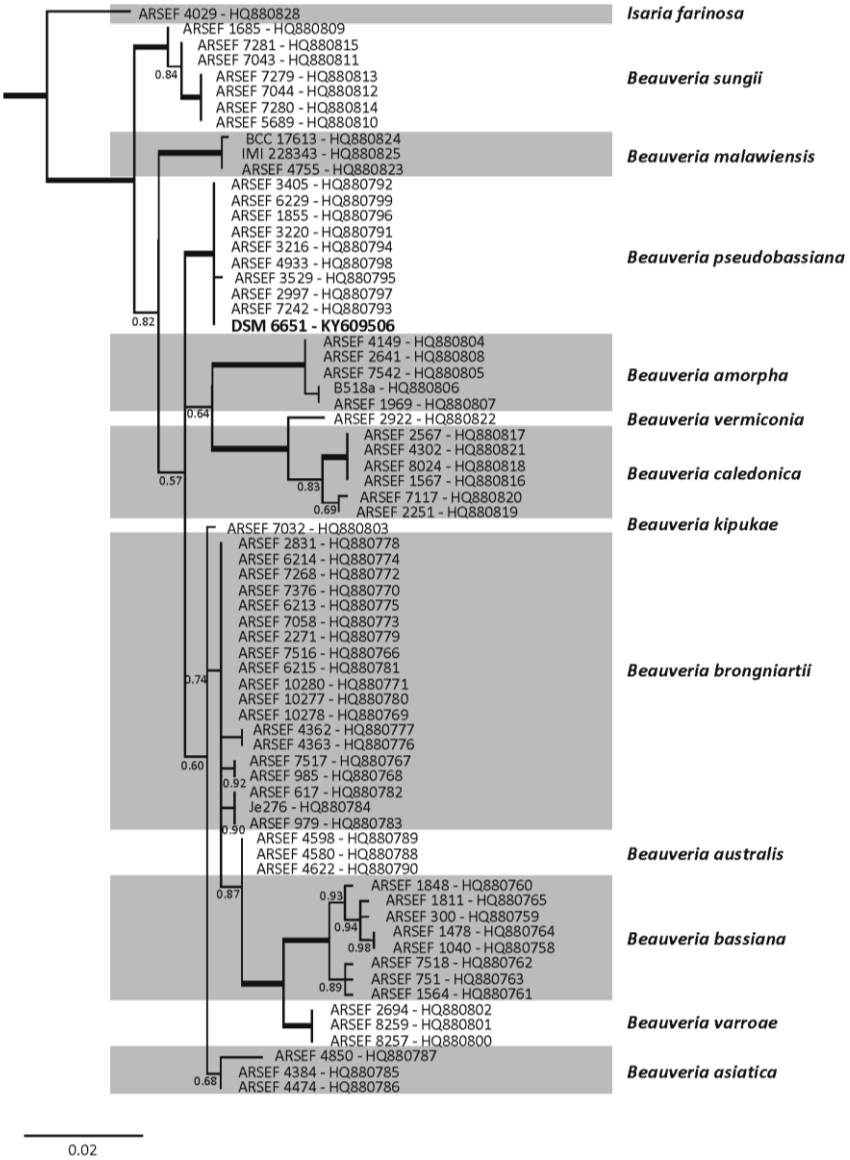

Figure 1. Phylogenetic analysis of 70 strains belonging to the genus *Beauveria* based on alignment of the ITS region. A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes 3.2.1. Bayesian posterior probability (BPP) values above 0.50 are shown at the nodes. The thickened nodes represent BPP of 1. Isolate used in this study (DSM 6651) is emphasized in bold. The scale bar represents the number of expected changes per site.

(Rehner *et al*., 2011) confirmed that the strain DSM6651 clustered within *B. pseudobassiana* (figure 1). Despite the original identification of the strain, both approaches indicate that the strain DSM6651 belongs to *B. pseudobassiana* taxon (figure 1).

Pathogenicity of *B. pseudobassiana* to *C. capitata*

The treatment with *B. pseudobassiana* conidia significantly lowered the hatching percentage of *C. capitata* eggs compared to the control (F = 40.484; d.f. = 1; P = 0.001). *B. pseudobassiana* treated eggs mortality was around three times the mortality of the control (55.67 ± 0.33 and, 19.00 ± 3.45%, respectively). Similarly, when the fungal spores were administered by the rearing medium, there was a significant larvicidal effect (F = 15.077; d.f. = 1; P = 0.018). Such effect persisted also after pupation, leading to death all the pupae in the *B. pseudobassiana* treated medium (table 1). A significant pathogenic effect was also observed when adults of *C. capitata* were treated with the *B. pseudobassiana* conidial suspension (F = 10.309; d.f. = 1; P = 0.015). The mortality of *B. pseudobassiana* treated flies was more than twice the mortality of the flies in the control treatment (63.86 ± 0.00 and, 31.42 ± 4.41%, respectively). Mycosis of unhatched eggs and dead adults was 46.67 ± 3.71, and, 54.00 ± 6.81%, respectively.

Insecticidal activity of *Beauveria*-laced bait

B. pseudobassiana-laced protein bait showed a good adulticidal activity, both *in vitro* and *in planta* tests. The exposition of *C. capitata* to the Bpb, carried out in the laboratory tests, showed a high virulence of the entomo-

Table 1. Larvicidal and pupicidal activity of *B. pseudobassiana* conidia against *C. capitata* (means ± SE).

Treatment	Larvae mortality %	Pupae mortality %	Adults emergence %
Control	41.67 ± 3.33 a	21.67 ± 7.26 a	36.66 ± 4.41 a
B. pseudobassiana	65.00 ± 5.00 b	35.00 ± 5.00 a	0.00 ± 0.00 b

Values are referred to the larvae submitted to the test. Values followed by different letters in each column are significantly different ($P \leq 0.05$).

Table 2. Adulticidal activity (% mortality means ± SE) of the protein bait laced with *B. pseudobassiana* conidia (Bpb), the protein bait-only (pb) and the control treatments against *C. capitata in vitro* and *in planta* tests, after 20 days.

Treatment	*In vitro*	*In planta*
Control	27.97 ± 8.76 a	37.78 ± 4.84 a
pb	42.47 ± 9.60 a	55.55 ± 7.77 ab
Bpb	95.83 ± 3.23 b	80.00 ± 9.62 b

Values followed by different letters in each column are significantly different ($P \leq 0.05$).

pathogenic fungus on the adults also when administrated as lacing agent in protein baits. After 20 d, mortality of treated flies was about three times the mortality of non-treated ones (control) ($F = 10.309$; d.f. = 1; $P = 0.015$) (table 2). A slightly higher mortality over control, although not significant (Tukey's; $P = 0.085$), was also observed when plants were treated with the protein bait only (table 2). A similar mortality was also observed *in planta* were the mortality of adults, after 20 days of treatment, was significantly affected by the presence of the entomopathogenic fungus in the protein bait ($F = 7.636$; d.f. = 2; $P = 0.022$) (table 2). *In planta*, Kaplan-Meier analysis of mortality rates (figure 2), showed a significantly different survival probability among treatments (Log-rank Mantel-Cox $\chi^2 = 39.31$; d.f. = 2; $P < 0.001$) with median survival time (MST) of flies in Bpb cages that was at least three times shorter than in control cages (table 3). Mycosis of dead flies was 72.33 ± 4.84 and 65.33 ± 2.91% *in vitro* and *in planta* tests, respectively.

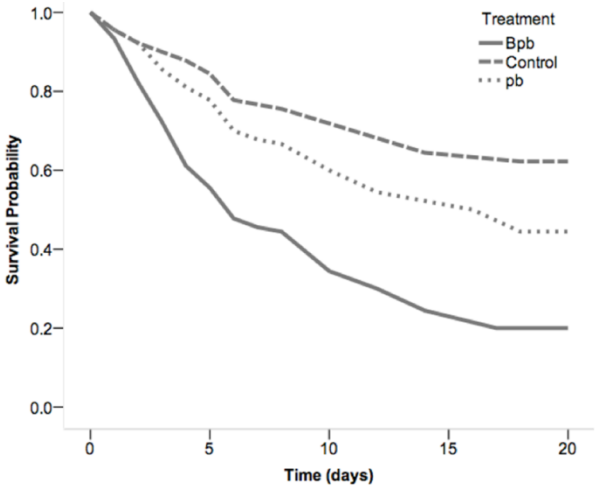

Figure 2. Kaplan-Meier estimates of survival probabilities of *C. capitata* populations exposed to the protein bait laced with *B. pseudobassiana* conidia (Bpb), the protein bait-only (pb) and the control treatments in greenhouse tests. Observations censored at 20 days.

Discussion

Entomopathogenic microorganisms represent a successful alternative to chemical pesticides in organic agriculture or where pesticide resistance and environmental concerns limit the use of synthetic products (Glare *et al*., 2012). This study indicates that *B. pseudobassiana* has significant ovicidal, larvicidal and adulticidal effects against *C. capitata* and that the conidia of the fungus could be successfully utilized as a biological insecticide in protein baits.

To the best of our knowledge, literature offers very limited data regarding the susceptibility of *C. capitata* to *B. pseudobassiana*. However, Lozano-Tovar *et al*. (2013) reported that *B. pseudobassiana* strains, inoculated in the soil, are able to infect and kill puparia and also affect, as a consequence, adult mortality of *C. capitata*. It should also be taken into account that, since *B. pseudobassiana* is morphologically not distinguishable from *B. bassiana*, some of the previous studies that report data on the pathogenicity of *Beauveria bassiana* against *C. capitata* may have been conducted with the species *pseudobassiana* only recently identified (Rehner *et al*., 2011). Actually, the fungal strain utilised in this

Table 3. Average and median survival time (days ± SE) of *C. capitata* adults exposed to the protein bait laced with *B. pseudobassiana* conidia (Bpb), the protein bait-only (pb) and the control treatments in greenhouse tests. Survival time limited to 20 days. CI = Confidence Interval.

Treatment	Average[1]	95% CI	Median[1]	95% CI
Control	15.23 ± 0.72 a	13.82-16.65	> 20 a	-
pb	13.30 ± 0.76 b	11.81-14.79	16.00 ± 2.71 b	10.68-21.31
Bpb	9.10 ± 0.72 c	7.68-10.50	6.00 ± 1.42 c	3.21-8.79

[1] Kaplan-Meyer estimates of survival. Means within columns with different letter are significantly different (log-rank Mantel-Cox test; $P < 0.05$).

experiment, originally isolated from *Holotrichia morose* Walker (Coleoptera Scarabaeidae) in China, was deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) as *B. brongniartii*. However, phylogenetic analysis of the ITS sequences showed that the strain DSM6651 corresponds to *B. pseudobassiana*, species morphologically similar to but phylogenetically distant from *B. bassiana*, *B. asiatica* and *B. australis*, which are sister lineages to *B. brongniartii* (Rehner *et al*., 2011).

Our study showed that *B. pseudobassiana* is able to affect all the stages of *C. capitata*. Most studies on fungal species as biocontrol agents of insect pests regards their effects on adults, and very few have studied the effect on other stages (Brabbs *et al*., 2015). This is justified by the fact that in most cases fungal spores cannot easily come into contact with eggs or larvae since they are protected by plant tissues. However, a survey of insect susceptibility in all stages can be useful for a complete description of the insect-pathogen interaction. Moreover, recent investigations revealed that insect pathogenic fungi could act as endophyte colonizing plant tissues (i.e. roots, stem, leaf) (Posada and Vega, 2005; Sasan and Bidochka, 2012; Behie *et al*., 2015). In this scenario, an interaction between insect eggs and larvae and the fungus within the plant tissue cannot be excluded.

In general, we observed a rather high mortality of larvae and adults in the control. This is not unexpected since high percentages of mortalities have been previously observed for *C. capitata* and other tephritid species under laboratory conditions (Conti, 1988; Papadopoulos and Katsoyannos, 2002; Kostantopoulou and Mazomenos, 2005). In particular, Papadopoulos and Katsoyannos (2002) find that, under laboratory conditions (25 °C and 65% RH), the total immature mortality (from newly hatched larvae to adults) ranged about from 53 to 77%, and Conti (1988) on artificial medium observed a cumulative mortality of *C. capitata* stages (eggs, larvae and pupae) until adult emergence, of about 84%. In line with our findings, Kostantopoulou and Mazomenos (2005) observed 28 and 22% of mortality of *Bactrocera oleae* (Rossi) and *C. capitata* adults, respectively, in the controls after 21 days of treatment.

In this study, *B. pseudobassiana* was also very effective against *C. capitata* larvae and pupae. In accordance, Yaginuma *et al*. (2006) observed a high virulence of *B. brongniartii* against the first instar larvae of *Heptophylla picea* Motschulsky (Coleoptera Scarabaeidae) and Oreste *et al*. (2015) found that the treatment with *B. bassiana* strains leads to the death of about 50% of young (2 days old) medfly puparia. It is noteworthy that, in our experiment, *B. pseudobassiana* infection contracted by the larval instar, persisted also in the pupae leading to 100% mortality of treated individuals. A similar persistence of entomopathogenic fungi infection along the development stages has been previously observed for the medfly, as well as for other insect species (Poprawski *et al*., 1985; Ekesi *et al*., 2002; Goble *et al*., 2011; Lozano-Tovar *et al*., 2013; Imoulan and Elmeziane, 2014). This persistence of the mycosis could be due to the relative duration of the fungal infection process respect to the insect cycle and could have a practical importance, increasing the total impact of the fungal pathogen among the population (Beris *et al*., 2013).

Effective eradication of potentially damaging pests often requires the use of pesticides by conventional broadcast aerial spraying (Hosking *et al*., 2003). In the case of fungal biocontrol agents, this implies the release in the environment of a large quantity of fungal spores with consequent off-target effects. In accordance with the attract and kill strategy (Stetter and Folker, 2000), the administration of the fungal spores coupled with protein baits allows the administration of the fungus in spots on the fruit trees, allowing the minimum dispersion of the fungus in the environment and the higher inoculation efficiency (Strand *et al*., 2014). In this study, the efficacy of *B. pseudobassiana*-laced protein baits against adults of *C. capitata* was tested both *in vitro* and *in planta*. *In vitro*, Bpb showed a very high efficacy against *C. capitata* adults causing the death of almost all the fly population after 20 days. This result is consistent with the observation of Konstantopoulou and Mazomenos (2005) who found that, among several isolates, *B. bassiana* and *B. brongniartii* were the most pathogenic fungi to *C. capitata* with mortality of 85.6 and 97.4%. Similarly, Beris *et al*. (2013) obtained mortality rates of 46.8, 56.3 and, 88.0% of medfly adults by contact with conidia of *B. bassiana*, *Isaria fumosorosea* (Wize) and *M. anisopliae*. The observed slightly higher mortality of flies treated with pb in comparison with the control is not surprising since a similar effect has been already observed in previous experiments (Bazzoni *et al*., 1997; Mangan *et al*., 2006).

The effectiveness of the Bpb as infection source was confirmed also by the *in planta* tests, performed in the greenhouse, where the survival time of the flies in treated cages was around three times shorter than the one of control. The observed survival time (MST = 6 d) was very similar to that found in laboratory conditions by Yousef *et al*. (2014) using conidia of *M. brunneum* (Petch) sprayed directly onto the flies (LT_{50} = 5.6 d).

Overall, our results are in accordance with previous experiments performed with entomopathogenic fungi showing that the insecticidal activity, observed in laboratory trials, could be obtained also in practical applications, with effectiveness similar to or higher than those obtained by conventional chemical treatments (Ortu *et al*., 2009; Navarro-Llopis *et al*., 2015).

Conclusions

Overall, the current study shows that *B. pseudobassiana* used as biological insecticidal agent in protein baits could have substantial impacts on fly populations. The observed effectiveness of the treatments raises the possibility of develop an attractive-infective tool based on spot surface applications of entomopathogenic fungi by protein baits to control tephritids and, consequently, to reduce yield losses in intensive orchards systems and greenhouses. However, further experiments are needed to confirm the potential of *B. pseudobassiana* in open field and to delineate the best formulation to enhance its effectiveness as bioinsecticide in protein baits.

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