Available online at www.sciencedirect.com
ScienceDirect
Journal of Invertebrate Pathology 97 (2008) 237–244
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Evaluation of *Metarhizium anisopliae* (Metsch) Sorok. to target larvae and adults of *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) in soil and fiber band applications

Pierdomenico Marannino [a,b], Cándido Santiago-Álvarez [a], Enrico de Lillo [b], Enrique Quesada-Moraga [a,*]

[a] *Departamento de Ciencias y Recursos Agrícolas y Forestales, ETSIAM, Universidad de Córdoba, Campus de Rabanales, Edificio C4 Celestino Mutis, Cordoba 14071, Spain*
[b] *Dipartimento di Biologia e Chimica Agro-forestale e Ambientale, Università degli studi di Bari, Via Amendola 165/a, Bari 70126, Italy*

Received 29 March 2007; accepted 10 September 2007
Available online 15 September 2007

Abstract

The aim of this work has been to evaluate in the laboratory the potential of entomopathogenic fungi against adults and larvae of *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) through fiber band application and a potted plant bioassay with soil application, respectively. Our previous findings revealed that *Metarhizium anisopliae* EAMa 01/58-Su isolate was the most virulent against neonate larvae of the buprestid. In the present work, *M. anisopliae* EAMa 01/58-Su isolate has been also shown to be highly virulent against adult beetles by immersion in a conidial suspension; thus it was selected to accomplish our objectives. When adult beetles were stimulated to climb 100×200 mm non-woven commercial fiber bands impregnated with conidia of *M. anisopliae* EAMa 01/58-Su isolate, total mortality rates varied from 85.7% to 100.0%; whereas no significant correlation was detected between the time needed to cross the band (mean value 648.7 ± 22.4 s) and the time of death, with mean average survival time ranging between 10.3 and 16.0 days, compared to 28 days of the controls. Potted seedlings (5–6 months old) of cherry plum (*Prunus myrobalana* Lois.), a commonly used apricot rootstock, were used to study the efficacy of soil treatment with *M. anisopliae* EAMa 01/58-Su isolate against neonate *C. tenebrionis* larvae. The soil inoculation with *M. anisopliae* EAMa 01/58-Su isolate had a significant effect on the mean number of dead larvae recovered from the roots, with mean mortality ranging from 83.3% to 91.6%; whereas no significant differences were detected between the three fungal doses. In all cases, dead larvae found within roots exhibited external signs of fungal growth. Hence, it may be possible to use *M. anisopliae* EAMa 01/58-Su isolate in a biocontrol strategy targeting both adults and larvae of *C. tenebrionis*.

Keywords: *Beauveria*; *Metarhizium*; Soil treatment; Virulence; Neonate larvae; Endophitic; Biological control; *Capnodis tenebrionis*; Buprestidae; Fiber bands; Tree borers; Soil dwelling pest; Entomopathogenic fungi; Coleoptera

1. Introduction

The Mediterranean flatheaded peachborer, *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae), poses an enormous threat to stone-fruit crops of the family Rosaceae in the Mediterranean basin due to the feeding behavior of both adults and larvae (Alfaro-Moreno, 2005). Adults cause defoliation by feeding on twigs and young branches throughout the warm season (Garrido, 1984). Neonate boring larvae, which hatch from eggs deposited on the ground close to the base of the tree stem, reach the host by crawling through the soil (Marannino and de Lillo, 2007a) and may cause tree death or severe plant weakening in consequence of root/collar tunnelling under the bark. In consideration of the high injury it produces, *C. tenebrionis*

* Corresponding author. Fax: +34 957218440.
E-mail address: cr2qumoe@uco.es (E. Quesada-Moraga).

0022-2011/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2007.09.006

has been included on the European list of harmful organisms impairing quality of stone-fruit propagating material (Comm. Dir. 93/48/EEC).

To date, no effective methods for controlling this jewel beetle are available in organic fruit production, and the only *C. tenebrionis* management tactic in conventional fruit production is nonselective chemical insecticide application by repeated foliar sprays against adults and by soil dusting against neonate larvae. This approach is generally unsatisfactory due to its adverse effects on humans, non-target fauna and environment ecological balance (Ben-Yehuda et al., 2000); thus biological control might represent a viable option. Neonate larvae of *C. tenebrionis* are susceptible to steinernematid and heterorhabditid nematodes (Marannino et al., 2004; García del Pino and Morton, 2005); whereas the use of these nematodes against the adults in the aerial part of the plant is highly limited, not only by the lack of efficacy, but mainly by desiccation (Begley, 1990). Despite the possible use of nematodes, the most important natural enemies of *C. tenebrionis* are the entomopathogenic fungi (Marannino and de Lillo, 2007b), which are attractive potential biocontrol agents for *C. tenebrionis* because they may be economically produced in large quantities and they can be formulated in a variety of ways (Wraight et al., 2001). Moreover, they could provide an additional advantage as they could be used in a biocontrol strategy targeting both adults and larvae. Such a strategy will require the identification of a fungal isolate active against both insect stages.

In a previous study, we found five isolates of the fungal pathogens *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* to be very effective for the control of *C. tenebrionis* neonate larvae using a bioassay method simulating their natural endophytic growing conditions (Marannino et al., 2006). However, the pathogenicity of these candidate isolates against *C. tenebrionis* adults has not yet been investigated.

In order to develop a reliable *C. tenebrionis* microbial control strategy, not only is the lethality of the candidate fungal isolate to larvae and adults important, as revealed by laboratory bioassays, but also critical is its ability to operate satisfactorily when applied in the soil, to target larvae, or to the aerial part of the plant, to target the adults. When emerging from soil, *C. tenebrionis* adults must feed intensively before mating and ovipositing; due to their weakness and to the lack of optimal temperature, especially in springtime after over-wintering, they are not able to fly and are thus forced to reach the host-tree canopy by climbing the trunk (Caponero et al., 2006). On the basis of this behavioral feature, the efficacy of entomopathogenic fungi could be tested through application on the stem. To this end, as shown by several authors (Nobuchi, 1993; Higuchi et al., 1997; Dubois et al., 2004), fiber bands impregnated with the mycopathogen conidial powder and wrapped around the trunk represent the most effective microbial control measure available to date to combat tree-boring Cerambycidae and Buprestidae.

Consequently, the first objective of this work was to select from among our best isolates for *C. tenebrionis* larvae, those that are also pathogenic against adults. Among them, we selected a candidate fungal isolate for further evaluation against both stages, administering it by soil application and by fungal bands, respectively.

2. Materials and methods

2.1. Entomopathogenic fungi

The isolates used in the experiment belong to the fungal collection of the Agricultural and Forestry Sciences and Resources (AFSR), Department of the University of Córdoba (Spain). *Beauveria bassiana* isolate EABb 04/01-Tip was obtained from *Timaspis papaveris* (Hymenoptera; Cynipidae) at Sevilla (Spain) and *M. anisopliae* EAMa 01/58-Su isolate from the soil of a wheat crop at Córdoba (Spain). Both isolates were chosen due to their high level of virulence against peach borer neonate larvae (Marannino et al., 2006). Fungal cultures were grown on Malt Agar (12.75 g/l malt extract, 2.75 g/l dextrine, 2.35 g/l glycerol, 0.78 g/l gelatine peptone and 15.0 g/l agar) at 25 °C in the dark. Conidial suspensions for experiments were prepared by scraping conidia from 15-day-old well sporulated cultures into an aqueous solution of 0.2% Tween-80. Suspensions were then filtered through several layers of cheesecloth to remove mycelium and the concentrations of viable conidia were estimated as colony forming units, using a dilution plate count method.

2.2. Insects

About 500 recently emerged adults (both males and females) of *C. tenebrionis* were collected from heavily infested stone-fruit orchards in the provinces of Córdoba and Murcia in Southern Spain during September and October 2006. The beetles were fed on fresh apricot twigs under laboratory conditions (28–30 °C, 40–50% RH, 16:8 L/D photoperiod). This allowed them to reach sexual maturity, mate and lay eggs in Petri dishes filled with sifted sand, according to the method described by Garrido et al. (1987) to separate the soil from the eggs. Incubation took place under the same conditions. Adults of approximately the same size were selected for the experiments. In those experiments performed with larvae, we selected neonates from the rearing boxes within 24 h after hatching.

2.3. Susceptibility of C. tenebrionis adults to B. bassiana and M. anisopliae

The beetles were immersed individually for 10 s in a conidial suspension (1.0×10^8 conidia/ml) or in 0.2% Tween 80 aqueous solution (for controls). They were then moved to clean rearing boxes (240 × 80 mm) kept at 25 °C, fed every day on fresh apricot twigs and monitored daily for mortality. There were three replicates (boxes) per

treatment (two isolates and the controls) and 10 individuals in each box (5 males and 5 females). Adult mortality was assessed within the 4 weeks following the application. The cadavers were removed daily, and when external signs of fungal infection were not directly observed, cadavers were immediately surface sterilized with 1% sodium hypochlorite for 1 min followed by three rinses with sterile distilled water, placed on sterile wet filter paper in sterile Petri dishes, sealed with parafilm and kept at room temperature.

2.4. Band exposure bioassay

On the basis of data resulting from the previous susceptibility tests, we selected *M. anisopliae* EAMa 01/58-Su isolate for the band exposure assay. For that, we used Taotec® bands (Soften S.p.A., Italy), which are a commercially available pest control product designed to be applied around the collar or trunk, acting as a proven mechanical barrier to prevent crawling insects (mainly weevils) from climbing onto host plants from the ground. Non-woven fiber bands obtained from Taotec® were composed of PEGT [poly (ethylene glycol terephtalate)] (density of 10 kg/m^3 and 0.03% hygroscopicity).

All bands used in this bioassay measured 100×200 (h) mm. The bands were impregnated by dragging on a bed of conidia and then they were gently shaken to remove excess conidia. Initial conidial density on bands, which was quantified by blending four 30 cm^2 pieces of bands and counting conidia using a hemocytometer, was $4.45 \times 10^8 \pm 5.0 \times 10^7$ conidia/cm^2.

The fiber bands were wrapped around the central area of each of the six 400 mm long and 15 mm diameter pieces of apricot branches cut for the experiment, which were then placed vertically in a box containing sterilised soil, leaving approximately 5 cm of uncovered branch between the ground and the base of the band. One beetle was placed close to each stem and allowed to climb. During the experiment, a 60 W light bulb was switched on over the stems and, when needed, the buprestids were also mechanically stimulated to start climbing. The test ended 15 min after the beginning of climbing, or before if the insect had crossed the barrier. Individuals were discarded if they did not start climbing within 5 min or did not walk far enough to reach the Taotec® strip within a limit of 10 min; individuals that fell down to the ground during the test were placed again close to the stem to restart climbing. The time of contact with the fungal band was recorded. Test ($n = 120$) and control ($n = 40$) adults were then individually reared in sterile 50 ml conical centrifuge tubes (JLC, Spain) with the opening covered by 1-mm-mesh window screen stopped with a rubber band. They were fed on fresh apricot twigs and monitored daily for mortality. When external signs of fungal infection were not directly observed, cadavers were immediately surface sterilized with 1% sodium hypochlorite for 1 min followed by three rinses with sterile distilled water, placed on sterile wet filter paper in sterile Petri dishes, sealed with parafilm and kept at room temperature. Mortality was assessed for 4 weeks after exposure.

2.5. Lethal time of C. tenebrionis larvae inoculated with M. anisopliae EAMa 01/58-Su isolate

The neonate larvae were immersed individually for 10 s in a spore suspension (1.0×10^8 conidia/ml) of *M. anisopliae* EAMa 01/58-Su isolate or in 0.2% Tween 80 aqueous solution (for controls). They were then gently transferred with a small soft paintbrush (no. 2) into a 10-mm-long cut made at one end of 1-cm-diameter branch pieces collected from an organic orchard and allowed to start boring. The infested material was kept in rearing boxes at 25 °C and 65% RH, in the dark. Each day after the application, 8 branch pieces were stripped of their bark and examined for larvae using a dissecting microscope, until all larvae were found to be dead.

2.6. Potted plant bioassay with soil application of M. anisopliae EAMa 01/58-Su isolate

The bioassay was performed with 5–6-month-old seedlings of cherry plum (*Prunus myrobalana* Lois.), a commonly used apricot rootstock, grown in $5 \times 5 \times 5$ cm pots. These nursery plants (average diameter of the stem base 3–4 mm) were selected mainly to allow a simpler and faster detection of the larvae in the root system. The plants were maintained at 25 ± 5 °C and 60 ± 5% RH and regularly supplied with sterile distilled water. Five millilitres of a spore suspension (1.0×10^6, 1.0×10^7, 1.0×10^8 conidia/ml) or of 0.2% Tween 80 aqueous solution (for control) were spread onto the soil (85% organic matter, pH 6.5). Irrigation was then stopped for 4 days to make the roots more accessible to the neonate larvae of *C. tenebrionis*. Two days after the application, 4 larvae were placed in each pot near the base of the stem and allowed to settle under the soil surface. Three weeks later, the roots were checked for the presence of larvae inside them to assess larval mortality (Fig. 1A and B). Each treatment consisted of 4 replicates, with 10 plants per replicate, each replicate being performed with new conidial suspensions.

To evaluate the persistence of *M. anisopliae* EAMa 01/58-Su isolate in the soil, each spore suspension was also applied to an additional batch of 7 potted plants cultivated as described above. Once a week for 21 days, a 1-g soil sample (consisting of seven 43 mg sub-samples from the 1.5 top first cm of each pot) was taken for isolation of *M. anisopliae*. This sample was suspended in 9 ml of sterile distilled water, shaken for 3 h at 120 rev min^{-1}, serially diluted, and plated in quadruplicate in Sabouraud dextrose agar medium (Cultimed, Barcelona, Spain) containing chloramphenicol (0.05 g/l). Numbers of *M. anisopliae* colony forming units (c.f.u.) were determined after incubation for 12 days at 25 °C.

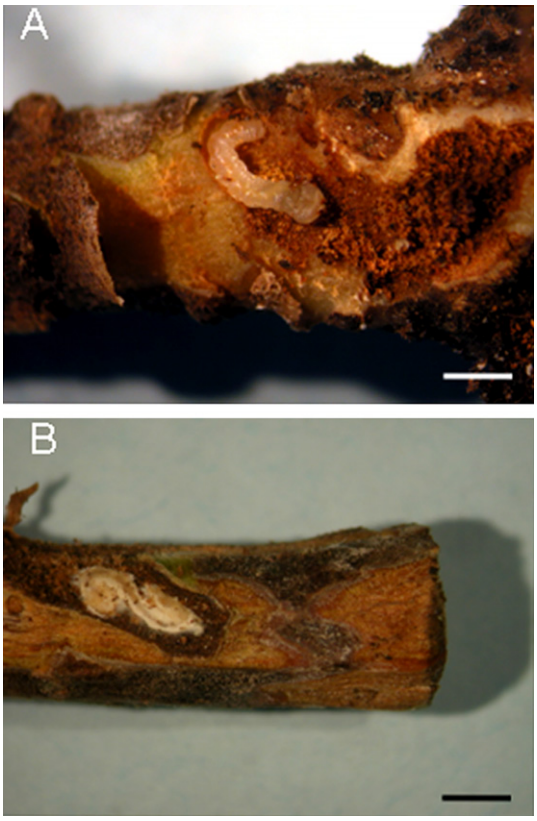

Fig. 1. Potted plant bioassay with soil application of *M. anisopliae* EAMa 01/58-Su isolate. (A) Control *C. tenebrionis* control larva that has endophitically colonised the roots. Bar = 10 mm; (C) Neonate larva of *C. tenebrionis* mycosed by *M. anisopliae* within a gallery in a root. Bar = 20 mm.

2.7. Statistical analysis

Both larval and adult mortality data were analyzed using analysis of variance (ANOVA), and the Tukey (HSD) test was used to compare means. Before conducting ANOVA, all percentages were transformed using the arcsin transformation. One way ANOVA was also used for analysis of conidial densities. In the adult band exposure assays, the cumulative mortality response across the assessment period was analyzed with Kaplan–Meier survival analysis. In the larval lethal time evaluation bioassays, the LT_{50} values were determined using the probit analysis method for correlated data (Throne et al., 1995). All analyses were carried out using the SPSS 12.0 for Windows (SPSS 2002), except the probit analysis for correlated data that was performed using a test version of the program Mathematica 5.0. Correlation (Pearson) between time to cross the band and time of death was performed using SPSS.

3. Results

3.1. Susceptibility of C. tenebrionis adults to B. bassiana and M. anisopliae

There was a significant effect of the fungal treatment on adult mortality ($F_{2,17} = 494.0$; $P < 0.0001$), with 100.0% and 86.7% mortality rates for isolates *B. bassiana* EABb 04/01-Tip and *M. anisopliae* EAMa 01/58-Su respectively, compared with uninoculated individuals, which showed 3.3% mortality (Table 1). After inspection, all dead insects exhibited mycosis. There was no significant interaction between treatment and beetle sex ($F_{2,17} = 2.0$; $P = 0.25$), with no significant differences between male and female mortalities within each treatment (Tukey test; $P < 0.05$) (Table 1). Average Survival Time (AST) of treated adults differed slightly (log-rank = 4.51; $P = 0.03$), with mean values of 11.1 and 11.6 days for isolates EABb 04/01-Tip of *B. bassiana* and EAMa 01/58-Su of *M. anisopliae*, respectively (Table 1).

Due to its high activity against larvae and adults of *C. tenebrionis* (Marannino et al., 2006), we selected the *M. anisopliae* EAMa 01/58-Su isolate for further evaluation against both stages.

3.2. Efficacy of fiber bands impregnated with M. anisopliae EAMa 01/58-Su isolate against C. tenebrionis adults

Of the 120 assayed individuals, 29 did not finish climbing the band within the time limit of 15 min. The mean period (±SE) required for the remaining 91 insects to cross the 200 mm of band was 648.7 ± 22.4 s, with a minimum of 72 s and a maximum of 878 s. This period was statistically similar to the one of the control group (623.3 ± 18.3 s, with a minimum of 67 s and a maximum of 845 s). When the time needed to cross the band was correlated with the time of death, a correlation coefficient of −0.0013 ($P = 0.99$) was obtained, indicating that there was no relationship between exposure time and survival time. In addition, neither total mortality nor mortality due to mycosis were correlated with exposure time, with total mortality rates ranging between 85.7% and 100.0% and mycosis rates varying from 33.3% to 100.0%. No mortality was observed in the controls. Average Survival Time values did not differ (Log rank test; $P < 0.05$) between exposures, with mean values ranging between 10.3 to 16.0 days (Table 2).

3.3. Lethal time of C. tenebrionis larvae inoculated with M. anisopliae EAMa 01/58-Su isolate

The LT_{50} of treated neonate larvae was calculated for a dose of 1.0×10^8 conidia/ml, which gave a value of 4.22 d [(3.62–4.78 d) (slope ± SE = 16.5 ± 3.8, $\chi^2 = 0.095$ ($P = 0.758$)].

3.4. Efficacy of soil inoculation with M. anisopliae EAMa 01/58-Su isolate against C. tenebrionis neonate larvae

Of the 160 seedlings used, 158 were successfully infested by *C. tenebrionis* neonate larvae. Percentages of applied larvae recovered from the roots varied from 45.0% to 50.0% (Table 3), with significant differences observed neither between control and fungal treatments ($F_{3,159} = 0.33$; $P = 0.8050$) nor among the three assayed fungal doses

Table 1
Insecticidal activity of *Beauveria bassiana* and *Metarhizium anisopliae* isolates on *C. tenebrionis* adults 15 days after treatment

Isolate	Mortality[a,b] (%) ± SE			Kaplan–Meier survival analysis	
	Total	Male	Female	AST[c] d (+SE)	Confidence interval (d)
Control	3.3 ± 3.3a	6.6 ± 6.6a	0.0 ± 0.0a	15.0 ± 0.0a	15.0–15.0
B. bassiana EABb 04/01-Tip	100.0 ± 0.0b	100.0 ± 0.0b	100.0 ± 0.0b	11.1 ± 0.2b	10.6–11.6
M. anisopliae EAMa 01/58-Su	86.7 ± 6.6b	93.3 ± 6.6b	80.0 ± 11.5b	11.6 ± 0.5c	10.8–12.5

[a] There were three replicates per treatment, with 10 individuals per replicate (5 males and 5 females). Means within columns with the same letter are not significantly different ($P < 0.05$) according to the Tukey (HSD) test. After inspection, all dead insects exhibited mycosis.
[b] For all isolates and control, there were no significant differences between male and female mortalities.
[c] Average Survival Time (AST) limited to 15 days. Means within columns with the same letter are not significantly different ($P < 0.05$) according to the log-rank test.

Table 2
Kaplan–Meier survival analysis of field collected *C. tenebrionis* adults inoculated by walking on non-woven fiber bands impregnated with *M. anisopliae* EAMa 01/58-Su isolate

Time on band[a] (s)	Number of insects	Kaplan–Meier survival analysis	
		AST[b] (mean ± SE) d	95% Confidence interval (d)
60–120	3	11.0 ± 2.0a	7.1–14.9
120–180	3	11.0 ± 1.2a	8.7–13.2
180–240	0	—	—
240–300	2	13.0 ± 0.0a	13.0–13.0
300–360	5	14.0 ± 1.5a	11.1–16.9
360–420	5	10.8 ± 1.8a	7.3–14.3
420–480	3	16.0 ± 5.1a	5.9–26.1
480–540	2	14.0 ± 1.0a	12.0–15.9
540–600	7	13.4 ± 2.5a	8.6–18.3
600–660	6	12.5 ± 1.4a	9.8–15.2
660–720	10	10.3 ± 0.8a	8.8–11.8
720–780	15	12.2 ± 0.8a	10.7–13.6
780–840	18	12.8 ± 0.7a	11.4–14.2
840–900	41	14.4 ± 0.7a	13.0–15.9

[a] Initial conidial density on bands was $4.45 \times 10^8 \pm 5.0 \times 10^7$ conidia/cm^2. No mortality was observed in the controls.
[b] Average Survival Time (AST) data with the same letter are not significantly different (log rank statistic; $\alpha = 0.05$). AST limited to 28 days.

Table 3
Effect of soil application with *M. anisopliae* EAMa 01/58-Su isolate on *C. tenebrionis* neonate larvae

Dose (conidia/ml)	Larvae recovered per plant[a] (all data are mean ± SE)		
	Total	Dead larvae[b]	
		Number	Percentage
0	2.0 ± 0.1a	0.0 ± 0.0a	0.0 ± 0.0a
10^6	1.8 ± 0.1a	1.5 ± 0.1b	83.3 ± 4.6b
10^7	1.9 ± 0.2a	1.6 ± 0.1bc	83.7 ± 4.8b
10^8	2.0 ± 0.1a	1.9 ± 0.1c	91.6 ± 3.3b

[a] Four *C. tenebrionis* neonate larvae were placed in each of 10 pots. The effect of the treatment was evaluated for 21 days post-inoculation.
[b] All dead larvae displayed *M. anisopliae* fungal outgrowth.

($F_{2,119} = 1.14$; $P = 0.3240$). However, soil inoculation with the *M. anisopliae* EAMa 01/58-Su isolate had a significant effect on the mean number of dead larvae recovered from the roots ($F_{3,159} = 58.91$; $P < 0.0001$), with mean mortality ranging from 83.3% to 91.6%. No significant differences (Tukey test; $\alpha = 0.05$) were detected in larval mortality among the three fungal doses (Table 3). In all cases, dead larvae found within roots exhibited external signs of fungal growth (Fig. 1B).

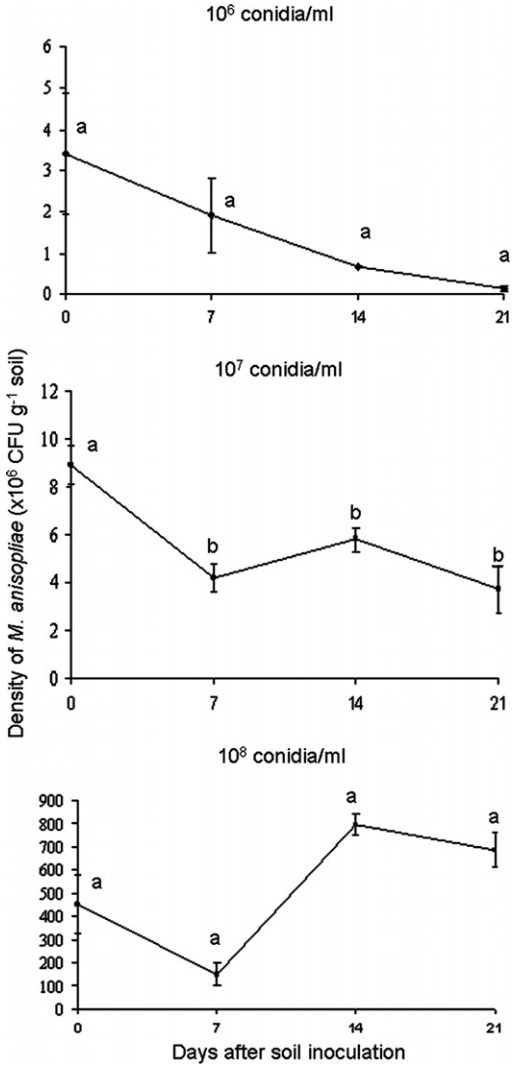

Fig. 2. Changes in density of *M anisopliae* (mean ± EE) of colony forming units assessed on the selective medium in soil samples taken from the top 15 mm of the potted plants for 21 days. Data followed by the same letter are not significantly different ($P < 0.05$) according to the Tukey (HSD) test.

Changes in the density of *M. anisopliae* conidia were obtained over 21 days following soil inoculation (Fig. 2). In general, there was a trend towards a reduction in the conidial density. For conidial concentrations of 1.0×10^6 and 1.0×10^7 conidia/ml, time had a significant effect on conidial densities ($F_{3,15} = 4.33$; $P = 0.0378$ and $F_{3,15} = 12.74$; $P = 0.0014$, respectively), with mean values ranging between 1.47×10^5 and 3.45×10^6 and between 3.75×10^6 and 5.85×10^6 conidia/g soil, respectively. For the concentration of 1.0×10^8 conidia/ml, however, this effect was not significant ($F_{3,15} = 2.19$; $P = 0.15$), with mean values varying from 1.50×10^7 to 7.95×10^7 conidia/g soil (Fig. 2).

4. Discussion

The main difficulty in managing wood-boring insects in the field comes from the fact that they are protected under the tree bark during much of their life cycle. By considering the case of *C. tenebrionis*, adults and newly hatched larvae represent the stages exposed and vulnerable to control measures. In a previous study (Marannino et al., 2006), the pathogenicity of *B. bassiana* and *M. anisopliae* autochthonous strains was established against neonate larvae. The present study demonstrated, through a direct immersion bioassay and using the same conidial concentration (1.0×10^8 conidia/ml), that also the peachborer adults are highly susceptible to both fungi. The results achieved are consistent with those reported for other buprestid, *Agrilus planipennis* Fairmaire, which showed no adult mortality for the first 3 days after exposure and a cumulative mortality ranging from 97.5% to 100.0% using *B. bassiana* and *M. anisopliae* at a concentration of 10^7 conidia/ml (Liu and Bauer, 2006). However, the second measure of virulence, the Average Survival Time (AST) of treated adults, was longer for *C. tenebrionis* (11–12 d) than for the the emerald ash borer (4.2–4.7 d), even though the conidial suspension was more concentrated (10^8 conidia/ml). The larger size of *C. tenebrionis* individuals (15–25 mm long versus 7.5–13.5 mm) and the greater cuticle hardiness might account, at least in part, for these differences. In adult test, *M. anisopliae* EAMa 01/58-Su isolate, while highly effective, proved to be not as virulent as *B. bassiana* EABb 04/01-Tip isolate but it outperformed the latter and all other isolates against larvae (Marannino et al., 2006) whose feeding activity causes undoubtedly the most important economic losses, even if prevention of feeding by adults is also necessary. Thus, judging by our previous findings and by the new outcomes, we selected the former isolate for evaluation in the subsequent bioassays.

Our experiment with Taotec® bands impregnated with *M. anisopliae* EAMa 01/58-Su isolate showed that the AST of treated beetles (in the range of 11–14 days) was significantly shorter than the controls, with mortality rates approaching between 85.0% and 100.0%. There are scarce data addressing the use of fiber bands to apply entomopathogenic fungi, and most of them have being obtained under field conditions (Hajek et al., 2006). The data obtained in the laboratory by Dubois et al. (2004) may be compared to those presented here because they used similar assay protocols with the mycopathogen *Beauveria brongniartii* (Saccardo) Petch against the cerambycid *Anoplophora glabripennis* (Motschulsky). These authors obtained median survival times ranging from 9.0 to 10.0 days for *A. glabripennis* adults using non-woven fiber bands impregnated with the fungus, numbers that are in the same range as ours. Further work under field conditions is needed in order to study the efficacy of these fiber bands, directly, by monitoring adult longevity and efficacy to prevent *C. tenebrionis* adult feeding, and also indirectly, by estimating the effect of the fungal treatment on reproduction. From the present data, and considering that the period from adult beetle emergence to oviposition is longer than 2 months under natural conditions (Garrido, 1984), we could expect a decrease of the number of females reaching the onset of oviposition, and also a possible reduction of the biotic potential of fungally challenged females (Quesada-Moraga et al., 2004; Quesada-Moraga et al., 2006).

Our experimental data also demonstrated that the inoculum of *M. anisopliae* EAMa 01/58-Su isolate contained in the soil in the potted plant bioassay was capable of producing infections among *C. tenebrionis* neonate larvae. This result is particularly important since we use a non-sterile soil with high organic matter content, conditions that are not optimal for entomopathogenic fungi to express their maximum insecticidal potential. The high content of organic matter in the soil may reduce its abrasive nature, which does not favor host penetration by the fungus and instead promotes fungistasis due to microorganisms. Our potted plant bioassay that simulates both natural endophytic conditions of the larvae and natural rates of larvae colonizing plant tissues (Marannino et al., 2004) proved to be suitable; whereas looking for larvae in all roots from each potted plant was extremely highly time consuming.

The behavior of neonate larvae that must crawl through soil to reach plants probably facilitated the initiation of infection by promoting the exposure of a greater proportion of the cuticle surface to the soil inoculum thereby increasing the effective dosage to a host. Application of *M. anisopliae* EAMa 01/58-Su isolate conidia to the top 20 mm of soil provided nearly complete control of beetle larvae at each of the three assayed dosages. It is important to point out that in nature eggs are laid in the ground within the first 7 mm (average length of the ovipositor) (Guessous, 1950). These results are coherent with what reported on *Delia radicum* (L.) by Chandler and Davidson (2005) who established that most conidia drenched onto the surface of compost remained in the top layer, increasing the exposure of *D. radicum* neonates to fungi as they moved to the root zone. Therefore, concentrating fungal inoculum at the soil surface may be a possible strategy for larval control, as suggested by Vanninen et al. (1999).

On the basis of the results obtained, a strong correlation between fungal concentrations in soil and the degree of reduction in larval mortality could not be demonstrated, in agreement with Dolci et al. (2006), who also found this lack of correlation in *B. brongniartii* soil treatment against *Melolontha melolontha* L. From our data, it seems that even the lower conidial dose was above the minimum inoculum concentration for *C. tenebrionis* larval fatal infection. Thus, a concentration lower than 10^6 conidia/ml could be sufficient for good larval control; whereas the possible establishment and recycling of the fungus in the soil for long term control seems to be favored by a higher dose, which it is also corroborated by our results on the evolution of conidial density in the soil during the experiment. It must be taken into account the existence of *M. anisopliae* rhizosphere competent isolates, as recently ascertained (Bruck, 2005). In this regard, when selecting an isolate, exploring factors associated with entomopathogen biology outside the insect host may be as much important as to determine its virulence in laboratory bioassays. Such knowledge could serve as starting point for further investigations aimed to assess the potential to use colonized roots as a delivery system for fungal biological control agents as already happens in the field of plant pathology with the application of rhizosphere competent organisms for the microbial control of plant diseases.

The results of the present work demonstrate that *C. tenebrionis* larvae and adults are susceptible to *M. anisopliae* EAMa 01/58-Su isolate when treated with conidia in the soil and with fiber bands impregnated with the mycopathogen, respectively. However, while not a subterranean insect, *C. tenebrionis* has a natural life cycle and behavior that include considerable exposure to soil that could potentially lead to additional infections. For example, adult overwintering occurs in/on the soil (Garrido, 1984); adults may walk across the treated soil surface, from plant to plant, to feed and lay eggs, an activity that might contaminate tarsi. At the time of oviposition in the soil, females may be contaminated in the ovipositor and abdominal sterna. In addition, conidia adhering to ovipositors and terminal abdominal segments may be transferred to plant surfaces and eggs, causing subsequent infection of adults and larvae, respectively. Infection could take place also during mating, which lasts several minutes (Chrestian, 1955), and involves a large contact between the partners' bodies (Bari et al., 2004). In conclusion, the results of our research efforts, focused on the evaluation of *M. anisopliae*, are encouraging and indicate that this approach could be used to protect fruit bearing orchards, greenhouse trees and nurseries from *C. tenebrionis* attack.

Acknowledgments

This research was supported by Project no. 36 of the I.F.A.P.A. (expdte 92162/1) from the regional government of Andalusia (Junta de Andalucía). Pierdomenico Marannino is a fellowship holder at University of Bari. We thank Michele Lassandro and Inmaculada Garrido Jurado for his valuable help in the collection and rearing of the beetles, and in carrying out the bioassays.

References

Alfaro-Moreno, A., 2005. Entomología Agraria. Los Parásitos Animales de las Plantas Cultivadas. Edition of Cándido Santiago Álvarez. Diputación Provincial de Soria, pp. 219–221.

Bari, G., De Cristofaro, A., de Lillo, E., Germinara, S., 2004. Studio preliminare sulle interazioni intraspecifiche ed interspecifiche in *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae). Atti XIX Congr. Naz. It. Entomol., Catania, 725–731.

Begley, J.W., 1990. Efficacy against insects in habitats other than soil. In: Gaugler, R., Kaya, H.K. (Eds.), Entomopathogenic Nematodes in Biological Control. CRC Press Inc., Florida, pp. 215–220.

Ben-Yehuda, S., Assal, A., Mendel, Z., 2000. Improved chemical control of *Capnodis tenebrionis* and *C. carbonaria* in stone -fruit plantations in Israel. Phytoparasitica 28, 27–41.

Bruck, D.J., 2005. Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management. Biological Control 32, 155–163.

Caponero, A., Marannino, P., Bari, G., de Lillo, E., 2006. Il capnode delle drupacee preoccupa in Basilicata. Informatore Agrario 16, 66–68.

Chandler, D., Davidson, G., 2005. Evaluation of entomopathogenic fungus *Metarhizium anisopliae* against soil-dwelling stages of cabbage maggot (Diptera: Anthomyiidae) in glasshouse and field experiments and effect of fungicides on fungal activity. Journal of Economic Entomology 98, 1856–1862.

Chrestian, P., 1955. Le Capnode noir des Rosacées. Protectorat de la République Française au Maroc, Service de la Défense des Végétaux, Travaux originaux 6, 141 pp.

Dolci, P., Guglielmo, F., Secchi, F., Ozino, O.I., 2006. Persistence and efficacy of *Beauveria brongniartii* strains applied as biocontrol agents against *Melolontha melolontha* in the Valley of Aosta (northwest Italy). Journal of Applied Microbiology 100, 1063–1072.

Dubois, T., Zengzhi, L., Jiafu, H., Hajek, A.E., 2004. Efficacy of fiber bands impregnated with *Beauveria brongniartii* cultures against the Asian longhorned beetle, *Anoplora glabripennis* (Coleoptera: Cerambycidae). Biological Control 31, 320–328.

García del Pino, F., Morton, A., 2005. Efficacy of entomopathogenic nematodes against neonate larvae of *Capnodis tenebrionis* (L.) (Coleoptera: Buprestidae) in laboratory trials. BioControl 50, 307–316.

Garrido, A., 1984. Bioecología de *Capnodis tenebrionis* L. (Col.: Buprestidae) y orientaciones para su control. Boletín Servicio Plagas 10, 205–221.

Garrido, A., del Busto, T., Malagón, J., 1987. Método de recogida de huevos de *Capnodis tenebrionis* L. (Coleop.; Buprestidae) y algunos factores abióticos que pueden condicionar la puesta. Boletín de Sanidad Vegetal Plagas 13, 303–309.

Guessous, A., 1950. Recherches sur la ponte du Capnode noir des arbres fruitiers (*Capnodis tenebrionis* L.). Revue de Pathologie Végétale 29, 137–151.

Hajek, A.E., Huang, B., Dubois, T., Smith, M.T., Li, Z., 2006. Field studies of control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) using fiber bands containing the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria brongniartii*. Biocontrol Science and Technology 16, 329–343.

Higuchi, T., Saika, T., Senda, S., Mizobata, Y., Nagai, J., 1997. Development of biorational pest control formulation against longicorn beetles using a fungus *Beauveria brongniartii* (Sacc.) Petch. Journal of Fermentation and Bioengineering 84, 236–243.

Liu, H., Bauer, L.S., 2006. Susceptibility of *Agrilus planipennis* (Coleoptera: Buprestidae) to *Beauveria bassiana* and *Metarhizium anisopliae*. Journal of Economic Entomology 99, 1096–1103.

Marannino, P., Tarasco, E., de Lillo, E., 2004. Biological notes on larval hatching in *Capnodis tenebrionis* (L.) (Coleoptera Buprestidae) and evaluation of entomopathogenic nematodes in controlling neonate larvae. Redia 86 (2003), 101–106.

Marannino, P., de Lillo, E., 2007a. *Capnodis tenebrionis* (L. 1758) (Coleoptera: Buprestidae): morphology and behaviour of the neonate larvae, and soil humidity effects on the egg eclosion. Annales de la Société Entomologique de France 43, 145–154.

Marannino, P., de Lillo, E., 2007b. The peach flatheaded rootborer, *Capnodis tenebrionis* (L.), and its enemies. IOBC/wprs Bulletin 30, 197–200.

Marannino, P., Santiago-Álvarez, C., de Lillo, E., Quesada-Moraga, E., 2006. A new bioassay method reveals pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against early stages of *Capnodis tenebrionis* (Coleoptera; Buprestidae). Journal of Invertebrate Pathology 93, 210–213.

Nobuchi, A., 1993. An automatic releasing equipment of *Beauveria* contaminated bark beetle for microbial control of *Monochamus alternatus*. Forest Pests 42, 213–217.

Quesada-Moraga, E., Santos-Quirós, R., Valverde-García, P., Santiago-Álvarez, C., 2004. Virulence, horizontal transmission and sublethal reproductive effects of *Metarhizium anisopliae* (Anamorphic fungi) on the German cockroach (Blattodea: Blattellidae). Journal of Invertebrate Pathology 87, 51–58.

Quesada-Moraga, E., Ruiz-García, A., Santiago-Álvarez, C., 2006. Laboratory evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against puparia and adults of *Ceratitis capitata* (Diptera: Tephritidae). Journal of Economic Entomology 99, 1955–1966.

Throne, J.E., Weaver, D.K., Chew, V., Baker, J.E., 1995. Probit analysis of correlated data: Multiple observations over time at one concentration. Journal of Economic Entomology 88, 1510–1512.

Vanninen, I., Hokkanen, H., Tyni-Juslin, J., 1999. Screening of field performance of entomopathogenic fungi and nematodes against cabbage root flies (*Delia radicum* L. and *D. floralis* (Fall.); Diptera, Anthomyiidae). Acta Agriculturae Scandinavica B-S P 49, 167–183.

Wraight, S.P., Jackson, M.A., Kock, S.L., 2001. Production, stabilization and formulation of fungal biocontrol agents. In: Butt, T.M., Jackson, C.W., Magan, N. (Eds.), Fungi as Biocontrol Agents Progress, Problems and Potential. CABI Publishing, pp. 253–288.