Biocontrol of *Bactrocera oleae* (Diptera: Tephritidae) With *Metarhizium brunneum* and Its Extracts

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**ABSTRACT** The susceptibility of preimaginal and adult olive fruit fly, *Bactrocera oleae* (Gmelin) (Diptera: Tephritidae), to a strain of the mitosporic ascomycete *Metarhizium brunneum* (Petch) (Hypocreales: Clavicipitaceae) and the insecticidal activity of its crude extract to olive fruit fly adults were investigated. Strain EAMb 09/01-Su caused 60% mortality to *B. oleae* adults, with average survival time (AST) of 8.8 d. In soil treatments against pupariating third-instar larvae, preimaginal *B. oleae* mortality reached 82.3%, whereas preimaginal mortality targeting puparia was 33.3%. The crude extract of EAMb 09/01-Su strain caused 80.0% adult mortality when administered per os, with AST of 27.7 h. The crude extract was demonstrated to be quite thermostable and photoresistant. These results indicate that *M. brunneum* EAMb 09/01-Su strain and its crude extract show potential to be used in an integrated pest management olive fruit fly management strategy targeting both adults and preimaginals.

**KEY WORDS** biological control, secondary metabolite, microbial control, crude extract, olive fruit fly

The olive (*Olea europaea* L.) tree is planted in all regions of the globe located between 30 and 45° latitude of the two hemispheres, in which it counts among the most important oil-producing crops (IOOC 2010). Spain is the leading country in olive production, with >300 million olive trees spread over 2,580 million ha (21% of the world total and 51% of the European Union total). Spain is also the largest producer of table olives and oil, with 30 and 40% of production, respectively, >80% of which is from Andalusia (MARM 2011).

There are more than a hundred species feeding or developing on olive tree, polyphagous, oligophagous, and a small group of monophagous species representing the greatest threat to the crop and its environment (De Andrés Cantero 2001, Tzanakakis 2006). The harvestable portion of the crop is the fruit and is used to produce oil or for table consumption. Therefore, although the general health of the tree is the concern of every good grower, the protection of the fruit is a practice of critical importance. The fruit of the olive tree is attacked by a diverse set of species, whereas the species that poses the greatest threat to the olive crop throughout its range is the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae) (De Andrés Cantero 2001, Alfaro 2005, Tzanakakis 2006). This tephritid causes a reduction in oil quality, along with a reduction in the yield per unit, that together account for approximately €500 million/yr, with an annual expenditure on control measures of €100 million (Haniotakis 2005). There have even been reported losses of 100% of some table varieties and up to 80% of oil value due to *B. oleae* (Zalom et al. 2003).

The females of this species thrust their ovipositor to penetrate the skin of the fruit, usually depositing a single egg inside the fruit. After hatching, the larva feeds on the mesocarp, and when it reaches the end of its development, it forms a chamber and becomes a pupa inside the fruit or falls to the ground to pupate beneath the tree. This species completes between two and five generations per year (Santiago-Alvarez and Quesada-Moraga 2007). The feeding of the larvae at the expense of the olive pulp results in a damage of both quantity (by decreasing the amount of the crop) and quality (by facilitating the entry of fungi that affect the quality and stability of the resulting oil) (Santiago-Alvarez and Quesada-Moraga 2007, Genç and Nation 2008).

The economic importance of this dipteran pest has led to the assessment of most available tools in controlling the field against *B. oleae*, without practical results in most cases. Currently, the use of insecticides is the most widely used measure of control, either by bait adulticide treatments or by larvicidal treatments. Other strategies against *B. oleae* have a much more limited application; these strategies include mass trapping and preventative spraying with kaolin (physical barrier) and copper (Belcari et al. 2005, Haniotakis 2005, Johnson et al. 2006, Daane and Johnson 2010).
Insecticide treatments, not being selective when applied over large areas and throughout much of the foliage, cause severe effects on ecosystems (Ruano et al. 2001, Haniotakis 2005, Pascual et al. 2010). These effects are coupled with the loss of efficiency through the development of resistance, particularly due to the repeated and abused use of a very limited number of active materials. Already, some populations of *B. oleae* have been found to be resistant to dimethoate and other organophosphates, pyrethroids, and more recently spinosad (Kakani et al. 2008, 2010; Margaritopoulos et al. 2008).

Therefore, it is essential to investigate the development of alternative methodologies for an integrated control of the olive fruit fly, emphasizing biological control through entomophagous predators and parasitoids (research to date has devoted much effort with little effect) or microbes to exploit the full potential of entomopathogenic microorganisms.

To date, *Bacillus thuringiensis* strains with adequate insecticidal activity against *B. oleae* are not available for practical use (Navrozidis et al. 2000). In the absence of baculovirus diseases and protozoa in the olive fruit fly, the possibilities of using entomopathogenic microorganisms are restricted to entomopathogenic fungi, whose unique tegumentary mode of action puts them at the forefront of the global development of alternative control strategies of tephritids (Castillo et al. 2000, Quesada-Moraga et al. 2006b, Ekesi et al. 2007); however, their use against *B. oleae* has not yet been studied. Furthermore, there is potential for the use of the mitosporic entomopathogenic ascomycetes for tephritid control; not only are they virulent against both preimaginal and adult flies but also they secrete new molecules with natural insecticidal properties for the control of adults (Konstantopoulou et al. 2006, Ekesi et al. 2007). Our previous studies demonstrate the use of the insecticidal activity of a protein fraction of *Metarhizium anisopliae* (Met.) Sorok. in the control of the adult Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) (Quesada-Moraga et al. 2006b, Ortiz-Urquiza et al. 2009). However, to date, we have not evaluated the possible existence of an active molecule against *B. oleae*. The aim of this study was to evaluate the potential of a strain of the ascomycete mitosporic *Metarhizium brunneum* (Petch) (Hyphocreales: Clavicipitaceae) to infect and suppress preimaginal and adult *B. oleae* as well as to evaluate the insecticidal activity of the crude extract of this fungus against adults of the species. This strain was selected based on previous unpublished studies that indicate its high virulence against *C. capitata*.

**Materials and Methods**

**Insects.** Wild *B. oleae* were obtained from naturally infested fruit collected from September to December 2011 in the olive groves that enclose the experimental farm “La Alameda del Obispo” belonging to Instituto de Investigación y Formación Agraria y Pesquera (Junta de Andalucía) in Córdoba, Spain. The infested olives were placed in round cages of 115 by 50 mm (40–50 olives in each cage). The cages were maintained in an environmental chamber programmed at 25 ± 2°C, 50–60% RH, and photoperiod of 16:8 (L:D) h. The puparia from pupariating larvae that left the olives to attain the pupal stage on the cage floor were collected every 7 d and placed in methacrylate round dishes (30 mm) and in petri plates (55 mm) (an average 10–15 puparia in each plate) containing a layer of cotton. Two milliliters of distilled water was added to the cotton to keep high-humidity conditions to promote adult emergence. However, puparia were placed in the 30-mm dishes to avoid direct contact with watered cotton, which promotes growth of saprotrophic fungi.

**Fungal Preparation.** To prepare the inoculums for the experiments, slant cultures of the isolates were subcultured on malt agar (BioCult B. Laboratories, Madrid, Spain) for 12 d at 25°C in darkness. The petri plates were sealed with Parafilm. Conidial suspensions were prepared by scraping conidia from the petri plates into a sterile aqueous solution of 0.1% Tween 80. This initial suspension was sonicated for 5 min and then filtered through several layers of cheesecloth to remove mycelial mats and thus to collect pure conidia. The conidial suspension used for the bioassays was adjusted by diluting the conidia with 0.1% Tween 80 to a final concentration of 1.0 × 10^5 conidia per ml. The number of conidia was estimated using a Malassez hemocytometer (Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany). The suspension was stored in a refrigerator at 4°C for no longer than 24 h.

**Initial Pathogenicity Assay of EAMb 09/01-Su Strain Against Newly Emerged B. oleae Adults.** Cold-anesthetized newly emerged adults were treated with 1 ml of a 1.0 × 10^5 conidia per ml suspension by using a Potter tower (Burkard Rickmansworth Co. Ltd., United Kingdom), resulting in coverage of 1.57 ± 10^5 conidia cm^-2 for a standard tank of 0.06 mg cm^-2 and 0.7 bars of pressure. The controls were treated with the same volume of a sterile aqueous solution of 0.1% Tween 80. The treated adults were placed in methacrylate cages (80 by 80 by 60 mm; Resopal, Alcalá de Henares, Madrid, Spain) with covers containing a circular hole (20 mm in diameter) covered with a net cloth; a liquid diet (0.1 g of hydrolyzed protein and 0.4 g of sucrose with 1.5 ml of distilled water) was provided every 48 h. The bioassay was conducted at 26 ± 2°C, 50–60% RH, and a photoperiod of 16:8 (L:D) h. Mortality was monitored for 12 d. Dead flies were removed daily to prevent horizontal transmission of the fungal inoculum. The dead flies were immediately surface sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water. They were then placed on sterile
wet filter paper in sterile petri plates that were sealed with Parafilm and kept at room temperature to be inspected for fungal outgrowth on the cadavers (Quesada-Moraga et al. 2006a). Each treatment was replicated five times with five adult flies per replicate.

**Initial Pathogenicity Assays of EAMb 09/01-Su Strain Against B. oleae Preimaginals.** To evaluate the pathogenicity of *M. brunneum* against *B. oleae* preimaginals, two bioassays were performed. In the first bioassay, we used pupariating larvae. Bioassay cages similar to those used for the adult bioassays were prepared with 30 g of soil that covered the entire floor. The soil used in this study was collected from a farm in Córdoba and was characterized as sandy loam (78.0% sand, 17.0% silt, 5.0% clay, and 0.2% organic matter) with a pH of 8.3. The soil was sieved (2-mm mesh) and stored in a dry place at 20°C with a humidity of 80%RH. The soil was then sterilized at 121°C for 20 min and dried in an oven at 105°C for 24 h. One milliliter of fungal suspension containing 1.0 × 10^6 conidia per ml and 1.7 ml of sterile distilled water containing 0.1% Tween 80 was added to the bioassay cages to attain a water potential of −0.01 MPa (9.0% [wt:wt] measured with a Decagon WP4 psychrometer, Decagon Devices, Pullman, WA). The bioassay was arranged as a randomized complete block design with two treatments, fungal isolate and control, and four replicates per treatment. Puparia were gathered daily until a reasonably sized population (63 puparia) was achieved. The experimental unit was a transparent plastic box (80 by 80 by 60 mm).

After the treatment, the cages were covered with Parafilm membrane with a 20 mm hole in the center for ventilation and they were placed in a plastic box (330 by 250 by 140 mm) that was covered with damp filter paper that was moistened periodically to maintain a ±3% loss of the initial soil water content. The boxes were placed at 25°C until adult emergence (Garrido-Jurado et al. 2011). Puparia that failed to emerge were placed as indicated with adults to be inspected for fungal outgrowth.

In the second pathogenicity bioassay, *B. oleae* puparia were immersed in a fungal suspension for 10 s. The control was immersed in sterile distilled water containing 0.1% Tween 80. There were three replicates of each treatment, fungus and control, and 10 puparia per replicate. The bioassay end point was four days after the first adult emergence in the control. Puparia that failed to emerge were removed and prepared as described previously for the diagnosis of cause of death (Quesada-Moraga et al. 2006b).

**Production of Fungal Extract of EAMb 09/01-Su Strain in Liquid Culture.** The fungal extract was produced in a liquid medium consisting of 40 g of glucose and 20 g of mycological peptone per liter. A primary culture of this medium inoculated with 1 × 10^5 conidia per ml of the *M. brunneum* strain (EAMb 09/01-Su) was cultivated on a rotatory shaker at 110 rpm for 4 d at 25°C. Then, 2 ml of the primary culture was transferred to flasks containing 250 ml of liquid medium. As described above, the secondary culture was cultivated for 15 d before removing the mycelia by filtration through filter paper (Whatman No. 3 Chr filter paper, Whatman, Kent, United Kingdom), and this extract was centrifuged at 9000 rpm for 20 min and concentrated 15 times in a continuous flow chamber at 30°C (Quesada-Moraga and Vey 2004, Ortiz-Urquiza et al. 2010).

**Evaluation of Insecticidal Activity of EAMb 09/01-Su Strain Crude Extract Against Newly Emerged B. oleae Adults.** The insecticidal activity of EAMb 09/01-Su crude extract was tested in a diet test. One-day-old male and female flies were collected randomly, placed in cages (80 by 80 by 50 mm: Resopal, Alcalá de Henares, Madrid, Spain), and fed an artificial diet and crude extract (test suspension: 0.4 g of sucrose and 0.1 g of protein hydrolysate per 1.5 ml of distilled water) on a 1.5-ml centrifuge tube cap. Controls were treated with an equivalent volume of the liquid medium with the above proportions of sucrose and protein hydrolysate (control suspension). Five replicates of ten insects each were used for the control and the treatments, totaling 100 insects for the full experiment. For both treated and control insects, the microtube cap was filled with a 100-μl aliquot of test or control suspension. The experiment was conducted at 26 ± 2°C, 60 ± 5% RH, and a photoperiod of 16:8 (L:D) h. The number of dead flies was recorded at 24 h.

*B. oleae* adult survival was determined to the highest concentration of crude extract (10%). Newly emerged adults were fed according to the bioassay method described above. Since the beginning of the photophase in the rearing chamber, the control and treated insects were fed for 48 h with a mortality register every 3 h. The bioassay was arranged with five replicates, with five insects per replicate. The entire procedure was repeated after 45 d with a new extract and a new generation of adult flies, gathering in total 50 insects for the full experiment.

**Biological Activity of EAMb 09/01-Su Strain Crude Extract Against Newly Emerged B. oleae Adults.** The insecticidal activity of the crude extract was tested against adults in a diet test. For this experiment, a diet was prepared containing 0.4 g of sucrose and 0.1 g of protein hydrolysate per 1.5 ml of distilled water, and the fraction of the crude extract was added as indicated in Table 1.

**Table 1. Crude extract (15 d of fermentation) concentration range used in per os bioassay against B. oleae adults**

<table>
<thead>
<tr>
<th>Concna (extract: water)</th>
<th>Vol (μl) (extract:water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:0</td>
<td>1,500:0</td>
</tr>
<tr>
<td>7:3</td>
<td>1,050:450</td>
</tr>
<tr>
<td>6:4</td>
<td>900:600</td>
</tr>
<tr>
<td>4:6</td>
<td>600:900</td>
</tr>
<tr>
<td>2:8</td>
<td>300:1,200</td>
</tr>
</tbody>
</table>

a Concentration was defined using a 0–10 rating scale, i.e. 10 parts of extract and 0 part of water and 2:8, 2 parts of extract and 8 parts of water.
with an equivalent volume of the water with the above-mentioned proportions of sucrose and protein hydrolysate. Five replicates of five insects each were used for the control and the treatments, totaling 175 insects for the full experiment. For treated and control insects, the microtube cap was filled with a 100-µl aliquot of test or control suspension, respectively. The experiment was conducted at 26 ± 2°C, 60 ± 5% RH, and a photoperiod of 16:8 (L:D) h. The number of dead flies was recorded at 24 h.

Effect of B. oleae Adult Exposure Time on EAMb 09/01-Su Strain Crude Extract Insecticidal Activity. The effect of exposure time on the chronic insecticidal activity of the crude extract of EAMb 09/01-Su was studied using an experimental protocol that included five exposure times and the controls. One-day-old male and female flies were collected randomly and placed in the bioassay cages. Control groups were fed with 1.5-ml microtube caps containing 100 µl of a liquid diet lacking treatment. In contrast, the treatment groups were acutely exposed to 100 µl of crude extract containing the same proportions of sucrose and yeast hydrolysate. Starting at the beginning of the photophase in the rearing chamber, both control and treatment groups were fed for 1, 3, 6, 9, and 24 h. After the groups were fed as described above, a liquid diet lacking treatment was offered to the flies until the end of the experiment. The total experimental time was 48 h, and mortality data were recorded every 24 h. For every exposure time, five replicates consisting of five insects each were used, totaling 150 flies for this experiment. The mean time of exposure (MET), that is, the exposure time causing 50% of mortality, was calculated by correlating the time with the mortality values reached at every time of exposure.

Effect of Temperature Treatments on EAMb 09/01-Su Strain Crude Extract Insecticidal Activity Against Newly Emerged B. oleae Adults. The crude extract was incubated at two temperatures: 60°C for 2 h and 120°C for 20 min. The treated solutions were offered to adults as previously described. Control flies were fed the liquid medium with the same temperature treatments. Five replicates of five insects each were used for treatments and controls, totaling 100 insects for the full experiment. The experiment was conducted and monitored at 26 ± 2°C, 60 ± 5% RH, and a photoperiod of 16:8 (L:D) h.

Effect of Irradiance on EAMb 09/01-Su Strain Crude Extract Insecticidal Activity Against Newly Emerged B. oleae Adults. To evaluate the effect of ultraviolet (UV) radiation on the insecticidal activity of the crude extract, it was exposed for 2, 4, and 6 h at UV-B (920 mW/cm²). The treated solutions were offered to B. oleae adults as described previously. Control flies were fed the liquid medium, water, and crude extract without UV-B irradiance treatment. Five replicates of five insects each were used for treatments and controls, totaling 150 insects for the full experiment. The experiment was conducted and monitored at 26 ± 2°C, 60 ± 5% RH, and a photoperiod of 16:8 (L:D) h.

Statistical Analysis. Mortality data were analyzed using the one-way analysis of variance (Statistix 9.0, Analytical Software, Tallahassee, FL); Tukey’s honestly significant difference (HSD) test was used to compare means. If data did not conform to a normal distribution, the following transformation was used:

\[
\text{arc} = 180 \times \arcsin \left( \left( \frac{\text{dead insects}}{100} \right)^{0.5} \right) \times \frac{360}{2 \times \Pi}
\]

The values of average survival times obtained by the Kaplan–Meier method and compared using the log-rank test were calculated with SPSS 15.0 Software for Windows (SPSS, Inc., Chicago, IL). The mean lethal concentration (LC₅₀) was estimated by Probit analysis (Finney 1971), as performed with SPSS 15.0.

Results

Initial Pathogenicity Assay of EAMb 09/01-Su Strain Against Newly Emerged B. oleae Adults. There was a significant effect of the fungal treatment with M. brunneum (F = 12; df = 1, 7; P < 0.001) on total adult mortality, with a mean value of 60% compared with uninoculated adult flies, for a mortality of 6.66% (Table 2). The fungal treatment also had a significant effect on mortality with fungal outgrowth (F = 28.87; df = 1, 7; P < 0.001), reaching 38.78%, with no signs of fungal outgrowth in the controls. The AST of adults treated with M. brunneum EAMb 09/01-Su strain was 8.8 d, significantly shorter than that of the controls (12 d; Table 2).

Initial Pathogenicity Assays of EAMb 09/01-Su Strain Against B. oleae Preimaginals. The treatment with the EAMb 09/01-Su strain targeting pupariating third-instar larvae had a significant effect on B. oleae preimaginal survival (F = 22.78; df = 1, 7; P = 0.0031), with 82.27% of preimaginals unable to reach the adult stage compared with 35.45% in the controls, with a significant (F = 8.55; df = 1, 7; P = 0.0265) percentage

### Table 2. Pathogenicity of M. brunneum EAMb 09/01-Su strain against B. oleae newly emerged adults

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total mortality (mean ± SE)%</th>
<th>Mortality with fungal outgrowth (mean ± SE)</th>
<th>Kaplan–Meier survival analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. brunneum</td>
<td>60.00 ± 10.95a</td>
<td>38.78 ± 5.41a</td>
<td>AST (d, mean ± SE)b 95% confidence interval</td>
</tr>
<tr>
<td>Control water</td>
<td>6.66 ± 6.66b</td>
<td>0b</td>
<td>6.66 ± 0.72a</td>
</tr>
</tbody>
</table>

* Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the Tukey’s HSD test.

b AST limited to 12 d. Means within columns with the same letter are not significantly different (P ≤ 0.05) according to the log-rank test.
of nonviable preimaginals, 15.8%, showing fungal outgrowth (Table 3). Although the fungal treatment targeting *B. oleae* puparia did not cause a significant mortality (33.3%) compared with the controls (13.3%) \((F = 1.8; \text{df} = 1, 5; P = 0.250)\), 20% of them showed fungal outgrowth (Table 3).

**Evaluation of Insecticidal Activity of EAMb 09/01-Su Strain Crude Extract Against Newly Emerged *B. oleae* Adults.** The crude extract of *M. brunneum* EAMb 09/01-Su strain (after 15 d of fermentation) exhibited a highly significant insecticidal effect on adult *B. oleae* \((F = 468.33; \text{df} = 1, 9; P < 0.001)\), with 70.16% mortality after 24 h of treatment compared with mortality in the controls that was 0 and 1.52% in the liquid medium and water controls, respectively. In a further experiment with a new batch of insects and a crude extract obtained from new inoculum, *B. oleae* adult mortality reached 80% after 48 h, with an AST of 27.72 h (95% confidence interval, 22.07–33.36 h) (Fig. 1).

**Biological Activity of EAMb 09/01-Su Strain Crude Extract Against Newly Emerged *B. oleae* Adults.** The mortality of adults fed for 24 h with EAMb 09/01-Su strain crude extract was dose related, ranging from 32.0 to 64.0% (Table 4). These dose-related mortality values were submitted to concentration–mortality response regression analysis (Probit); the regression coefficient was 1.23 and the \(\chi^2\) value was not significant (1.028, with 3 df), indicating good fit of the regression line. The LC\textsubscript{50} value was 49.98% crude extract.

**Effect of *B. oleae* Adult Exposure Time on EAMb 09/01-Su Strain Crude Extract Insecticidal Activity.** The exposure time significantly affected the adult mortality caused by the crude extract at 48 h \((F = 16.44; \text{df} = 4, 24; P < 0.001)\) in a dose-dependent manner (Table 5). The highest mortality value, 92.0%, and the lowest AST, 30.7 h, were obtained after 24 h of exposure to the crude extract. The mortality values recorded for each exposure time were fitted to the following first-degree linear equation: \(Y = 3.6062X + 5.7863\) \((R^2 = 0.99)\), where \(Y\) is the percentage of mortality and \(X\) is the exposure time. The period of exposure required to achieve 50% mortality was 12.26 h.

**Effect of Temperature Treatments on EAMb 09/01-Su Strain Crude Extract Insecticidal Activity Against Newly Emerged *B. oleae* Adults.** The temperature treatment had a significant effect on the insecticidal activity of the crude extract of EAMb 09/01-Su strain \((F = 4.91; \text{df} = 2, 14; P < 0.001)\). Although a significant reduction in adult mortality, from 60% to 48%, was observed after incubation of the crude extract for 20 min at 120°C, its activity was not significantly reduced after incubation at 60°C (72% adult mortality).

**Effect of Irradiance on EAMb 09/01-Su Strain Crude Extract Insecticidal Activity Against Newly Emerged *B. oleae* Adults.** The UV-B irradiance exposure was significantly \((F = 25.66; \text{df} = 5, 29; P < 0.001)\) affected EAMb 09/01-Su strain crude extract insecticidal activity, with adult mortalities of 60, 60, 48, and 56% after 0, 2, 4, and 6 h at UV-B (920 mW/cm\(^2\)) exposure, respectively, with no mortality in the two controls, medium, and water.

**Discussion**

The results of this work indicate high susceptibility of the preimaginal- and adult-state *B. oleae* to the

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**Table 3. Pathogenicity of the *M. brunneum* EAMb 09/01-Su strain against *B. oleae* third-instar pupariating larvae and puparia**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pupariating larvae</th>
<th>Puparia</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mortality (mean ± SE)%</td>
<td>Mortality (mean ± SE)%</td>
</tr>
<tr>
<td></td>
<td>With fungal growth</td>
<td>Without fungal growth</td>
</tr>
<tr>
<td></td>
<td>(mean ± SE)</td>
<td>(mean ± SE)</td>
</tr>
<tr>
<td><em>M. brunneum</em></td>
<td>82.27 ± 10.26a</td>
<td>15.80 ± 6.21a</td>
</tr>
<tr>
<td>Control water</td>
<td>35.45 ± 4.24b</td>
<td>0b</td>
</tr>
</tbody>
</table>

Means within columns with the same letter are not significantly different \((P \leq 0.05)\) according to the Tukey’s HSD test.
effects of conidia and the crude extract obtained from *M. brunneum* EAMb 09/01-Su strain. Adult mortality reached 60% at the dose we used, whereas the AST was 8.8 h, helping to reduce the adult population in the field before the first egg laying, a critical moment of insect attack (Santiago-Álvarez and Quesada-Moraga 2007). To further contribute to this goal, the fungal inoculum may be disseminated by adults before being killed (Quesada-Moraga et al. 2008). Similar results have been obtained by Konstantopoulo and Mazomenos (2005), who, in evaluating different entomopathogenic fungi against adults of *B. oleae*, obtained mortalities between 57.8 and 63.3% after 14 d of treatment. However, these mortalities were obtained by walking bioassay on fungal colonies, where exposure time to conidia is higher than in spraying bioassay. Besides, Siebeneicher et al. (1992) reported that some application methods of conidial suspension could be more effective than others because they cover more insect body area. The similarity between both results is striking, considering the different methods used. Only Mahmoud (2009) used an application method similar to the method used in this work. With three commercial products of entomopathogenic fungi, Mahmoud (2009) obtained mortalities in the range of 47.2–70.4% after 15 d of treatment. These mortalities are quite similar to ours, even if our product was not a commercial product.

Preimaginal stages of tephritids, particularly puparia, are less susceptible to entomopathogenic fungi (Ekési et al. 2007). According to Vanninen et al. (1999), stages of insects living in the soil may have developed high levels of resistance to infection by natural selection because entomopathogens, especially fungi, are widespread in soil. Furthermore, the cuticle of the third stage larvae remains in the tephritids to form the puparium conferring a barrier to penetration and output of these fungal agents of microbial control. Despite such apparent mechanisms conferring resistance to fungal infection in the third instars and puparium, our results indicate high efficacy of the strain EAMb 09/01-Su against the pupariating third instars (82.3% mortality) but less so against puparia (33.3%). De la Rosa et al. (2002) found that the strains of *B. bassiana* effective against adults were not pathogenic against *Anastrepha ludens* (Loew) puparia, whereas Cossentine et al. (2010) also obtained highly virulent strains of *B. bassiana* against the adult western cherry fruit fly, *Rhagoletis indifferentens* Curran, but low efficacy against its larvae and puparia. Investigations of Kaaya and Munynyi (1995) in tsetse fly (Diptera: Glossinidae; Glossina Wiedemann) also indicated low susceptibility of puparia to entomopathogenic fungi. In previous studies, only the EAMa 01/58-Su strain of *M. anisopliae* from our collection of indigenous strains and the Bb-1333 strain of *B. bassiana*, originating from adults of *B. oleae*, have shown greater virulence against tephritid puparia used in this work (Quesada-Moraga et al. 2006b). Therefore, strain EAMb 09/01-Su of *M. brunneum* shows potential against *B. oleae* in treatments against adults in aerial part of the tree and against third-instar pupariating larvae and puparia in soil beneath the tree canopy. However, the fact that we used sterile soil in our work should be taken into account in future studies on controlling pupariating larvae in the soil environment.

Table 4. Percentage of mortality of newly emerged *B. oleae* adults after being fed for 24 h with EAMb 09/01-Su crude extract at different concentrations

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Mortality (mean ± SE)%</th>
<th>Coefficient of variance (CV) %</th>
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</thead>
<tbody>
<tr>
<td>20</td>
<td>32.00 ± 8.00</td>
<td>Linear**</td>
</tr>
<tr>
<td>40</td>
<td>40.00 ± 8.94</td>
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</tr>
<tr>
<td>60</td>
<td>56.00 ± 9.79</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>64.00 ± 7.48</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>60.00 ± 6.32</td>
<td></td>
</tr>
</tbody>
</table>

**P < 0.01.**

*It is indicated the percentage of crude extract after being water diluted. Control mortality was zero and was not included in the analysis.

* Coefficient of variance.

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The crude extract of the strain EAMb 09/01-Su of *M. brunneum* also has per os insecticidal activity against adults of *B. oleae*, as already observed with the crude protein extract of strain EAMA 01/58-Su (Ortiz-Urquiza et al. 2009). Specifically, the value of AST was 27.7 h and 50% mortality of *B. oleae*. These results place this extract among the most toxic against tephritid adults derived from both plants and microorganisms (Konstantopoulo and Mazomenos 2005, Quesada-Moraga et al. 2006a, Zapata et al. 2006) and represent the first report of an extract from an entomopathogenic fungus with activity against *B. oleae*.

Adult mortality was directly related to crude extract dose, with a mean lethal concentration LC₅₀ of 49.9% crude extract. Dosages are expressed in terms of crude extract dilution. Dose-related insecticidal effects have been observed before with other fungal secreted secondary metabolites, such as destruxins, efrapeptins, and cordycepin (Amiri et al. 1999, Bandani et al. 1999, Kim et al. 2002, Konstantopoulo et al. 2006), *Photobacterium* toxins (Blackburn et al. 1998, Gerritsen et al. 2005), a few plant-derived compounds (Zapata et al. 2006), and spinosad (Raga and Sato 2005).

Table 5. Percentage of mortality and AST of newly emerged *B. oleae* adults exposed to EAMb 09/01-Su crude extract for various exposure times and then transferred to control diet

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Mortality (48 h) (mean ± SE)%</th>
<th>Kaplan–Meier survival analysis (AST (mean ± SE)b)</th>
<th>95% confidence interval b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.0 ± 8.89</td>
<td>12.0 ± 1.59a</td>
<td>42.95–49.21</td>
</tr>
<tr>
<td>3</td>
<td>12.0 ± 8.00</td>
<td>12.0 ± 1.59a</td>
<td>42.95–49.21</td>
</tr>
<tr>
<td>6</td>
<td>25.0 ± 8.00</td>
<td>43.20 ± 2.07ab</td>
<td>39.14–47.26</td>
</tr>
<tr>
<td>9</td>
<td>40.0 ± 6.32</td>
<td>42.24 ± 2.16b</td>
<td>38.00–46.48</td>
</tr>
<tr>
<td>24</td>
<td>92.0 ± 4.89</td>
<td>30.72 ± 2.20c</td>
<td>26.40–35.04</td>
</tr>
</tbody>
</table>

Means within columns with the same letter are not significantly different (P ≥ 0.05) according to the log-rank test.

a Control mortality was zero and was not included in the analysis.

b AST limited to 48 h days.
It is interesting to highlight the acute activity of the crude extract after 15 d of fermentation of \textit{M. brunneum} EAMb 09/01-Su strain against \textit{B. oleae}, with a mortality of 50\% after 12.2 h of exposure. These results are in the range of those reported in our previous work for the crude extract of the strain EAMa 01/58-Su of \textit{M. anisopliae} against laboratory \textit{C. capitata} adults, 8.3 h (Ortiz-Urquiza et al. 2010), these insects are very seldom more susceptible than field-collected insects such as those used in this work. This observation shows the potential of this extract for use as a bait-spray or even to develop a new “lure-and-kill” olive fruit fly control strategy.

Another aspect to highlight in this study is the photo-resistance and thermo-stability of the crude extract of the \textit{M. brunneum} EAMb 09/01-Su strain that seems to guarantee its environmental competence. The exposure at 60°C for 2 h did not significantly reduce the insecticidal activity of the extract (72.0\% mortality among adults of \textit{B. oleae}): on the contrary, the exposure tended to increase the activity. Furthermore, exposure to 120°C for 20 min only reduced the insecticidal activity of the extract by 20\%. These results are slightly different from those obtained in our previous work (Ortiz-Urquiza et al. 2009) that showed a significant reduction in the insecticidal activity of the EAMa 01/58-Su strain after exposure at 120°C for 20 min; this reduction is undoubtedly related to the proteaceous nature of this active compound. Our results also reveal the photostability of EAMb 09/01-Su \textit{M. brunneum} crude extract, which again highlights its potential for olive fruit fly control, as UV-B exposure limits the practical use of numerous natural insecticides. This aspect is even more relevant if the heat-stroke conditions prevailing in the olive tree distribution areas are considered. Further studies regarding purification of the active fraction (or compound) and its mode of action are being carried out. The use of the pure active fraction could highly reduce the exposure time needed to achieve substantial mortality as observed in our previous studies (Ortiz-Urquiza et al. 2009). Besides, we are exploring different lure and kill devices to optimize the active adult intake ratio to accelerate the speed of kill.

In conclusion, this study reveals the potential of the EAMb 09/01-Su \textit{M. brunneum} strain for the microbial control of \textit{B. oleae} adults in aerial sprays and pupariating third-instar larvae and puparia in soil treatments beneath the tree canopy. In addition, the crude extract of this isolate also shows potential to be used against the tephritids as a new insecticidal compound of natural origin. Presently, we are investigating the combined use of the fungus and its extract, and we have progressed in the purification of the active fraction of the extract.

Acknowledgments

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