Entomopathogenic fungi as promising biocontrol agents for the rosaceous longhorn beetle, Osphranteria coerulescens

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Entomopathogenic fungi as promising biocontrol agents for the rosaceous longhorn beetle, *Osphranteria coerulescens*

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Abstract During a survey for natural enemies of the rosaceous longhorn beetle (RLHB), *Osphranteria coerulescens*, two isolates of entomopathogenic fungi (EPF) were found in Razavi Khorasan province of Iran. These isolates were determined to be of the species *Beauveria bassiana* and *Metarhizium anisopliae* using morphological characteristics and molecular analysis. Subsequently, the mycosis process of the isolates on larvae of RLHB was documented using scanning electron microscopy (SEM). In order to assess virulence of these indigenous isolates of EPF, a series of trials were conducted against RLHB larvae, pupae and adult stages. In laboratory bioassays the pest was susceptible to both EPF isolates, but the *B. bassiana* isolate caused significantly higher mortality than the *M. anisopliae* isolate in all life stages tested. In a semi-field trial EPF suspension containing 2.5×10^{10} viable conidia per ml was sprayed directly against pest larvae beneath tree bark under two conditions: apricot branches covered with a polyvinyl cover and without cover. The *B. bassiana* isolate showed a higher infection rate than *M. anisopliae* and the cover had significant effect on larval mortality, ranging from 67 % for covered infested apricot branches to 31 % without cover in the *B. bassiana* treatments. Our findings revealed that polyvinyl covers were necessary to improve conidial persistence under field conditions. In a supplementary survey on the semi-mass production potential of the fungal conidia, the ability of *B. bassiana* and *M. anisopliae* to germinate was studied using three solid media. Sugar beet pulp provided the highest yield of conidia for both EPF species, and can be considered as an effective solid medium for fungal growth. These findings highlighted the potential of EPFs as biocontrol agents of *O. coerulescens*.

Keywords Biocontrol · *Beauveria bassiana* · *Metarhizium anisopliae* · Insect pathology · SEM · Virulence

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Introduction

The rosaceous longhorn beetle (RLHB), *Osphranteria coerulescens* Redtenbacher (Coleoptera:

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Cerambycidae), is a native wood borer in Iran and poses a serious threat to stone-fruit trees in the family Rosaceae in Iran, Turkey, and Syria (Sharifi et al. 2014). Its cylindrical and vermiform larvae have a soft integument and reach about 32 mm length (Esmaeili 1983). The adult insects are active from late May to mid-June and prefer annual herbaceous plants. They feed on foliage and oviposit in the upper sections of twigs (Sharifi et al. 1970). Newly hatched larvae enter directly into the bark until reaching the hardwood where they feed until maturity. *Osphranteria coerulescens* is univoltine and overwinters within larval galleries as immature larvae (Behdad 1984). In spring, larval feeding near the phloem interrupts the flow of plant sap, reducing the commercial value of fruits (Esmaeili 1983), and weakens branches that can then be easily broken by wind (Sharifi et al. 2014). This pest poses unique management challenges because of its inaccessible habitats and cryptic behavior during the immature stages under the bark of host trees. Application of chemical pesticides against this pest is problematic due to the unpredictable translocation of insecticides within trees (Esmaeili 1983). Moreover, the larvae exit from eggs asynchronously, and this allows them to escape treatment. Currently, the main control method for *O. coerulescens* is cutting and burning the infested branches. This is not considered a sustainable environmental approach, because abandoned larval galleries throughout serve as shelters for many groups of natural enemies, and many species of parasitoids and predators take refuge in galleries during the winter. Due to wood boring insects causing significant damage worldwide, many researchers have addressed the use of entomopathogenic fungi (Ascomycota: Hypocreales) as an alternative method of controlling these key pests (Liu and Bauer 2008). These agents commonly infect a broad spectrum of insect pests such as Lepidoptera, etc. (Lee et al. 2015). Among the natural enemies of *O. coerulescens* larvae, several species of entomopathogens, including fungi were found infecting larval populations in Mashhad, eastern part of Iran (Karimi, unpublished data). The potential of entomopathogenic nematodes (EPNs) against this pest has already been determined (Sharifi et al. 2014). In this study, we investigated the potential of entomopathogenic fungi (EPFs) to be incorporated into management programs of this pest. The EPFs are a major group of insect pathogens that have physiologically different modes of action and are utilized as microbial pesticides. These pathogens can infect their hosts without being ingested, which is an important factor, because microbes that require ingestion to infect a host are not appropriate for use against wood borer larvae (Hajek and Bauer 2007). Extensive research has been done on the successful application of EPFs against wood boring insect pests. *Beauveria bassiana* caused 75 % mortality of *Monochamus alternatus* Hope (Coleoptera: Cerambycidae) larvae (Hajek and Bauer 2007). In other cases, fiber bands containing EPFs were applied to control *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) (Hajek et al. 2006), and *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) conidia were sprayed on trees infested with *Enaphalodes rufulus* Haldeman (Coleoptera: Cerambycidae) (Meyers et al. 2013). EPFs are promising and feasible biocontrol agents, because they can be formulated in various ways with reasonable shelf life and used against both adults and larvae of numerous pests (Marannino et al. 2008). The main objectives of the current work were (1) to isolate and characterize naturally occurring EPFs within larval population of *O. coerulescens*, (2) to study the infection process of the isolates fungi against RLHB using SEM images, (3) to assay virulence of these fungi against different life stages of *O. coerulescens* under laboratory conditions, (4) to perform semi-field trials to determine the potential of native EPF isolates against the RLHB, and (5) to address the effect of different natural substances as culture media for propagating the fungi.

Materials and methods

Insect sources

Last instar larvae of *O. coerulescens* were collected from infested shoots of cherry and apricot trees in fruit orchards of Mashhad in Razavi Khorasan province, which is located in North East of Iran (36°4N′, 59°7E′) from late November 2011 to February 2012. The larvae were removed from galleries, placed in perforated plastic containers (175 × 85 mm), and then maintained at 4 °C until use. Average larval weight was 0.4 ± 0.02 g. For all bioassays, insects of similar weights were used equally among treatments following the technique of Dubois et al. (2008). Pupae and adult insects were removed from their galleries, placed

in perforated plastic containers and kept at 4 °C until use. A group of pupae (about 250) were kept at room temperature until sclerotized and adult beetles emerged.

Isolation of fungi

Two fungal isolates used in the current study were collected from *O.coerulescens* larvae of Astan Qods orchards, Mashhad. Those larvae which seemed to be infected by EPFs were removed and surface sterilized by submerging for 30 s in 70 % ethanol followed by 2 min in 1 % hypochlorite. Each cadaver was then washed twice with sterile distilled water and incubated in sterilized glass Petri dishes containing moist cotton and one Whatman No. 1 filter paper at 25 ± 2 °C, 60 ± 5 % RH with a photoperiod of 16:8 h (L:D) to allow for sporulation of the fungi. After four days, cadavers on which spores had formed were removed, cultured on Potato Dextrose Agar (PDA) plus 1 % peptone, and incubated under controlled conditions [25 ± 2 °C, 60 ± 5 % RH with a photoperiod of 16:8 h (L:D)] until sporulation within two weeks. To confirm Koch's postulates, virulence of the fungal isolates was determined by dipping healthy larvae in fungal suspension with 0.05 % Tween 80. The treatments were incubated at controlled conditions for seven days. In the control group, the insects were submerged in sterilized 0.05 % Tween 80 for an equivalent time period and under the same controlled conditions as the treated insects. Signs of mycosis were recorded after four days when the cadavers were surface sterilized with 0.2 % sodium hypochlorite solution.

Characterization of fungi isolates

Morphological identification

Morphological identification was conducted from macro- and micro-morphological features of fungal colonies. In the macroscopic characterization, the colour of conidia and growth of the fungal colony in pure fungal cultures were determined. In microscopic observations, features including the size and shape of conidia were investigated and the species were characterized according to Humber's key (Humber 2012). Twenty specimens were mounted and stained in aniline blue, then they were examined for the size, arrangement and shape of hyphal and conidial cells.

Molecular characterization

For molecular analysis, the fungal isolates were cultured on PDA plates and incubated under controlled conditions for 15 days. Subsequently, the mycelial mass was removed and transferred to Erlenmeyer flasks, containing 50 ml sterile PDB liquid medium (200 g potato and 10 g dextrose in 1 l of sterilized water plus 0.035 g streptomycin). The flasks were placed on a shaker at room temperature for ten days. Then the mycelia were harvested, eluted with sterile distilled water, transferred into a sterile 2-ml micro-tube, and finally stored at 4 °C until use. Genomic DNA was extracted using the QIAGEN DNeasy® kit (Venlo, the Netherlands). The ITS region was subjected to PCR with the ITS4 and ITS5 primers (White et al. 1990). Each PCR reaction contained 10 × *Taq* DNA polymerase reaction buffer 5 µl, 2 mM dNTPs 5 µl, 25 mM MgCl2 5 µl, 10 pmol each of the primers, 2 unit *Taq* DNA polymerase and 5–20 ng genomic DNA. Amplification was conducted using a Biometra thermal cycler with one cycle of denaturation for 2 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 s, an annealing step at 52 °C for 30 s, and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 10 min. The PCR products were sent to the Macrogen sequencing service facilities (Macrogen Inc., Seoul, Korea) for direct sequencing of double strands of DNA. All sequences were assembled by DNA Baser software. The consensus sequence was compared with the database using the nBLAST search tool from NCBI Genbank. The most similar sequences were retrieved from the Genbank and among them, valid and verified data were used to reconstruct the phylogenetic tree. Multiple alignments were performed using the Clustal W program. Phylogenetic trees were reconstructed using the neighbor-joining method in Molecular Evolutionary Genetic Analysis (MEGA6) software (Tamura et al. 2013). Bootstrap analysis was carried out with 1000 replicates.

Scanning Electron Microscopy (SEM) studies

In SEM studies, RLHB larvae were inoculated with 20 µl of 1×10^6 conidia ml^{-1} suspension of each

fungus. Then the infected larvae were assayed with SEM following the technique of Campos et al. (2005). Briefly, 48 h after inoculation (h.a.i.), the larvae were fixed overnight at 4 °C with 4 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH—7.3) and rinsed three times with 0.1 M sodium cacodylate buffer (pH—7.3) for 15 min. Next, the fixed samples were dehydrated in a series of ethanol solutions for 5 min, and then dried using the critical point technique. Finally, the specimens were mounted on stubs, coated with gold–palladium in a sputter coater (SC 7620) for 3 min, and photographed in a LEO 1450 VP SEM at 20 kV.

Bioassays

Conidia suspension preparation and viability test

Viability of the conidia was confirmed on PDA and all bioassays were performed using cultures which had more than 90 % conidial viability. The germination percentage of conidia for both isolates was estimated in three replicates. After preparing the stock cultures of conidia, the numbers of conidia were determined using a Neubauer haemocytometer (Superior, Marienfeld). The stock culture was diluted to make other concentrations.

Virulence assays

For virulence analysis, six concentrations of both EPF isolates were selected based on the preliminary assays and tested against RLHB larvae, pupae, and adults using the dipping method as described by Dubois et al. (2008). Briefly, larvae and adults were directly immersed individually for 30 s in conidial suspension ranges (1×10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 conidia ml^{-1}) and then placed in a sterilized Petri dish lined with a Whatman No. 1 filter paper and kept under controlled conditions. After treatment, the adult and larvae were transferred to plastic containers (150 × 70 mm) containing fresh apricot twigs as food and kept under controlled conditions [25 ± 2 °C, 60 ± 5 % RH with a photoperiod of 16:8 h (L:D)]. Five larvae or adults were used for each treatment in a completely randomized design with four replicates. For the assay with pupae, pupae were immersed individually in 30 ml of a conidia suspension (10^8 viable conidia ml^{-1} in sterile deionized water with 0.05 % Tween 80). There were five in each of four replicates. Then they were placed in sterilized Petri dishes containing filter paper and transferred to controlled conditions. In all treatments, the controls treatments consisted of sterile water with 0.05 % Tween 80 without fungal conidia. The treatments were checked daily for eight days. Any insects found dead were surface sterilized, washed three times with sterile distilled water, transferred into sterilized Petri dishes lined with filter paper, and kept under controlled conditions [25 ± 2 °C, 60 ± 5 % RH with a photoperiod of 16:8 h (L:D)] to investigate for mycosis.

Semi-field assessment: direct spray

To determine the efficacy of the EPF isolates on *O. coerulescens* larvae in infested branches, the fungal suspension against infested apricot branches containing the last instar larvae was investigated by two methods. In the first trial, 28 infested apricot branches directly were sprayed with suspensions of 2.5×10^{10} viable fungal conidia ml^{-1} plus 0.05 % Tween 80, using a knapsack manual sprayer equipped with a flat Flan nozzle ISO white 08F110 until the bark surface became completely wet. The treated apricot branches were left in the research orchard of Ferdowsi University of Mashhad outside the polyvinyl cover. The second trial was conducted under a polyvinyl cover to coat of sprayed infested branches with same conditions to first trial, such as conidia suspension, sprayer and time. Control treatment was sprayed with sterile distilled water with 0.05 % Tween 80. Ten days later, the treatments were checked for mycosis on dead larvae.

Potential of various solid media

One liter sterile liquid PDB was distributed into 250 ml Erlenmeyer flasks (for each fungus) and autoclaved. A fresh suspension of approximately 6×10^7 conidia ml^{-1} of both fungal isolates was prepared from four-week-old cultures on PDA (Jenkins and Prior 1993). Four flasks were inoculated with 1 ml of conidia suspension of each fungal isolate. The flasks were shaken on a rotary shaker at room temperature and allowed to grow for five days. During the process, neutral pH, moisture level (60 ± 5 % RH), and temperature (25 ± 2 °C) were kept constant. The solid media were prepared using three different substances: paddy rice, beet pulp, and wheat flour (Arian Trade service company) (100 g of each). Each medium was moistened slightly, and then distributed

into autoclavable bags. All bags were filled with equal amounts of substance and then autoclaved. They were then transferred to a sterile microbiological hood and inoculated with liquid culture (50 ml per 100 g substances) (Jenkins et al. 1998). After uniform inoculation, the bags were incubated under controlled conditions (25 ± 2 °C, 60 ± 5 % RH) for 21 days until sporulation. The conidia were extracted by pouring 1 ml 0.05 % Tween 80 in 350 ml distilled water into the bags, and the number of conidia was estimated using a haemocytometer. This experiment was conducted in a completely randomized design within factorial arrangement with three replicates.

Data analysis

Statistical analyses for all bioassays (larvae, pupae, and adult insects) and pot trials were conducted with the SAS software version 9.1 (SAS Institute 2002). Analysis of variance of mean numbers was performed using General Linear Model (PROC GLM). Mortality data were control corrected (Abbott 1925). Percentages data were normalized by arcsine square root transformation if necessary. In the larva and adult bioassays, the data were analyzed in a completely randomized design based on a factorial design including fungal isolate and EPF concentrations. In the pupa bioassays, pupae mortality was analyzed by *t* test. Analysis of field trials was also conducted based on a completely randomized design in a factorial arrangement including fungal isolates and trial conditions (covered and uncovered status). The mortality data were analyzed by two-way ANOVA and means separated using Tukey (HSD) test ($p = 0.05$). In the experiment on the potential of different solid substrate, the mean germination values on the three mediums were compared by Tukey tests. The median lethal concentration (LC_{50}) values (fiducial 95 %) for the larvae and adult insects exposed to EPFs were calculated using the procedure Probit in IBM-SPSS statistics, version 20 (SPSS Inc. IBM Company 2010).

Results

Entomopathogenic fungus identification

Two EPFs isolates were found in *O. coerulescens* larvae population from Astan Qods orchards, Mashhad, Razavi Khorasan province, Iran. The isolates were determined to be *Beauveria bassiana* and *Metarhizium anisopliae*. The conidial cells of *B. bassiana* had a spherical appearance and were usually densely clustered (or solitary). Mean size of conidia was 3.5 ± 0.25 µm in length and 2.35 ± 0.14 µm in width. The colony of *B. bassiana* was usually white to pale yellow in colour. The conidial cells of *M. anisopliae* were cylindrical shaped with rounded to conical apices, and conidial chains were arranged side by side. Mean size of conidia for *M. anisopliae* was 8.35 ± 0.05 µm in length and 2.89 ± 0.19 µm in width. The colour of the *M. anisopliae* colony was initially white and changed gradually to green. For molecular identification, the ITS sequences of both fungal isolated were used to reconstruct the phylogenetic tree. The obtained DNA sequences of both isolated species were deposited in the NCBI Genbank under accession numbers KP213287 for *B. bassiana* and KP213288 for *M. anisopliae*. A nBLAST search for *Beauveria* isolate (FUM 01) indicated the highest similarity to *B. bassiana* KC 753385.1 (data not shown). For the *Metarhizium* isolate, nBLAST results confirmed its 100 % similarity to another *Metarhizium* isolate. The *Beauveria* sp. FUM 01 isolate was most closely related to *B. bassiana* AB 576868 and *B. bassiana* KC 753385. The *Metarhizium* isolate was placed in the similar clade of *M. anisopliae* with a high bootstrap value. Overall, two species of hypocrealean fungi were identified by sequence analysis of ITS as well as morphological identification.

SEM studies

SEM clearly showed the details of events in the mycosis from the initial stages of conidia adhesion and subsequent penetration in *O. coerulescens* larvae treated with *B. bassiana* and *M. anisopliae* (Fig. 1). Evidence of conidial arrangement and mycelial growth was found on different regions of the insect bodies. From SEM studies, it was confirmed that the fungal conidia adhered to the cuticle while surrounding trichoid sensilla. This revealed the frequency of fungal conidia which adhered to the throughout the surface of larval body (Fig. 1a). The germ tube of fungal conidia was formed gradually (Fig. 1b), and following its formation the appressoria appeared in some regions of larval epicuticle (Fig. 1c). Subsequently, degradation of the cuticle began, and

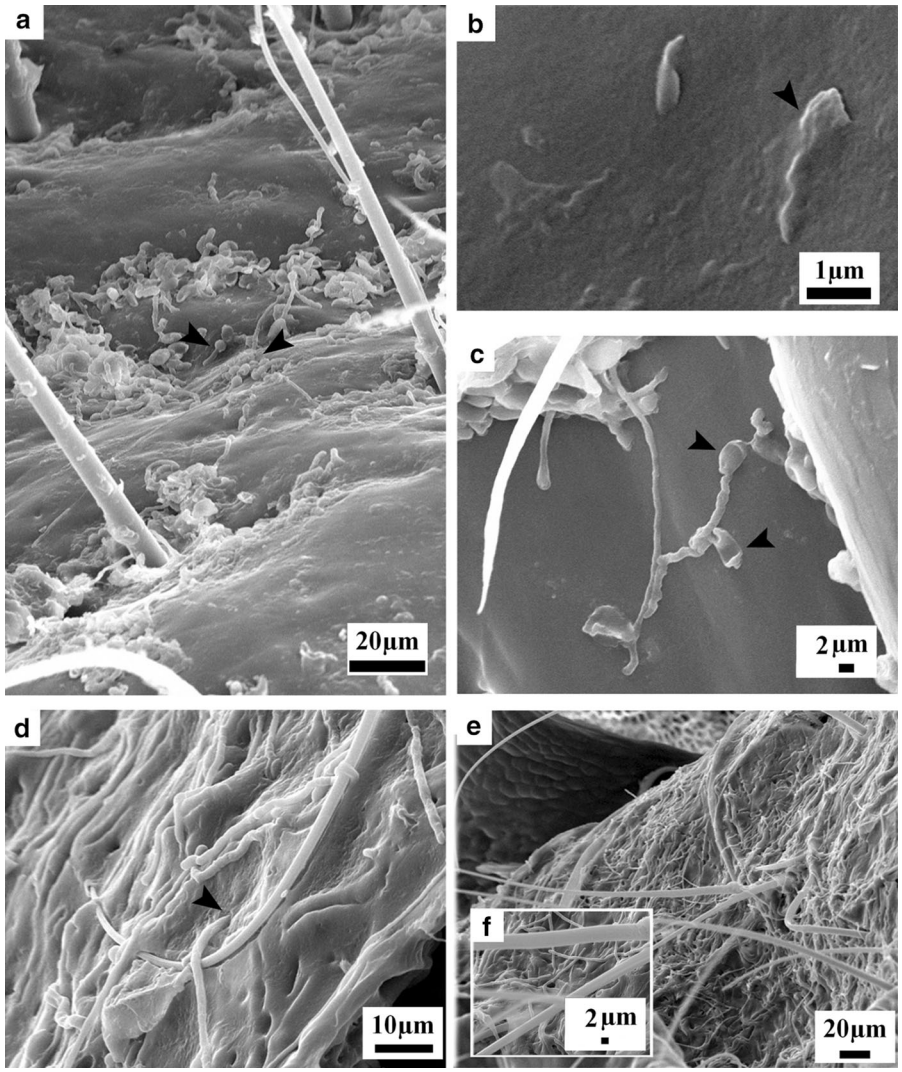

Fig. 1 Scanning electron microscopy pictures from surface body of *Osphranteria coerulescens* larva inoculated with conidia suspension of *Beauveria bassiana* and *Metarhizium anisopliae*. **a** Infected larva with *Metarhizium anisopliae* showing adhesion and germination of fungal conidia in the base of sensory sensilla. **b** Detail of germinated conidium of *Beauveria bassiana* showing signs of germ tube formation 48 h after inoculation (h.a.i.). **c** Appressorium showing fungal adhesion to the cuticle of *Osphranteria coerulescens* larvae 48 h.a.i. **d** Detail of fungal colonization on the larval body showing direct cuticle penetration by fungal hyphae from cuticle. **e** Hyphal network of *Metarhizium anisopliae* over the larval sensory surface 48 h.a.i. **f** Detail of hyphal development on *Osphranteria coerulescens* cuticle 48 h.a.i

penetration of other structures such as appressoria and infective pegs were observed through various areas, like the larval epicuticle (Fig. 1d). Extensive networks of hyphae were obvious over the body surface of the larvae which showed signs of fungal vegetative growth at 72 h.a.i. (Fig. 1e). Ventral parts of the larval body indicated a high density of mycelia (Fig. 1f).

Bioassays

The germination percentages of the *B. bassiana* and *M. anisopliae* isolates 48 h.a.i. were 90.3 ± 2.1 % and 86.1 ± 1.3 %, respectively. Results from bioassays confirmed that three stages of RLHB (larva, pupa, and adult) were susceptible to both EPFs species. The results also showed that an increase in conidial

concentration led to increased mortality rate. In all dead insects, a mycelial mass was observed on the sterilized surface of cadavers six days post-inoculation, and thus Koch's postulates were fulfilled.

Virulence assays

The EPFs had the highest virulence against larvae, especially in high concentrations (10^8 conidia ml^{-1}) at five days post-inoculation. In the larval bioassay, significant effects on mortality were observed for fungal species ($F_{1,42} = 46.56, p < 0.0001$) and fungal concentrations ($F_{6,42} = 66.57$, $p < 0.0001$). There was no interaction between species and concentrations ($F_{6,42} = 2.79$, $p < 0.202$). *B. bassiana* caused a higher infection rate, ranging between 25 % at the lowest rate and 90 % at the highest rate (Fig. 2a). In contrast, *M. anisopliae* caused no mortality at the lowest concentration (10^3 and 10^4 conidia ml^{-1}), and caused significantly lower mortality than *B. bassiana* at three of four higher rates (Fig. 2a). The corresponding LC_{50} values for both EPF species on the larvae were calculated five days post-inoculation and used to compare the virulence of applied fungal species (Table 1). RLHB pupae suffered greater mortality from *B. bassiana* (79 %) than from *M. anisopliae* (55 %) at five days post treatment (data not shown). No mycosis was found in the control. For adult RLHB, mortality at five days after inoculation was significantly affected by fungal isolates ($F_{1,42} = 4.45$, $p < 0.0409$) and concentrations ($F_{6,42} = 79.01$, $p < 0.0001$). There was no interaction between isolates and concentrations ($F_{6,42} = 1.02, p < 0.427$). *B. bassiana* caused greater mortality than *M. anisopliae* with the highest mortality observed at the highest concentration (10^8 conidia ml^{-1}) being 75 % for *B. bassiana* and 65 % for *M. anisopliae* (Fig. 2b). The LC_{50} values also indicated that the *B. bassiana* isolate was more virulent than the *M. anisopliae* isolate against adult insects of *O. coerulescens* (Table 1).

Semi-field assessment: direct spray

Larval mortality in infested apricot branches at ten days post-treatment was significantly affected by fungal isolate ($F_{2,18} = 12.29$; $p < 0.004$) and presence/absence of cover after application ($F_{1,18} = 8.92$; $p < 0.007$). There was no significant interaction between isolate and cover ($F_{2,18} = 3.37$; $p = 0.057$). *Beauveria bassiana* caused greater mortality than *M. anisopliae* and mortality was higher when the branches were covered. However, covering the branch only significantly increased mortality for *B. bassiana* (67 vs. 32 %) but not mortality by *M. anisopliae* (22 vs. 17 %) (Fig. 3).

Potential of various solid media

There was a significant effect of substrate ($F_{2,12} = 326.62$; $p < 0.0001$) and EPF species ($F_{1,12} = 27.90$; $p = 0.0002$) but no interaction between substrate and species ($F_{2,12} = 1.36$; $p = 0.2939$). Sugar beet produced the greatest number of conidia reaching 6.33×10^9 conidia g^{-1} substrate for *B. bassiana* and 5.44×10^9 conidia g^{-1} substrate for *M. anisopliae* (Fig. 4). Conidial production was significantly lower in wheat flour and was the lowest in paddy rice. *Beauveria bassiana* produced more conidia than *M. anisopliae* but significantly so only in sugar beet pulp and wheat flour.

Table 1 Probit regression lines adjusted to mortality data of *Osphranteria coerulescens* larvae/adult exposed to *Beauveria bassiana* and *Metarhizium anisopliae* in laboratory

Fungal species	Stage	n	LC_{50} (conidia ml^{-1})	95 % FL[a]	Slope ± SE[b]	χ^2 (df)	*P* value [c]
Beauveria bassiana	Larva	120	4.8×10^5	9.4×10^4–3.0×10^6	0.35 ± 0.08	9.57 (22)	0.99
	Adult	120	4.8×10^6	1.8×10^6–1.5×10^7	0.69 ± 0.12	9.44 (22)	0.99
Metarhizium anisopliae	Larva	120	8.6×10^6	3.0×10^6–3.4×10^7	0.64 ± 0.11	5.75 (22)	1.0
	Adult	120	1.5×10^7	5.1×10^6–8.0×10^7	0.62 ± 0.12	6.25 (22)	1.0

[a] Fiducial limits 95 %

[b] In linear regression analyses, all slope coefficients were significantly different from zero ($p < 0.001$)

[c] Since significance level is greater than 0.15, no heterogeneity factor was used in the calculation of fiducial limits

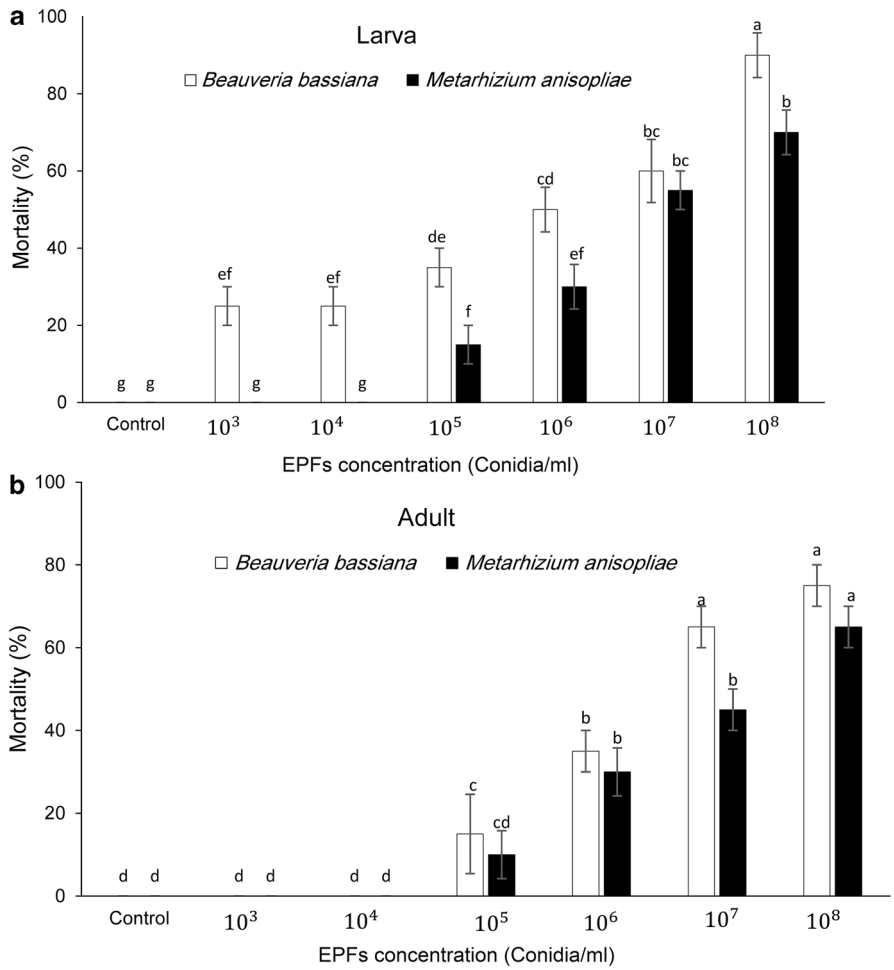

Fig. 2 Mean corrected mortality (% ±SE) of *Osphranteria coerulescens* **a** larvae and **b** adults, five days after treatment with *Beauveria bassiana* and *Metarhizium anisopliae* at six concentrations (1×10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 conidia ml^{-1}). Different letters indicate significant difference between fungal species and concentration, Tukey test ($p < 0.05$)

Discussion

Two native hypocrealean species were isolated from *O. coerulescens* larvae in orchard, characterized and used in a trail series to examine their interactions with their natural host insect. Occurrence of *B. bassiana* and *M. anisopliae* within the larvae of *O. coerulescens* is a new report for incidence of EPFs as natural entomopathogen on the *O. coerulescens.* Studies on occurrence, isolation and characterization of native EPF species is important (Bidochka et al. 1998), as exotic species/strain of EPFs have often failed in biocontrol programs due to poor adaptation of exotic fungal strains to local environmental conditions or factor related to fungus species like host species (Lacey et al. 2015). Recent research highlights the important and diverse ecological functions of EPFs. Significant attention has been paid to EPF use in integrated pest management programs (Lacey et al. 2015). It is well-known that native EPF isolates may have greater environmental persistence and compatibility with the local environment than exogenous ones (Kim and Kim 2008).

The main goal of this study was to investigate the potential of local isolates of two EPF species against larval, pupal and adult stages of the rosaceous longhorn beetle. Considering the "disease triangle" representing the insect host/pathogen/environment relationship (Tanada and Kaya 1993; Scholthof 2007), we attempt to explain the role of main elements of each component involved. Based on this theory, various factors, related to insect, fungus, or host plant are involved in EPF infection of insect pests (Shapiro-Ilan et al. 2012). The bioassay focused on three life stages of RLHB, which indicated maximum mortality occurred in the larval stage when exposed to

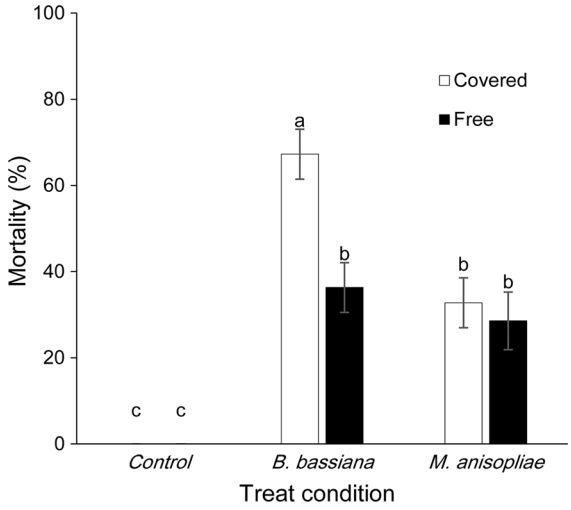

Fig. 3 Mean corrected mortality (% ±SE) of *Osphranteria coerulescens* larvae in infested apricot branches ten days post-treatment. Branches were covered after treatment or left uncovered. *Different letters* indicate significant differences between the fungal species and cover status of the branches, Tukey test ($p < 0.05$)

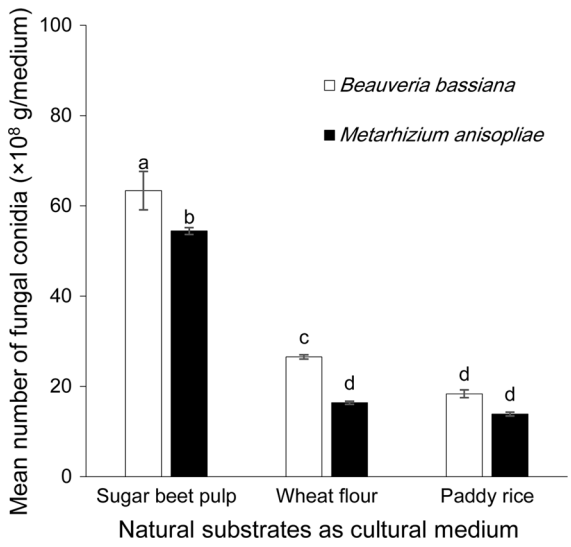

Fig. 4 Mean number of conidia (±SE) produced in three different solid substances (paddy rice, beet pulp, and wheat flour) for *Beauveria bassiana* and *Metarhizium anisopliae*. *Different letters* indicate significant differences between interactive fungal species and substance, Tukey test ($p < 0.05$)

10^8 conidia ml^{-1}, causing 67 and 93 % mortality by *M. anisopliae* and *B. bassiana,* respectively. Based on the high susceptibility of the larva rather than pupa and adult, this study focused on the larval stage of *O. coerulescens* as the target stage. The susceptibility of an insect host to fungal infection plays a critical role in determining whether or not a successful control is possible. Host density as well as genetic and behavioral characters (related to resistance) are involved in interactions between host, pathogen and environment. For example, the thickness of the wax layer (Shimazu et al. 2002) and amino acid properties of the insect cuticle (Tanada and Kaya 1993) play critical roles in preventing hyphal invasion. Applying EPFs in the laboratory bioassays provided a high mortality rate of *O. coerulescens* larvae, because each larva was immersed in pure EPF suspensions.

In the semi-field trials, however, EPF suspension was sprayed on infested apricot branches. It seems that fruit orchards infested with *O. coerulescens* are potential targets for application of EPFs. The efficacy of fungal conidia is not determined by a single factor. Liu and Bauer (2008) observed that longitudinal bark crevices and mosses attached to the tree bark provide the required moisture for conidia germination on pest larvae and protection of the inoculum (Meyers et al. 2013). RLHB larvae are considered difficult to control because the damaging stage lives in cryptic and inaccessible habitats. Our study indicated that *B. bassiana* was able to penetrate infested apricot branches satisfactorily and showed the potential of EPFs incorporation in management plans for RLHB. Liu and Bauer (2008) found possible effects of *B. bassiana* on larvae of *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) under tree bark, using topical spray in a field trial. Their results showed that the fungal infection caused 8 % mortality of fourth instar larvae and delayed larval development after fungal treatment. In Japan, *B. bassiana* has been developed as a microbial control agent against *M. alternatus* larvae since spraying of fungal conidia caused 75 % larval mortality (Hajek and Bauer 2007).

The second element of the disease triangle is EPF characteristics. Several items including fungus species, concentration, origin of fungus, nutritional status of fungus, etc., play a critical role for the interaction between fungi, RLHB and environment. The current experiments showed that *B. bassiana* caused higher mortality rates than *M. anisopliae*. Different species of wood boring insects have shown various levels of susceptibility to EPF species. Shimazu et al. (2002) showed that *B. brongniartii* has strong virulence against *M. alternatus* adults but *B. bassiana* only

moderate virulence. Carrillo et al. (2015) determined the susceptibility of *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae) to three commercial strains of entomopathogenic fungi by immersing female beetles in conidial solution. Results showed that female beetles which were exposed to *B. bassiana* solution died faster than those treated with *Isaria fumosorosea* Wize (Hypocreales: Cordycipitaceae). They also found that a higher number of *B. bassiana* conidia attached to female beetle bodies than *I. fumosorosea*. In another study, Glare et al. (2002) examined the potential of three EPF strains as biological agents against the pin-hole borer (*Platypus* spp.) and reported that *B. brongniartii* and *B. bassiana* were similar in virulence against the adult wood borer, causing 100 % mortality within 6–8 days. Although, their results were similar to those of the current study, some previous studies have shown that *M. anisopliae* was more virulent against a wood boring insect compared with *B. bassiana* (Dubois et al. 2008).

Another factor related to EPF virulence is its concentration. We confirmed that the highest mortality rates of RLHB were observed at highest concentrations of both EPF species. Previous studies suggested that mortality occurs quickly by applying higher concentrations of fungal conidia (Shanley et al. 2009; Castrillo et al. 2008). Shanley et al. (2009) stated that calculating the LC_{50} values is an essential criterion in determining the activity of EPFs. In our experiment, the LC_{50} of *B. bassiana* was estimated as 4.8×10^5 and 4.8×10^6 conidia ml^{-1} for larvae and adults, respectively, whereas the LC50 for *M. anisopliae* were higher (Table 1).

Another important element which relates to conidial effectiveness is nutritional contents of the fungus. Shah et al. (2005) reported that the nutritional conditions of fungi, including carbon and nitrogen, could affect germination and quality of the conidia. We showed that sugar beet pulp was the most efficient solid medium for conidia production. Therefore, different natural solid substrates as nutrient enriched the media and provided optimal conditions for fungal germination. Therefore, it is necessary to optimize the nutritional condition for better EPF growth and sporulation. A main principle is enriching media to yield high levels of spores at a low cost (Dorta et al. 1990). Optimizing nutritional conditions could affect fungus virulence (Tanada and Kaya 1993; Shah et al. 2005).

The application of a polyvinyl cover provided suitable condition of humidity for delivering conidia into infested apricot branches. Hajek and Bauer (2007) hypothesized that water flow from rain and other factors (such as microorganisms) could move the fungal conidia. Generally, different application techniques of conidial suspensions have various results (Hajek et al. 2006). The application of the polyvinyl cover as a successful technique improved conidial persistence by protection from unsuitable environmental conditions such as UV radiation and desiccation. Reay et al. (2007) suggested that the development of a biopolymer coating as a formulation technique for protecting conidia from UV radiation can play an important role in fungal virulence. The result suggests that applying conidial fungal suspensions associated with a polyvinyl cover against infested branches could be useful as an efficient biological strategy against *O. coerulescens* in stone-fruit orchards. This technique requires less fungal suspensions and equipment than broad spectrum spraying in small parts of an orchard. However, further studies are needed to determine the best method for using EPF preparations. This could be related to third component of the disease triangle, i.e., environment.

An understanding of the infection process in the host body can be useful to understand the mechanism of EPF influences (Kumar et al. 2015). Güerri-Agullo et al. (2010) reported that SEM studies play an important role in identifying possible approaches for development of EPF formulations. SEM images of *O. coerulescens* larvae showed that sensilla hairs and spines are desirable sites for conidia adhesion. Güerri-Agullo et al. (2010) also reported that sensilla structures are important for inoculum activation. Furthermore, cuticular degradation occurred concurrently with appressoria formation, and these structures were formed in an adjacent zone intercalary suture (*e.g.,* head or joints of integument). The fungal appressorium structures are well known as being important factors involved in the mortality of pests by direct penetration into the body cavity (Nahar et al. 2008; Güerri-Agullo et al. 2010). We found appressoria at hyphae while attached to insect cuticles impregnated with conidial suspensions of *B. bassiana* and *M. anisopliae*. The application of SEM was found to be a satisfactory technique for understanding the activity process of both EPF species.

In conclusion, applying native and virulent species of EPF, selecting the target life stage that has the highest susceptibility to EPFs, optimizing nutritional conditions of fungi and providing suitable cover after fungal treatment are critical in the microbial control of *O. coerulescens* using EPFs. Our results are in agreement with those of previous works (Meyers et al. 2013; Shimazu et al. 2002; Reay et al. 2007) and imply that native EPFs have significant potential as biocontrol agents of wood boring insects. The application of EPF suspensions under special climatic conditions or in special formulations warrants further studies.

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