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Efficacy of native isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against the invasive ambrosia beetle, *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae)

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Abstract

The invasive ambrosia beetle, *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae), is one of the most significant pests in hazelnut orchards in Turkey. Chemical control of the beetle is very difficult, because it spends the majority of its life in the sapwood of host trees. In this study, entomopathogenic fungi (EPF), *Metarhizium anisopliae* (TR-106 isolate), and *Beauveria bassiana* (TR-217 isolate) were tested against the female adults of *X. germanus* under laboratory conditions by two different methods. In the first method, conidial concentrations (1×10^6 and 1×10^8 conidia ml^{-1}) of the fungal isolates were directly applied to the beetles in Petri dishes (2 ml per dish), using a Potter spray tower. In the second method, the same conidial concentrations were applied on a sterile hazelnut branch placed in the Petri dishes, and then, beetles were released in dishes. The Petri dishes were incubated at $25 \pm 1^\circ\text{C}$ and $75 \pm 5\%$ RH, 16:8-h light:dark photoperiod. Lethal time values (LT_{50} and LT_{90}) for the isolates were calculated, using Probit analysis. As a result, LT_{50} and LT_{90} values of *M. anisopliae* isolate at 1×10^8 conidia ml^{-1} were 4.43 and 6.01 days, respectively, at the beetle application method and 3.97 and 5.68 days, respectively, at the branch application method. LT_{50} and LT_{90} values were 6.03 and 10.80 days, respectively, at 1×10^8 conidia ml^{-1} , when *B. bassiana* was applied directly on the beetles and 5.96 and 11.79 days at the same concentration, when it was applied on the branch application method. *M. anisopliae* at 1×10^8 conidia ml^{-1} caused 100% mortality rate at the end of the 8th day in the two application methods. The mortality rates when applying 1×10^8 conidia ml^{-1} of *B. bassiana* recorded 64% and 80% by the end of the 8th day on branch and insect application methods, respectively. The efficiency of 1×10^6 conidia ml^{-1} of the isolates was lower than 1×10^8 conidia ml^{-1} against *X. germanus* in both application methods. This study showed that the isolates of TR-106 and TR-217 had a significant potential as biological control agents against *X. germanus*.

Keywords: *Xylosandrus germanus*, Entomopathogenic fungi, Hazelnut, Turkey

Background

Ambrosia beetles (Coleoptera: Curculionidae: Platypodiinae and Scolytinae) are very important pests on many trees (Hulcr and Dunn 2011). They are wood boring insects that make tunnels through sapwood of trees and feed primarily on symbiotic fungi living in the tunnels (Knížek and Beaver 2007). Adults and larvae feed solely

with the fungi, except in some species where larvae also ingest fungus-infested wood (Biedermann 2007).

Turkey is the world's biggest hazelnut producer, supplying nearly 80% of the total global production. Ambrosia beetles including *Anisandrus dispar* Fabricius, *Xyleborinus saxesenii* Ratzeburg, and *Xylosandrus germanus* Blandford are widespread pests on hazelnut trees in Turkey. Due to their damage, most of the hazelnut trees die finally, especially in orchards at Black Sea coastline in which the ground water level is high (Ak 2016). *X. germanus* was firstly detected in Turkish hazelnut orchards in 2011

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(Knížek 2011) and became one of the major pests in hazelnut orchards with other ambrosia beetles. Afterwards, it was recorded as a pest of kiwi trees in Black Sea Region of Turkey (Ak et al. 2011).

Control methods against these ambrosia beetles, including *X. germanus* in hazelnut orchards, depend on the use of conventional insecticides against emerged adults, cultural methods (pruning and removing infested branches of trees), and mass trapping of emerged adults through red winged ethanol-baited sticky traps. When insecticide spraying is needed for control, it is necessary to use as many as 6–7 applications because of the beetle's long prevalence periods in orchards. But, chemical control is obviously unpractical due to its detrimental effects, high cost, and low efficacy in such a large area of hazelnut plantations. For this reason, there is a need for more effective and ecologically safe alternative control methods. Using EPF may have a potential to suppress the ambrosia beetles (Arthurs and Bruck 2017).

EPF are common natural enemies of arthropods worldwide, attracting attention as potential biological control agents. They are more than 700 species (Roy et al. 2006). EPF such as *Beauveria bassiana* (Bals.) Vuill., *Isaria fumosorosea* Wize, *Lecanicillium* spp., and *Metarhizium anisopliae* (Metch) Sorokin play an important role in the regulation of insect populations (Zimmermann 2007a, 2007b and 2008). Previous studies have showed that they were effective on ambrosia beetles including, *X. germanus*, *A. dispar*, *Xylosandrus crassiusculus* Motschulsky, and *Xyloborus glabratus* Eichhoff (Castrillo et al. 2013; Carrillo et al. 2015; Kushiyeve et al. 2018). Kushiyeve et al. (2018) determined that *I. fumosorosea* at 1×10^8 conidia ml⁻¹ against female adults of *X. germanus* applied on adults and on branches caused 100 and 90% mortality rates, respectively. EPF may affect not only adults outside the host trees but also larvae inside the beetle galleries (Castrillo et al. 2013).

The aim of the present was to evaluate the efficacy of two conidial concentrations (1×10^6 and 1×10^8 conidia ml⁻¹) of *M. anisopliae* (TR-106) and *B. bassiana* (TR-217) against the females of *X. germanus*, using two different application methods.

Material and methods

Isolation of EPF

The EPF, *M. anisopliae* (TR-106) and *B. bassiana* (TR-217), isolates used in this study were obtained from female cadavers of *X. germanus* collected from hazelnut orchards in Samsun provinces in Black Sea Region in Turkey. The cadavers were observed under a Leica EZ4 stereomicroscope at $\times 40$ – 70 magnification for fungal growth, and a piece of fungal mat was transferred to water agar (WA) by a sterile needle. The cultures were

incubated for 3–5 days at $25 \pm 1^\circ\text{C}$ and $75 \pm 5\%$ RH under 16: 8-h L:D photoperiods. Mycelial tips were cut from the edge of the 5-day-old fungal colonies, transferred to 2% malt extract agar (MEA) (Merck), and incubated for a period of 10–14 days under the same conditions. Single-spore isolates were obtained by serial dilution (Dhingra and Sinclair 1995), and the fungi were identified as *M. anisopliae* and *B. bassiana* according to Samson et al. (1988) and Humber (1997). The cultures were stored at 4°C on Sabouraud Dextrose Agar (SDA) (Merck Ltd., Darmstadt, Germany) slants and -80°C in cryogenic tubes containing 15% glycerol and deposited in the fungal culture collection of the Mycology Laboratory at the Ondokuz Mayıs University, Faculty of Agriculture's Department of Plant Protection in Samsun, Turkey.

Collection of insects

Hazelnut orchards in Samsun province (Turkey) were inspected to collect hazelnut branches infested with *X. germanus* during May 2016. Infested branches with beetles were cut (40-cm long) in the field, placed in plastic containers (20 \times 25 \times 40 cm), and then brought to the laboratory. Infested branches were dissected by pruning scissors, and females were collected from galleries. Collected females were inspected under the Leica EZ4 stereomicroscope at $\times 40$ – 70 magnification to separate the healthy *X. germanus* females in order to use them in bioassays. Like other ambrosia beetles, males of *X. germanus* are in very low sex ratio (about 10:1), flightless, and rarely seen outside the gallery. Thus, females were used in the tests of the study (Ranger et al. 2016).

Inoculum of entomopathogenic fungi isolates

The isolates of *M. anisopliae* (TR-106) and *B. bassiana* (TR-217) were propagated on SDA at $25 \pm 1^\circ\text{C}$ and $75 \pm 5\%$ RH for 15 days. Conidia were harvested by sterile distilled water containing 0.02% Tween 20. Mycelia were removed by filtering conidia suspensions through four layers of sterile cheesecloth. The concentrations of conidia were adjusted to both 1×10^6 and 1×10^8 conidia ml⁻¹, using a Neubauer hemocytometer under Olympus CX31 compound microscope (Olympus America Inc., Lake Success, NY, USA) for each isolate (Kushiyeve et al. 2018).

Assessment of conidial viability

The viability of conidia of the 2 isolates (TR-106 and TR-217) was determined by spreading 0.1 ml of concentration of 1×10^4 conidia ml⁻¹ on 3 separate 6-cm-diameter Petri dishes containing Potato Dextrose Agar (PDA: Oxoid Ltd., Basingstoke, UK) and incubating them at $25 \pm 1^\circ\text{C}$ for 24 h under a 14:10-h L:D photoperiod. After 24 h of incubation, germination was determined by counting 200 conidia/dishes with an Olympus

CX-31 compound microscope at $\times 400$ magnification. Conidia were regarded as germinated when they produced a germ tube at least half of the conidial length (Kushiyeve et al. 2018).

Virulence of the EPF versus *X. germanus* females

The experiments were carried out in 2 main tests. In the first, the conidial concentrations (1×10^6 and 1×10^8 conidia ml^{-1}) of TR-106 and TR-217 isolates were applied separately versus the females of *X. germanus* (2 ml per dish), using a Potter spray tower (Burkard, Rickmansworth, Hertz, UK) and one sterilized hazelnut branch (5 cm length and 1 cm in diameter) was added in each Petri dish. In the second test, the same concentrations of the isolates were sprayed on sterilized hazelnut branches in Petri dishes, and afterwards, the beetles were released on them. Another group of sterilized hazelnut branches was sprayed with sterile distilled water containing 0.02% Tween 20 (2 ml) for the control experiments. Petri dishes were loosely covered by a Parafilm after applications. Females of *X. germanus* were surface sterilized with 70% ethanol for 10 s before the experiments (Castriello et al. 2013). The hazelnut branches used in the experiments were autoclaved in autoclavable polyethylene bags (30×30 cm) for 1 h at 121°C on 2 successive days. The Petri dishes lined with moist filter paper (9 cm in diameter) at the bottom were used in all experiments. The spray tower was cleaned with 70% ethanol and sterile distilled water after each application of the fungus concentrations for disinfecting the apparatus. For 8 successive days of experimental observations, different groups of insects were used in 5 replications by 5 insects per Petri dish ($n = 25$ insects/day/concentration/application method) and so provided independence of the observations on the mortality on over time (Robertson et al. 2007). The same procedure was followed up at the control groups. Different control groups were set for each observation daily. Petri dishes were incubated at $25 \pm 1^\circ\text{C}$ and 95% RH, 16:8-h light:dark photoperiod for 8 days in a BINDER incubator (Model KBWF 240,

Germany). All dishes of each observation were inspected daily during 8 days, percent mortality was calculated on each observation day independently, and alive insects were discarded. Dead individuals in which the fungal sporulation is observed were counted under the Leica EZ4 stereo microscope at $\times 40$ – 70 magnification.

Statistical analysis

Independent-time mortality data from bioassays were analyzed by probity analysis program (POLO-PLUS Ver.2.0) to calculate 50% lethal time (LT_{50}) and 90% lethal time (LT_{90}). In lethal time analysis, Log-Probit analysis calculations were considered in the program. Slopes of regression lines were compared to each other by their standard errors. LT_{50} and LT_{90} values of two application methods and two conidial concentrations were compared based on overlapping of 95% fiducial limits.

Results and discussion

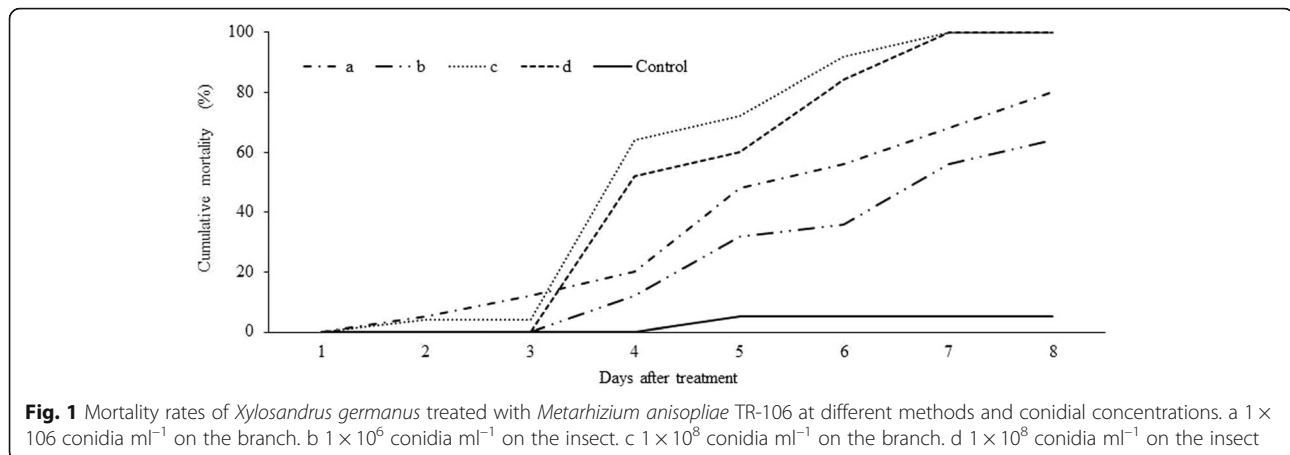
Conidial viability of *M. anisopliae* (TR-106) and *B. bassiana* (TR-217) was assessed before bioassays, and almost 100% of the conidia of the isolates germinated at the end of the 24 h. The results showed that the 2 isolates were pathogenic to females of *X. germanus* in the 2 different tested concentrations (1×10^6 and 1×10^8 conidia ml^{-1}) and application methods (on branch and direct on insect).

Considering the application method, a significant difference was observed between the 2 concentrations of the isolate (TR-106) applied to *X. germanus* ($P < 0.05$). The concentration (1×10^8 conidia ml^{-1}) was more effective than the other on *X. germanus* at both application methods (branch and insect). Following application with 1×10^6 conidia ml^{-1} of the TR-106 isolate of *M. anisopliae*, the LT_{50} value for *X. germanus* was 5.45 and 6.67 days, respectively, for branch and insect applications. Respective values at 1×10^8 conidia ml^{-1} were 3.97 and 4.43 days, respectively. However, the LT_{90} values for 1×10^6 conidia ml^{-1} of TR-106 isolate were 10.41 and 11.25 days, while the same values for 1×10^8 conidia ml^{-1} of the isolate were 5.68 and 6.01 days for

Table 1 Probit analysis data on mortality time of *Xylosandrus germanus* females after applications of two conidial concentrations of *Metarhizium anisopliae* TR-106 isolate on hazelnut branches and beetle females

Variables	1×10^6 conidia ml^{-1}		1×10^8 conidia ml^{-1}	
	On branch	On insect	On branch	On insect
LT_{50} (95% fiducial limit)	5.45 (4.85–6.21) b*	6.67 (6.06–7.59) b	3.97 (3.49–4.41) a	4.43 (4.10–4.73) a
LT_{90} (95% fiducial limit)	10.41 (8.54–14.88) b	11.25 (9.34–16.17) b	5.68 (5.04–6.90) a	6.01 (5.55–6.76) a
Slope \pm SE	4.56 \pm 0.68 b	5.63 \pm 0.99 b	8.25 \pm 1.06 a	9.66 \pm 1.35 a
Regression equation	$y = -3.36 + 4.56x$	$y = -4.64 + 5.63x$	$y = -4.94 + 8.25x$	$y = -6.24 + 9.66x$
χ^2	41.89	23.04	67.99	22.70
D_f	38	38	38	38
Heterogeneity	1.10	0.61	1.79	0.60

*Within rows, means followed by the same lower case letters do not differ significantly



application on branches and direct on insects, respectively (Table 1).

On the 1st day after treatment, there was no mortality for TR-106 isolate in the 2 tested concentrations applied in the 2 methods (Fig. 1). In the 2 methods, mortality rates were low during the 1st 3 days but death increased after the 3rd day (Fig. 1). Meanwhile, the mortality rates of *X. germanus* treated with 1×10^6 conidia ml^{-1} of the isolate reached 80 and 64% at the end of the 8th day on branches and directly on insects, respectively. The isolate caused 100% mortality rate at the end of the 8th day in the 2 application methods with 1×10^8 conidia ml^{-1} . Moreover, all applications of TR-106 isolate caused approximately 100% mycosis rate on dead adults of *X. germanus*.

The efficacy of *B. bassiana* (TR-217) used in this study was found to be lower than that of *M. anisopliae* (TR-106). The LT_{50} values of TR-217 isolate at 1×10^6 conidia ml^{-1} were 8.38 and 9.57 days, while the LT_{90} values were 16.78 and 20.30 days for treated branches and for application directly on the insects, respectively. Respective LT_{50} values for 1×10^8 conidia ml^{-1} of the isolate were 5.96 and 6.03 days, while they were 11.79 and 10.80 days for LT_{90} (Table 2).

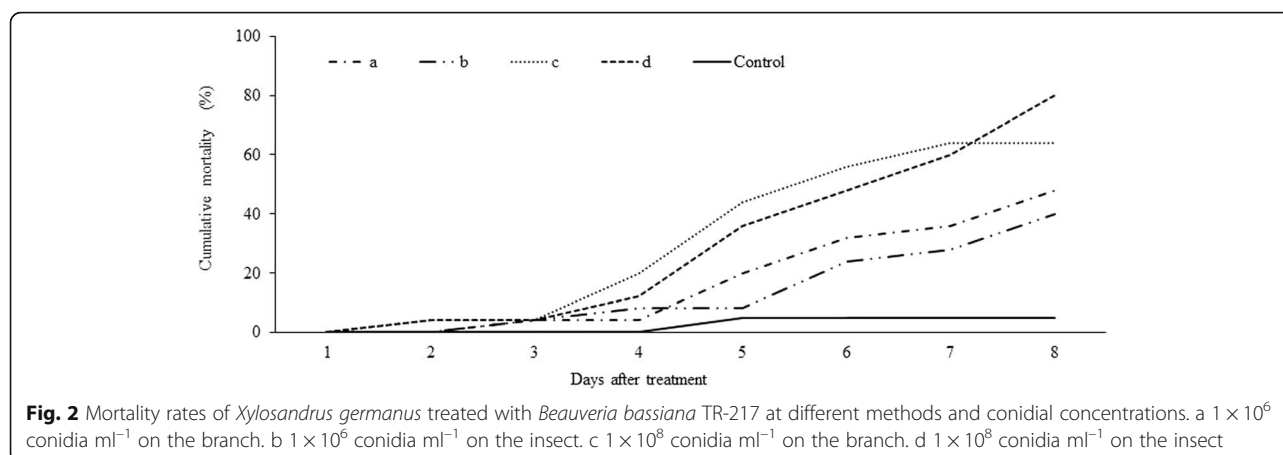
The mortality rates when applied at 1×10^8 conidia ml^{-1} of *B. bassiana* recorded 64 and 80% at the end of the 8th day on treated branches and insects, respectively. However, the efficacy of 1×10^6 conidia ml^{-1} *B. bassiana* (TR-217 isolate) against the beetle was lower than those of the highest concentration used in the study of the 2 application methods (Fig. 2). The mortality rates of *X. germanus* females at 1×10^6 conidia ml^{-1} of *B. bassiana* remained around 40.0 and 45.0%, even on the 8th day after treatment. Similar to *M. anisopliae*, *B. bassiana* isolate caused about 100% mycosis rate on females in all treatments.

LT_{50} and LT_{90} values of *M. anisopliae* (TR-106) and *B. bassiana* (TR-217) isolates decreased with increasing the conidial concentration at all applications. However, comparisons of the confidence intervals indicated insignificant differences between LT_{90} values at both concentrations of *B. bassiana* TR-217 isolate in the 2 application methods. The results of this study are in line with those of previous studies as many researchers have reported that efficacy of EPF increases according to concentration increase. Therefore, the LT_{50} and LT_{90} values of the fungi decreased (Kocaçevik et al. 2016 and Kushiyeve et al. 2017, 2018). Kushiyeve et al. (2017) found that LT_{50} and LT_{90} values of

Table 2 Probit analysis data on mortality time of *Xylosandrus germanus* females after applications of two conidial concentrations of *Beauveria bassiana* TR-217 isolate on hazelnut branches and beetle females

Variables	1×10^6 conidia ml^{-1}		1×10^8 conidia ml^{-1}	
	On branch	On insect	On branch	On insect
LT_{50} (95% fiducial limit)	8.38 (7.17–11.50) a*	9.57 (7.84–15.56) a	5.96 (5.32–6.85) b	6.03 (5.41–6.87) b
LT_{90} (95% fiducial limit)	16.78 (12.02–37.58) a	20.30 (13.37–63.78) a	11.79 (9.50–17.45) a	10.80 (8.91–15.38) a
Slope \pm SE	4.24 ± 0.92 a	3.92 ± 0.95 a	4.32 ± 0.69 a	5.06 ± 0.78 a
Regression equation	$y = -3.92 + 4.24x$	$y = -3.85 + 3.92x$	$y = -3.35 + 4.32x$	$y = -3.95 + 5.06x$
χ^2	36.89	23.95	29.61	40.78
D_f	38	38	38	38
Heterogeneity	0.97	0.63	0.78	1.07

*Within rows, means followed by the same lower case letters do not differ significantly



M. anisopliae TR-106 and *B. bassiana* TR-217 isolates applied to females of *A. dispar* decreased by increasing conidial concentration.

In the present study, there was insignificant difference between both application methods ($P > 0.05$) with regard to infectivity. Similarly, some researchers found insignificant differences in the mortality rates of *X. glabratus* females, treated with *B. bassiana* strain (GHA) and *I. fumosorosea* strains (Ifr 3581 and PFR), by dipping in fungal concentration or placing on treated avocado branches (Carrillo et al. 2015). In another study, Kushiye et al. (2018) investigated the efficacy of *I. fumosorosea* TR-78-3 at 1×10^6 and 1×10^8 conidia ml^{-1} against females of *A. dispar* by treating the plant branches and direct spray on insects and found insignificant differences between the two application methods.

Liu et al. (2017) stated that *B. bassiana* (12,108 isolates) was more effective than *M. anisopliae* (3,4607 isolate) on adults of *Curculio nucum* (Coleoptera: Curculionidae). The difference between obtained results and that of Liu et al. (2017) may be derived from using different isolates. Consequently, the efficacy of EPF may vary between species, within a genus, and even between isolates of the same species (Goettel et al. 2005).

EPF can be effective when applied directly on insect pests or when they are later acquired by the insects from treated surface; therefore, ambrosia beetles, which spent the majority of their life cycles inside the host trees, are ideal target for EPF (Castrillo et al. 2013) and it can be observed lately that studies on ambrosia beetle-EPF interactions were increased. Commercial strains of *B. bassiana* (GHA and Naturalis) and *M. brunneum* (F52) applied on females of *X. crassiusculus* caused 77.96 and 79% mortality rate, respectively, on the 5th day after application under laboratory conditions, and also, the beetle females exposed to peach stems treated with the fungi had low survival capacity (Castrillo et al. 2013).

Conclusion

The present findings determined that EPF, *M. anisopliae* (TR-106) and *B. bassiana* (TR-217), seemed to be promising one of biological control agent against the invasive ambrosia beetle, *X. germanus*. However, further studies are necessary to evaluate the efficacy of the isolates on the treated pest under field conditions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CT, RK, IE, IOO, and IS designed the study, supervised the work, and wrote the manuscript with input from all authors. CT, RK, IE, IOO, and IS carried out the experiments. CT analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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