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# Efficacy of native entomopathogenic fungus, *Isaria fumosorosea*, against bark and ambrosia beetles, *Anisandrus dispar* Fabricius and *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae)

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## Abstract

The efficacy of the native entomopathogenic fungus, *Isaria fumosorosea* TR-78-3, was evaluated against females of the bark and ambrosia beetles, *Anisandrus dispar* Fabricius and *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae), under laboratory conditions by two different methods as direct and indirect treatments. In the first method, conidial suspensions ( $1 \times 10^6$  and  $1 \times 10^8$  conidia  $\text{ml}^{-1}$ ) of the fungus 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 suspensions were applied on a sterile hazelnut branch placed in the Petri dishes. The  $\text{LT}_{50}$  and  $\text{LT}_{90}$  values of  $1 \times 10^8$  conidia  $\text{ml}^{-1}$  were 4.78 and 5.94/days, for *A. dispar* in the direct application method, while they were 4.76 and 6.49/days in the branch application method. Similarly,  $\text{LT}_{50}$  and  $\text{LT}_{90}$  values of  $1 \times 10^8$  conidia  $\text{ml}^{-1}$  for *X. germanus* were 4.18 and 5.62/days, and 5.11 and 7.89/days, for the direct and branch application methods, respectively. The efficiency of  $1 \times 10^6$  conidia  $\text{ml}^{-1}$  was lower than that of  $1 \times 10^8$  against the beetles in both application methods. This study indicates that *I. fumosorosea* TR-78-3 had a significant potential as a biological control agent against *A. dispar* and *X. germanus*. Further studies are necessary to evaluate the efficacy of the isolate on the pests under field conditions.

**Keywords:** Bark and ambrosia beetles, Biological control, Entomopathogenic fungus, *Isaria fumosorosea*, Hazelnut

## Background

Ambrosia beetles (Coleoptera: Curculionidae), having approximately 3400 species in the sub-families Scolytinae and Platypodinae, are among the major pests which threaten many fruit and forest trees (Hulcr and Dunn 2011). These beetles make galleries through sapwood (xylem) of host trees and cultivate symbiotic fungi such as *Ambrosiella* spp. and *Raffaelea* spp. in the galleries for their food (Harrington 2005). The symbiotic fungi which have co-evolved with ambrosia beetles provide nutrition required for the development of larvae and adults (Norris 1979). The

fungi are carried by females in a specialized structure known as mycangium or mycangia (Six 2003). Moreover, ambrosia beetles often inoculate secondary pathogenic fungi such as *Fusarium* spp. (Kessler 1974) or bacteria (Hall et al. 1982) during entry into trees (Oliver and Mannion 2001). Consequently, these beetles could harm the trees by carrying plant diseases, tunneling the trees, and farming symbiotic fungi. Among bark and ambrosia beetles, *Anisandrus dispar* Fabricius and *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae) are the most prevalent pest species all over the world (Oliver and Mannion 2001; Rabaglia et al. 2006; Ranger et al. 2016; Ak 2016). These pests are polyphagous and damage many perennial plants, including hazelnut (Bucini et al. 2005 and Ak et al. 2011).

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*A. dispar*, *X. germanus*, and *Xyleborinus saxesenii* Ratzeburg are among the significant pests of hazelnut in Turkey (Ak 2016; Tuncer et al. 2017). These beetles cause crop losses by draining hazelnut branches and trees, especially in orchards at coastline of the Black Sea region in the country, which has high groundwater level (Saruhan and Akyol 2012).

Ambrosia beetles are difficult to control as their majority of life is spent under the bark of host trees (Reding et al. 2010). Therefore, insecticides can be ineffective against these beetles unless applied repeatedly or application timing coincides with flight time of the beetles (Oliver and Mannion 2001). Keeping in view these facts, effective and eco-friendly alternative control methods are inevitable in the country. Considering environmental conditions, use of entomopathogenic fungi (EPF) against *A. dispar* and *X. germanus* could be an alternative pest management approach in hazelnut orchards. The Black Sea region receives frequent rainfall and has high humidity and low temperatures per year, and these environmental conditions are ideal for the development of entomopathogenic fungi (Erper et al. 2016).

EPF such as *Beauveria bassiana* (Bals.) Vuill., *Lecanicillium* spp., *Metarhizium anisopliae* (Metch) Sorok, and *Isaria fumosorosea* Wize are used to control several pests around the world (Zimmermann 2007a, 2007b, 2008; Gurulingappa et al. 2011). Among those, *I. fumosorosea* was known as *Paecilomyces fumosoroseus* since 30 years, and then transferred to the genus *Isaria* (Zimmermann 2008).

EPF are important control agents of ambrosia beetles, as they may affect larvae in the galleries as well as adults outside the host trees (Castrillo et al. 2013). Some EPF have been found associated with ambrosia and bark beetles (Popa et al. 2011). Several studies have indicated that *B. bassiana*, *M. anisopliae*, and *I. fumosorosea* were effective against ambrosia beetles such as *Trypodendron lineatum* Olivier, *X. germanus*, *Xylosandrus crassiusculus* (Motschulsky), and *Xyloborus glabratus* Eichhoff (Castrillo et al. 2011, 2013 and 2015). Castrillo et al. (2011) found that commercial strains of *B. bassiana* and *M. anisopliae* induced high mortality in females of *X. germanus*, as 100% of their offspring larvae was infected.

The present study shed a light on the efficacy of two conidial concentrations ( $1 \times 10^6$  and  $1 \times 10^8$  conidia ml<sup>-1</sup>) of *I. fumosorosea* TR-78-3 against females of *A. dispar* and *X. germanus*, using two different application methods under laboratory conditions.

## Material and methods

### Insect cultures

Hazelnut orchards in Terme district of Samsun province, Turkey, were surveyed to collect hazelnut branches infested with *A. dispar* and *X. germanus* during March–April 2017. The infested branches were cut into 30-cm-long pieces,

kept in plastic boxes (20 × 25 × 40 cm), and directly transferred to the laboratory (Ondokuz Mayıs University, Agriculture Faculty, Plant Protection Department, Samsun, Turkey). The branches were dissected by pruning scissors. Only females were collected from the galleries and inspected under Leica EZ4 stereomicroscope at × 40–70 magnification to separate healthy adults of *A. dispar* and *X. germanus* for use in bioassays. As in many EPF studies (Castrillo et al. 2015), only females were used in the present study. The males are rare in the population of ambrosia beetles (about 10:1 in favor of female), flightless, and rarely seen outside the gallery (Ranger et al. 2016).

### Fungal isolate

EPF isolate TR-78-3 used in the present study was isolated from the pupae of *Hyphantria cunea* (Lepidoptera: Erebidae) commonly called fall webworm, collected from hazelnut orchards (Samsun, Turkey). Single-spore isolate was obtained by serial dilution (Dhingra and Sinclair 1995) and identified as *I. fumosorosea* by Dr. Richard A. Humber, USDA-ARS Biological Integrated Pest Management Unit. The fungus was prepared and stored at 4 °C on Sabouraud dextrose agar (SDA; Merck Ltd., Darmstadt, Germany) slants and also in cryogenic tubes containing 15% glycerol at – 80 °C. The isolate was deposited in the fungal culture collection of the Mycology Laboratory, Ondokuz Mayıs University, Agriculture Faculty, Plant Protection Department, Samsun, Turkey, and in the USDA-ARS Entomopathogenic Fungal Culture Collection in Ithaca, NY (ARSEF 12173).

### Inoculum of *I. fumosorosea*

EPF, *I. fumosorosea* isolate TR-78-3 was plated on SDA and incubated (Binder KBWF 240; Germany) at 25 °C 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 cheese cloth. The suspensions of conidia were adjusted to  $1 \times 10^6$  and  $1 \times 10^8$  conidia ml<sup>-1</sup>, using a Neubauer hemocytometer under Olympus CX31 compound microscope (Olympus America Inc., Lake Success, NY) (Saruhan et al. 2015).

### Conidial germination assessment

The viability of conidia of the isolate was determined. A conidial suspension was adjusted to  $1 \times 10^4$  conidia ml<sup>-1</sup>, and 0.2 ml was sprayed onto Petri dishes (9 cm diameter), containing potato dextrose agar (PDA; Merck Ltd., Darmstadt, Germany). The Petri dishes were incubated at 25 ± 1 °C. After 24 h of incubation, percentage of germinated conidia was determined, using Olympus CX-31 compound microscope at × 400 magnification. Conidia were regarded as germinated when they produced a germ tube at least half of the conidial length (Saruhan et

al. 2015). The germination ratios of the fungus were determined by examining a minimum of 200 conidia from each of three replicate dishes.

### Bioassays

Females of *A. dispar* and *X. germanus* were surface disinfected with 70% ethanol for 10 s and placed on an autoclaved filter paper for drying (Castrillo et al. 2013). The hazelnut branches (5 cm length and 1 cm diameter), used in the bioassays, were sterilized in autoclavable polyethylene bags (30 × 30 cm) for 1 h at 121 °C on two successive days. The Petri dishes that were lined with the moist sterile filter paper at the bottom were used in all experiments. The bioassays were carried out by using two different methods; the first, two conidial suspensions ( $1 \times 10^6$  and  $1 \times 10^8$  conidia ml<sup>-1</sup>) of the isolate were directly sprayed to females of *A. dispar* and *X. germanus* in the Petri dishes (2 ml per dish), using a Potter spray tower (Burkard, Rickmansworth, Hertz UK), and a sterilized hazelnut branch was placed in each Petri dish, and the second method, same amount of conidial suspensions of the fungus was applied to Petri dishes containing a sterilized hazelnut branch with same method, and then the sterile females were released in the dishes. Control Petri dishes were treated by sterile distilled water (2 ml) containing 0.02% Tween 20. The spray tower was disinfected by 70% ethanol and sterile distilled water after each application. All Petri dishes were loosely covered by Parafilm and incubated at  $25 \pm 1$  °C and  $75 \pm 5\%$  RH, 16:8 h light to dark period in a Binder incubator (Model KBWF 240; Germany). The experiment had five replications (a Petri dish each), by placing five insects in each dish. Different groups of insects were used for 8 successive days, provided independence of the observations on mortality on over time (Robertson et al. 2007). The dead adults were removed daily and immediately surface disinfected by dipping in 1% sodium hypochlorite (NaOCl) for 3 min and in 70% ethanol for 3 min. Then, the dead insects were washed three times in sterile distilled water and placed in a moisture chamber. Mortality rates were confirmed by examination of hyphae

on the cadavers under Leica EZ4 stereomicroscope (Kocaçevik et al. 2016).

### Statistical analysis

Abbott correction was not used since mortality was lower more than 5% in the control. Independent time-mortality data from bioassays were analyzed by Probit analysis program (POLO-PLUS Ver.2.0) to calculate 50% (LT<sub>50</sub>) and 90% (LT<sub>90</sub>) lethal time. In lethal time analysis, Log-Probit analysis calculations were considered. Slopes of regression lines were compared to each other by their standard errors. LT<sub>50</sub> and LT<sub>90</sub> values of two application methods and two conidial suspensions were compared based on overlapping of 95% confidence intervals.

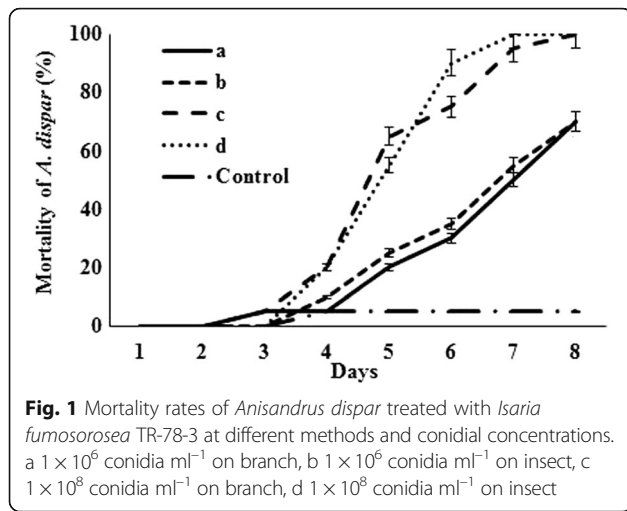
### Results and discussion

Conidia viability of *I. fumosorosea* isolate TR-78-3 was assessed before bioassays, and approximately (100%) germination was obtained. The LT<sub>50</sub> values of  $1 \times 10^6$  conidia ml<sup>-1</sup> for *A. dispar* were 6.92 and 6.66 days for branch and insect application, respectively. Correspondent LT<sub>90</sub> values, for the same concentration, were 11.16 and 10.57 days. The LT<sub>50</sub> values of  $1 \times 10^8$  conidia ml<sup>-1</sup> were 4.76 and 4.78 days, while the LT<sub>90</sub> values were 6.49 and 5.94 days for branch and insect application methods, respectively (Table 1). The isolate tested against *A. dispar* resulted in increasing mortality rate, starting from day 4 in all application methods, with both tested concentrations. The efficacy of  $1 \times 10^8$  conidia ml<sup>-1</sup> on *A. dispar* increased rapidly; by days 6 and 8, the mortality rates attained 75–90 and 100% in both methods, respectively. Nevertheless, the efficacy of  $1 \times 10^6$  conidia ml<sup>-1</sup> of the isolate was lower than that of  $1 \times 10^8$  conidia ml<sup>-1</sup> (Fig. 1). In addition, all applications of *I. fumosorosea* caused (100%) mycosis on dead females of *A. dispar*. Kushiyeve et al. (2017) estimated the LT<sub>50</sub> and LT<sub>90</sub> values of *M. anisopliae* TR-106 isolate at  $1 \times 10^8$  conidia ml<sup>-1</sup> concentration on females of *A. dispar* as 4.03 and 5.39 days at branch application and 3.67 and 4.22 days at insect application, respectively. Additionally, *M. anisopliae* TR-106 isolate applied with

**Table 1** Probit analysis data on mortality time of *Anisandrus dispar* females after applications of two conidial concentrations of *Isaria fumosorosea* isolate TR-78-3 on branch and insect

	$1 \times 10^6$ conidia ml <sup>-1</sup>		$1 \times 10^8$ conidia ml <sup>-1</sup>	
	On branch	On insect	On branch	On insect
LT <sub>50</sub> (95% CI)	6.92 (6.27–7.98) a*	6.66 (6.06–7.55) a	4.76 (4.37–5.14) b	4.78 (4.44–5.09) b
LT <sub>90</sub> (95% CI)	11.16 (9.22–17.12) a	10.57 (8.88–15.36) a	6.49 (5.91–7.60) b	5.94 (5.51–6.76) b
Slope ± SE	10.58 ± 2.21 b	10.96 ± 2.20 b	16.33 ± 2.72 a	23.23 ± 4.25 a
χ <sup>2</sup>	21.07	6.78	13.14	7.92
Df	30	30	30	30
Heterogeneity	0.70	0.23	0.44	0.26

\*Within rows, means followed by same lower case letter do not differ significantly



two methods against the beetle caused 100% mortality and about 95% mycosis after 8 days.

The  $LT_{50}$  values of ( $1 \times 10^6$  conidia  $\text{ml}^{-1}$ ) the isolate applied to *X. germanus* were 7.81 and 7.02 days in branch and insect application methods, respectively. Correspondent  $LT_{90}$  values were 11.03 and 11.46 days. The  $LT_{50}$  and  $LT_{90}$  values of  $1 \times 10^8$  conidia  $\text{ml}^{-1}$  of the isolate were 5.11 and 7.89 days for branch application method, while they were 4.18 and 5.62 days for insect application method (Table 2). The tested isolate caused mortality against *X. germanus* by day 4 in all treatments, except the  $1 \times 10^6$  conidia  $\text{ml}^{-1}$  at the branch application method. High concentration ( $1 \times 10^8$  conidia  $\text{ml}^{-1}$ ), using insect application method caused 40% mortality rate by day 4, reaching 100% by day 7. Likewise in the branch application method, an increasing mortality rate was observed by day 4, raised to 70% by day 6 and 90% by day 8. Conversely, efficacy of the low concentration ( $1 \times 10^6$  conidia  $\text{ml}^{-1}$ ) remained lower than that of the high concentration of the isolate (Fig. 2). Moreover, *I. fumosorosea* TR-78-3 isolate caused 95–100% mycosis on dead females of *X. germanus*. Similarly, Tuncer et al. (2016) reported

that  $LT_{50}$  and  $LT_{90}$  values of  $1 \times 10^8$  conidia  $\text{ml}^{-1}$  of *B. bassiana* TR-217 isolate against female adults of *X. germanus* were 5.96 and 11.79 days for branch method and 6.03 and 10.80 days for direct insect spray method.

As a result, the  $LT_{50}$  and  $LT_{90}$  values in the present study decreased with increasing conidial concentration of the isolate at all applications. However, comparisons of the confidence intervals indicated insignificant differences between  $LT_{90}$  values at both concentrations against females of *X. germanus* in branch application method. In addition, the isolate caused (100%) mortality rate with ( $1 \times 10^8$  conidia  $\text{ml}^{-1}$ ) in both methods by day 8 after inoculation against females of *A. dispar*. Same concentration of the isolate resulted to 90 and 100% mortality rate of *X. germanus* by day 8, in the branch and insect application methods, respectively. Some researchers have reported that the efficacy of EPF increases at high concentrations, which shorten the time required for insect mortality (Kocaçevik et al. 2016). Similarly, Tuncer et al. (2016) and Kushiyeve et al. (2017) found that  $LT_{50}$  and  $LT_{90}$  values of *M. anisopliae* TR-106 and *B. bassiana* TR-217 isolates applied to females of *A. dispar* and *X. germanus* decreased by increasing conidial concentration. The present study also indicated that all applications of *I. fumosorosea* caused 100 and 95–100% mycosis on dead females of *A. dispar* and *X. germanus*, respectively. Nevertheless, Demir et al. (2013) stated that *I. fumosorosea* isolate caused mortality to *Plagioderma versicolora* Laicharting (Chrysomelidae), but did not produce mycosis on dead cadavers.

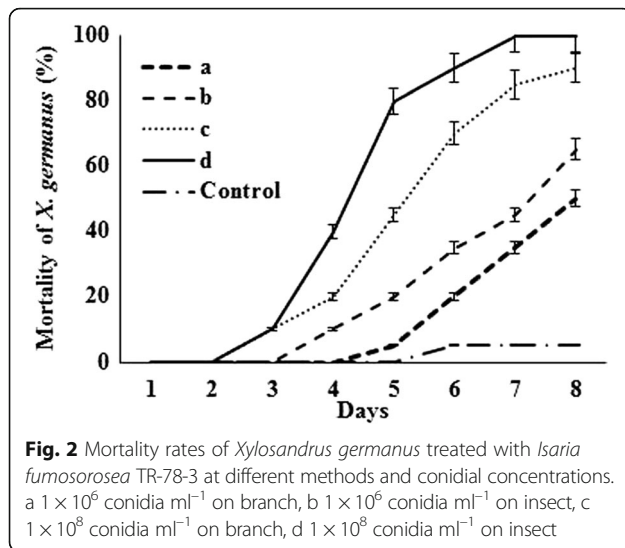
In the present study, significant difference was observed between the two application methods for  $1 \times 10^8$  conidia  $\text{ml}^{-1}$  applied to *X. germanus*; however, there was no difference between the methods for all other applications ( $P > 0.05$ ). Castrillo et al. (2015) reported insignificant differences in the mortality rates of *X. glabratus* females, exposed to *B. bassiana* strain (GHA) and *I. fumosorosea* strains (If 3581 and PFR), by dipping in fungal suspension or placing on treated avocado branches. Tuncer et al. (2016) investigated the efficiency

**Table 2** Probit analysis data on mortality time of *Xylosandrus germanus* females after applications of two conidial concentrations of the *Isaria fumosorosea* isolate TR-78-3 on branch and insect

	$1 \times 10^6$ conidia $\text{ml}^{-1}$		$1 \times 10^8$ conidia $\text{ml}^{-1}$	
	On branch	On insect	On branch	On insect
$LT_{50}$ (95% CI)	7.81(7.16–9.27)a*	7.02(6.34–8.18)a	5.11(4.63–5.60)b	4.18(3.82–4.52)c
$LT_{90}$ (95% CI)	11.03(9.28–17.83)a	11.46(9.38–18.10)a	7.89(6.95–9.85)a	5.62(5.12–6.58)b
Slope $\pm$ SE	14.66 $\pm$ 3.70 ab	10.32 $\pm$ 2.19 b	11.65 $\pm$ 1.93 b	17.12 $\pm$ 2.95 a
$\chi^2$	5.12	11.94	14.92	9.02
Df	30	30	30	29
Heterogeneity	0.17	0.40	0.50	0.31

\*Within rows, means followed by same lower case letter do not differ significantly





on insect and branch treatments of *M. anisopliae* TR-106 and *B. bassiana* TR-217 isolates at  $1 \times 10^6$  and  $1 \times 10^8$  conidia  $\text{ml}^{-1}$  against females of *X. germanus* and found insignificant differences between application methods and  $\text{LT}_{50}$  and  $\text{LT}_{90}$  values. In another study, Castrillo et al. (2013) demonstrated that *B. bassiana* (GHA and Naturalis) and *M. anisopliae* strain (F52) applied to females of *X. crassiusculus* caused 77, 96, and 79% mortality rates, respectively 5 days after treatment. Moreover, they stated that the females exposed to beech stems treated with same entomopathogen strains at  $1 \times 10^7$  conidia  $\text{ml}^{-1}$  had lower survival rates.

EPF can be effective when applied directly on the insect or when the insect contacts the applied surface. Moreover, they provided a good potential control for some pests living in wood of trees, such as ambrosia and bark beetles, as they could also be transported into the gallery by infected individuals (Castrillo et al. 2013). Castrillo et al. (2011) determined that treated females of *X. germanus* with commercial strains of *B. bassiana* (Naturalis and GHA) and *M. anisopliae* (F52) caused significantly higher mortality rates compared to control, and reduced gallery formation and brood production in rearing tubes. They also stated that some treated females had 100% infected broods. Treated male adults transferred fungal inocula to untreated females which reduced their breeding activity by 20% (Prazak 1991). In summary, EPF did not only cause mortality of adult beetles outside in the host trees but also decreased beetles' population through infecting next-generation broods in the galleries.

Sevim et al. (2013) indicated that EPF were more likely to have ecological compatibility with pests due to their geographical locations and habitats. In addition, The Middle and Eastern Black Sea region, which has largest hazelnuts growing area of Turkey, has favorable

environmental conditions to use EPF in biocontrol of *A. dispar* and *X. germanus*, because it is rainy and humid and has low annual temperatures (Erper et al. 2016).

## Conclusions

The present study showed that the EPF, *I. fumosorosea* isolate TR-78-3, seemed to be a promising biological control agent against *A. dispar* and *X. germanus*. However, further studies are necessary to evaluate the efficacy of the isolate on the pests under field conditions.

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## Availability of data and materials

All data are available at the end of the article, and the materials used in this work are of high quality and grade.

## Authors' contributions

IE, RK, CT, IOO, and IS designed the study, supervised the work, and wrote the manuscript with input from all authors. IE, RK, 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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