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# Virulence and horizontal transmission of *Beauveria pseudobassiana* S.A. Rehner & Humber in *Ips sexdentatus* and *Ips typographus* (Coleoptera: Curculionidae)

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**Abstract:** *Ips sexdentatus* (Boerner) and *I. typographus* (Linnaeus) (Coleoptera: Curculionidae) are considered to be important destructive pests of coniferous forests in Europe and Asia. In this study, the efficacy of *Beauveria pseudobassiana* strain ARSEF 9271 isolated from *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae) was evaluated against *I. sexdentatus* and *I. typographus* adults. Insects were treated with different conidial concentrations of the fungus to determine the virulence. A conidial suspension of $1 \times 10^8$ conidia $mL^{-1}$ caused 100% mortality in both *I. sexdentatus* and *I. typographus* within 5 and 7 days, respectively. Additionally, 100% mycosis was obtained in the concentration-response experiments for both insects. $LC_{50}$ values of the fungus were calculated as $3.94 \times 10^4$ conidia $mL^{-1}$ and $1.32 \times 10^4$ conidia $mL^{-1}$ for *I. sexdentatus* and *I. typographus*, respectively. *I. sexdentatus* and *I. typographus* adults were also inoculated with a conidial suspension of $1 \times 10^6$ conidia $mL^{-1}$ at inoculation rates of 0% (control), 25%, 50%, 75%, and 100% to determine the horizontal transmission of the fungus. Mortality values from horizontal transmission experiments were determined as 100% at all rates after 15 days at 20 °C under laboratory conditions. Our results indicate that *B. pseudobassiana* ARSEF 9271 is a promising microbial control agent against the tested *Ips* species and can horizontally spread among a population of both *I. sexdentatus* and *I. typographus*.

**Key words:** *Ips sexdentatus*, *Ips typographus*, *Beauveria pseudobassiana*, virulence, horizontal transmission

## 1. Introduction

Bark beetles (Curculionidae), including *Ips* species, are considered to have increasing importance as pests of coniferous trees and wood products. Among these pests, *Ips sexdentatus* (Boerner) and *Ips typographus* (Linnaeus) (Coleoptera: Curculionidae) are the most serious pests after *Dendroctonus micans* (Kugelann) (Coleoptera: Scolytinae) in Europe and Asia. They are known to be secondary bark beetles because they attack stressed, weakened, or dying trees (Raffa et al., 1993; Gitau et al., 2013). Some biotic and abiotic factors give rise to an enormous increase of both pests in infested spruce forests (Engesser et al., 2002; Wermelinger, 2004). Biotic factors include bark beetle population biology and the type, age, and distribution of tree species. Abiotic factors include climate, geographic location, avalanches, landslides, human-caused disturbances, and weather-related phenomena. A combination of the above factors must often occur before an outbreak develops (Samman and Logan, 2000). Following an outbreak, they can easily assume a primary position using a primary attack strategy and can cause great outbreaks in coniferous forests. Both pests affect a large number of conifer species belonging to the genera *Picea*, *Pinus*, *Abies*, and *Larix*. In addition, *I. sexdentatus* acts as a vector for a blue stain fungus (*Grosmannia clavigera* Robinson-Jeffrey & R.W. Davidson) that also damages conifer trees (Lieutier et al., 1989). While *I. sexdentatus* is a native pest for Turkey, an outbreak of *I. typographus* was first discovered in 1984 in Turkey, and nowadays these pests are widespread throughout Turkey, including the forests of the Black Sea Region (Öymen, 1992).

To date, some strategies have been used to control these pests, such as removing and debarking of infested trees, trap trees, insecticide treatments, phytosanitary measures, and pheromone trapping (Dubbel, 1993; Popa et al., 2011). In addition, several biological control agents, such as *Thanasimus formicarius* (Linnaeus) (Coleoptera: Cleridae) and *Rhizophagus dispar* (Paykull) (Coleoptera: Rhizophagidae), which are predators of these beetles, have been considered to prevent their damage and control the spread of *Ips* species (Yüksel, 1998). Moreover, many natural enemies including entomopathogens have been described and tested against *Ips* spp. (Nierhaus-

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Wunderwald, 1993; Wegensteiner et al., 1996; Weiser et al., 2006; Sevim et al., 2012a). Considering ecological and environmental conditions, the use of entomopathogenic fungi against *Ips* species would be an attractive alternative control approach in coniferous forests. Coniferous forests have heavy rainfall, warm summers, and humid climates (Kauffmann, 2012). Entomopathogenic fungi require moisture for sporulation and germination, and sometimes high humidity for infection. Rain also plays an important role in the transmission of entomopathogenic fungi (Goettel et al., 2005). Another important reason to use entomopathogenic fungi for control of *Ips* spp. is the life cycle of *Ips* species. Most *Ips* species have a polygynous mating system with harems of females (Reid, 1999). Typically, a male establishes a nuptial chamber in a log and produces an aggregation pheromone that attracts both males and females (Symonds et al., 2012). This increases the chance of horizontal transmission of fungal spores among beetles.

*Beauveria bassiana* (Bals) is a well-known entomopathogenic fungus for many insect pests and naturally occurs in *Ips* spp. (Sevim et al., 2012b; Wegensteiner, 1992; Nierhaus-Wunderwald, 1993). It was shown that this fungus has high virulence in bark beetles and *Ips* spp. in previous studies (Wegensteiner, 1992; Kreutz et al., 2004a; Sevim et al., 2010c; Steinwender et al., 2010; Tanyeli et al., 2010; Mudrončeková et al., 2013). Despite all control strategies, *Ips* species have still continued to spread and are still important pests of coniferous trees in Turkey. There has been no record about the efficacy of *B. pseudobassiana* on *I. sexdentatus* and *I. typographus* in the literature to date.

*Beauveria pseudobassiana* ARSEF 9271 is one of 12 isolates isolated from *Dendroctonus micans*, which is the most destructive pests of spruce forests in Turkey (Tanyeli et al., 2010). It was previously characterized in detail using molecular techniques by Rehner et al. (2011) and was identified as *B. pseudobassiana* (Kocacevik et al., 2015). This fungus was molecularly separated from *B. bassiana* by Rehner et al. (2011). The results from the study of Kocacevik et al. (2015) indicated that *B. pseudobassiana* seems to be a promising fungal biocontrol agent against *D. micans*, the major pest of conifers. In this study, we aimed to test this fungus (*B. pseudobassiana* ARSEF 9271) against *I. sexdentatus* and *I. typographus* using concentration-response and horizontal transmission experiments.

## 2. Materials and methods

### 2.1. Fungal isolate

*Beauveria pseudobassiana* ARSEF 9271 was selected for use in this study based on its pathogenic effects against *D. micans* (Kocacevik et al., 2015). The fungal isolate was propagated from a single conidium to obtain a pure culture. To achieve this, $1 \times 10^4$ conidia $mL^{-1}$ was plated on PDAY medium (potato dextrose agar + 1% yeast extract; Sigma, Leeds, UK) and incubated at 25 °C for 1 week. After that, a single colony was transferred to another PDAY medium.

### 2.2. Preparation of conidial suspensions

*Beauveria pseudobassiana* ARSEF 9271 was plated on PDAY agar and incubated at 25 °C for 4 weeks to obtain good sporulation. A conidial suspension of the fungal isolate was prepared by adding 10 mL of sterile 0.01% Tween 80 (AppliChem, Darmstadt, Germany) to the 4-week-old cultures. The obtained suspension was filtered through sterile two layers of muslin into a sterile 50-mL plastic tube (Falcon, Franklin Lakes, NJ, USA) to remove the medium and fungal debris and was then shaken for 5 min using a vortex for homogenization. The final suspension was adjusted to a desired concentration using an improved Neubauer hemocytometer at 400× magnification. The viability of conidia was determined by spreading the conidial suspension onto PDAY and assessing the germination after 24 h of incubation at 25 °C in the dark. Conidia were considered to have germinated if the germ tube was longer than the diameter of the conidium. Cultures with a viability above 95% were used for bioassay experiments.

### 2.3. Concentration-response tests

For virulence assay, *I. sexdentatus* and *I. typographus* adults were collected from naturally infested spruce forests in the vicinity of Trabzon, Turkey, between 2010 and 2012. Insects were put into plastic boxes (20 × 20 cm) containing a small piece of spruce bark. After collection, they were directly brought to the laboratory and kept for approximately 2–3 days at room temperature so that larvae would be acclimated to the laboratory conditions. After that, the healthy adults that did not show any disease symptoms were selected and used for bioassays. To determine the virulence of the fungus, 30 adults were dipped together into conidial suspensions of $1 \times 10^4$, $10^5$, $10^6$, $10^7$, and $10^8$ conidia $mL^{-1}$ for each pest and treatment. Control groups were treated with sterile water with 0.01% Tween 80. The adults were transferred thereafter into plastic boxes (20 × 20 cm) for each concentration after inoculation. A small piece of spruce bark was provided as food for the insects. All test groups were kept in rearing boxes at 20 ± 0.1 °C and 70 ± 5% relative humidity with a photoperiod of 12:12 h (L:D). Insects were monitored daily for 14 days and dead ones were surface-sterilized by dipping into 1% sodium hypochlorite for 3 min, followed by 70% ethanol for 3 min and washing three times in sterile distilled water. After that, they were put into a moisture chamber to stimulate fungal sporulation outside the cadaver. Mortalities of insects were confirmed by microscopic examination of hyphae and spores on the surface of the cadavers. All experiments were repeated three times on different occasions.

### 2.4. Horizontal transmission of *Beauveria pseudobassiana* among *Ips sexdentatus* and *Ips typographus* adults

To investigate the potential of fungal transmission within a population of *I. sexdentatus* and *I. typographus* adults, the suspension of $1 \times 10^6$ conidia $mL^{-1}$ was selected for the experiments since this concentration was determined to be sufficient for infection based on previous studies (Sevim et al. 2010c; Kocacevik et al., 2015). The suspension was prepared as described above. The treatments included a control treated with 0.01% Tween 80 and insects dipped in a $1 \times 10^7$ conidia $mL^{-1}$ spore suspension for 2–3 s with the number of inoculated insects within the cohorts of 36 being 9 (25%), 18 (50%), 27 (75%), and 36 (100%). Insects were then placed into plastic boxes (20 × 20 cm) including small pieces of spruce bark and incubated at 20 ± 0.1 °C and 70 ± 5% relative humidity with a photoperiod of 12:12 h (L:D). Dead insects were removed daily and were immediately surface-sterilized by dipping into 1% sodium hypochlorite for 3 min, followed by 70% ethanol for 3 min and washing three times in sterile distilled water. After that, they were put into a moisture chamber to stimulate fungal sporulation outside the cadaver. Mortalities of insects were confirmed by microscopic examination of hyphae and spores on the surface of the cadavers. All experiments were repeated three times on different occasions.

### 2.5. Data analysis

Mortality values were corrected according to Abbott's formula (Abbott, 1925) and percent mycosis values were calculated based on the mycelial growth outside cadavers. To determine differences among concentrations and treatment rates in horizontal transmission experiments, the data were subjected to ANOVA and subsequently to Tukey's post hoc test at the fifth day. Before performing ANOVA, all data sets were tested for homogeneity of variance using Levene's test and all percentage data were subjected to arcsine transformation. $LC_{50}$ values were calculated using probit analysis on mortalities 5 days after concentration-response tests. Computations for all experiments were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Concentration-response tests

In the concentration-response experiments, the increasing concentration of *B. pseudobassiana* ARSEF 9271 gradually increased the mortality in both *I. sexdentatus* and *I. typographus* adults. All concentrations caused 100% mortality in *I. sexdentatus* adults within different days. However, there was a significant difference among concentrations with respect to mortality of *I. sexdentatus* adults ($F = 222.93$; $df = 5, 12$; $P < 0.05$). The fastest mortality in *I. sexdentatus* was obtained from the concentration of $1 \times 10^8$ conidia $mL^{-1}$ within 5 days. The suspension of $1 \times 10^4$ conidia $mL^{-1}$ also caused 100% mortality in *I. sexdentatus* within 9 days (Figure 1). Mycosis rates of the infected *I. sexdentatus* adults in concentration-response tests reached 100%, except for $1 \times 10^4$ and $1 \times 10^5$ conidia $mL^{-1}$ (93 and 96%, respectively; Figure 2).

For *I. typographus*, all concentrations also caused 100% mortality within different days and there was a significant difference among concentrations ($F = 29.94$; $df = 5, 12$; $P < 0.05$). The fastest mortality in *I. typographus* was obtained from the concentration of $1 \times 10^8$ conidia $mL^{-1}$ within 7 days. The suspension of $1 \times 10^4$ conidia $mL^{-1}$ also caused 100% mortality in *I. typographus* within 12 days (Figure 3). Mycosis rates of the infected *I. typographus* adults in concentration-response tests reached 100%, except for $1 \times 10^4$, $1 \times 10^5$, and $1 \times 10^6$ conidia $mL^{-1}$ (86%, 93%, and 96%, respectively; Figure 4).

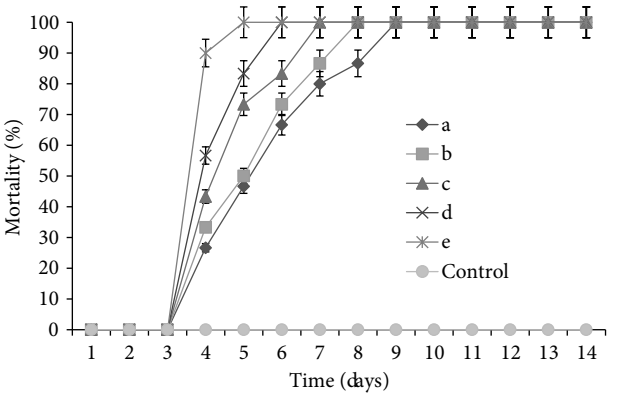


**Figure 1.** The cumulative mortality of *I. sexdentatus* adults caused by *Beauveria pseudobassiana* ARSEF 9271 in different concentrations of conidia at 20 °C within 14 days (N = 30). a: $1 \times 10^4$ conidia $mL^{-1}$, b: $1 \times 10^5$ conidia $mL^{-1}$, c: $1 \times 10^6$ conidia $mL^{-1}$, d: $1 \times 10^7$ conidia $mL^{-1}$, e: $1 \times 10^8$ conidia $mL^{-1}$. Bars show standard deviations.

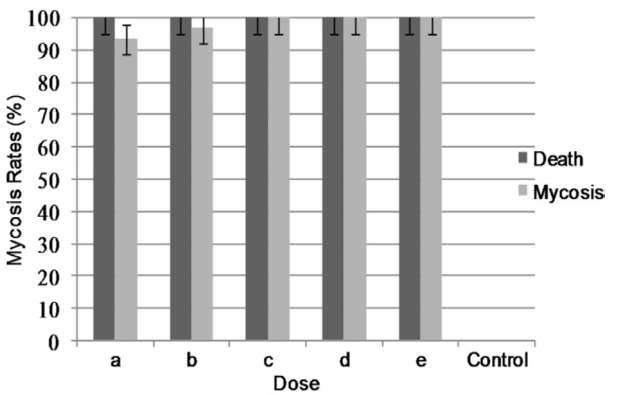


**Figure 2.** Mycosis rates of death of *I. sexdentatus* in dose-response tests after mycosis period. a: $1 \times 10^4$ conidia $mL^{-1}$, b: $1 \times 10^5$ conidia $mL^{-1}$, c: $1 \times 10^6$ conidia $mL^{-1}$, d: $1 \times 10^7$ conidia $mL^{-1}$, e: $1 \times 10^8$ conidia $mL^{-1}$. Bars show standard deviations.

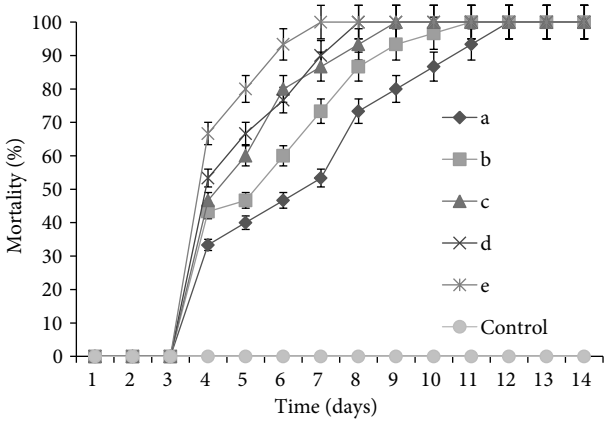


**Figure 3.** The cumulative mortality of *I. typographus* adults caused by *Beauveria pseudobassiana* ARSEF 9271 in different concentrations of conidia at 20 °C within 14 days (N = 30). a: $1 \times 10^4$ conidia $mL^{-1}$, b: $1 \times 10^5$ conidia $mL^{-1}$, c: $1 \times 10^6$ conidia $mL^{-1}$, d: $1 \times 10^7$ conidia $mL^{-1}$, e: $1 \times 10^8$ conidia $mL^{-1}$. Bars show standard deviations.

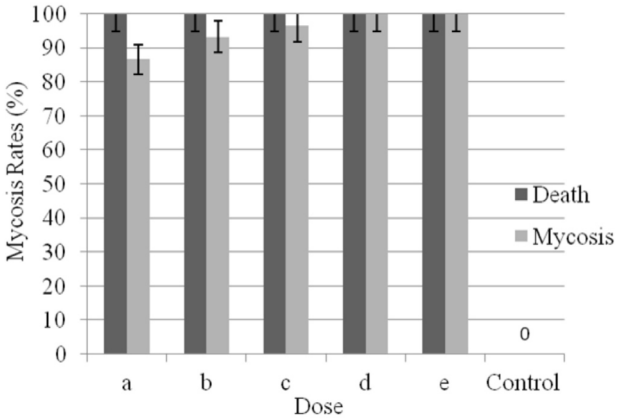


**Figure 4.** Mycosis rates of death of *I. typographus* in dose-response tests after mycosis period. a: $1 \times 10^4$ conidia $mL^{-1}$, b: $1 \times 10^5$ conidia $mL^{-1}$, c: $1 \times 10^6$ conidia $mL^{-1}$, d: $1 \times 10^7$ conidia $mL^{-1}$, e: $1 \times 10^8$ conidia $mL^{-1}$. Bars show standard deviations.

The $LC_{50}$ values of the fungus were calculated as $3.94 \times 10^4$ and $1.32 \times 10^5$ conidia $mL^{-1}$ in *I. sexdentatus* and *I. typographus*, respectively (Table).

### 3.2. Horizontal transmission of *Beauveria pseudobassiana* ARSEF 9271

In horizontal transmission experiments, increasing the number of infected insects in the population gradually increased the mortality and the horizontal transmission of spores among both *I. sexdentatus* and *I. typographus* adults and the mortalities of noninfected adults within each treatment. There was a significant difference among mortalities of treatments with different inoculated individual rates of *I. sexdentatus* adults with respect to the fifth day of the experiment ($F = 71.16$; df = 4, 10; $P < 0.05$) (Figure 5). There were no differences between mortalities on the 10th and 15th days of the experiment, while 100% mycosis was obtained from all treatments, except for the rate of 25%, for which mycosis was 89%.

For *I. typographus*, there was a significant difference between adult mortalities with respect to the 5th and 10th days of the experiment ($F = 33.84$; df = 4, 10; $P < 0.05$ and $F = 134.28$; df = 4, 10; $P < 0.05$, respectively) (Figure 6). However, there were no differences between mortalities on the 10th day of the experiment, and 100% mycosis was obtained from all treatments.

## 4. Discussion

Although some chemical, mechanical, and neoclassical biological control methods such as removal and debarking of infested trees, use of trap trees, insecticide treatments, phytosanitary measures, and pheromone trapping have been used against *Ips sexdentatus* and *I. typographus* in coniferous forests, the damage and spreading of these species could not yet be prevented. They are thus still serious pests of coniferous forests in Turkey and other countries (Dubbel et al., 1993; Popa et al., 2011). Therefore, there is a need to find new control methods against these pests.

The growing concern about ill effects of chemical insecticides has necessitated a change in strategies to manage insect pests in an ecologically acceptable manner. These concerns prompted scientists to

**Table**. Probit analysis parameters from the multiple concentration bioassays performed with the *Beauveria pseudobassiana* ARSEF 9271 against *Ips sexdentatus* and *I. typographus* adults after 5 days of concentration-response tests.

| Target pest | Intercept | Slope ± SE[a] | $LC_{50}$ (95% fiducial limits) | $\chi^{2b}$ | df |
|---|---|---|---|---|---|
| *I. sexdentatus* | –2.221 | 0.483 ± 0.94 | $3.94 \times 10^4$ $mL^{-1}$ ($5.9 \times 10^3$ to $1.26 \times 10^5$) | 3.71 | 3 |
| *I. typographus* | –1.373 | 0.268 ± 0.077 | $1.32 \times 10^5$ $mL^{-1}$ ($4.3 \times 10^3$ to $7.8 \times 10^5$) | 0.26 | 3 |

[a]SE: standard error.
[b]Pearson chi-square goodness-of-fit test on the probit model ($\alpha = 0.05$).

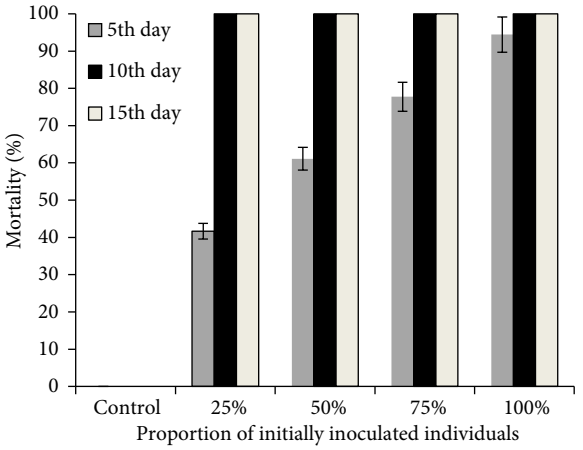


**Figure 5.** Mortality of *I. sexdentatus* adults after conidia transmission of *B. pseudobassiana* ARSEF 9271 from treated to untreated beetles at various rates using $1 \times 10^6$ conidia $mL^{-1}$ at 20 °C within 10 days (N = 30). Bars show standard deviations.

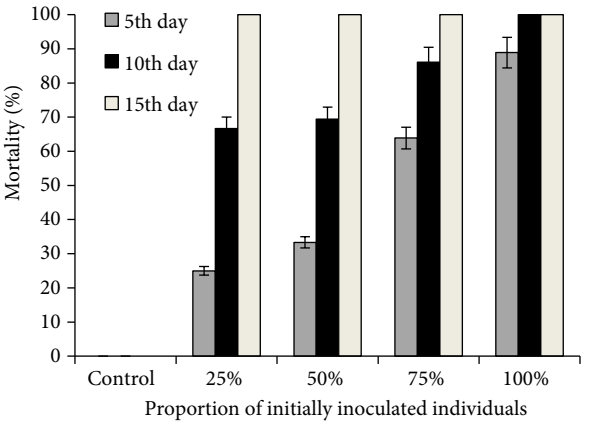


**Figure 6.** Mortality of *I. typographus* adults after conidia transmission of *B. pseudobassiana* ARSEF 9271 from treated to untreated beetles at various rates using $1 \times 10^6$ conidia $mL^{-1}$ at 20 °C within 10 days (N = 30). Bars show standard deviations.

look for biopesticides such as microbial pesticides. Entomopathogenic microorganisms are an attractive, effective, and environmentally safe alternative to control many pest species in both agriculture and forestry because they are safe for animals, plants, and the environment (Lacey, 2012; Vega and Kaya, 2012). To date, many pathogens have been isolated from insects and their insecticidal effects have been determined in agricultural and forest pests (Demir et al., 2002, 2012; Ince et al., 2007; Sevim et al., 2010a, 2010b, 2010d, 2013; Danismazoglu et al., 2012; Demirci et al., 2013). Among these, fungal entomopathogens have been improved as biopesticides, and they have been used effectively in pest management systems for a long time (Barlett and Jaronski, 1988; Flexner and Belnavis, 2000; Lacey and Kaya 2007; Zimmermann, 2007; Vega and Kaya, 2012).

Considering the environmental conditions of the Black Sea Region of Turkey, fungal entomopathogens could be the most significant microbial control agents against *I. sexdentatus* and *I. typographus* because of the humid climate, lower annual temperatures, and high rainfall in this region (Sevim et al., 2010c, 2012b; Tanyeli et al., 2010; Kocacevik et al., 2015). In this sense, in order to find an effective biocontrol agent against bark beetles, we have begun some studies about controlling *D. micans* using possible fungal entomopathogens isolated from soil and insect samples (Sevim et al., 2010c; Tanyeli et al., 2010; Kocacevik et al., 2015). One of these isolates, *B. pseudobassiana* ARSEF 9271, had significant mortality against both larvae and adults of *D. micans* (Tanyeli et al., 2010; Kocacevik et al., 2015). Therefore, in this study, this fungus isolate was selected for use against *I. sexdentatus* and *I. typographus* since they share the same habitat with *D. micans*.

Previously, some researchers showed that *Beauveria* spp. are natural fungal pathogens of many different forest insects including *D. micans*, *I. sexdentatus*, and *I. typographus* (Goettel et al., 1990; Kreutz et al., 2004a; Batta, 2007; Sevim et al., 2010c; Steinwender et al., 2010; Tanyeli et al. 2010). Kreutz et al. (2004a) tested *B. bassiana* against *I. typographus* under laboratory conditions. Among the tested *B. bassiana* isolates, they found that some isolates (138 and Z) caused 100% mortality within 7 days. Draganova et al. (2007) tested three *B. bassiana* strains (two of them from lepidopteran larvae and one from *I. sexdentatus*) against *I. sexdentatus*, and they showed that the highest mortality rate (96%) was obtained with the strain that was isolated from *I. sexdentatus*. Mudrončeková et al. (2013) also showed that *B. bassiana* caused 99% mortality against *I. typographus* using a conidial suspension of $1 \times 10^8$ conidia $mL^{-1}$ within 14 days. Finally, Kocacevik et al. (2015) tested *B. pseudobassiana* ARSEF 9271 against *D. micans* and they found that a conidial suspension ($1 \times 10^8$ conidia $mL^{-1}$) of this fungus caused 100% mortality in both larvae and adults of *D. micans* within 5 and 6 days, respectively. They also found that this fungus could horizontally spread within *D. micans* populations. In our study, we also obtained excellent results from *B. pseudobassiana* ARSEF 9271 against *I. sexdentatus* with 100% mortality using even the lowest concentration ($1 \times 10^4$ $mL^{-1}$) within 9 days.

In horizontal transmission experiments, we showed that the lowest treatment rate (25%) also caused 100% mortality within 10 days. Horizontal transmission of *B. bassiana* is a phenomenon occurring in several orders of insects (Kreutz et al., 2004b; Klinger et al., 2006; Lopes et al., 2011). Some researchers investigated the transmission of this fungus between treated and untreated bark beetles (*I. typographus*

and *D. micans*) and showed the effective horizontal transmission of the fungus among insect populations (Kreutz et al., 2004b; Kocacevik et al., 2015). Kreutz et al. (2004b) conducted a study related to the horizontal transmission of *B. bassiana* among *I. typographus* adults in both the laboratory and the field. In the laboratory experiment, they showed that conidia transfer between treated and untreated adults resulted in 96% mortality. In a second laboratory experiment, the efficacy of *B. bassiana* was investigated after transmission from contaminated to healthy beetles within 5 days. Two weeks later, the mortality of treated and untreated beetles was 99%, while the mycosis rate was 79%. In the field experiments, significant reductions were observed in the length of maternal galleries, the number of larvae and pupae, and the number of bore holes. Kocacevik et al. (2015) also showed that mortality values of horizontal transmission experiments between larvae and adults of *D. micans* that were contaminated with $1 \times 10^6$ conidia $mL^{-1}$ spore suspension at the rates of 25%, 50%, 75%, and 100% were determined as 100% within 15 days at 20 °C under laboratory conditions. In the current study, we also observed a horizontal transmission among adults of *I. sexdentatus* and *I. typographus* populations after application of a conidial suspension of $1 \times 10^6$ $mL^{-1}$ at the rates of 0% (control), 25%, 50%, 75%, and 100%. Mortality values from horizontal transmission experiments were determined as 100% within 10 days at 20 °C under laboratory conditions. All these studies suggest that *B. pseudobassiana* ARSEF 9271 can horizontally spread among bark beetles of spruce trees.

Consequently, the results of this study indicate that *B. pseudobassiana* ARSEF 9271 seems to be a promising and environmentally friendly alternative against *I. sexdentatus* and *I. typographus*. Additional research is needed to determine the effectiveness of the isolate in the field. Moreover, application methods and long-term effects of this fungus should also be investigated.

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