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ORIGINAL CONTRIBUTION

Molecular characterization, virulence and horizontal transmission of *Beauveria pseudobassiana* from *Dendroctonus micans* (Kug.) (Coleoptera: Curculionidae)

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Abstract

The great spruce bark beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae), has been a potential threat for Turkey and the entire Eurasian spruce forests for many years. Control strategies which have been applied so far are still insufficient to prevent its damage. A previous study has shown that a *Beauveria* isolate (ARSEF 9271) proved to be an efficient microbial control agent against the great spruce bark beetle. In this study, this isolate was identified as *B. pseudobassiana* based on the partial sequence of EF1-α and ITS sequence. A conidial suspension (1×10^8/ml) of this fungus caused 100% mortality on both larvae and adults of *D. micans* within 5 and 6 days, respectively. Also, it caused 100% mycosis value on both larvae and adults. Mortality values of horizontal transmission experiments between larvae and adults which were contaminated with 1×10^6/ml spore suspension at 25%, 50%, 75% and 100% rates were determined as 100% after 15 days at 20°C under the laboratory conditions. We also determined the decrease of the damage in spruce wood block (15×25 cm) when the contamination rate of the larvae was increased. Our results indicate that *B. pseudobassiana* ARSEF 9271 seems to be a very promising biocontrol agent against *D. micans*.

Introduction

The great spruce bark beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae), is a main pest of spruce (*Picea* spp.) and some other conifers, from eastern Siberia to the west of Europe including Turkey (Fielding and Evans 1997). This pest breeds under bark and causes destruction of the cambium and debilitation of trees. Finally, it can cause tree death in extreme cases. Control strategies utilized so far for controlling of this pest are still insufficient to prevent its damage. Biological control of insect pests with entomopathogenic fungi is an attractive alternative to the usage of conventional pesticides, as they are generally safer for plants, animals and the environment (Khetan 2001; Uygun et al. 2010). Entomopathogenic fungi such as *Beauveria* spp., *Metarhizium* spp. and *Isaria* spp. have shown the great potential for the management of various insect pests, and most of these fungi are associated with insects in diverse habitats (Askary and Brodeur 1999; Cuthbertson and Walters 2005; Sevim et al. 2010a,b; Saito et al. 2012). *Beauveria* is a cosmopolitan anamorphic genus of arthropod pathogens that includes argonomically significant species. One of the species, *B. bassiana*, is used widely as mycoinsecticides for microbial control of several agricultural pests (Goettel et al. 2005). In a previous study, Tanyeli et al. (2010) conducted a study related to isolation of entomopathogenic fungi from *D. micans*. The results of that study indicated that

B. bassiana ARSEFF 9271 seemed to have promising potential for the control of *D. micans*. Therefore, in this study, we characterized this fungus in detail and tested it against the larvae and adults of *D. micans* using the concentration response and horizontal transmission experiments.

Materials and Methods

Collection of insect samples

Dendroctonus micans larvae and adults were collected from different localities of the vicinity of Trabzon, Turkey between 2010 and 2012. Larvae and adults, collected from infested *Picea orientalis* trees by opining galleries under barks with an axe, were put into plastic boxes (20 mm) including a small piece of spruce bark and brought to the laboratory. They were waited for approximately 2–3 days at room temperature, so that larvae were acclimated to the laboratory conditions. Insects were fed with spruce bark and checked daily with regard to any fungal infection. The healthy last instar larvae and adults were randomly selected and used for bioassays.

Fungal isolate

Beauveria pseudobassiana ARSEF 9271 (formerly characterized as *B. bassiana* by Tanyeli et al. (2010)) was selected for use in this study based on its pathogenic effect on *D. micans* (Tanyeli et al. 2010). The fungal isolate was propagated from single conidia to obtain pure culture. To achieve this, 100 μl of conidial suspensions (1×10^4 conidia per mililitre) was plated on PDAY agar (Potato dextrose agar + 1% Yeast extract) (Sigma, Leeds, UK) and incubated at 25°C for 1 week. Following incubation, a single colony was transferred to another PDAY medium. Pure culture of the isolate was plated on PDAY agar and incubated at 25°C for 1–2 weeks in darkness for further sporulation.

DNA extraction

To extract genomic DNA from *B. pseudobassiana* ARSEF 9271, 100 μl of conidial suspension (1×10^4 conidia per mililitre) of the isolate was plated on PDAY and incubated at 25°C for 1–2 weeks to select colonies derived from single conidia. After the incubation period, mycelium growing from a single colony was transferred on another PDAY plate and incubated at 25°C for 1–2 weeks. The resultant mycelium was inoculated into 100 ml flasks containing 50 ml PDB (Potato dextrose broth) (Difco Laboratories, Detroit, MI). Culture was shaken at 230 rpm on a rotary shaker (GFL 3031, Burgwedel, Germany) at 25°C for 1 week under 12 : 12 photoperiod. Mycelial samples were collected on filter paper, and then all mycelia samples were frozen in liquid nitrogen before being crushed with a mortar and pestle. Approximately, 50 mg of crushed mycelium was used for DNA extraction. Remaining mycelium was stored at −20°C. DNA extraction was carried out using the DNeasy Plant mini Kit (Qiagen, Venlo, the Netherlands) and Nucleospin Plant Kit (Clontech, Otsu, Shiga, Japan) according to manufacturers' recommendations. Isolated DNA was stored at −20°C until use.

Gene sequencing

The sequence of ITS1-5.8S-ITS2 gene region of the fungal isolate was amplified using primer pairs of ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) as forward primer and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as reverse primer (White et al. 1990) in a 50 μl reaction volume containing the following ingredients: 5 μl 10× *Taq* DNA polymerase reaction buffer, 200 μM of each dNTPs, 50 pmol of each opposing amplification primers, 2.5 unit *Taq* DNA polymerase (Fermentase, Waltham, MA) and 50 ng of genomic DNA. PCR condition was as follows: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 55 s and 72°C for 2 min; and the final extension at 72°C for 10 min.

In addition to ITS gene region, the partial sequence of elongation factor1-α (*EF1-α*) (1.150 bp) was determined for the further characterization of *B. bassiana* ARSEF 9271 according to the study of Rehner et al. (2011) using primer pairs of EF1T (5′-TGGGTAAGGARGACAAGAC-3′) as forward and 1567R (5′-CHGTRCCRATACCACCSATCTT-3′) as reverse. Reaction mixtures were prepared in 50 μl reaction volume containing the following ingredients: 10 μl 5× *Phusion* HF reaction buffer, 200 μM of each dNTPs, 50 pmol of each opposing amplification primers, 1 unit *Phusion*-DNA polymerase and 50 ng of genomic DNA. PCR conditions were as follows: initial denaturation at 98°C for 30 s; 35 cycles of 98°C for 30 s, 55°C for 30 s and 72°C for 30 s; and the final extension at 72°C for 10 min. PCR products was separated on 1.0% agarose gels, stained with ethidium bromide and viewed under UV light. After checking PCR products, they were purified using QIAquick PCR purification kit (Qiagen) and then sent to Macrogen (Amsterdam, the Netherlands) for sequencing. Obtained sequences were subjected to BLAST searches using the NCBI GenBank database to compare with other species

(Altschul et al. 1990; Benson et al. 2012). Finally, the sequences were used to construct phylogenetic tree to verify isolate identification.

Preparation of conidial suspensions

Conidial suspensions of the fungal isolate ARSEF 9271 were prepared by adding 10 ml of sterile 0.01% Tween 80 (Applichem, Darmstadt, Germany) into the 4-week-old cultures. The conidial suspensions were filtered through two layers of muslin into sterile 50 ml tube (Falcon, Franklin Lakes, NJ) and then vortexed for 5 min to ensure homogeneous mixing. The final suspensions were adjusted to a concentration of 1×10^6 conidia per mililitre after conidial counts using an improved Neubauer haemocytometer at 400× magnification. The viability of conidia was determined by inoculating them onto PDAY and incubated at 25°C for 24 h in the dark. The isolate with a viability of above 95% was used for bioassay experiments.

Concentration–mortality response test

The concentration–mortality response test was conducted with *B. pseudobassiana* ARSEF 9271 based on its significant pathogenic effects on larvae and adults of *D. micans* according to the screening test by Tanyeli et al. (2010). To determine the virulence of the isolate on this pest, ten larvae and adults per replicate were treated with the conidial suspensions of 10^4, 10^5, 10^6, 10^7 and 10^8 conidia per mililitre by dipping them into the different spore concentrations for 2–3 s, separately. Control groups were treated with sterile 0.01% Tween 80. While the larvae were put between spruce barks including a square shaped hole in one of them, the adults were put into plastic boxes (20 mm) after inoculation of spore suspension. A small piece of spruce bark was provided as food for adults. All larval and adult test groups were kept in rearing boxes at 20°C under 12 : 12 photoperiod. Dead insects were monitored daily for 10 days. Mortality data were corrected using Abbott's formula (Abbott 1925), and LC_{50} values were calculated by probit analysis. All experiments were repeated three times on different days.

Horizontal transmission of *B. pseudobassiana* ARSEF 9271

To investigate the potential of fungal transmission between treated and untreated *D. micans* larvae and adults, 12 larvae and adults of *D. micans* per replicate were contaminated at the rate of 0 (control), 25%, 50%, 75% and 100% with 1×10^6/ml conidial suspension by dipping them into the spore suspensions for 2–3 s. Conidia production and preparation of suspensions were performed as described above. After that, larvae were placed between spruce barks, and adults were placed in the plastic boxes including a small spruce bark as in the concentration–mortality response test and incubated at 20°C under the laboratory conditions. Dead insects were monitored daily for 15 days. Mortality data were corrected using Abbott's formula (Abbott 1925). Experiments were repeated three times on different days.

Wood block experiments

This test was conducted to determine the efficiency of fungal transmission between treated and untreated *D. micans* larvae at wood blocks and to show the effect of fungal infections on damage of *D. micans* larvae at spruce wood blocks. Thirty six larvae per replicate were contaminated with 1×10^6/ml conidia suspension by dipping for 2–3 s at the rate of 0 (control), 25%, 50%, 75% and 100%. Then, they were put on spruce wood blocks (15 × 25 cm) including a rectangle shaped hole (1–1.5 × 1–1.5 × 15–20 cm) in each of them. Wood blocks were surrounded with tulle to prevent insect escape. After 2 days, entering sites of larvae into wood blocks and all other holes were filled with cotton and they were covered with wax to prevent insect escape. All wood blocks were incubated at 20°C under the laboratory conditions for 30 days. At the end of this period, wood blocks were opened with an axe and live and death larvae were counted. Mortality values were corrected using Abbott's formula (Abbott 1925). Furthermore, the damage of larvae infected with different rates in wood blocks was also measured. Experiments were repeated three times on different days.

Data analysis

DNA sequences of the isolate ARSEF 9271 were compound, edited and aligned with BioEdit (Hall 1990). Cluster analyses of the sequences were performed using BioEdit (version 7.09) with Clustal W (Hall 1990; Thompson et al. 1994). Genomic analyses using the minimum evolution were performed using the Kimura two-parameter model with MEGA 5.2 software (Kimura 1980; Tamura et al. 2011). The reliability of the dendrogram was tested by bootstrap analysis with 1,000 replicates using MEGA 5.2 (Tamura et al. 2011).

Mortality values were corrected according to Abbott's formula (Abbott 1925), and per cent mycosis values were calculated based on the mycelia growth outside cadaver. The effect of the different concentrations of *B. pseudobassiana* ARSEF 9271 spores on larval and adult mortalities of *D. micans* was analysed using Kaplan–Meier survival analysis in GraphPad Prism 5.04 using a Log-rank test. Difference between adult and larval horizontal transmissions, and difference between adult and larval mortality were assessed with chi-square test. In the wood block experiment, to determine difference among different contamination rates (25%, 50%, 75% and 100%), the data were subjected to ANOVA (analysis of variance) and subsequently to LSD multiple comparison test. Before performing the ANOVA, all data set were tested for homogeneity of variance using Levene's statistic. Finally, LC_{50} values were calculated with probit analysis using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA).

Nucleotide sequence accession numbers

The accession number of the sequences of ITS and EF1-α is KM875708 and KM875709, respectively.

Results

Phylogenetic analysis

The isolate ARSEF 9271 which was previously identified as *B. bassiana* by Tanyeli et al. (2010) was further characterized using ITS and *EF1-α* gene sequences. These sequences were used to construct a phylogenetic tree and to compare the isolate ARSEF 9271 with representative isolates from the study of Rehner et al. (2011). Based on these analyses, the isolate ARSEF 9271 was identified as *B. pseudobassiana* (figs 1 and 2).

Concentration–response test

In the concentration–response experiments, the increase in the spore concentration of *B. pseudobassiana* ARSEF 9271 gradually increased the mortality of both larvae and adults. All concentrations caused 100% mortality on the larvae and adults of *D. micans,* and it was determined that there was no significant difference between larvae and adults with respect to the fungal infection (fig. 3) ($P > 0.05$). Differences were calculated between the survival curves for the different doses using Kaplan–Meier analysis, and it was found that there was a significant difference

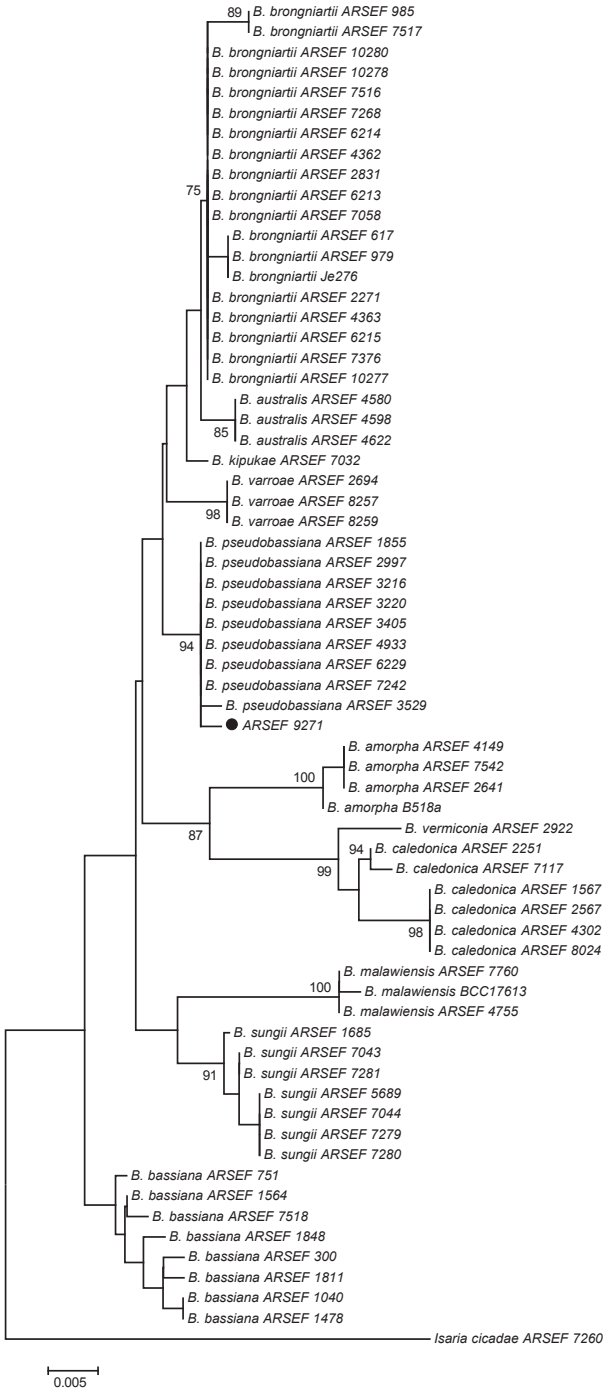

Fig. 1 The minimum evolution tree of the isolate ARSEF 9271 and the representative strains from the study of Rehner et al. (2011) based on ITS sequence. The approximately 500-bp sequence of the ITS1-5.8S-ITS2 gene region was used to construct the dendrogram. Bootstrap values based on 1000 replicates were indicated above nodes. Bootstrap values C ≥ 70 are labelled. *B. pseudobassiana* ARSEF 9271 was indicated with black circle. The scale on the bottom of the dendrogram indicates the degree of dissimilarity.

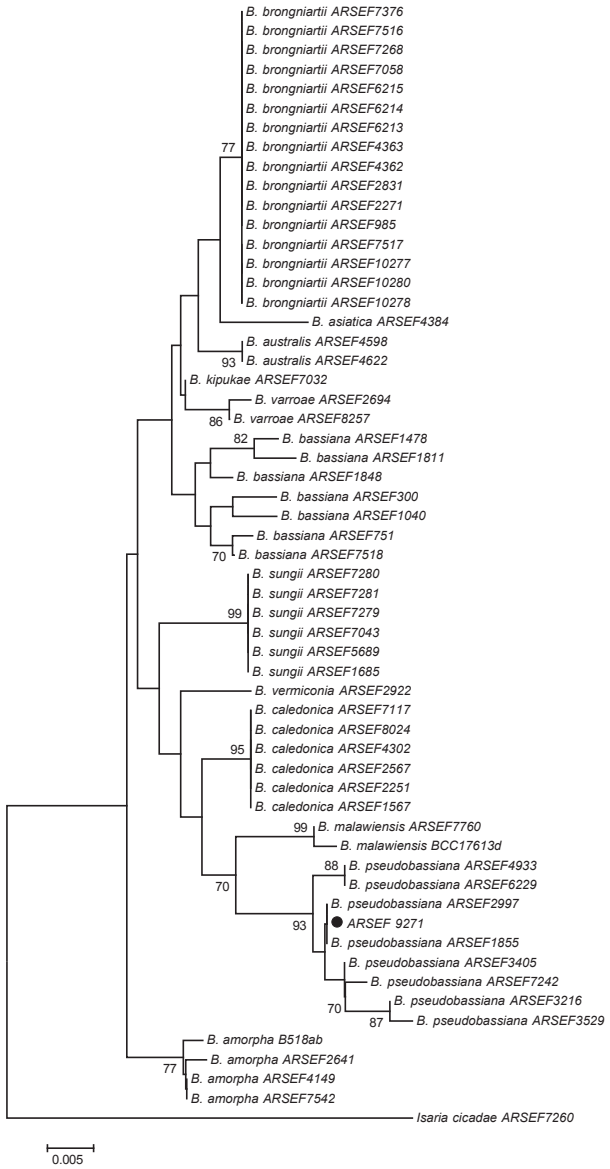

Fig. 2 The minimum evolution tree of the isolate ARSEF 9271 and the representative strains from the study of Rehner et al. (2011) based on the partial sequence of *EF1-α*. The approximately 1150-bp sequence of the *EF1-α* was used to construct the dendrogram. Bootstrap values based on 1000 replicates were indicated above nodes. Bootstrap values C ≥ 70 are labelled. *B. pseudobassiana* ARSEF 9271 was indicated with black circle. The scale on the bottom of the dendrogram indicates the degree of dissimilarity.

between all doses for both larvae and adults ($P < 0.05$). The fastest mortality on the larvae and adults was obtained from 1×10^8/ml conidial concentration ($P < 0.05$) (fig. 3). Additionally, the LC_{50} of the fungus was calculated as 2.79×10^5 conidia per mililitre on larvae and as 1.36×10^4/ml conidia on adults based on probit analysis (table 1).

Horizontal transmission of *Beauveria pseudobassiana* ARSEF 9271

In horizontal transmission experiments, the increase in the number of contaminated insect in population gradually increased the horizontal transmission of spores on both larvae and adults, and mortalities of non-contaminated insects. It was observed that there is no significant difference between larvae and adults with respect to horizontal transmission ($P > 0.05$) (fig. 4). Mortality values of larvae and adults which were contaminated at the rates of 25%, 50%, 75% and 100% with 1×10^6/ml conidial suspension of *B. pseudobassiana* were determined as 100% after 15 days at 20°C under the laboratory conditions (fig. 4).

Wood block experiment

In the wood block experiment, the increase in the number of contaminated larvae gradually increased also the horizontal transmission of spores among larvae and mortalities of non-contaminated larvae in the test population (fig. 5). There is a significant difference between contamination rates of larvae with respect mortality and mycosis ($F = 69.74$, d.f. = 3, $P < 0.05$). During the experiment, some escape holes were observed on the woods which included contaminated larvae at rate of 25%, 50% and control groups. The mortality and mycoses values of larvae were 100% in the wood blocks in which all larvae were infected. At the contamination rates of 75%, only three larvae survived and turned into adult. The rest of the larvae died and got mycosis, resulting in 97% mortality. At the contamination rates of 50%, while one pupae and 24 adults were determinated in woods, the mortality was calculated as 70%. At the contamination rates of 25%, 25 living adults and 73 died larvae were determined and the mortality was determined as 62%. In the control groups, while none of the larvae died and got mycosis, it was determined that all adults escaped from woods, except for 16 adults. We also determined that contaminated larvae at the rates of 25%, 50%, 75% and 100% with 1×10^6/ml conidia of *B. pseudobassiana* caused different damages rates such as 68, 52, 45 and 29 cm^2 in woods, respectively.

Discussion

Although some chemical, mechanical and neo-classical biological control methods have been developed

Table 1 Summary of probit analysis parameters from multiple-concentration bioassays performed with the *B. pseudobassina* ARSEF 9271 against larvae and adults of *D. micans*

Bioassay	Intercept	Slope (±SE)*	LC_{50} (95% Fiducial limits)	$\chi^{2\dagger}$	d.f.
Larvae	−3.56	0.65 (0.45–0.85)	2.79×10^5 ($0–3.53 \times 10^7$)	11.37	3
Adults	−2.44	0.59 (0.36–0.82)	1.36×10^4 (1.74×10^3 –4.25×10^4)	2.22	3

*Slope of the concentrations (±SE) response of larvae and adults of *D. micans* to the *B. pseudobassina* ARSEF 9271.
†Pearson's chi-square goodness-fit test on the probit model ($\alpha = 0.05$).

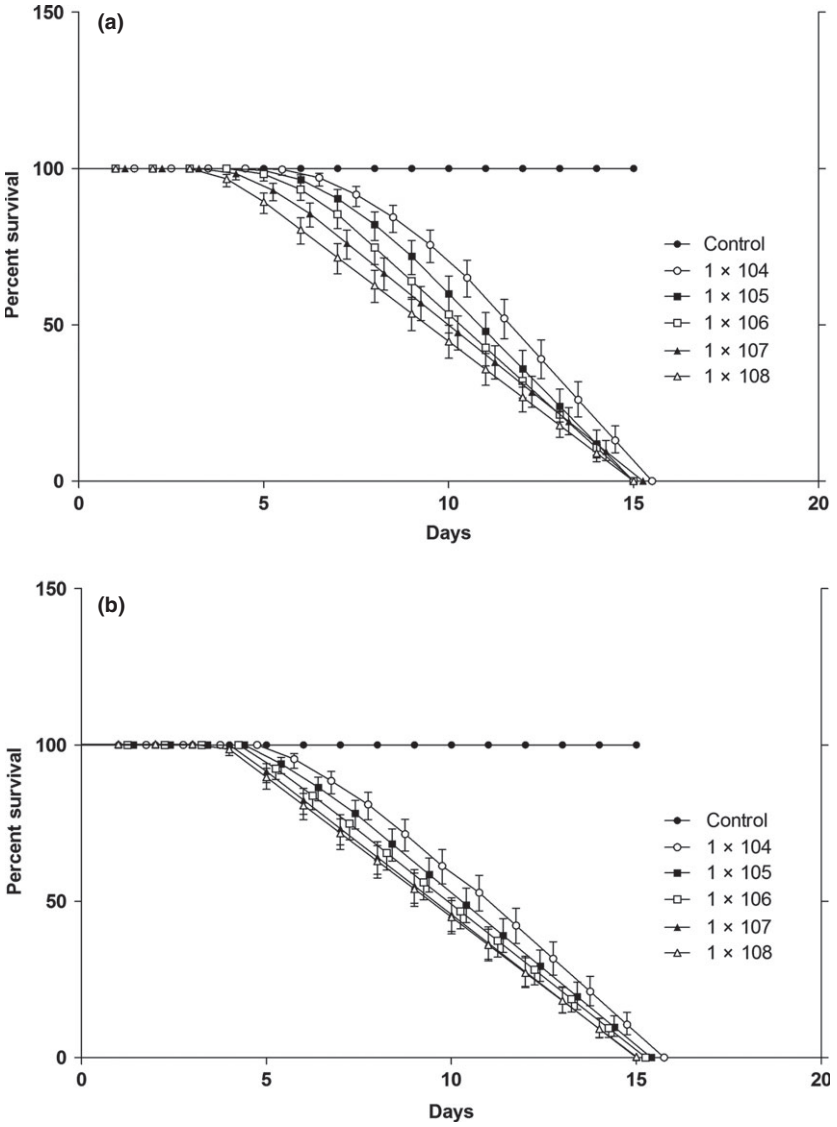

Fig. 3 The survival of *D. micans* after application of different concentrations (1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8/ml) of *B. pseudobassiana* ARSEF 9271 spores. (a) Larvae, (b) adults.

against *D. micans*, spreading of this pest could not be prevented yet, and it is still the most serious pest of oriental spruce forest in Turkey and Europe. More people pay close attention to microbial control agents against the pest. Up to now, so many pathogens have been isolated from insects and their insecticidal effects have been determined on agricultural and forest pests. (Ince et al. 2007; Gokce et al. 2010; Sevim et al. 2010b,c; Danismazoglu et al. 2012; Demir et al. 2012). In addition, fungal pathogens have been improved as biopesticide and still used effectively in pest management system for a long time (Bartlett and

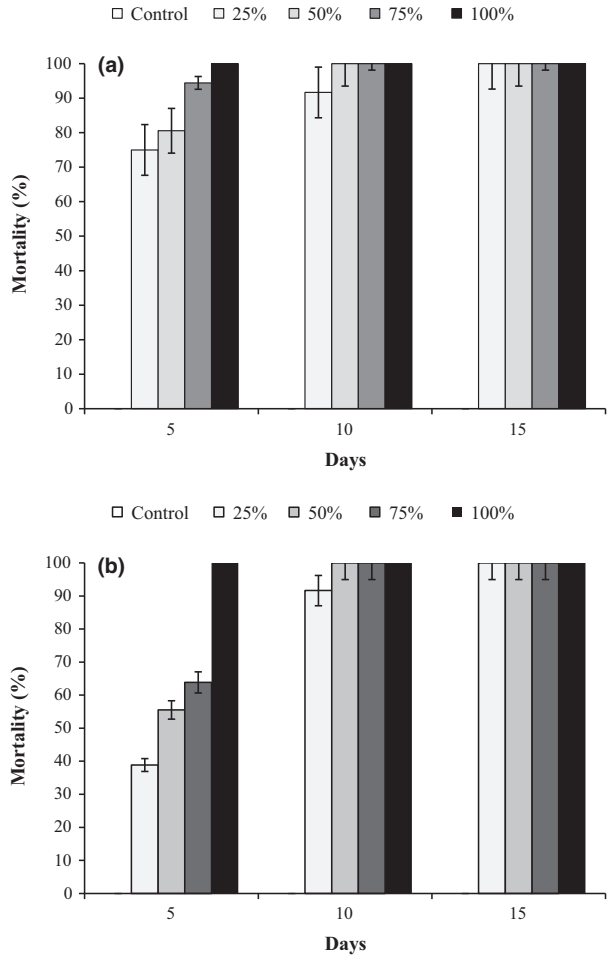

Fig. 4 The mortality of *D. micans* after conidial transmission of *B. pseudobassiana* ARSEF 9271 from the treated to the untreated beetles at various rates after application of 1×10^6 conidia per mililitre. (a) Larvae, (b) adults.

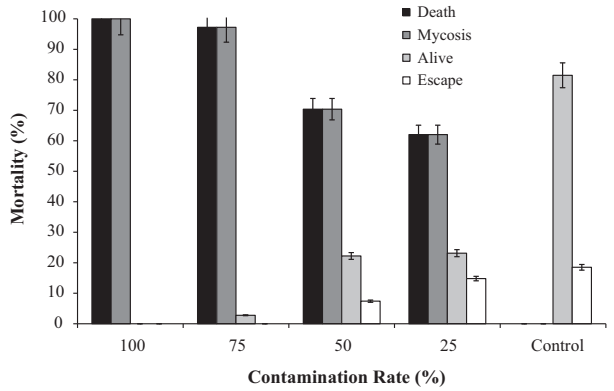

Fig. 5 The mortality and mycosis rates of *D. micans* after conidial transmission of *B. pseudobassiana* ARSEF 9271 from the treated to the untreated larvae at various rates after application of 1×10^6 conidia per mililitre at 20°C in wood block experiment.

Jaronski 1988; Flexner and Belnavis 2000; Lacey and Kaya 2007; Zimmermann 2007; Vega et al. 2012). To find more effective and safe fungal biocontrol agent against *D. micans*, we collected some soil and insect samples in the Eastern Black Sea Region of Turkey. In one of these studies by Sevim et al. (2010a), we tested the pathogenicity and virulence of nine highly pathogenic fungal strains isolated from soil samples and different insects such as *Rhynchites baccus* L. (Coleoptera: Attelabidae) and *Thaumetopoea pityocampa* (Den. and Schiff.) (Lepidoptera: Thaumetopoeidae) on the larvae and adults of *D. micans* under laboratory conditions. In that study, we obtained the highest mortality from *B. bassiana* KTU-53 and *M. anisopliae* KTU-60 with 100%. However, it is known that microbial pathogens directly obtained from target pests could be better in terms of microbial control of pest species due to ecological compatibility with pest species and reduced risk of significant impact on non-target organisms (Gulsar Banu et al. 2004; Takatsuka 2007). Therefore, we preferred to continue native fungal isolates from *D. micans* larvae and adults collecting from naturally infested spruce forests in Turkey. In the study of Tanyeli et al. (2010), a total of twelve fungal strains including *Lecanicillium muscarium*, *Isaria farinosa*, *Fusarium*, *B. bassiana* and *Beauveria* sp. were isolated from *D. micans*, and it was determined that *B. bassiana* ARSEF 9271 produced high mortality and mycosis value against the larvae and adults of *D. micans*. Hereby, this isolate have been selected as subject in this study. This is the first study for detail characterization and virulence of *B. bassiana* against *D. micans*.

Previously, some researchers showed that *Beauveria* sp. is a natural fungal pathogen of many different pest species in both agriculture and forestry (Goettel et al. 1990; Kreutz et al. 2004; Batta 2007; Er et al. 2007; Sevim et al. 2010b,d). Different species and strains of *Beauveria* genus, which were isolated from different geographical locations and host insects, vary in performance, and it is the most common enthomopathogenic fungi in agricultural fields (Meyling and Eilenberg 2006). Scientific researchers showed that *B. bassiana* can infect many bark beetles and displayed significant potential to control of forest pests such (Kreutz et al. 2004; Draganova et al. 2006; Batta 2007). Besides observations of fungal occurence in collected insect cadavers, relatively little research has been conducted on the use of *B. pseudobassiana* for environmentally safe control of *D. micans*.

Up to now, horizontal transmission of *B. bassiana* between bark beetles such as *Ips typographus* L. (Coleoptera: Curculionidae) which is the secondary pest of spruce trees have been investigated, and it was shown

that this fungus displayed effective transmission among beetles (Kreutz et al. 2004; Lopes et al. 2011). In this paper, we tested the horizontal transmission of *B. pseudobassiana* ARSEF 9271 between adults of *D. micans*. It was also shown that conidia transfer of *B. pseudobassiana* ARSEF 9271 among the treated and the untreated larvae and adults of *D. micas* was successful and resulted in 100% mortality at all test groups (25%, 50%, 75% and 100%) within 15 days. Moreover, we also obtained good results from wood block experiment. The mortality of adults reached 60% after application of 25% contamination rate in wood block. We also considered mycosis rate because sporulation is very important for fungi to disseminate in the field. It was determined that *B. pseudobassiana* ARSEF 9271 showed good mycelium formation and sporulation on dead cadavers.

Entomopathogenic fungi require moisture for sporulation and germination of conidia; some even need high humidity to initiate infection. In addition, rain plays an important role in transmission of entomopathogenic fungi (Goettel et al. 2005). Considering this information, it is possible to say that *B. pseudobassiana* ARSEF 9271 seems to be good candidate for controlling *D. micans* in the Eastern Black Sea Region of Turkey. Because this region has favourable environmental conditions to use fungal entomopathogens as it has a wet, humid climate and lower annual temperatures (Sevim et al. 2013).

All these results suggest that *B. pseudobassiana* ARSEF 9271 seems to be the most promising and environmental friendly alternative isolate against *D. micans* for biological control purpose. Additional research is needed to determine the effectiveness of isolates in the field. Moreover, mass production studies, application methods and long-term effects of this fungus should be investigated. Finally, the side effects of the isolate ARSEF 9271 against natural enemies of *D. micans* and beneficial insects should be also investigated.

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