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ORIGINAL ARTICLE

Entomopathogenic fungal endophytes from *Pinus sylvestris* needles and their potential in controlling *Diprion pini*

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Abstract

Entomopathogenic fungi (EPFs) are important factors in the biological control of many insect pests, and these microorganisms often cause widespread epizootics in pest populations. In addition to the use of EPFs as microbial control agents, recent studies show that these fungi can live endophytically with plants. These endophytes are also known to play a role in plants to protect them against insects, pathogens, and nematodes. In this study, it was aimed to isolate and molecularly characterize endophytic entomopathogenic fungi from yellow pine forests in Kırşehir province, Türkiye, and test them against *Diprion pini* L. (Hymenoptera: Diprionidae). Endophytic entomopathogenic fungus isolation was carried out using a selective medium from a total of 234 healthy needle samples (three from each branch) from 117 branches (two from each tree) from 39 yellow pine trees. After the isolation studies, a total of 24 fungi (10.25% based on the needle number) were isolated and purified. The isolated fungi were then characterized by gene sequencing (*Bloc* gene for *Beauveria* spp. and *β-tubulin* for *Metarhizium* spp.) and phylogenetic analysis. Twenty isolates were identified as *B. bassiana*, and four isolates were identified *Metarhizium majus*. All isolates were also tested against *D. pini* larvae, and different mortalities were recorded. The highest mortality values were obtained from *Beauveria bassiana* AD-9, AD-12, and AD-16 with 96.66, 76.66, and 96.66%, respectively. The highest mycoses were obtained from *B. bassiana* AD-9 and AD-16 with 96.66 and 90%, respectively. These results should be beneficial and provide new opportunities in the control of pine pests using endophytic entomopathogenic fungi.

Keywords *Pinus sylvestris* · Endophytic fungi · Microbial control · Beauveria · Metarhizium

Introduction

Entomopathogenic fungi (EPFs) are important agents in the biological control of many insect pests, and these microorganisms frequently cause wide-spreading natural epizootics in insect populations. EPFs have been used as microbial control agents for more than 100 years, and today, there are many commercial products available based on entomopathogenic fungi worldwide (Roberts 1989; Goettel et al. 2005). For example, *Beauveria bassiana* (Bals.-Criv.) Vuill. (1912) has been used against pine caterpillar (*Dendrolimus* spp.) in China and there are many studies showed that these fungi could be successfully used against some other forest pests such as *Thaumetopoea pityocampa* Den. & Schiff. (Lepidoptera: Thaumetopoeidae) and *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae) (Goettel et al. 2005; Sevim et al. 2010a, b). In addition, there are many products which are sold in the market under trade names such as Bio-Power (Biorin), Bas-Eco, and Ditera against many pests around the world (Ruiu 2018). In addition to the use of entomopathogenic fungi as microbial control agents against insect pests, recent studies also show that these fungi can live endophytically and epiphytically with plants. The term endophyte was first described by German scientist Heinrich Anton De Bary (1884) as bacteria or fungus that live in plant tissues and do not cause any visible symptoms in the plant (Wilson 1995). Fungal endophytes belonging to many plants have been identified in the studies carried out so far, and most of these plants include agriculturally important plants such as wheat, bananas, and tomatoes. At the same time, there are some studies showing that these fungi can live endophytically inside some forest trees such as *Pinus*

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radiata (Reay et al. 2010; Vega 2018). The fungal endophytes have many important roles in terms of plant health, and they can protect plants against insects, pathogens, and nematodes. At the same time, endophytes have recently been important for plant health by promoting plant development. In addition, fungal endophytes have become the point of interest of biotechnology due to their potential to be used as genetic vectors, being a source of secondary metabolites and being used as biological control agents (Vega 2018). Among entomopathogenic fungi, *Beauveria* spp. (especially *B. bassiana*) and *Metarhizium* spp. (*Metarhizium robertsii* (Metchnikoff) Sorokin (1883)—formerly known as *M. anisopliae*) are the two most studied species in terms of commercial production. Although *Beauveria* species are predominant in forest ecosystems, *Metarhizium* species are widely found on farmlands (Meyling and Eilenberg 2007).

Entomopathogenic fungi are widely found in forest lands worldwide (such as soil and leaf residues), and the species diversity of these fungi is seen to be lower in warm forests compared to tropical forests. But it is possible to say that even in the forests, there is a more variety of entomopathogenic fungal species than in agricultural areas. Differences in prevalence and species diversity may also vary by forest type (Augustyniuk-Kram and Kram 2012). It is also known that these fungi, which are commonly found in forests, can form large-size epizootics in various forest pests and are used as biological control agents against many important forest pest insects (Dara et al. 2019). However, it has been showed that a few entomopathogenic fungi (especially *B. bassiana*) can be found endophytically in many forest trees such as *Pinus radiata* D. Don (Reay et al. 2010; Brownbridge et al. 2012; Lefort et al. 2016), *Pinus monticola* D. Don (Ganley and Newcombe 2006), *Quercus robur* L. (Gorzkowska et al. 2016; Kwaśna et al. 2016), *Carpinus caroliniana* Walter (Bills and Polishook 1991), *Theobroma gileri* Cuatrec. (Evans et al. 2003), and *Fagus sylvatica* Zlatia (Unterseher and Schnittler 2010) by endophytic plant inoculation method or natural isolation method (Vega 2018). Considering all these studies, it is possible to say that *B. bassiana* can live endophytically in *Pinus* species and that locally obtained isolates have a chance of success in biological control of various pine diseases or pests.

Chemical insecticides used against insect pests in both agriculture and forestry are known to cause the resistance to these drugs in insects, to kill beneficial insects, honeybees, birds, and fish in the environment, and to reach humans and animals through the food chain, and finally may cause many permanent or fatal diseases (Fenemore 1984; Ecevit 1988; Sevim 2010). In this sense, there is a need to discover and use more effective and environmentally friendly control methods. That is why, it is known that biological control can eliminate many problems caused by chemical control, and is prioritized in all developed countries and studies on this subject are taken further. Biological control agents have been developed worldwide, commercially produced, and sold in many countries. In many countries around the world, and especially in Türkiye, the use of chemical insecticides against insects in forests is prohibited and is applied subject to special permission in very difficult cases (Sevim 2010). In this sense, it has become desirable to find effective biological control agents that can be used against forest diseases and pests in our country.

Entomopathogenic fungi have many advantages in terms of biological control. They do not have any toxic effects on mammals, do not cause resistance in insects, have a high potential for biotechnological developments, provide long-term control by staying in the environment for a long time after application, infect all development stages of their hosts, generally act synergistically with insecticides and easily overcome mass production problems (Wan 2003; Sevim 2015). Among the entomopathogenic fungi, *Beauveria* spp. (especially *B. bassiana*) and *Metarhizium* spp. (especially *M. majus*) are the two most studied species and the most focused on in terms of commercial production. These two species are known to have cosmopolitan species and infect many insect species worldwide (Goettel et al. 2005; Meyling and Eilenberg 2007). Also, it is important to search for new and more potentially effective local fungal isolates against the target pest because the choice and application of local isolates may reduce future environmental impacts and may be better adapted to survive local conditions, and this may provide a long-term control after application (Bilgo et al. 2018; Keçili et al. 2022).

As a first step in the process to investigate and develop any products based on endophytic entomopathogenic fungi against pine pests, a survey was conducted in Kırşehir, Türkiye, to determine the prevalence and distribution of EPFs in yellow pine forests. The isolated fungi were molecularly characterized by gene sequencing and tested against one of target pests (*D. pini*) under laboratory conditions. To our knowledge, this is the first study to determine the distribution of endophytic EPFs in yellow pine forests.

Materials and methods

Collection of needles

Endophytic fungal (especially entomopathogenic *Beauveria* spp. and *Metarhizium* spp.) isolation was performed by taking needle samples from yellow pine forests in the study area (Kırşehir, Türkiye). The yellow pine forests in the study area consist entirely of plantation areas. In isolation studies, only needle samples were used, assuming that endophytic entomopathogenic fungi are found intensively in needle based on the literature (Reay et al. 2010). The needle

collection was performed randomly in the study area. A total of 234 healthy needle samples from 39 yellow pine trees were collected in the spring of 2021 and 2022. Two branches were randomly selected from each tree, and three needles from each branch were analyzed in terms of the presence of endophytic EPFs. Trees were also randomly selected. Preferably 2- or 3-year-old and 6–10-cm-long needles were taken from the lower sides of trees, as they are assumed to contain more endophytic fungi (Johnson and Whitney 1992).

Endophytic EPF isolation

The needles were first rinsed in 10% sodium hypochlorite for 5 min and then rinsed in 96% ethanol for 1 min for surface sterilization. After that, they were twice washed with sterile distilled water. In terms of proving the accuracy of the surface sterilization process in the needle samples, 100 µl from the last water sample used in the washing of plant materials was plated on PDAY (potato dextrose agar + 1% yeast extract) and incubated in the dark at 25–28 °C for two weeks. At the end of the incubation, the examples that do not show growth were considered successful in terms of surface sterilization (Gurulingappa et al. 2010). After surface sterilization, the needle samples were placed on sterile filter papers and dried under sterile conditions in the laminar cabinet. After drying, the needles were cut into at least three parts with a sterile scalpel. Finally, the needle parts were placed on PDAY medium containing 50 µg/ml tetracycline and 75 µg/ml ampicillin to prevent bacterial growth and were incubated at 25–28 °C in the dark for 20 days (Reay et al. 2010; Allegrucci et al. 2017). A different PDAY plate was used for each needle sample. During incubation, PDAY plates were monitored daily, and the growing fungus colonies were transferred to another antibiotic-free PDAY and purified by single conidia isolation. The purified fungi were stocked in 15% glycerin for later use. In addition, the needle parts were placed on the selective PDAY (for 1 L, 10 g peptone, 20 g glucose, 12 g agar prepared and cooled to 55 °C after autoclave, 600 µg/ml streptomycin, 50 µg/ml tetracycline, 100 µg/ml dodine, and 50 µg/ml cycloheximide) to selectively isolate *Beauveria* spp. and *Metarhizium* spp., considering that other endophytes can grow fast and intensively and suppress these two species (Inglis et al. 2012). All petri dishes were incubated for 2 weeks at 28 °C in dark, and as a result, the growing colonies were purified by single conidia isolation and stored in sterile 15% glycerin at − 20 °C for subsequent use.

Species identification

All fungal isolates were first morphologically identified, and then, molecular characterization studies were performed basically using gene sequencing according to specific gene regions for all species. Morphological examination of the isolated fungi was carried out first, and microscopic preparations for further examination were made according to the 'tape touch method' technique and stained with acetoorcein. After that, the preparations were examined under the microscope with respect to the characteristics of the hypha, reproductive organs, the structure of the conidiophore, and the shape and color of spores and they were used for species determination (Humber 1997; Harris 2000). Based on the colony morphology, white colonies were considered as *Beauveria* spp. and yellowish colonies were considered ad *Metarhizium* sp.

Morphological species identification of the fungal isolates was also molecularly confirmed using various gene sequences. For this purpose, genomic DNA isolation was carried out in accordance with the recommendations of the manufacturer using "Powersoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA)." Since it is difficult to break down the fungal cell wall during DNA isolation, a tissue grinder with stainless steel beads were used for this purpose. The isolated DNAs were stored at − 20 °C until use.

Following DNA isolation, *Bloc* (for *Beauveria* spp.) and β-*tubulin* (for *Metarhizium* spp.) gene regions were amplified by polymerase chain reaction (PCR) using the primers specified in Table 1. PCR conditions were carried out according to the references indicated in Table 1. After PCR, 5 µl of the PCR products was electrophoresed at 90 V

Table 1 Primers and their sequences used in the species identification of endophytic *Beauveria* spp. and *Metarhizium* spp. from *Pinus sylvestris* needles

Gene	Primer name	Sequence	PCR conditions	References
Bloc	B5.1F	5′-CGACCCGGCCAACTACTTTGA-3′	94 °C for 5 min, 95 °C for 1 min, 57 °C for 45 s, 72 °C for 1.5 min for 35 cycles and 72 °C for 10 min for 1 cycle	Rehner et al. (2006)
	B3.1R	5′-GTCTTCCAGTACCACTACGCC-3′		
β-*Tubulin*	T1	5′-AACATGCGTGAGATTGTAAGT-3′	94 °C for 5 min, 95 °C for 1 min, 57 °C for 45 s, 72 °C for 1.5 min for 35 cycles and 72 °C for 10 min for 1 cycle	O'Donnell and Cigelnik (1997)
	T2	5′-TCTGGATGTTGTTGGGAATCC-3′		

for 30 min with 1-kb DNA ladder in 1% agarose gel with 0.5 µg / ml ethidium bromide. The remaining PCR products were sent to MACROGEN (Seoul, Republic of Korea) for sequencing. Approximately 1.000 bp length of both gene regions was sequenced. The obtained DNA sequences were compared with the known DNA sequences in the NCBI GenBank to verify the species identification and then used in phylogenetic analysis (Benson et al. 2013). *Beauveria* isolates were compared to the reference species and strains in the study of Rehner et al. (2011) with phylogenetic analysis, and *Metarhizium* isolates were compared to the reference species and strains in the study of Bischoff et al. (2009) with phylogenetic analysis.

Virulence tests against *Diprion pini*

Insecticidal activity of entomopathogenic fungal endophytes from yellow pine trees against *D. pini* larvae (3rd or 4th instar) was determined under laboratory conditions. For this purpose, 100 µl conidial suspension from a stock solution of 1×10^5 conidia / ml of endophytic fungal isolates was streaked on PDAY and incubated at 28 °C for 2–3 days in dark. At the end of the incubation period, single colonies were selected and transferred to another PDAY medium and incubated at 28 °C for 4 weeks in dark. After sporulation, 10 ml sterile 0.01% Tween 80 was added to the petri dishes and the conidia was provided to pass into the water by scraping with glass baguette. After that, the conidia suspensions were filtered into 50-ml sterile falcon tubes with a two-layer sterile cheesecloth to remove mycelium and agar pieces. The obtained suspensions were vortexed for 5 min to homogenize conidia suspensions, and the conidia concentrations were adjusted to the desired concentrations with a Neubauer hemocytometer. The viability of conidia was tested by spreading 100 µl of conidia suspension on PDAY and determining germination after 24-h incubation. The germ tube which was greater than spore diameter was considered as germinated. As a result, spores that germinate 90% or over were used in virulence tests (Sevim et al. 2010a).

Conidial suspensions of the endophytic fungal isolates were used in virulence tests against *D. pini* larvae (3rd or 4th instar). The larvae collected from pine forests in Kırşehir and its surrounding regions in May–November were used in virulence tests. Ten larvae were used for each repetition in virulence tests. Pine needles and fresh shoots as nutrition of larvae were collected from forests, and all tests were carried out in the climate cabinet (28 °C, 70% humidity, and 12:12 (light:dark) light period). The larvae collected from the forest were brought to the laboratory on the same day and were fed for 2 days to ensure the use of healthy larvae in tests. Ten healthy larvae (3–4. instar) were placed in plastic boxes ($20 \times 20 \times 20$ cm), and the conidial concentration of 1×10^8 conidia/ml was exposed to larvae with an aerosol type sprayer (airbrush) for 2–3 s. Freshly collected pine needles and shoots were used as food and were changed daily according to the situation. Different boxes and 10 larvae were used for each dose and repetition. All trials were repeated 3 times at different occasions. The control group were inoculated with only sterile 0.01% Tween 80. After inoculation, all boxes were left to incubate for 15 days in the climate cabinet at 28 °C, 70% humidity, and 12:12 (L:D) period. After incubation period, dead larvae were counted, and the percentage mortality values were calculated. The percent mycosis values were also calculated to determine whether the cause of death is fungus or not. For this, dead larvae were surface sterilized with 1% sodium hypochlorite solution for 3 min. After being subjected to surface sterilization, they were washed with the sterile distilled water three times and taken into sterile petri dishes with moist filter paper and left to incubate at 28 °C and in the dark (Sevim et al. 2010b). Samples showing external fungal growth were considered as mycosis.

Data analysis

All DNA sequences were edited with the BioEdit 7.09 (Hall 1999) software, and their percentage similarities with other known DNA sequences in GenBank were determined by blasting them in NCBI GenBank (Benson et al. 2013). The data obtained from here were then used to verify the morphological identification of the isolates. Cluster analysis of DNA sequences was done with the ClustalW program packed in the BioEdit, and the data obtained from this were used in neighbor-joining (NJ) analysis with the help of the MEGA X phylogenetic software (Kumar et al. 2018). Alignment gaps were considered as missing data. The reliability of the generated dendrograms were tested with 1.000 replicates by bootstrap analysis using the MEGA X.

The data obtained from the virulence tests were interpreted using the Abbott formula, and the percent mortality were calculated. In addition, the percent mycosis values were calculated by keeping the dead insects in the moisture section (Abbott 1925). In virulence tests against the common sawfly, one-way analysis of variance (ANOVA) was used to compare fungal isolates with respect to mortality and mycoses. In the variance analysis test, Fisher test was used to compare the isolates with the control group and each other. Before performing variance analysis, all data were evaluated in terms of variance homogeneity using Levene statistics, and all percentage (%) data (in case some of them come out 0 (zero)) were subjected to arcsin transformation. All data obtained were analyzed using Minitab 17 statistical software.

GenBank accession numbers

GenBank accession numbers for the fungal isolates were provided by GenBank genetic sequence database. *Bloc* gene sequences were deposited under the accession numbers of PP717793-PP717812, and β-*tubulin* gene sequences were deposited under the accession numbers of PP717813-PP717816.

Results

Species identification and distribution of entomopathogenic fungal endophytes

A total of 24 fungal isolates were obtained from needle samples, and the frequency of distribution of endophytic EPFs in yellow pine forests (when evaluated based on the needle examined) was found to be 10.25%. Only colonies growing from the cut end of the needles were examined and considered as endophytes (Reay et al. 2010). Initially, white colonies were considered *Beauveria,* and yellowish-green colonies were considered as *Metarhizium*. Colonies reproducing in the initial medium were purified by single conidia isolation method and compared with reference species by performing phylogenetic analyzes by subjecting them to sequence analysis (Bischoff et al. 2009; Rehner et al. 2011). After phylogenetic analysis, 20 isolates were identified as *B. bassiana* (8.54%) and 4 isolates as *M. majus* (1.7%) (Figs. 1 and 2).

Mortalities of entomopathogenic fungal endophytes against *Diprion pini*

All isolates caused different mortality values against *D. pini* larvae, and all were significantly different from the control group, except for *M. majus* AD-3, *B. bassiana* AD-7, and AD-19 ($F = 8.5$, df = 24, $p < 0.05$). The highest mortalities were obtained from AD-9, AD-12, and AD-16 with 96.66, 76.66, and 96.66%, respectively ($F = 8.5$, df = 24, $p < 0.05$). Also, all isolates produced different mycoses values, and all were different from the control, except for AD-1, AD-3, AD-7, AD-19, and AD-23 ($F = 8.77$, df = 24, $p < 0.05$). The highest mycoses were obtained from AD-9 and AD-16 with 96.66 and 90%, respectively ($F = 8.77$, df = 24, $p < 0.05$) (Fig. 3).

Discussion

Entomopathogenic fungi generally cause infection in many insect species and have been used in the control of insect pests for many years. However, there have been many recent studies, showing that these fungi can live endophytically with plants, thus providing various advantages to the plant (Vega 2018). Our study is the first showing the distribution and prevalence of entomopathogenic fungi as endophytes in yellow pine forests in Türkiye. Even if the sampling were carried out from a small number of trees within the study area, it is thought that the results could be useful in the control of pine pests and even in the control of pine diseases, considering the antagonistic properties of these fungi (Kutalmış et al. 2023).

All fungal isolates were tested against *D. pini* larvae under laboratory conditions and caused different levels of mortality. Many of *B. bassiana* isolates produced relatively higher mortality rates compared to *M. majus* isolates. *B. bassiana* is the most widely distributed species in the genus *Beauveria* and can be isolated from insects, soils, and various plant specimens (surface and interior parts) in both temperature and tropical regions around the world. This species is also used to control various insect pests from different insect orders in agriculture, veterinary, and forestry, and many commercial products are available based on this species (Zimmermann 2007). This fungus was also isolated from many different forest pests, known to cause epizootics, and studies showed that it has a great potential in biological control of forest pests (Dara et al. 2019; Skrzecz et al. 2023). For example, in China, *B. bassiana* has been produced since 1990 to control *Dendrolimus* spp. in *Pinus* spp. forests and has been used in very large areas (about 500,000 ha per year). After these applications, it has been noted that they provided a long-term suppression of the pest for at least 3–5 years (Li 2007; Skrzecz et al. 2023). In addition, *B. bassiana* has been used under different strategies and techniques (such as the use of sawyer traps impregnated with *B. bassiana* as well as the direct inoculative release) to control *Monochamus alternatus* (the Japanese pine sawyer) (Hope 1842) (Coleoptera: Cerambycidae), which acts as a vector of pine with nematode in pine forests, and promising results have been obtained (Okitsu et al. 2000; Kim et al. 2022). Apart from these, there are many studies involving the use of other Hypocreales fungi against forest pests and successful results have been demonstrated in terms of field application (Skrzecz et al. 2023). For instance, *B. pseudobassiana* Rehner et Humber has been shown to have a good potential against *D. pini* after application of different doses of conidia under semi-field conditions using pot experiments (Sevim et al., unpublished). *Beauveria brongniartii* (Sacc.) Petch has been also successfully used against *Melolontha melolontha* L. (Coleoptera, Scarabaeidae), which is serious pasture, forest, and orchard pest, and some reduction in the pest population was achieved (Keller et al. 1997). Virulence tests under laboratory conditions also indicate that many Hypocreales fungi, including *B. bassiana*, have good potential in the biological control of various forest pests (Sevim

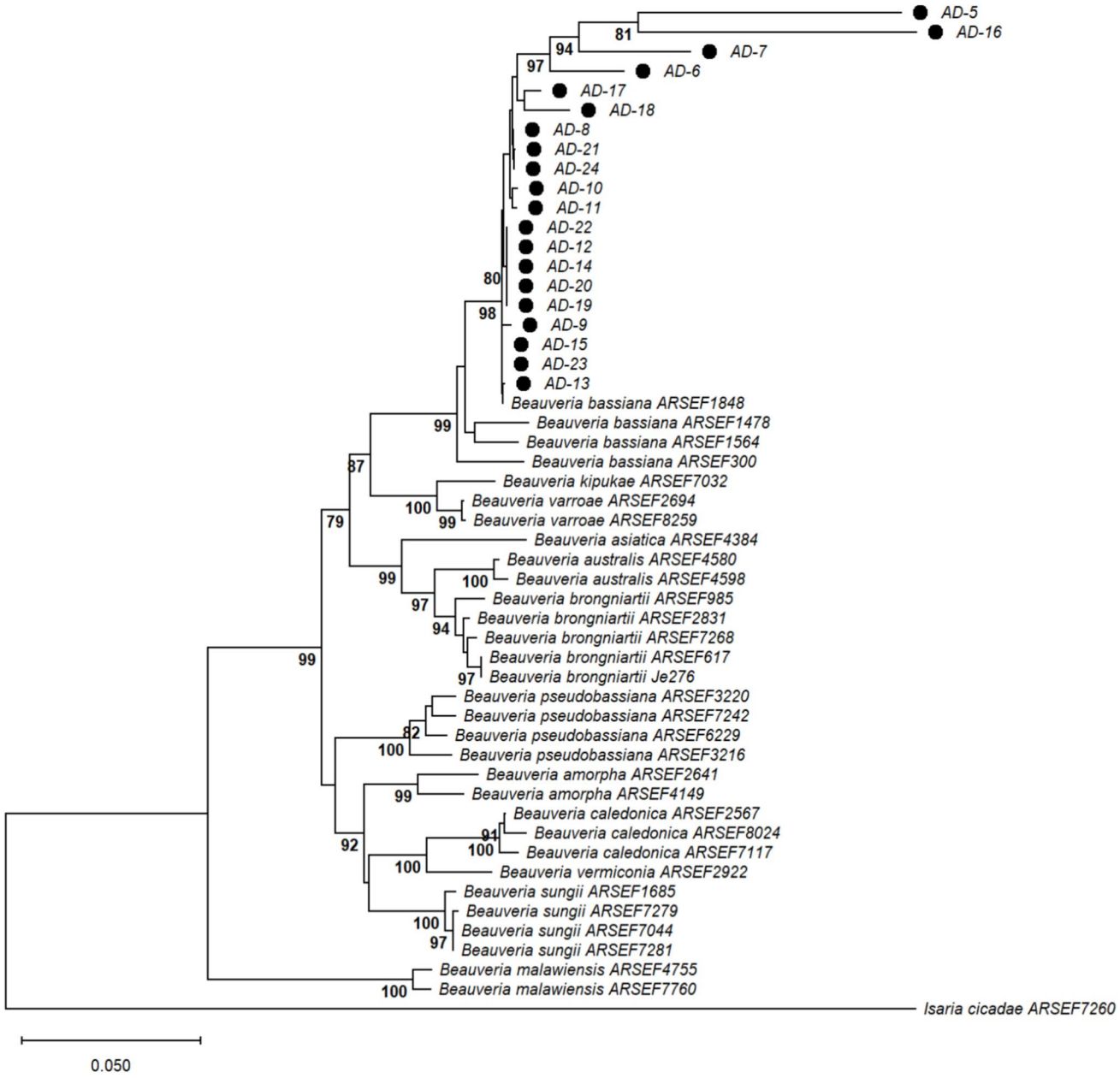

Fig. 1 Phylogenetic comparison of *Beauveria* isolates with reference species/isolates indicated in the study of Rehner et al. (2011). The tree was generated based on neighbor-joining (N-J) analysis with p-distance correction using the partial sequence of *Bloc* gene region. Bootstrap values from 1.000 bootstrap replicates are shown above the branches, and only values above 70% are indicated. *Beauveria* isolates obtained from this study were marked by a solid black circle. *Isaria cicadae* ARSEF7260 was used as outgroup

et al. 2010a, b). In this study, two *B. bassiana* isolates (AD-9 and AD-16), which have a strong endophytic potential with *P. sylvestris*, produced a good mortality and mycoses values against *D. pini* under controlled laboratory conditions. The results obtained should be beneficial in the biological control of pine pests for future studies.

Knowing the species occurrence and distribution of entomopathogenic fungi present in a specific ecosystem or area is of great important in terms of conservative biological control. When the species occurrence and distribution of the entomopathogenic fungi isolated in our study is examined, it is seen that 20 of the 24 fungi obtained are *B. bassiana* and the remaining 4 are *M. majus*. Although both species have a cosmopolitan distribution throughout the world, it is noteworthy that *Beauveria* species are mostly associated with forest ecosystems and *Metarhizium* species are more associated with agricultural areas (Meyling and Eilenberg 2007). In the study area, Sevim et al. (unpublished) also studied

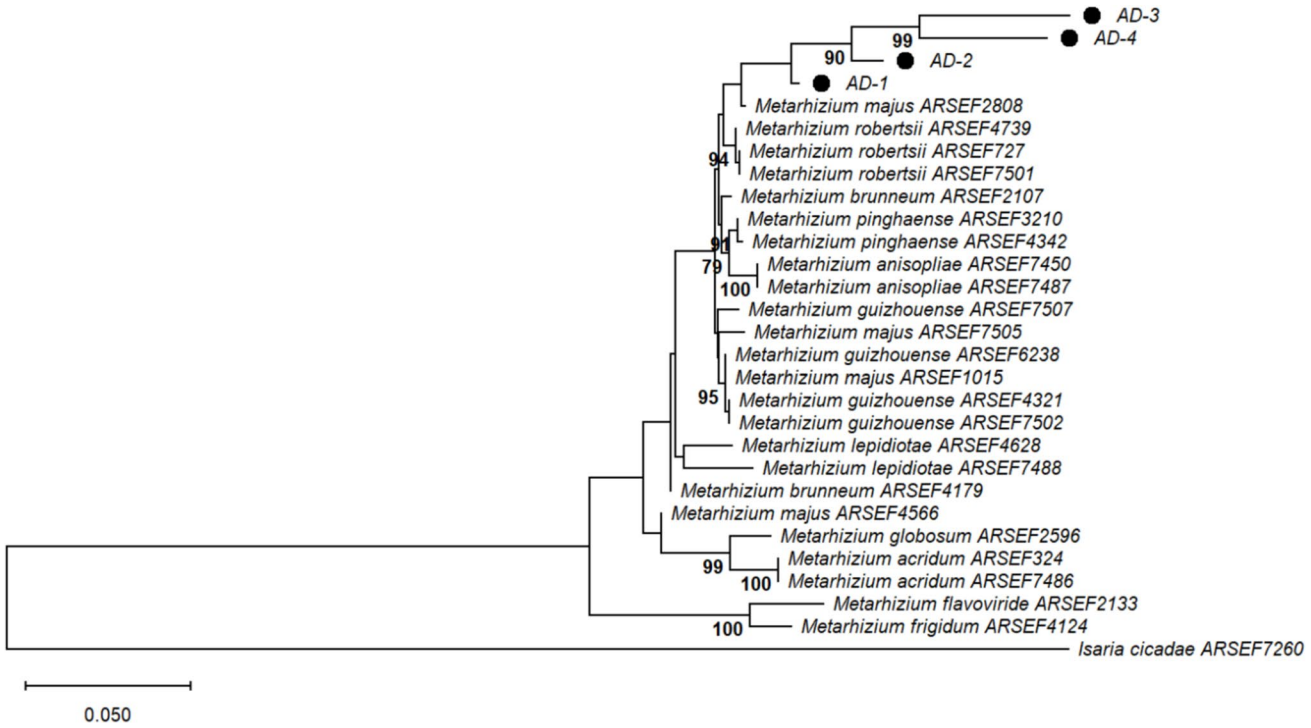

Fig. 2 Phylogenetic comparison of *Metarhizium* isolates with reference species/isolates indicated in the study of Bischoff et al. (2009). The tree was generated based on neighbor-joining (N-J) analysis with p-distance correction using the partial sequence of *β-tubulin* gene region. Bootstrap values from 1.000 bootstrap replicates are shown above the branches, and only values above 70% are indicated. *Metarhizium* isolates obtained from this study were marked by a solid black circle. *Isaria cicadae* ARSEF7260 was used as outgroup

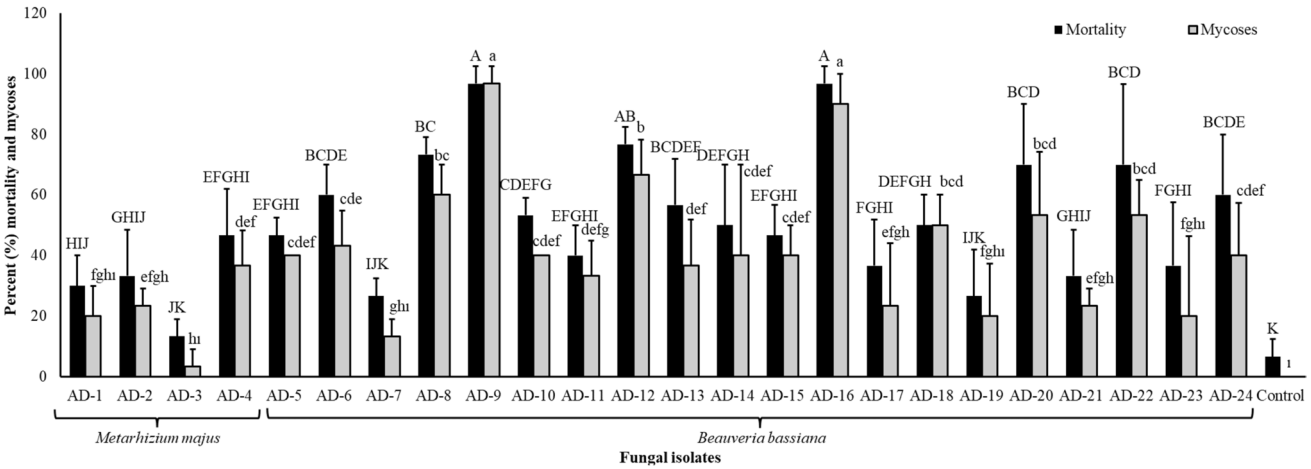

Fig. 3 Percent (%) mortality and mycosis values (±SE) of the endophytic fungal isolates against 3rd/4th instar larvae of *Diprion pini* within 15 days. The conidial concentration of 1×10^8 conidia/ml was applied to the larvae by aerosol type sprayer for each isolate. Mortality values were corrected according to Abbott's formula (Abbott 1925). The same capital and lowercase letters indicate no significant difference between treatments in mortality and mycosis levels, respectively, according to Fisher test ($p < 0.05$). 0.01% Tween 80 was used as negative control

the occurrence and distribution of entomopathogenic fungi from soil samples in pine forest and they mostly isolated *Beauveria* species (*B. bassiana* and *B. pseudobassiana*) by insect bait method using *D. pini* larvae. Kovač et al. (2021) investigated the occurrence, diversity, and distribution of entomopathogenic fungi in forest soils in Croatia and they identified five entomopathogenic fungal genera and the most common were *Beauveria* species. Majchrowska-Safaryan

and Tkaczuk (2021) determined the species composition of entomopathogenic fungi in the leaf litter and at different soil depths in different types of forests during different seasons in Poland. They found that Beauveria spp. were the most prevalent fungi among leaf litter and soil samples compared to other entomopathogenic fungi. However, Sánchez-Peña et al. (2011) conducted a study about determining of occurrence of entomopathogenic fungi in soils in four adjacent habitats (oak forest, agricultural soil, pine reforestation, and chaparral habitat) in Saltillo, México. In their study, *B. bassiana* was significantly more frequent in forest soil while *M. anisopliae* was significantly more frequent in agricultural soil. In this study, we found that *B. bassiana* was frequently associated with *P. sylvestris* as endophyte compared to *M. majus* and all data indicated here may be having considerable importance with respect to the use of fungal biocontrol agents against pine pests under different biological control strategies (especially conservative biological control).

In addition to the use of entomopathogenic fungi in the microbial control of insect pests, these fungi can colonize on many parts of plants, including their green parts, and provide many beneficial properties such as plant growth promoter, stress tolerance, water uptake enhancer, and resistance to pests and diseases (Bamisile et al. 2021). So far, there have been many studies showings that many entomopathogenic fungi (especially *B. bassiana* and *M. anisopliae*) live endophytically with various plants and most of these plants include agriculturally important plants such as wheat, bananas, and tomatoes (Vega et al. 2008; Vega 2018). However, there are also some studies showing that these fungi can live endophytically inside parts of some forest trees such as *Pinus radiata* D. Don, Trans. Linn. Soc. London 17: 442 (1836) (Reay et al. 2010). Reay et al. (2010) conducted a survey study for the presence of *B. bassiana* in needles, seeds, and roots of *P. radiata* (the Monterey pine) throughout New Zealand. They isolated 21 *B. bassiana* isolates; of these, 19 were obtained from needle samples. They also showed that these fungi were pathogenic to *Hylurgus ligniperda* (F.) (Coleoptera: Scolytidae) adults and *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) larvae. In other studies, *B. bassiana*, formerly isolated from commercially planted *P. radiata*, was found to be successfully establish in pine seedlings using both root dip and seed coating and vertically transmitted (Brownbridge et al. 2012; Lefort et al. 2016). Kim et al. (2022) also showed that *B. bassiana* can colonize on pine tree bark and the pine tree logs pre-treated with *B. bassiana* can kill *Monochamus alternatus* (pine sawyer beetle) (Hope) (Coleoptera: Cerambycidae). Ganley and Newcombe (2006) studied fungal endophytes in *P. monticola* (Douglas) needles (western white pine) using a sequence-based approach and found that *B. bassiana* was one of those fungal isolates. Apart from pine species, *B. bassiana* and some other EPFs were isolated from many forest trees as endophyte (Bills and Polishook 1991; Fisher et al. 2011; Mantzoukas and Eliopoulos 2020). In this study, we obtained 20 *B. bassiana* isolates from *P. sylvestris* needles as a possible endophyte and confirmed their pathogenicity against *D. pini*. The results obtained can provide a new dimension for the biological control of pine pests as well as other forest pests.

Many *Metarhizium* species have been identified naturally in relation to the roots of many plants (grasses, shrubs, herbs, and trees) as root endophytes under field conditions (Fisher et al. 2011; Flonc et al. 2021; Ahmad et al. 2022). In the studies conducted so far, it has been reported that 6 species in the *Metarhizium* genus (*M. anisopliae* (Metchnikoff) Sorokin, *M. robertsii* (Metchnikoff) Sorokin, *M. brunneum* Petch, *M. marquandii* (Massee) Kepler et al., *M. guizhouense* Q.T.Chen & H.L.Guo, and *Metarhizium flavoviride* var. *pemphigi* Gams & Roszypal) live as endophytes with plant roots and it has been suggested that they may have different beneficial roles such as nutrient intake in plants (Fisher et al. 2011; Branine et al. 2019; St. Leger and Wang 2020). In this study, we isolated four *M. majus* isolates from P. *sylvestris* needles as endophyte and tested their pathogenicity against *D. pini* larvae. Among four isolates, *M. majus* AD-4 caused moderately good mortality with 46.66%. This is the first study reporting pathogenicity of *M. majus* isolates as a needle endophyte of *P. sylvestris*.

Conclusion

Studying the roles of fungal endophytes in plants is of great importance since they may provide a new dimension for the biological control of plant pests and diseases in both agriculture and forestry. Apart from fungal endophytes' direct roles in plants, they have become the point of interest of biotechnology due to their potential to be used as genetic vectors, being a source of secondary metabolites and being used as biological control agents. The first step to investigate the roles of these microorganisms in plants is to isolate, characterize them, and determine their prevalence and distribution in related plants. In this study, several endophytic insect pathogenic fungi were identified from *P. sylvestris* needles, and their virulence were determined against one of the most important pine pests (*D. pini*). It is thought that the results obtained here could be useful in the control of pine diseases and especially pests. However, further studies are needed to determine experimentally endophytic colonization of these isolates in *P. sylvestris* needles. Additionally, studies about the determination of the roles of these endophytes in yellow pine are also warranted.

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Data availability All data and materials used for the research are available upon request.

Declarations

Conflict of interest The authors declare that they have no financial or personal competing interests in this paper.

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