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ORIGINAL ARTICLE

Beauveria pseudobassiana: A good candidate for controlling *Diprion pini* L. (Hymenoptera: Diprionidae)

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Abstract

The common pine sawfly, *Diprion pini* (Linnaeus, 1758) (Hymenoptera: Diprionidae), is a well-known defoliating pest of various pine forests almost all over the world, including Europe. It can cause damage to many pine species but usually opts for *Pinus sylvestris* Linnaeus and *P. nigra* subsp. *laricio* (Poiret) Maire. The prohibition of the use of chemical insecticides in forests (at least for Türkiye) has led to the fact that other control methods have come to the fore in the control of this pest. In this respect, biological control agents, which are eco-friendly and can persist in the field over time, providing long-term control for plant protection, have an important potential in the control of *D. pini*. Therefore, in this study, entomopathogenic fungi were isolated from pine forest soils and identified by gene sequencing and phylogenetic analysis. Ten isolates (DP-37, DP-38, DP-45, DP-46, DP-49, DP-53, DP-54, DP-57, DP-58 and DP-63) were identified as *Beauveria pseudobassiana*, four isolates (DP-35, DP-41, DP-52, and DP-61) were identified as *B. bassiana*, and only one isolate was identified as *Metarhizium robertsii* (DP-15). All isolates were tested against the larvae of the pest under laboratory conditions, and the highest mortality and mycosis values (96.6% and 63.3%, respectively) were obtained from *B. pseudobassiana* DP-57. This isolate was also tested against the pest under outdoor conditions using different conidial concentrations. Based on probit analysis, the LC_{50} and LC_{90} values were estimated to be 1.309×10^7 and 1.21×10^{10} conidia/ml, respectively. The results showed that *B. pseudobassiana* DP-57 could be a good candidate in the biological control of *D. pini*.

KEYWORDS
entomopathogenic fungi, microbial control, soil, the common sawfly

1 | INTRODUCTION

Diprion pini (Linnaeus, 1758) (Hymenoptera: Diprionidae), the common pine sawfly, is a species of sawfly in the family Diprionidae. This species is one of the most important pests in economic forestry and can cause defoliation of large pine forests almost all over the world (Barre et al., 2002). It can cause outbreaks resulting in considerable damage to Scots pine (*Pinus sylvestris* L.) and is also known to cause damage to some other pine species such as *P. nigra* (Arnold) (Géri, 1988; Tunca et al., 2009). Although the pest is mainly found in Europe, it has also been found in North Africa and Eastern Europe (Barre et al., 2002; Pschorn-Walcher, 1982). In the northern regions and at high altitudes, it has one generation per year, while throughout central and southern Europe, two generations are apparent annually (Novak, 1976). Generally, in regions where outbreaks occur frequently, it is recommended to monitor the populations of this fly with various sampling methods (such as sampling of egg clusters from pine twigs, collection of cocoons from vegetation or forest litter and visual inspection of larval

colonies) in early spring and to implement management when the threshold value (20 vital cocoons/m^2, six vital females/m^2 or one larval colony/tree) is exceeded (Feemers, 1997). The damage caused by this pest in pine forests varies, but in some cases, it results in the death of pine trees. In Russia, outbreaks have been reported to occur during hot and dry summers, usually at intervals of 3–6 years (Sharov, 1993). In the study of Lyytikäinen-Saarenmaa and Tomppo (2002), it was shown that the increment of Scots pine and the timber yield decreased after *D. pini* damage. In the same study, it was reported that volume growth decreased by 86% after moderate defoliation and 94% after heavy defoliation and 30% tree death estimation was reported in defoliated trees. It was also stated that the fecundity of *P. sylvestris* was significantly reduced when it was defoliated by *D. pini* in the previous year after a moderate infestation (Géri et al., 1993).

In the control of this pest, mechanical control methods such as hand collection and destruction are often recommended in cases where the larval populations are low. Apart from this, there are some biological control methods, such as the transportation of nests of the red forest ant (*Formica rufa* L. (Hymenoptera: Formicidae)), an important predator of *D. pini*, as well as hanging the nests of insectivorous birds within the forest stand (Eroğlu, 2017). Also, the experimental use of the gregarious cocoon parasitoid *Dahlbominus fuscipennis* (Zetterstedt 1838) (Hymenoptera: Eulophidae) for controlling *D. pini* was successfully performed (Schwenke, 1964). However, these methods have not yet reached sufficient effectiveness. In cases where larval populations are high, the use of chemical insecticides is applied subject to special permission (Çelik & Sevim, 2022; Eroğlu, 2017). However, the non-target impacts or ecotoxicological risks associated with the use of insecticides in forests pose possible threats to the delicate balance of forest ecosystems (Thompson, 2011; Zhou et al., 2025). Additionally, the use of chemical insecticides in Türkiye's forests is legally prohibited, and without special permission in certain cases. Considering all these, it has become imperative to use environmentally friendly and effective control methods against this pest and to conduct research in this direction (Nicolopoulou-Stamati et al., 2016).

Entomopathogenic fungi (EPFs) are pathogenic microorganisms that contain different members from Oomycota, Ascomycota, Microsporidia, Chytridiomycota, Basidiomycota and Entomophthoromycota groups and kill their hosts by infecting them. They are taxonomically distributed across different fungal phyla and the most relevant groups for insect biocontrol are Ascomycota and Entomophthoromycotina subphylum (phylum Entomophthoromycota). Some species in these groups (especially several species in Entomophthoromycotina which are known from only one host) contain only a few species with narrower host range (Goettel et al., 2005; Hong et al., 2024). Members of EPF differ from each other phylogenetically, morphologically and ecologically and have great potential in microbial control of insect pests in both agriculture and forestry (Angelo et al., 2022; Bich et al., 2021; Islam et al., 2021; Litwin et al., 2020). These microorganisms have been used for more than 100 years in the control of harmful insects in many parts of the world (Roberts, 1989). Until now, more than 700 entomopathogenic fungi species belonging to at least 90 genera have been identified, and species such as *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *Metarhizium anisopliae* (Metsch) Sorok, *Isaria fumosorosea* (Wize) Brown and Smith (formerly known as *Paecilomyces fumosoroseus* (*Wize*) *A.H.S. Br.* and *G. Sm.*) and *Lecanicillium lecanii* R. Zare & W. Gams, 2001 (previously known as *Verticillium lecanii* (Zimmerman) Viegas) can be commercially produced in many ways (Rath, 2000). EPFs can infect all developmental stages of their hosts, and some species can persist in the environment (especially in soil) for many years after application for biological control (Keçili et al., 2022; Rajula et al., 2021). Although there are many different species within this group, two species that are of the greatest commercial interest are *M. anisopliae* and *B. bassiana* (Zimmermann, 2007a, 2007b). For example, *B. bassiana* has been used against pine caterpillar (*Dendrolimus* spp.) in China and many studies have shown that these fungi could be successfully used against other forest pests such as *Thaumetopoea pityocampa* Den. & Schiff. (Lepidoptera: Thaumetopoeidae) and *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae) (Goettel et al., 2005; Sevim et al., 2013; Sevim, Demir, & Demirbağ, 2010; Sevim, Demir, Höfte, et al., 2010; Sevim, Demir, Tanyeli, & Demirbağ, 2010). In addition, there are many products which are sold in the market against many pests around the world (Ruiu, 2018). Globally, the research on the control of *D. pini* with entomopathogenic fungi is limited with only a few studies carried out at the laboratory level. Sierpińska (Sierpińska, 1998) isolated different species (mostly *Isaria farinosa* (Holmsk.) Fries) of entomopathogenic fungi from *D. pini* samples collected in Poland and found that these species caused 30%–80% mortality in wintering larvae. Tkaczuk and Mietkiewski (1998), Tkaczuk et al. (1998) investigated the infection rates of *D. pini* cocoons with entomopathogenic fungi and found that fungal infections in cocoons ranged from 12.3% to 52.2%. In these studies, *B. bassiana* has been identified as the most commonly isolated species and they detected infection rates of up to 85.2% with this fungus in some regions.

When it is thought that local isolates adapt to their environmental and climatic conditions, the use of local EPF isolates in microbial control programs may provide significant advantages over exotic EPF including the ability to survive and persist under local environmental conditions (Bilgo et al., 2018). For this purpose, in this study, we isolated different entomopathogenic fungi from yellow pine forest soils of Kırşehir, Türkiye, and identified them by gene sequencing and phylogenetic analysis. Also, the isolated fungi were initially tested against *D. pini* larvae under laboratory conditions and different doses of the selected most effective isolate (*B. pseudobassiana* DP-57) were applied to the larvae under outdoor conditions. Some isolates (especially DP-57) obtained in this study could have a good potential for use in the control of *D. pini* in future studies.

2 | MATERIALS AND METHODS

2.1 | Collection of soil samples

To find effective fungal biocontrol agents against *D. pini*, a total of 78 soil samples were collected from 13 different regions (six sites

TABLE 1 Entomopathogenic fungal species isolated from yellow pine forests in Kırşehir, Türkiye, and their locality, GPS coordinates and GenBank accession numbers.

			Locality			GenBank accession numbers		
No	Isolate	Species	City	County	Geographic coordinates	*ITS*	*Bloc*	*β-tubulin*
1	DP-15	*Metarhizium robertsii*	Kırşehir	City centre	39°09′10.3″ N 34°14′33.6″ E	OR294274	–	OR338289
2	DP-35	*Beauveria bassiana*	Kırşehir	City centre	39°10′56.7″ N 34°15′00.3″ E	OR294275	OR338290	–
3	DP-37	*B. pseudobassiana*	Kırşehir	City centre	39°11′33.9″ N 34°14′32.7″ E	OR294276	OR338291	–
4	DP-38	*B. pseudobassiana*	Kırşehir	City centre	39°12′00.1″ N 34°13′54.8″ E	OR294277	OR338292	–
5	DP-41	*B. bassiana*	Kırşehir	City centre	39°11′16.4″ N 34°16′45.1″ E	OR294278	OR338293	–
6	DP-45	*B. pseudobassiana*	Kırşehir	City centre	39°12′23.9″ N 34°12′28.3″ E	OR294279	OR338294	–
7	DP-46	*B. pseudobassiana*	Kırşehir	City centre	39°12′44.1″ N 34°12′59.0″ E	OR294280	OR338295	–
8	DP-49	*B. pseudobassiana*	Kırşehir	City centre	39°13′13.4″ N 34°13′25.5″ E	OR294281	OR338296	–
9	DP-52	*B. bassiana*	Kırşehir	City centre	39°12′48.9″ N 34°12′15.6″ E	OR294282	OR338297	–
10	DP-53	*B. pseudobassiana*	Kırşehir	City centre	39°13′40.6″ N 34°11′42.7″ E	OR294283	OR338298	–
11	DP-54	*B. pseudobassiana*	Kırşehir	City centre	39°14′22.8″ N 34°12′59.4″ E	OR294284	OR338299	–
12	DP-57	*B. pseudobassiana*	Kırşehir	City centre	39°12′00.2″ N 34°13′04.8″ E	OR294285	OR338300	–
13	DP-58	*B. pseudobassiana*	Kırşehir	City centre	39°12′10.9″ N 34°13′08.7″ E	OR294286	OR338301	–
14	DP-61	*B. bassiana*	Kırşehir	City centre	39°12′31.3″ N 34°13′11.0″ E	OR294287	OR338302	–
15	DP-63	*B. pseudobassiana*	Kırşehir	City centre	39°13′15.2″ N 34°13′56.1″ E	OR294288	OR338303	–

for each region) in Kırşehir (located in Central Anatolia, Türkiye) (Table 1). Soil samples were taken randomly from each region in pine forests and were obtained at a depth of approximately 5–20 cm after the top of the soil was removed. A hoe was used to collect soil samples and was sterilized with 70% alcohol after each collection. During the soil collection from a region, sampling was done from five different points (at least 5 m between them) and after mixing the collected soils in a plastic bag, approximately 750 g of soil sample from the mixture was placed in polyethylene bags, brought to the laboratory and examined for the presence of entomopathogenic fungus within approximately 7 days after collection (Meyling, 2007). Soil samples that were not analysed were kept at +4°C in case they were needed in the future.

2.2 | Insect bait method

The *Galleria* bait method was used to isolate entomopathogenic fungi from the soil samples with minor modifications (Meyling, 2007; Zimmermann, 1986). The third or fourth instar *D. pini* larvae were used as bait insects. Each soil sample was placed in a 1 L plastic container, and 20 *D. pini* larvae were added. If the soil was too dry, sterile water was added to provide the necessary moisture. After that, the containers were sealed and inverted to keep the larvae under the soil. The containers were then kept at room temperature (20–25°C) and, during the first 2 weeks, were inverted frequently to ensure maximum contact of insects with the soil. Finally, the containers were examined within 20 days at 5-day intervals and fungal isolation was done from the dead and mycosed larvae (Meyling & Eilenberg, 2006; Sevim, Demir, Höfte, et al., 2010).

Entomopathogenic fungal isolation from larval samples showing external mycelial growth was done using an inoculation loop and PDAY (potato dextrose agar + yeast extract (1%)) medium. To prevent bacterial growth, ampicillin (100 μg/mL), tetracycline (50 μg/mL) and streptomycin (100 μg/mL) were added to the medium after cooling to 50°C (Sevim, Demir, & Demirbağ, 2010). All fungal isolates were purified from a single conidium and stored in 15% glycerol at −20°C.

2.3 | Fungal identification

The obtained fungi were identified according to morphological and molecular techniques. Morphological identification was performed according to the identification key described by Humber (1997). Species identity was also molecularly confirmed using various gene sequences. For this, genomic DNA extraction from the fungal isolates was performed with the E.Z.N.A. Soil DNA kit (OMEGA-BIO-TEK). The extracted DNA samples were preserved at −20°C for future studies. After DNA extraction, the ITS gene region including ITS1, 5.8S rRNA and ITS2 was amplified by PCR using the universal primer pairs of ITS5: 5′-GGAAGTAAAAGTCGTAACAAGG-3′ as forward and ITS4: 5′-TCCTCCGCTTATTGATATCG- 3′ as reverse and sequenced for all isolates (White et al., 1990). The PCR conditions were adapted and optimized based on the study of Sevim et al. (2022).

In addition, the *Bloc* gene region (approximately 1571 bp length) was amplified and sequenced for all *Beauveria* isolates using the oligonucleotide primers of B5.1F (5′-CGACCCGGCCAACTACTTTGA-3′) as forward and B3.1R (5′-GTCTTCCAGTACCACTACGCC-3′) as reverse. PCR conditions were adapted according to the study of Rehner et al. (2006). Moreover, the *β-tubulin* gene region (approximately 1400 bp)

for *Metarhizium* isolates was amplified and sequenced using the primer pairs of T1 (5′-AACATGCGTGAGATTGTAAGT-3′) as forward and T2 (5′-TCTGGATGTTGTTGGGAATCC-3′) as reverse and PCR conditions were adapted according to the study of O'Donnell and Cigelnik (1997).

PCR products were sent to MACROGEN (Netherlands) for sequence analysis. The obtained DNA sequences were initially compared with the known DNA sequences in the NCBI GenBank using BLAST search to verify the species identification (Benson et al., 2012). Finally, *Beauveria* isolates were phylogenetically compared with the reference isolates mentioned in the study of Rehner et al. (2011) and *Metarhizium* isolates were compared with the reference isolates in the study of Bischoff et al. (2009).

2.4 | Screening tests against *Diprion pini*

The mortality of the fungal isolates against *D. pini* larvae under laboratory conditions was determined. We particularly chose the larval stage of the pest for experiments considering the life cycle of the pest and ease of application, and also because only larvae actively feed on needles. For this, 100 µL conidial suspension from a stock solution of 1×10^6 conidia/ml of the fungal isolates was spread on PDAY and incubated at 28°C for 2–3 days. At the end of the growth period, single colonies were selected and transferred to another PDAY and incubated at 28°C for 4 weeks. At the end of the incubation period, 10 mL sterile 0.01% Tween 80 was added to each Petri dish and the conidia were provided to pass into the water by scraping them with glass baguette. After that, the conidial suspensions were filtered into 50 mL sterile Falcon tubes using a sterile two-layer cheesecloth to remove unwanted mycelia and agar pieces. The obtained suspensions were vortexed for 5 min to homogenize conidial suspensions, and the conidial concentrations were adjusted to 1×10^7 conidia/ml with a Neubauer haemocytometer. The viability of conidia was tested by spreading 100 µL conidial suspension on PDAY and determining germination after 24 h incubation. Germination was considered successful if the germ tube length was greater than the diameter of the conidia. As a result, the conidia that germinate 90% or over were used in mortality tests (Sevim, Demir, & Demirbağ, 2010).

The prepared conidial suspensions were used in mortality tests against *D. pini* larvae. The larvae collected from pine forests in Kırşehir and its surrounding regions were used in tests. Ten larvae were used for each repetition in mortality tests, and all experiments were repeated three times. Needles and fresh shoots required for mortality tests as a nutrition source for larvae were collected from forests and provided to larvae just at the beginning of the incubation period. All tests were carried out at room temperature under 12:12 photoperiod. 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 (3rd or 4th instar) were placed in plastic boxes (20 × 20 cm) and 2 mL of the conidial concentration of 1×10^7 conidia/ml for each isolate was applied to larvae with an aerosol type sprayer (airbrush). Freshly collected pine needles and shoots were used as food and were changed daily according to the situation. The control group was inoculated with 2 mL of only sterile 0.01% Tween 80 using an aerosol type sprayer (airbrush). *Metarhizium robertsii* ARSEF 2575 isolated from *Curculio caryae* (Coleoptera: Curculionidae) in South Carolina, USA, was obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (RW Holley Center for Agriculture and Health, Ithaca, NY) and used as exotic isolate. After inoculation, all boxes were left to incubate for 20 days. The percentage mortality was calculated at the end of the 20-day incubation period. The percentage mycosis values were also calculated to determine whether the cause of death was fungus or not. For this, dead larvae were surface sterilized with 1% sodium hypochlorite solution for 3 min, then rinsed with sterile distilled water three times, placed in sterile Petri dishes with moist filter paper and left to incubate at 2°C in the dark (Sevim, Demir, Tanyeli, & Demirbağ, 2010). The samples with external fungal growth for the isolate in question were considered as mycosed.

2.5 | Outdoor pot experiments

According to the initial mortality tests, the isolate *B. pseudobassiana* DP-57 was selected for the outdoor test since it had the highest mortality and mycosis percentage (Figure 3). For the outdoor test, conidial suspensions were prepared as described above. *D. pini* larvae collected from yellow pine plantations in Kırşehir, and its surrounding regions were used in tests. The collected larvae were brought to the laboratory on the same day, and the healthy ones showing no sign of disease symptoms were used. In the outdoor tests, yellow pine saplings (average 5–6 years old) which were pre-transplanted to pots received ~100 larvae each and three replicate pots were established for each conidial concentration ($n = 18$ pots). Larvae were directly added to pine foliage. Later, the conidial suspensions (1×10^4, 1×10^5, 1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia/ml) were applied to the larvae for 10 s (roughly 40 mL) using the aerosol type sprayer. Only sterile 0.01% Tween 80 (roughly 40 mL) was applied to the control group which had three replicates also. The saplings were watered once to the pot capacity at the beginning of the experiment with tap water. After inoculation, the upper part of each sapling was wrapped with a sterile cheesecloth to prevent the larvae from escaping and to protect them from other factors. Then, all saplings were left to incubate for 20 days under outdoor conditions, after which the dead larvae were counted, and the percentage death based on Abbott's formula was calculated for each concentration (Abbott, 1925). These values were used in the calculation of LC_{50} (the concentration of conidia causing death in 50% of test larvae) and LC90 (the concentration of conidia causing death in 90% of test larvae) values by probit analysis. Weather data during the date range when pot experiments were performed was obtained from https://www.timeanddate.com/ and the average temperature and relative humidity values were 22.65°C ± 4.01 (18–32°C) and 56.45% ± 10.35% (23%–76%), respectively.

TABLE 2 Per cent identity of the isolated entomopathogenic fungi with their most related fungal species/isolates according to BLAST search in GenBank using ITS gene sequences and their GenBank ID numbers (Benson et al., 2012).

Isolate	GenBank ID number for ITS	The most related species	GenBank ID number	Query coverage (%)	Identity (%)
DP-15	OR294274	*Metarhizium anisopliae* isolate Eg18	MK942509	100%	99.61%
		Metarhizium anisopliae strain CBS 127632	MH864645	100%	99.61%
		Metarhizium sp. isolate W11LA	MF872441	100%	99.61%
		Metarhizium sp. isolate G3LB	MF872437	100%	99.61%
DP-35	OR294275	*Beauveria bassiana* isolate SBI-Bb04	MT586632	100%	99.82%
		Beauveria bassiana isolate SBI-Bb03	MT584885	100%	99.82%
		Beauveria bassiana isolate SBI-Bb01	MT584878	100%	99.82%
		Beauveria bassiana clone EF_102	MT528751	100%	99.82%
DP-37	OR294276	*Beauveria pseudobassiana* isolate B3	MH374534	100%	99.63%
		Beauveria pseudobassiana isolate E1067	MH165262	100%	99.63%
		Beauveria pseudobassiana isolate E1175	MH165261	100%	99.63%
		Beauveria pseudobassiana isolate E1083	MH165257	100%	99.63%
DP-38	OR294277	*Beauveria pseudobassiana* isolate E1080	MH165260	99%	100%
		Beauveria pseudobassiana strain E51	MH259852	100%	99.82%
		Beauveria pseudobassiana strain BB36	MH185851	100%	99.82%
		Beauveria pseudobassiana strain ANKO38	MH185843	100%	99.82%
DP-41	OR294278	*Beauveria bassiana* isolate EABb07_06Rf	KC753375	100%	99.82%
		Beauveria bassiana isolate ArgB10	KT378227	100%	99.63%
		Beauveria bassiana isolate SASRI BWR1	JX110376	100%	99.63%
		Beauveria bassiana isolate 2486	EU821491	100%	99.63%
DP-45	OR294279	*Beauveria pseudobassiana* isolate E1080	MH165260	100%	100%
		Beauveria pseudobassiana strain E51	MH259852	100%	99.82%
		Beauveria pseudobassiana strain BB36	MH185851	100%	99.82%
		Beauveria pseudobassiana strain BB41	MH185846	100%	99.82%
DP-46	OR294280	*Beauveria bassiana* strain BXY01	KU214583	99%	99.81%
		Beauveria pseudobassiana isolate E1175	MH165261	99%	99.81%
		Beauveria bassiana isolate GC07	JN379794	99%	99.81%
		Beauveria pseudobassiana strain E51	MH259852	99%	99.81%
DP-49	OR294281	*Beauveria pseudobassiana* isolate E1175	MH165261	100%	99.26%
		Beauveria bassiana isolate GC07	JN379794	100%	99.26%
		Beauveria pseudobassiana strain E51	MH259852	99%	99.26%
		Beauveria pseudobassiana strain BB36	MH185851	99%	99.26%
DP-52	OR294282	*Beauveria* sp. voucher JLF7482	MT101858	98%	99.61%
		Fungal sp. isolate OTU42	MK415879	98%	99.61%
		Beauveria pseudobassiana isolate C5	MK142275	98%	99.80%
		Beauveria tenella strain CBS	MH864341	98%	99.61%
DP-53	OR294283	*Beauveria pseudobassiana* isolate E1175	MH165261	100%	99.45%
		Beauveria bassiana strain BXY01	KU214583	100%	99.45%
		Beauveria bassiana isolate GC07	JN379794	100%	99.45%
		Beauveria pseudobassiana strain E51	MH259852	99%	99.44%
DP-54	OR294284	*Beauveria pseudobassiana* strain E51	MH259852	100%	99.63%
		Beauveria pseudobassiana strain BB36	MH185851	100%	99.63%
		Beauveria pseudobassiana strain ANKO38	MH185843	100%	99.63%
		Beauveria pseudobassiana isolate E1175	MH165261	100%	99.63%
DP-57	OR294285	*Beauveria pseudobassiana* isolate E1175	MH165261	100%	99.82%
		Beauveria bassiana isolate GC07	JN379794	100%	99.82%

(Continues)

TABLE 2 (Continued)

Isolate	GenBank ID number for ITS	The most related species	GenBank ID number	Query coverage (%)	Identity (%)
		Beauveria pseudobassiana strain E51	MH259852	99%	99.82%
		Beauveria pseudobassiana strain BB36	MH185851	99%	99.82%
DP-58	OR294286	*Beauveria bassiana* isolate GC21	JN379808	100%	100%
		Beauveria sp. voucher JLF7482	MT101858	99%	100%
		Fungal sp. isolate OTU42	MK415879	99%	100%
		Beauveria tenella strain CBS 126934	MH864341	99%	100%
DP-61	OR294287	*Beauveria bassiana* isolate EABb07_06Rf	KC753375	100%	100%
		Beauveria bassiana isolate KJ4	KY515369	100%	99.81%
		Beauveria bassiana isolate KJ1	KY515355	100%	99.81%
		Beauveria bassiana isolate TS5	KY515351	100%	99.81%
DP-63	OR294288	*Beauveria pseudobassiana* isolate E1175	MH165261	100%	100%
		Beauveria bassiana isolate GC07	JN379794	100%	100%
		Beauveria pseudobassiana strain E51	MH259852	99%	100%
		Beauveria pseudobassiana strain BB36	MH185851	99%	100%

2.6 | Data analysis

All DNA sequences obtained from the fungal isolates were edited with the BioEdit 7.09 (Hall, 1999). For ITS sequences, the percentage similarities of the fungal isolates with their most related fungal species or isolates were determined using BLAST search in GenBank database (Altschul et al., 1990; Benson et al., 2012). The ClustalW software packed in BioEdit was used to perform cluster analysis of the DNA sequences. The obtained data from here was used in neighbour-joining (NJ) analysis in MEGA 11.0.10 to construct phylogenetic trees (Tamura et al., 2021). Alignment gaps were considered as missing data. The reliability of the generated phylograms was tested by bootstrap analysis using the MEGA 11.0.10 based on 1000 pseudoreplicates.

Abbott's formula was used to correct for natural mortality (Abbott, 1925). Also, the per cent mycosis values were calculated as described above. Analysis of variance (one-way ANOVA) was used to compare the fungal isolates with respect to mortality and mycoses. LSD multiple comparison test as *post hoc* was used for all pairwise comparisons. All data were tested with respect to variance homogeneity using Levene statistics, and all percentage data were subjected to arcsine transformation to stabilize variances before statistical analysis. LC_{50} and LC_{90} values were calculated by probit analysis. All data were analysed using SPSS 16.0 statistical software.

3 | RESULTS

A total of 78 soil samples were collected and 15 fungal isolates were obtained from these soil samples (Table 1). About 19.23% of the samples were found to be positive with respect to the presence of entomopathogenic fungi. The isolated fungi were first morphologically identified, and it was found that all of them belonged to *Beauveria*, except for DP-15, which was *Metarhizium*. Sequencing of the ITS region confirmed this (Table 2). Sequencing of the *Bloc* region further identified these as *B. pseudobassiana* (DP-37, DP-38, DP-45, DP-46, DP-49, DP-53, DP-54, DP-57, DP-58 and DP-63) and *B. bassiana* (DP-35, DP-41, DP-52 and DP-61) (Figure 1). In addition, sequencing of the *β-tubulin* region further identified the isolate DP-15 as *M. robertsii* (Figure 2).

3.1 | Mortality of *Diprion pini* larvae

All isolates caused different mortality values in comparison with each other ($F(16, 34) = 33.16$, $p < .05$). The highest mortalities were obtained from *B. pseudobassiana* DP-46 ($M = 90$, $SD = 10$) and DP-57 ($M = 96.67$, $SD = 5.77$) with 90% and 96.6%. All isolates were also significantly different from the control group, except for DP-45 ($M = 0$, $SD = 0$), DP-49 ($M = 0$, $SD = 0$), DP-52 ($M = 0$, $SD = 0$) and DP-58 ($M = 0$, $SD = 0$) (Figure 3). Also, all isolates caused different mycosis values in comparison with each other ($F(16, 34) = 10.31$, $p < .05$). The highest mycosis value was obtained from *B. pseudobassiana* DP-57 with 63.3%, and it was significantly different from the control ($M = 63.33$, $SD = 15.27$). The second highest mycosis which was significantly different from the control was obtained from *B. bassiana* DP-35 with 33.3% ($M = 33.33$, $SD = 5.77$).

LC_{50} value for *B. pseudobassiana* DP-57 in the outdoor tests was estimated as 1.309×10^7, and LC_{90} value was estimated as 1.21×10^{10} conidia/ml for larvae (Table 3).

4 | DISCUSSION

Entomopathogenic fungi have been successfully used for many years in the control of insect pests in both agriculture and forestry.

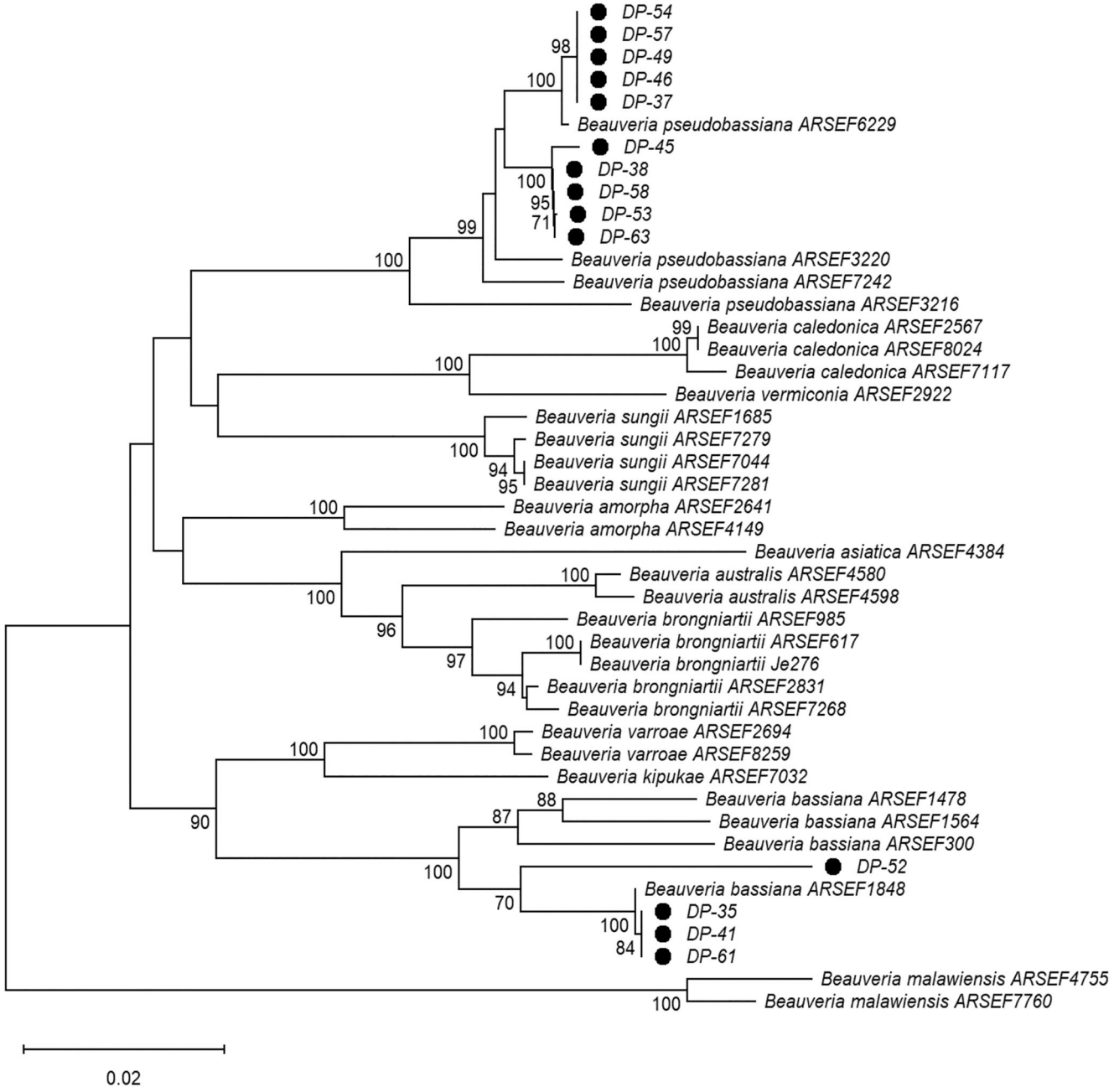

FIGURE 1 Phylogeny of *Beauveria* isolates inferred from neighbour-joining (N–J) analysis with p-distance correction using the *Bloc* gene region. The reference species/isolates were labelled according to the USDA-ARS Collection of Entomopathogenic Fungi (ARSEF) accession numbers and taken from the study of Rehner et al. (2011). Bootstrap values above 70% (percentages of 1000 bootstrap replicates) are indicated above the branches of the tree. *Beauveria* isolates from this study were indicated by the solid black circle.

Considering the negative effects of chemical insecticides on human and environmental health, entomopathogenic fungi can be considered good candidates as sustainable biological control agents against insect pests, especially in forests. Recently, it has been shown that *D. pini* had a widespread distribution in the study area (Kırşehir, Türkiye), causing serious damage to yellow pine trees. This pest also causes serious economic damage to other pine forests in Türkiye, and we believe that the fungal isolates obtained in this study may have a good potential against pine pests in Türkiye, especially *D. pini*.

Entomopathogenic fungi can frequently be isolated from soil samples by various methods (such as bait insect and the use of selective media) and soil is a suitable habitat for these fungi, allowing them to maintain their viability for many years (Meyling & Eilenberg, 2007). The studies so far have shown that these fungi were isolated from soil samples belonging to agriculture, forests and some other habitats and the isolation rate varies according to studies, and it seems that there is no general rule regarding this rate (Imoulan et al., 2011; Keller et al., 2003; Meyling & Eilenberg, 2007; Sevim, Demir, Höfte,

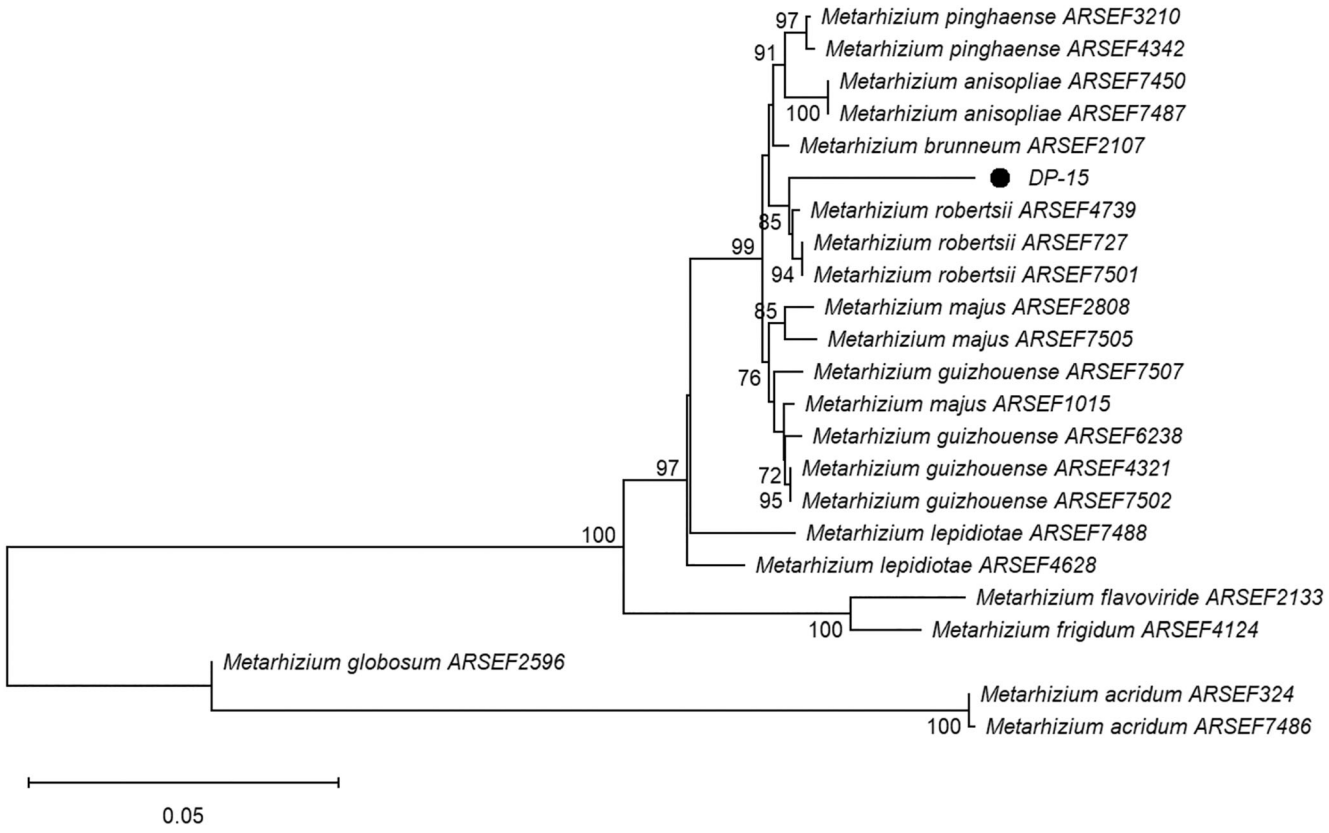

FIGURE 2 Phylogeny of the isolate DP-15 inferred from neighbour-joining (N–J) analysis with p-distance correction using the *β-tubulin* gene region. The reference species/isolates were labelled according to the USDA-ARS Collection of Entomopathogenic Fungi (ARSEF) accession numbers and taken from the study of Bischoff et al. (2009). Bootstrap values above 70% (percentages of 1000 bootstrap replicates) are indicated above the branches of the tree. The isolate DP-15 was indicated by the solid black circle.

et al., 2010). In addition, the isolation rate and the species diversity of entomopathogenic fungi from soil samples vary according to the soil and habitat type, geographical origin, the isolation method and the species of bait insect (Meyling & Eilenberg, 2007; Sharma et al., 2018). Some studies have shown that the genus *Metarhizium* is more common in soils associated with agricultural areas, while the genus *Beauveria* is more predominant in forested soils (Augustyniuk-Kram & Kram, 2012; Bidochka et al., 1998; Keller et al., 2003; Kovac et al., 2021; Majchrowska-Safaryan & Tkaczuk, 2021; Meyling & Eilenberg, 2006; Meyling & Eilenberg, 2007). In our study, a total of 15 entomopathogenic fungi were isolated from 78 soil samples from yellow pine forests (19.23% the isolation rate) by the *Galleria* bait method (*D. pini* as bait insect) and 14 of them were included in the genus *Beauveria* (mostly *B. pseudobassiana*), while only one isolate was identified as *Metarhizium*. Although our results are consistent with the literature in terms of species diversity, the isolation rate seems to be slightly lower. The reason for this is thought to be the use of a direct target pest larvae (*D. pini* in this case) as bait insect. Because traditional bait insects used for EPF isolation from soils such as *Galleria mellonella* L. (Lepidoptera: Pyralidae) and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) could be more susceptible to fungal infections than *D. pini* because of adaptations that the pest has developed naturally to local host species (Dubovskiy et al., 2013).

The fungal isolates tested in this study showed different levels of mortality on *D. pini* larvae in a single-dose application (1×10^7 conidia/ml) in the laboratory. Several isolates caused mortality of 50% or more. The degree of mycosis on the larvae differs between isolates. The most effective isolate selected for outdoor testing (*B. pseudobassiana* DP-57) caused 96.6% mortality and 63.3% mycosis in the initial screening test when applied directly to larvae. The field effectiveness of entomopathogenic fungi is affected by many biotic and abiotic factors, and therefore the field or outdoor application is of great importance in testing the efficacy of any biocontrol agent (Jaronski, 2010).

The studies on the biological control of *D. pini* using entomopathogenic fungi are very limited. The studies done so far have aimed only at determining the natural rate of infection in wintering larvae and cocoons, and there have been no studies to determine the biological control potential of entomopathogenic fungi against the pest (Sierpińska, 1998; Tkaczuk et al., 1998; Tkaczuk & Mietkiewski, 1998). However, some studies showed that entomopathogenic fungi have a great potential in the control of many forest pests belonging to different insect orders in field applications. Liu and Bauer (2008) tested the lethal effect of the commercial formulation of *B. bassiana* GHA on *Agrilus planipennis* (Coleoptera: Buprestidae) adults and larvae in the greenhouse and field. They showed that the efficacy of *B. bassiana*

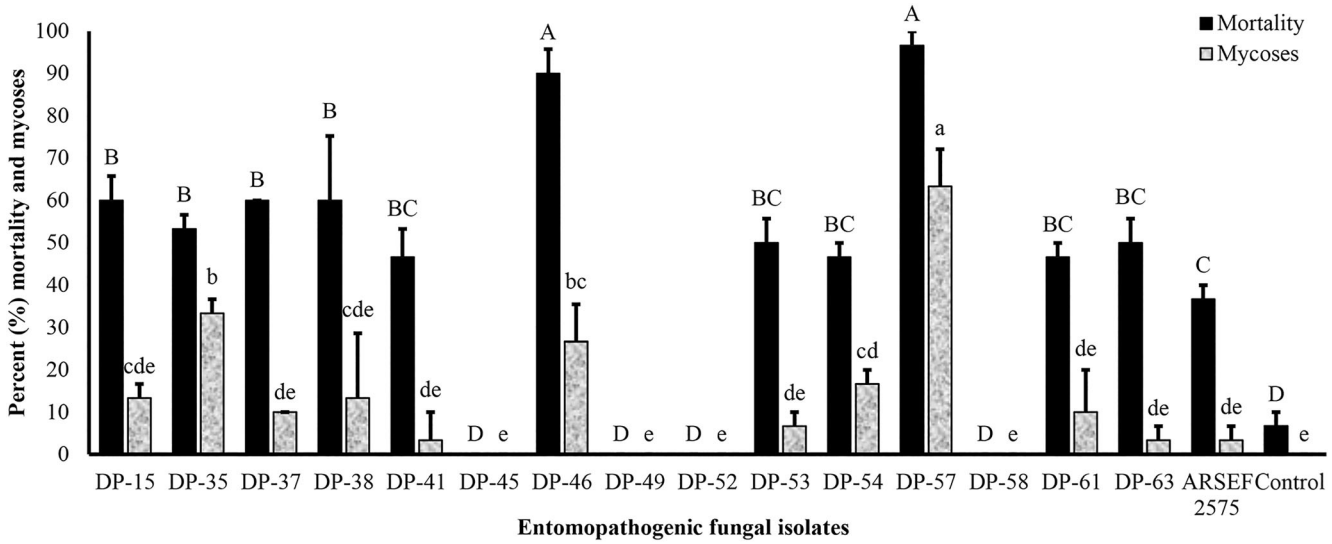

FIGURE 3 Per cent mortality and mycosis values (± SE) of the fungal isolates obtained from forest soils against 3rd or 4th instar larvae of *Diprion pini* within 20 days. Conidial concentration of 1×10^7 conidia/mL was applied to the larvae for each isolate. Abbott's formula was used to calculate for natural mortalities (Abbott, 1925). The same capital and lowercase letters indicate no significant difference between treatments in mortality and mycosis levels, respectively according to LSD multiple comparison test ($p < .05$). *Metarhizium robertsii* ARSEF 2575 was used as exotic isolate. 0.01% Tween 80 was used as negative control.

TABLE 3 Summary of probit analysis parameters from the multiple concentration bioassays performed with *Beauveria pseudobassiana* isolate DP-57 against *Diprion pini* larvae in outdoor pot experiments.

Development stage	Intercept ± SE	Slope ± SE[a]	LC_{50} (95% fiducial limits)	LC_{90} (95% fiducial limits)	Pearson goodness of fit test[b]		
					χ^2	*Df*	*p*
Larvae	−3.075 ± 0.453	0.432 ± 0.066	1.309×10^7 (4.43×10^6–4.64×10^7)	1.21×10^{10} (1.596×10^9–4.164×10^{11})	0.52	4	>.05

[a]Slope of the concentration ± standard error response of larvae of *D. pini* to *B. pseudobassiana* isolate DP-57.
[b]Pearson chi-square goodness-of-fit test on the probit model ($p < .05$) indicated a significant fit between the observed and expected regression models.

GHA was improved in the field application by spraying directly on trunk surfaces before adult emergence. The adult infection rates in the field ranged from 58.5% and 83% at two application rates of 10 and 100×10^{13} conidia/ha. Lalık et al. (2021) evaluated the effectiveness of *B. bassiana* against *Hylobius abietis* (Coleoptera: Curculionidae) under laboratory, semi-natural and field conditions. In the field application, they used a carrier which was produced from a mixed material (flour, cereal brands and egg) and on which the fungus can grow and sporulate, producing about $1.7 \times 10^9 \pm 0.4 \times 10^9$ conidia. They determined the mortality rate up to 58% for adults trapped in the plot with carriers which were placed on pieces of trap bark. In addition, the efficacy of *B. bassiana* on *Dendroctonus ponderosae* (Hopkins) (Coleoptera: Curculionidae), an important pest of *Pinus contorta* in Canada, was tested in field experiments. The treatment with a high dose (1×10^9 cfu/cm^2) resulted in shorter larval galleries and significantly reduced offspring production (Fernandez et al., 2023). The above-mentioned studies were generally aimed at determining the effectiveness of *B. bassiana* against forest pests with true field application where the EPFs were applied to the tree system. In our study, the fungus was directly applied to the larvae which were on saplings under outdoor conditions. Our preliminary field evaluation suggests that EPFs (especially DP-57) may have good potential as sustainable biocontrol agents in the control of *D. pini*. Additional studies under more realistic field conditions and application scenarios will help us to evaluate the full effectiveness of these fungi against *D. pini*.

The LC_{50} and LC_{90} values for *B. pseudobassiana* DP-57 were estimated to be 1.309×10^7 and 1.21×10^{10} conidia/ml, respectively, against larvae of the pest under outdoor conditions. *B. pseudobassiana* was described as a new species in the genus *Beauveria* in 2011 (Rehner et al., 2011), and there are currently no commercial products based on this species that can be used to control forest pests in any country. However, we think that *B. pseudobassiana* DP-57 tested in this study has a good potential in the biological control of *D. pini* and at least it may be further investigated for commercial product development with future studies such as mass production and formulation.

The use of local isolates in the biological control of insect pests is of great importance since they can be evolutionarily adapted to the geographical region, habitats, local climatic, environmental conditions

and host insects (Bayman et al., 2021; Bidochka et al., 2002; Fernandes et al., 2009; Maurer et al., 1997; Sevim et al., 2012). These adaptations increase the survival and persistence abilities of native isolates under local environmental conditions (Islam et al., 2023; Sain et al., 2019). In addition, it is always desirable to search new biocontrol agents to find more effective and safer agents and, in this way, the control of target insect pest with local fungal isolates can be effectively addressed considering insecticide resistance and renascence issues (Islam et al., 2023). In this study, one exotic isolate (*M. robertsii* ARSEF 2575) was used against larvae of the pest under laboratory conditions, and it did not show good efficacy (36.6% mortality). Entomopathogenic fungi, even within the same species, show the natural variation of virulence against the target pest (Sutanto et al., 2021). For all these reasons, it is important to study the natural variation of virulence of local isolates against the target pests since this seems to be a high impact on the choices for selecting the optimal fungal agents (Valero-Jiménez et al., 2014). Hereby, the local isolates obtained from this study (especially *B. pseudobassiana* DP-57) should be good candidates for controlling *D. pini* and it is believed that this isolate will also increase the chances of success against the pest, considering all these factors.

All isolates and one exotic isolate (*M. robertsii* ARSEF 2575) were screened against the *D. pini* larvae in the laboratory, and the most effective isolate was determined to be *B. pseudobassiana* DP-57 according to the mortality and mycosis values. It was further investigated by testing against the pest in outdoor conditions. As a result, the isolate DP-57 seems to be good candidate in the control of *D. pini* considering its outdoor efficacy and local origin. However, further studies are needed to determine the efficacy of DP-57 in field trials in which the fungus is applied on larger trees infested with the larvae using the same equipment that pest managers would use. Moreover, the susceptibility of DP-57 to certain environmental factors and predisposition to mass production and formulation should be investigated. The detailed horizontal transmission studies between larvae are also warranted.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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