Sakarya University Journal of Science

ISSN : 2147-835X
Publisher : Sakarya University

Vol. 28, No. 2, 381-391, 2024
DOI: https://doi.org/10.16984/saufenbilder.1382917

Research Article

Morphological, Molecular Identification and Virulence of Entomopathogenic Fungi Isolated From *Dendroctonus micans* (Kugelann,1794) (Coleoptera: Curculionidae)

Seda Biryol[1*], Ali Soydinç[2], Sevda İşık[2]

[1] Trabzon University, Tonya Vocational School, Medical Laboratory Techniques, Trabzon, Türkiye, sedabiryol@trabzon.edu.tr
[2] Trabzon Regional Directorate of Forestry, Trabzon, Türkiye, alisoydinc@ogm.gov.tr, sevdaisik@ogm.gov.tr
[*]Corresponding Author

ARTICLE INFO

Keywords:
Dendroctonus micans
Entomopathogenic fungus
Metarhizium
Microbiology
Mycology

Article History:
Received: 31.10.2023
Accepted: 26.12.2023
Online Available: 24.04.2024

ABSTRACT

In this study, to determine an effective fungal agent against *Dendroctonus micans* (Kugelann, 1794) (Coleoptera: Curculionidae), which causes significant economic losses in forested areas, *Picea orientalis* (L.) Link in Artvin between 2021-2022. *Dendroctonus micans* larvae and adults were collected from the trees, and 18 fungi were isolated from larvae and adult insects. Morphological (infection type, colony morphology, spore form) and molecular (ITS1-5.8S ITS2 gene region) characterization determined that the isolates were *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae), *M. robertsii*, *M. pinghaense* and *Clonostachys rosea* Samuels & Rossman, 1999 (Hypocreales: Bionectriaceae). Isolates *M. robertsii* (OZM4) and *M. pinhaense* (OZM9) have been isolated from this pest for the first time. As a result of insecticidal activity tests performed on *D. micans* larvae and adults of $1x10^7$spore/ml spore suspension, the larvae, *M. anisopliae* (OZM2), showed a mortality rate of 92% within 7 days and adults mortality was determined 100% at the end of the experiment, and mycosis rates were found to be consistent with mortality rates. These results show that isolates with high virulence are promising in microbial and integrated control applications against important forest pests.

1. Introduction

Bark beetles (Coleoptera: Curculionidae, Scolytinae) are among the most harmful forest beetles in North America and Europe and settle on the host tree via aggregation pheromones establish brood systems in the phloem layer, resulting in tree death [1- 3]. According to Armendáriz-Toledano et al. [4], twenty species of the genus *Dendroctonus* have been recognized as having a significant negative impact on conifer forests [5]. Invasion pest *Dendroctonus micans* (Kugelann,1974) (Coleoptera: Curculionidae), sometimes known as the great spruce bark beetle, has expanded throughout practically all of Türkiye *Picea orientalis* (L.) Link. forests, where it aggressively damages numerous trees [6].

Dendroctonus (Coleoptera: Curculionidae) species, which especially like to attack living trees, colonize the tree and can kill all or part of their hosts due to their aggressive behavior during gallery formation [7, 8], and when the population reaches significant levels, they cause widespread tree death [9]. Changes in forest populations (abiotic and biotic) are known to impact species population dynamics. Climate change, degraded forest structure, water stress and drought, and other abiotic factors all contribute to increased bark beetle outbreaks in spruce forests [10- 12].

Chemical, biological, and physical control (or a mix of these approaches) are popular methods for managing various sorts of insects. Initially,

Cite as: Biryol S., Soydinç A., İşık S. (2024). Morphological, Molecular Identification and Virulence of Entomopathogenic Fungi Isolated From Dendroctonus micans (Kugelann,1794) (Coleoptera: Curculionidae), Sakarya *University Journal of Science, 28*(2), 381-391. https://doi.org/10.16984/saufenbilder.1382917

certain management measures were employed to control this pest, such as the using chemical pesticides and mechanical approaches, but effective results were not attained [13]. Several studies have focused on the pathogens and parasitoids of bark beetles searching for effective biocontrol agents in some parts of the world and Türkiye lately [14- 19].

Entomopathogenic fungi are parasitic microorganisms capable of infecting and killing arthropods. They are primarily employed in ecological farming as biopesticides as a safer alternative to hazardous chemical insecticides [20]. Around 750 entomopathogenic fungal species are identified, but the most researched and economically produced are *Beauveria bassiana* (Bals. -Criv.) Vuill. (Ascomycota: Hypocreales) and *Metarhizium anisopliae* (Metschn.) Sorokin, 1883 (Hypocreales: Clavicipitaceae) [21].

The average summer temperature in the Eastern Black Sea Region is 25-30°C, with a RH of 70-80%. At the levels of temperature and humidity explained above, entomopathogenic fungus (EPF) development produces the most the ideal environment for virulence, germination, and continuance. Therefore, entomopathogenic fungi have the potential to be used especially in microbial control against bark pests. The primary objective of this study is to identify the EPF related to *D. micans* and investigate their pathogenicity in adults and larvae in the laboratory.

2. General Methods

2.1. Insect samples

Between 2021 and 2022, *D. micans* larvae and adults were collected from *Picea orientalis* (L.) Link trees in Artvin were obtained from an ax to open tunnels beneath the bark and transferred to the laboratory. They were subsequently put in 20 x 20 cm plastic cages and fed spruce bark for 2-3 days at room temperature. Pest larvae and adults, which were assumed to be signs of infection other than a natural death, were tested regularly for fungal infestations.

2.2. Isolation of fungal strains

Insects collected as cadavers in the forest or died in the laboratory while growing on spruce branches were studied to see whether the fungal infection was the cause of death. To induce mycosis, surface sterilized cadavers were cultured in Petri dishes and kept in an incubator under 25°C for 1-2 weeks to promote fungal growth (Figure 1) [22]. Transfer affected insects to PDAY (Potato dextrose agar + 1% Yeast extract) (Sigma, USA) with 50 g/ml ampicillin (AppliChem, Darmstadt, Germany) to prevent bacterial infection.

A single colony was transplanted to another PDAY medium for a pure culture of the fungal isolate. The isolate's pure culture was plated on PDAY agar and incubated in the dark at 25°C for 1-2 weeks for additional sporulation [19]. After ensuring the purity of the cultures and preparing stock culture. The samples were kept at -80°C in 20% glycerol (BioChemica, A0970).

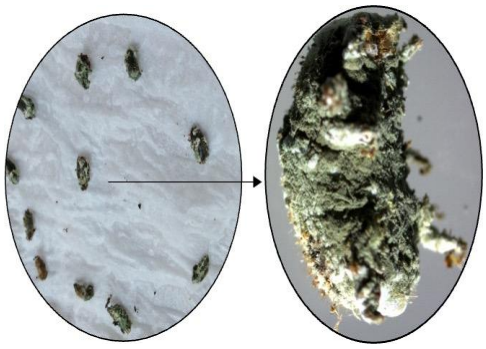

Figure 1. Fungus sporulating on *Dendroctonus micans* adults infected by *Metarhizium anisopliae*.

2.3.Morphological identification

For the morphological identification of fungal strains, shape, surface, edge, consistency, and height were first observed for each colony trait, such as macroscopic features on the PDA of colonies, and color on the top and bottom of the colonies [23]. The EPF strains were stained with Lactophenol blue solution (Merck, 113741) with slide culture methods [24]. The stained samples showed spore shape, color, mycelium type, and colony height features under light microscopy. All isolates were identified using Humber's identification key [23].

2.4. DNA extraction and amplification

Following the manufacturer's recommendations, EPF was resuscitated from stock culture and DNA was obtained using the Quick-DNA Fungal/Bacterial MiniPrep Kit (Zymo Research, Irvine, CA, USA). ITS5 (5'-GGAAGTAAAATCGTAACAAGG-3') and ITS4 (5'-TCCCCGCTTGATATGC-3') primers were used in the PCR reaction for the ITS1-5.8S-ITS2 region [25]. Amplified areas were detected on 1.0% agarose gel electrophoresis and purified using a NucleoSpin gel and PCR cleaning kit from MAchery-Nagel in Duren, Germany. Samples were sent for sequence analysis to Macrogen Inc. (Amsterdam, Netherlands), and their phylogenetic relationships were determined using BioEdit and MEGA software version 7.0.26 [26]. *Candida albicans* CBS562 was used as an external group in phylogenetic trees.

2.5. Prepared spore suspension and insecticidal activity tests

Bioassay tests were conducted using 4-week-old cultures. These cultures were treated with 10 ml of 0.01% Tween 80 on top of the spores for the spore solution, and the spores were recovered from the Petri surface. The spore suspension was passed through a sterile double-layer cheesecloth to remove mycelial pieces. Conidia viability was determined by spreading spore suspensions on a PDAY medium and evaluating germination after 24 hours of incubation at 25°C in the dark. For bioassay tests, cultures with conidia viability of more than 95% were chosen.

Larvae and adult beetles to be used in the bioassay were obtained from the Trabzon Regional Directorate of Forestry Forest Pests and Biological Control Laboratory. Biotest experiments were also performed on healthy last-instar larvae and same-age adults selected randomly. For evaluation of the virulence of the isolates on *D. micans* larvae and adults, ten larvae and adults (larvae and adults were used in separate experimental setups for each experiment) from all of the groups were sprayed with a hand-operated sprayer and conidial solutions containing 10^7 spore/ml. The control groups were given sterilized 0.01% Tween 80. Adults received feed and shells in plastic boxes (20 cm), while larvae were inserted (square-shaped) between the shells. Spruce bark and meal were used as food for adult insects. All larval and adult test groups were kept in plastic boxes and were conducted in a climate-controlled cabinet at 20°C and %65 RH under L12:D12. For 15 days, dead insects were observed daily. Each treatment was replicated 3× on separate days.

2.6. Statistical analysis

The mortality statistics gathered in bioassay experiments were calculated using Abbott's method [27]. For bioassay validation, percent mycosis values were determined using cadaveric mycelial growth. The results were subjected to one-way ANOVA, followed by Duncan's post-hoc tests to compare test isolates with each other and the control group in terms of mortality ($p<0.05$). SPSS 28.0 (IBM Corp., United States) was used to perform every graphic and statistical analysis for the experiments.

3. Results and Discussion

3.1. Morphological identification and molecular characterization

Eighteen isolates belonging to two genera from the parasitic fungi family Bionectriaceae and Clavicipitaceae were obtained from *D. micans*. The isolates *Metarhizium* sp. and *Clonostachys* sp. isolates OZM1, OZM2, OZM3, OZM4, OZM6, OZM7 OZM8, OZM9, OZM10, OZM12, OZM18, OZM19, OZM22b OZM25 and OZM26 were determined as *Metarhizium* sp. and OZM23 was determined as *Clonostachys* sp. All *Metarhizium* sp. isolates generated the characteristic greenish conidial masses on the culture plate with smooth plate reversal, according to the preliminary characterization (Figure 2). Such cultural morphology played a crucial role in separating the desired EPF from its other cousins [28]. Additionally, partial identification of the isolates was supported by microscopic inspection of spore characteristics (spore shape and size). The obtained isolates generated ellipsoid, tiny, and intermediately sized spores (Figure 2). This study noted that the spore characteristics of different *Metarhizium* strains might occasionally differ in terms of color and size [29] (Figure 2).

OZM1, OZM2, OZM3, OZM6, OZM7 OZM8, OZM10, OZM12, OZM18, OZM19, OZM22b OZM25 and OZM26 were determined as *Metarhizium anisopliae*, OZM4 was determined as *Metarhizium robertsii*, OZM9 as *Metarhizium pinghaense* (Figure 3), OZM23 was determined as *Clonostachys rosea* (Figure 3). In particular, OZM4 isolate *M. roberstii* ARSEF 2575 was determined to be closely related and OZM9 isolates to be closely related to *M. pinghaense*.

3.2. Insecticidal activity tests *D. micans* larvae and adults

In bioassay, all isolates were tested *D. micans* larvae and adults at concentrations of 1×10^7 spore/ml. The larvae mortality rates ranged from 30-100% testing within 15 days ($F = 256.377$, $df = 18$, $p < 0.05$) (Figure 4). The adult mortality rates varied from 28-100% after 15 days of testing ($F = 273.041$, $df = 18$, $p < 0.05$) (Figure 5). It was established that mortality happened, particularly within three days, and the mortality rate rapidly increased.

It was determined that OZM2, OZM4, OZM6, and OZM22b isolates were extremely effective on larvae ($p<0.05$). These isolates 7-day larvae mortality rates were 92.2%, 82.2%, 80% and 88.9%, while their 15-day mortality rates were 100%, respectively ($p<0.05$) (Figure 4). Mycosis rates after death were calculated to be 96.4%, 97.3%, 93.3 and 94.0, respectively ($p<0.05$).

As a result of the adult experiments, it was determined that the mortality rates increased after seven days and at the end of the experiment, most of the isolates exhibited 100% mortality ($p<0.05$) (Figure 5). OZM2, in particular, was shown to be the most effective strain on both larvae and adults, with an 80% mycosis value ($p<0.05$). The mortality rate of *D. micans* larvae and adults in control treatment ranged between 5 and 15% and mycosis was not observed.

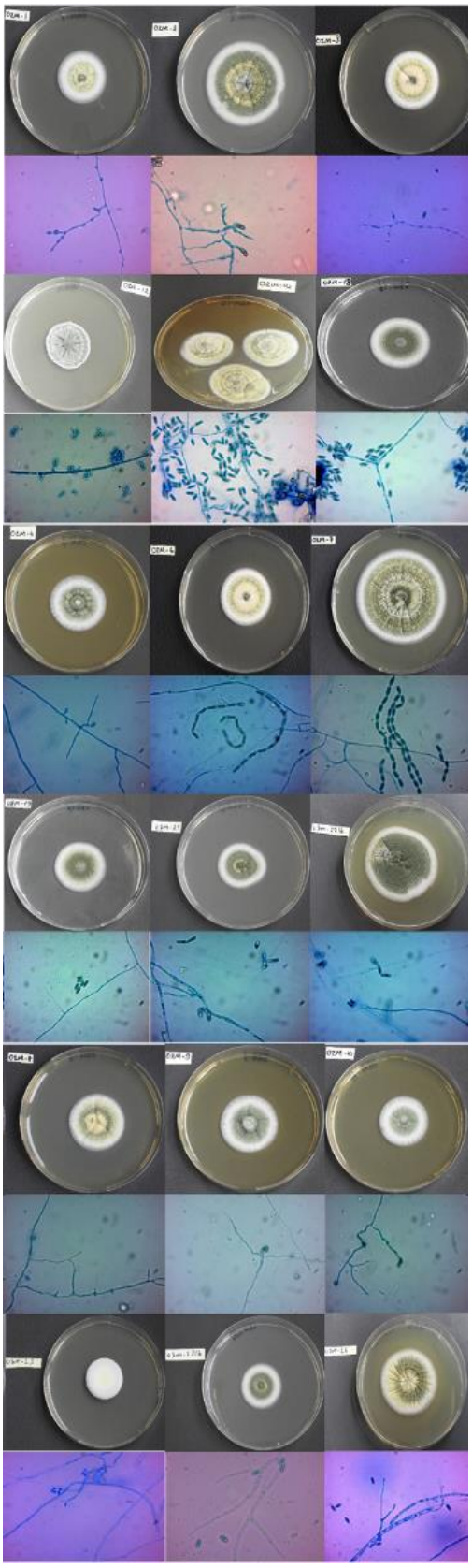

Figure 2. PDA colony growth, morphological structures of entomopathogenic fungus isolates and microphotographs of samples stained with lactophenol blue cotton.

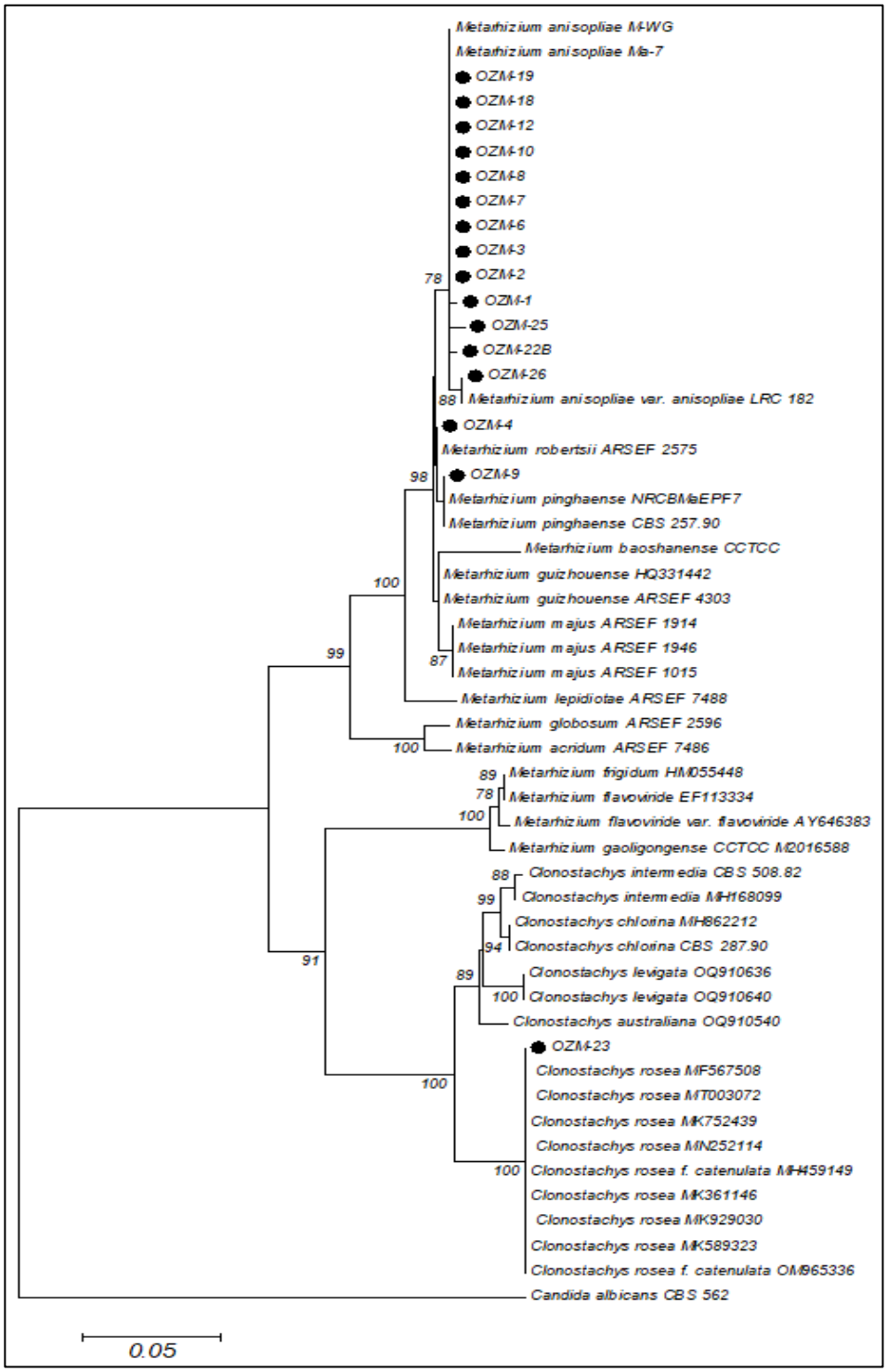

Figure 3 Phylogenetic tree of strains isolated from *Dendroctonus micans*. The approximately 580-bp sequence of the ITS1-5.8S-ITS2 gene region was used to construct the dendrogram. Bootstrap values based on 1000 replicates were indicated above the nodes. Bootstrap values C ≥ 70 are labeled. All isolates were indicated with a black circle. The scale on the bottom of the dendrogram indicates the degree of dissimilarity [26].

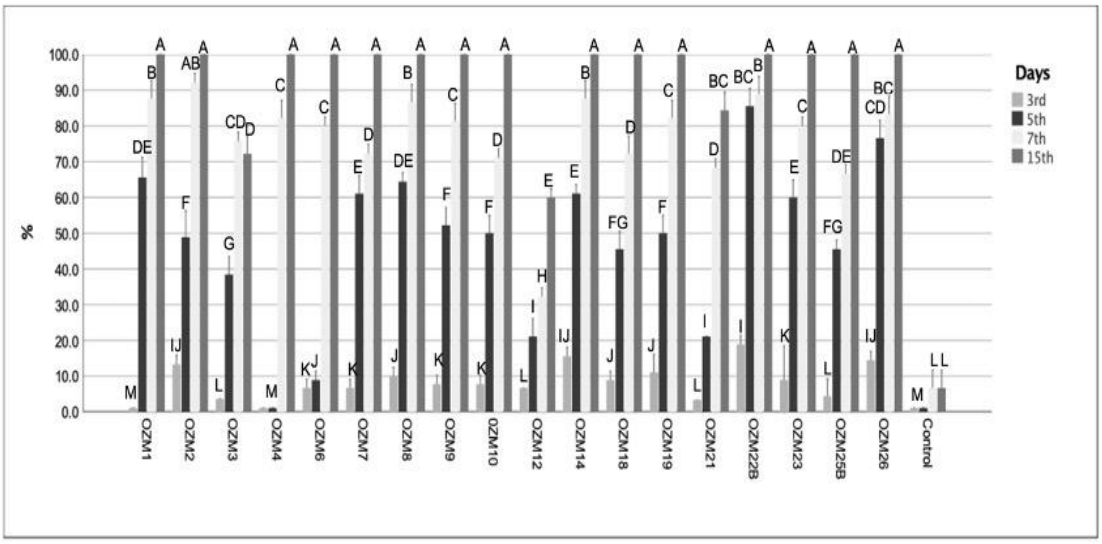

Figure 4 Mortality of *Dendroctonus micans* larvae after application of 18 entomopathogenic fungal isolates within 15 days after application of 1×10^7 spore/ml. Mortality data were corrected according to Abbott's formula [26]. Different uppercase letters represent statistically significant differences among treatments concerning mortality according to Duncan's post-hoc tests ($p < 0.05$). Bars show standard deviation.

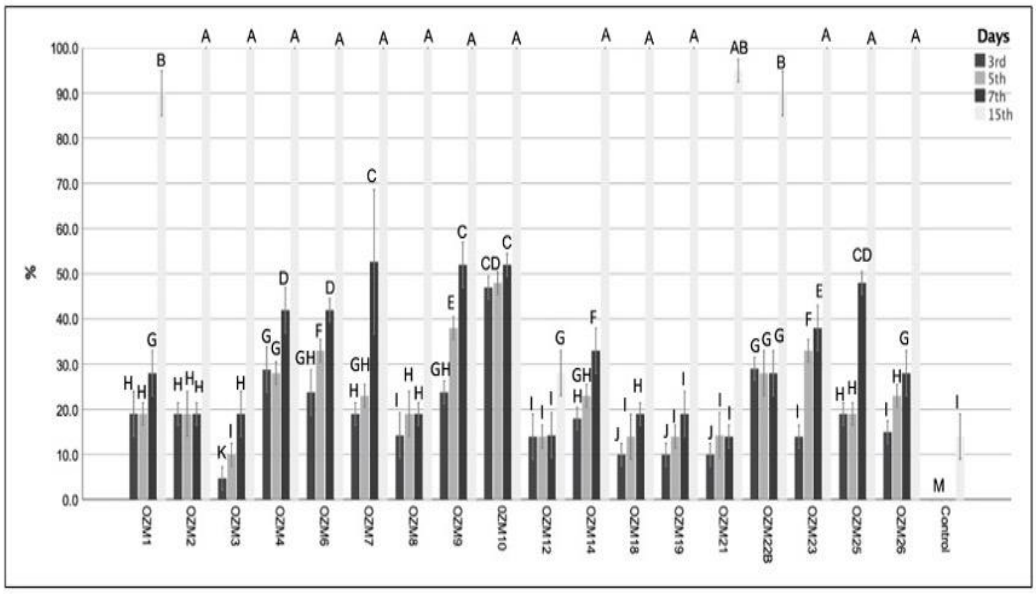

Figure 5 Mortality of *Dendroctonus micans* adults after application of 18 entomopathogenic fungal isolates within 15 days after application of 1×10^7 spore/ml. Mortality data were corrected according to Abbott's formula [27]. Different uppercase letters represent statistically significant differences among treatments concerning mortality according to Duncan's post-hoc tests ($p < 0.05$). Bars show standard deviation.

Bark beetles cause significant infestations, especially in needle forests, and often cause the death of older trees [30, 31]. Especially, *D. micans* is one of the important bark beetle species that cause significant economic damage in spruce forests in Europe and Asia [32, 33]. By this time, the most effective method of control among the methods of control is the production and release of the predator beetle of the pest. Biological methods of controlling the pests have also been tried. In particular, the potential of EPF to be used in the control against pests is higher than other microorganisms.

According to Sevim et al. [17], *Metarhizium anisopliae* (2), *Beauveria cf. bassiana* (2), *Beauveria bassiana* (2), *Isaria fumosorosea* (1), *Metarhizium* sp. (1), and *Evlachovaea* sp. (1) fungi were identified from this pest. In particular, it has been emphasized that there may be efficacy

potential of *Metarhizium* sp. strains in which *B. bassiana* KTU-53 isolate has high lethal activity on the pest. In our study, seventeen species of *Metarhizium* were identified especially in *D. micans* and it was determined that *Metarhizium* isolates were effective on the pest.

Beuaveria bassiana, an EPF species, has been found in studies to be effective against various bark beetles, particularly spruce bark beetles [34-36]. There are few studies on the use of *M. anisopliae*, particularly against spruce bark beetles. Some research has been carried out with *Ips typographus* Linnaeus [37] and *D. micans* [17]. Due to its powerful spore-producing ability and virulence, *M. anisopliae* is recognized to be an effective biocontrol agent against several pests [38, 39]. This investigation demonstrated that *M. anisopliae* OZM-2 is also effective against *D. micans*. Especially among bark beetles, in his study, Moore [40], found two species of *M. anisopliae* and 37 *Penicillium* sp., especially from the *D. frontalis* pest. Pabst and Sikorowski [41], also described *Paecilomyces viridis* and also *B. bassiana*, and *M. anisopliae* species. In another study, on the *Ips typographus* adults, Takov et al. [42], for the first time evaluated the virulence of *M. pemphigi* (isolated from unidentified carabid beetle) and found the cumulative mortality caused by *M. pemphigi* at four different conidial concentrations (2×10^4-2×10^7spore/ml) should range from 75% to 100% ten days after treatment, with an LC_{50} value of 2.9×10^3conidia/ml and an LC_{90} value of 6.4×10^4conidia/ml. In our study, *M. robertsii* and *M. pinhaense* were isolated for the first time, especially from *D. micans*, for the first time, and in the bioassay studies, it was determined that they showed 100% mortality at the end of the application (15 days) on both larvae and adults.

In another study conducted by Draganova et al. [35], different fungi species showed high efficacy in both laboratory and natural environments, especially in a laboratory environment (4. day 100% mortality) log experiments performed in the natural environment of *M. anisopliae* 619 strain determined that it was especially *I. typographus* and *Hylastes cunicularius*.

4. Conclusion

Worldwide, *Metarhizium* spp. has been used in the biological control of Coleoptera species. However, in our study and other studies, it was also revealed that spruce bark beetles can cause high mortality. Although the isolation and identification of *Metarhizium* species from the soil is remarkable, it has been shown to have a high potential for use, especially on bark beetles. Of course, the high virulence of the tested strains is optimistic, as our study was carried out under laboratory conditions, but field trials are necessary to determine the performance of bark beetles under their true ecological conditions.

Article Information Form

Acknowledgments
We thank the Trabzon Regional Directorate of Forestry for their support in the making of this study.

Funding
The authors have no received any financial support for the research, authorship or publication of this study.

Authors' Contribution
Conceptualization, S. B.; methodology, S. B., A. S. and S. İ.; software, S. B.; validation, S. B., A. S. and S. İ.; formal analysis, S. B.; investigation, S.B., A. S. and S. İ.; resources, S. B., A. S. and S. İ.; data curation, S. B.; writing—original draft preparation, S. B.; writing—review and editing, S.B., A. S. and S. İ.; visualization, S.B., A. S. and S. İ.; supervision, S.B.; All authors have read and agreed to the published version of the manuscript.

The Declaration of Conflict of Interest/ Common Interest
No conflict of interest or common interest has been declared by the authors.

The Declaration of Ethics Committee Approval
This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

References

[1] D. L. Wood, ‘‘The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles,’’ Annual Review of Entomology, vol. 27, pp. 411-446, 1982.

[2] S. Seybold, D. Huber, J. Lee, A. Graves, J. Bohlmann, ‘‘Pine monoterpenes and pine bark beetles: a marriage of convenience for defense and chemical communication,’’ Phytochemistry Reviews, vol. 5, pp. 143-178, 2006.

[3] A. Martín, I. Etxebeste, G. Pérez, G. Álvarez, E. Sánchez, J. Pajares, ‘‘Modified pheromone traps help reduce bycatch of bark-beetle natural enemies,’’ Agricultural and Forest Entomology, vol. 15, no. 1, pp. 86-97, 2013.

[4] F. Armendáriz-Toledano, A. Niño, B. T. Sullivan, L. R. Kirkendall, G. Zúñiga, ‘‘A new species of bark beetle, *Dendroctonus mesoamericanus* sp. nov. (Curculionidae: Scolytinae), in southern Mexico and Central America,’’ Annals of the Entomological Society of America, vol. 108, no. 3, pp. 403-414, 2015.

[5] D. L. Six, R. Bracewell, ‘’*Dendroctonus*,’’ in Bark beetles: Biology and ecology of native and invasive species, Academic Press, ed., London: Vega FE, Hofstetter RW, 2017 pp. 305-350.

[6] A. Büyükterzi, G. E. Özcan, O. E. Sakici, ‘‘Variations in the attack pattern of *Dendroctonus micans* and the colonization rate of *Rhizophagus grandis* in *Picea orientalis* stands,’’ Biologia, vol. 77, no. 9, pp. 2475-2485, 2022.

[7] J. D. Reeve, F. E. Anderson, S. T. Kelley, ‘‘Ancestral state reconstruction for *Dendroctonus* bark beetles: evolution of a tree killer,’’ Environmental Entomology, vol. 41, no. 3, pp. 723-730, 2012.

[8] H. Alkan Akıncı, F. E. Bak, B. A. Çalışkan, ‘‘Some tree features affecting host selection by *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae, Scolytinae): experimental results from Artvin spruce forests,’’ Artvin Çoruh University Journal of Forestry Faculty, vol. 19, no. 2, pp. 186-193, 2018.

[9] CABI, ‘‘*Dendroctonus micans* (great spruce bark beetle),’’ Feb. 16, 2021. [Online]. Available:https://www.cabi.org/isc/datasheet/18352.

[10] G. Rouault, J. N. Candau, F. Lieutier, L. M. Nageleisen, J. C. Martin, N. Warzée, ‘‘Effects of drought and heat on forest insect populations in relation to the 2003 drought in Western Europe,’’ Annals of Forest Science, vol. 63, no. 6, pp. 613-624, 2006.

[11] J. Hushaw, ‘‘Forest pests and climate change,’’ Apr. 1, 2021. [Online]. Available: https://www.manomet.org/wpcontent/uploads/old-files/Forest Pests-and-Climate Change_FullBulletin.pdf).

[12] D. Kulakowski, ‘‘Managing bark beetle outbreaks (*Ips typographus*, *Dendroctonus* spp.) in conservation areas in the 21st century,’’ Forest Research Papers, vol. 77, no. 4, pp. 352-357, 2016.

[13] B. Yuksel. ‘‘The Damaging Insect Species at Oriental Spruce Forests and Their Predators and Parasite Species. II. Eastern Black Sea Forestry Research Institute Technical Notes, Trabzon, Turkey, vol. 6, pp. 46, 1997.

[14] D. W. Langor, ‘‘Arthropods and nematodes co-occur with the eastern larch beetle, *Dendroctonus simplex* (Col.: Scolytidae), in Newfoundland,’’ Entomophaga, vol. 36, pp. 303-313, 1991.

[15] H. Yılmaz, K. Sezen, H. Kati, Z. Demirbag, ‘‘The first study on the bacterial flora of the European spruce bark beetle, *Dendroctonus micans* (Coleoptera: Scolytidae),’’ Biologia Bratislava, vol. 61, pp. 679-686, 2006.

[16] M. Yaman, R. Radek, ‘‘Pathogens and parasites of adults of the great spruce bark beetle, *Dendroctonus micans* (Kugelann) (Coleoptera: Curculionidae, Scolytinae) from Turkey,’’ Journal of Pest Science, vol. 81, pp. 91-97, 2008.

[17] A. Sevim, İ. Demir, E. Tanyeli, Z. Demirbag, ‘‘Screening of entomopathogenic fungi against the European spruce bark beetle, *Dendroctonus micans* (Coleoptera: Scolytidae),’’ Biocontrol Science and Technology, vol. 20, pp. 3-11, 2010.

[18] M. Yaman, Ö. Ertürk, İ. Aslan, ‘‘Isolation of some pathogenic bacteria from the great spruce bark beetle, *Dendroctonus micans* and its specific predator, *Rhizophagus grandis*,’’ Folia Microbiologica, vol. 55, pp. 35-38, 2010.

[19] S. Kocacevik, A. Sevim, M. Eroglu, Z. Demirbag, I. Demir, ‘‘Molecular characterization, virulence and horizontal transmission of *Beauveria pseudobassiana* from *Dendroctonus micans* (Kug.) (Coleoptera: Curculionidae),’’ Journal of Applied Entomology, vol. 139, no. 5, pp. 381-389, 2015.

[20] B. Lovett, R. J. St Leger, ‘‘The insect pathogens,’’ Microbiology Spectrum, vol. 5, no. 2, pp.10-1128, 2017.

[21] L. A. Lacey, D. Grzywacz, D. I. Shapiro-Ilan, R. Frutos, M. Brownbridge, M. S. Goettel, ‘‘Insect pathogens as biological control agents: back to the future,’’ Journal of Invertebrate Pathology, vol. 132, pp. 1-41, 2015.

[22] M. S. Ali-Shtayeh, A. B. B. Marai, R. M. Jamous, ‘‘Distribution, occurrence and characterization of entomopathogenic fungi in agricultural soil in the Palestinian area,’’ Mycopathologia, vol. 156, no.3, pp. 235-244, 2003.

[23] R. A. Humber, ‘‘Fungi: identification,’’ İNManual of techniques in insect pathology, Academic Press, 1997, pp. 153-185.

[24] J. L. Harris, ‘‘Modified method for fungal slide cultures,’’ Journal of Clinical Microbiology, vol. 24, no. 3, pp. 460-461, 1986.

[25] T. J. T. White, S. Bruns, J. Taylor, “Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics,’’ in PCR Protocols: A Guide to Methods and Applications, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White. Academic Press, San Diego,1990, pp. 482.

[26] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, ‘‘MEGA6: molecular evolutionary genetics analysis version 6.0.,’’ Molecular Biology and Evolution, vol. 30, no. 12, pp. 2725-2729, 2013.

[27] W. S. Abbott, ‘‘A method of computing the effectiveness of an insecticide,’’ Journal of Economic Entomology, vol. 18, pp. 265-267, 1925.

[28] C. L. Du, B. Yang, J. H. Wu, S. Ali, ‘‘Identification and virulence characterization of two *Akanthomyces*

attenuatus isolates against *Megalurothrips usitatus* (Thysanoptera: Thripidae),'' Insects, vol.10, no. 6, pp. 168, 2019.

[29] N. Tangthirasunun, S. Poeaim, K. Soytong, P. Sommartya, S. Popoonsak, ''Variation in morphology and ribosomal DNA among isolates of *Metarhizium anisopliae* from Thailand,'' Journal of Agricultural Technology, vol. 6, no. 2, pp. 317-329, 2010.

[30] B. J. Bentz, A. M. Jönsson, ''Modeling Bark Beetle Responses to Climate Change, 533-53,'' in Reviews the Modeling Approach to The Bark Beetle Dynamics Under A Climate Change Scenario. ed., F. E. Vega, R. W. Hofstetter. San Diego, Academic Press, 2015, pp. 533-53 pp.

[31] K.F. Raffa, B. H. Aukema, B. J. Bent, A. L. Carroll, J. A. Hicke, M. G. Turner, W. H. Romme, ''Cross-scale drivers of natural disturbances prone to anthropogenic amplification: the dynamics of bark beetle eruptions,'' Bioscience, vol. 58, no. 6, pp. 501-517, 2008.

[32] C. I. Fraser, O. Brahy, P. Mardulyn, L. Dohet, F. Mayer F, J. C. Grégoire, ''Flying the nest: male dispersal and multiple paternity enables extrafamilial matings for the invasive bark beetle *Dendroctonus micans*,'' Heredity, vol. 113, no. 4, pp. 327-333, 2014.

[33] F. Mayer, F. B. Piel, A. Cassel-Lundhagen, N. Kirichenko, L. Grumiau, B. Økland, P. Mardulyn, ''Comparative multilocus phylogeography of two Palaearctic spruce bark beetles: influence of contrasting ecological strategies on genetic variation,'' Molecular Ecology, vol. 24, no. 6, pp. 1292-1310, 2015.

[34] J. Kreutz, O. Vaupel, G. Zimmermann, ''Efficacy of *Beauveria bassiana* (Bals.) Vuill. against the spruce bark beetle, *Ips typographus* L, in the laboratory under various conditions,'' Journal of Applied Entomology, vol. 128, pp. 384–389, 2004.

[35] S. A. Draganova, D. D. Doychev, D. K. Pilarska, D. I. Takov, ''Bioassays of entomopathogenic fungi against xylophagous insects in Bulgaria: laboratory and field experiments,'' Acta Zoologica Bulgarica, vol. 69, no. 3, pp. 411-419, 2017.

[36] Y. A. Batta, ''Biocontrol of almond bark beetle (*Scolytus amygdali* Geurin-Meneville, Coleoptera: Scolytidae) using *Beauveria bassiana* (Bals.) Vuill. (Deuteromycotina: Hyphomycetes),''Journal of Apply Microbiology, vol. 103, pp. 1406–1414, 2007.

[37] S. Keller, C. Epper, B. Wermelinger, ''*Metarhizium anisopliae* as a New Pathogen of the Spruce Bark Beetle *Ips typographus*,'' Mitteilungen der Schweizerischen Entomologischen Gesellschaft, vol. 77, pp. 121 – 123, 2004.

[38] F. Ihara, M. Toyama, T. Sato, ''Pathogenicity of *Metarhizium anisopliae* to the chestnut weevil larvae under laboratory and field conditions,'' Applied Entomology and Zoology, vol. 38, no. 4, pp. 461-465, 2003.

[39] P. Marannino, C. Santiago-Álvarez, E. de Lillo, E. Quesada-Moraga, ''A new bioassay method reveals pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against early stages of *Capnodis tenebrionis* (Coleoptera; Buprestidae),'' Journal of Invertebrate Pathology, vol. 93, no. 3, pp. 210-213, 2006.

[40] G. E. Moore, ''Mortality factors caused by path- bacteria and fungi of the southern pine beetle in North Carolina,'' Journal of Invertebrate Pathology, vol. 17, pp. 28-37, 1971.

[41] G. S. Pabst, P. P. Sikorowski, ''Susceptibility of southern pine beetle (*Dendroctonus frontalis*) on oligidic medium to *Paecilomyces viridis* and also *Beauveria bassiana*, and *Metarhizium anisopliae*,'' Journal of Georgia

Entomology Socienty, vol. 15, pp.235-241, 1980.

[42] D. Takov, M. Barta, T. Toshova, D. Doychev, D. Pilarska, ‘‘On the Pathogenicity of *Metarhizium pemphigi* against *Ips typographus* L,’’ Proceedings of the Bulgarian Academy of Sciences, vol. 75, no. 4, pp. 554-560, 2022.