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Entomology

Diversity of Entomopathogenic Fungi in Hazelnut Plantations in the Black Sea Region of Georgia

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The commercial production of hazelnuts requires the control of its pests. Entomopathogenic fungi are a potential option for the suppression of their numbers and the reduction of the human health and environmental impacts of synthetic pesticide use. The aim of our research was to isolate entomopathogenic fungi from hazelnut plantations in the Black Sea Region of Georgia and to identify the species. Plantations in the Guria, Samegrelo and Adjara regions of West Georgia were selected for the study. Species of entomopathogenic fungi were identified by their morphology and by sequencing of the rDNA region with ITS4 and ITS5 primers and a mitochondrial nad1 gene fragment. BLASTN analysis of the sequenced isolates showed that the isolates had between 95% and 100% similarity to the isolates of entomopathogenic fungi in the database. The identified fungi were *Beauveria bassiana*, *Hirsutella thompsonii*, *Lecanicillium lecanii*, *Metarhizium brunneum*, *Metarhizium* sp. and *Purpureocillium lilacinum*. *Hirsutella thompsonii* and *L. lecanii* were isolated from the hazelnut big bud mite, *Phytoptus avellanae*, and *B. bassiana*, *M. brunneum*, *Metarhizium* sp. and *P. lilacinum* were isolated from different soil samples. Specifically, *M. brunneum* and *P. lilacinum* were isolated from fluvisols of Samegrelo and from nitisols in Adjara, respectively, and *B. bassiana* was isolated from cambisols in Guria. *© 2022 Bull. Georg. Natl. Acad. Sci.*

hazelnut, entomopathogenic fungi, ITS4 and ITS5, nad1

Hazelnut is a traditional crop and an export product for Georgia, and therefore is of great economic importance. In Europe, approximately 200 species of pests are known in hazelnut plantation and 150 species have been found in the Black Sea Region of Turkey [1]. In Georgia, a total of 9 pest species can be considered economically important hazelnut pests [2, 3]. The control of pests is essential for the commercial production of hazelnuts [1]. Protection from harmful pests is important in order to minimize losses and attain the best quality yield. The use of chemical pesticides reduces the numbers of pest mites but can damage human health and cause environmental problems, including harmful

effects on non-target fauna. Thus, the use of biological control agents, e.g., entomopathogenic fungi (EPF), to control pests is essential.

Approximately 800 entomopathogenic species have been reported to cause infection in a wide array of insects and mites, with individual fungal species and strains being very target specific [4]. The soil environment is a major reservoir for entomopathogenic fungi, which are also found on insects and mites. Four species of entomopathogenic fungi were isolated from soils of the hazelnut growing areas in the eastern Black Sea Region of Turkey, namely *Metarhizium anisopliae, Beauveria bassiana, Isaria fumosorosea* and *Evlachovaea* sp. [5]. Also*, Verticillium lecanii* [6]*, Paecilomyces eriophyes* [7] and *Cephalosporium* sp. [8] were found on big bud mites inside hazelnut big buds in Turkey and Italy. In addition, species of *Hirsutella* and *Sporothrix* were observed on eriophyoid mites in India [9].

The knowledge of entomopathogenic fungi in hazelnut plantations in Georgia is sparse. Therefore, the aim of our research was the isolation and identification of entomopathogenic fungi from hazelnut plantations, including those on hazelnut big bud mites, in the Black Sea Region of Georgia.

Materials and Methods

Collection of soil and big bud samples. Soil and big bud samples were collected from hazelnut plantations in Western Georgia during May, June and September, and May, respectively, of 2020 and 2021. A total of four hazelnut plantations were sampled. From each plantation, 10 samples were collected from randomly selected sites; 5 soil samples from 0-10 cm depth and 5 shoots with fungus-infected big buds were collected and put in separate, labeled plastic bags, then stored in cooler boxes and transferred to the laboratory within 12 h.

Isolation of entomopathogenic fungi from soil. Isolation of entomopathogenic fungi from soil was done with the "Galleria bait" method [10]. From each soil sample, four 100 g sub-samples were sifted and placed in 6 cm wide × 9 cm high plastic containers. The soil in each container was dampened with sterilized distilled water. Four, fifth instar *Galleria mellonella* (L.) larvae were then placed on the soil surface in each container and incubated at 25°C for 7 days. The containers were checked regularly in the laboratory for dead larvae which were collected and placed on separate Petri dishes containing a selective medium, Sabouraud Dextrose Yeast Agar (SDYA), for the isolation of entomopathogenic fungi. The fungi were sub-cultured to obtain pure cultures and maintained on Potato Dextrose Agar (PDA) slants [11]. The fungal taxa were morphologically identified by light microscopy (400x, 1000x magnification).

Isolation of entomopathogenic fungi from big bud mites. The collected hazelnut big buds were screened for the presence of big bud mites infected with entomopathogenic fungi under a dissecting microscope (40×). The samples with confirmed fungal infection were used for the isolation of pure cultures which were obtained by incubating individual mites with fungal infection on PDA [11]. The plates were incubated at 22 ± 2°C for 15 days. The fungal taxa were morphologically identified under a light microscope at 400× and 1000× magnification. The obtained cultures were stored at 4°C.

Molecular characterization For DNA extraction, each fungal isolate was inoculated in YPG medium in a 2 ml tube and then placed on a shaker at 200 rpm at 25°C for 7 days. The fungal mycelia were then collected with the centrifuge method (10,000 rpm for 10 minutes). The DNA was extracted with an Easy-DNA™ kit (Invitrogen). Specifically, approximately 100 mg of mycelia were transferred to a 2 ml tube and 400 µl of lysis buffer was added. DNA was extracted according to the standard protocol recommended by the kit manufacturer. Amplifications of the internal transcribed spacer (ITS) region, ITS1-5,8S_ITS2, of 18S-

26S nuclear ribosomal DNA (nrDNA) of the fungal isolates were conducted by using the ITS5/4 primer pair, 5'-TCCTCCGCTTATTGATATGC-3' and 5'- GAAAGTAAAAGTCGTA ACAAGG-3') [12] and the mitochondrial NADH dehydrogenase subunit (nad1) gene with the following nad1A/nad1B primer pair, 5′-ATGGCSAGTATGCAAAGAAGA-3' and 5′-GCATGTTCTGTCATAAASCCACTAAC-3' [13, 14]. The PCR tests were performed in a total volume of 25 µl containing 12.5 µl of 2X PCRBIO TagMix (PCR Biosystems); 0.4 µM of each primer and 3 µl of DNA template. A thermocycler (SimpliAmp ThermalCycler) was used for the PCR testing. Typical cycle conditions for the ITS4/ITS5 primers were initial denaturation at 94°C for 3 min., followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 52°C for 1 min. and extension at 72°C for 1 min. followed by a final extension at 72°C for 10 min. For the nadH1 region, PCR conditions included initial denaturation at 94°C for 3 min. followed by 30 cycles of denaturation at 94°C for 1 min., annealing at 50°C for 1 min. and extension at 72°C for 2 min., followed by a final extension step at 72°C for 5 min. The obtained PCR products were studied with horizontal electrophoresis on agarose gel [15]. The sequencing of PCR products was conducted commercially in the Laboratory Services Division of the University of Guelph in Ontario Province, Canada. The obtained sequences were analyzed with the computer programs BLASTN and MEGA 7.

Results and Discussion

The entomopathogenic fungi, *Beauveria bassiana, Hirsutella thompsonii, Lecanicillium lecanii, Metarhizium brunneum. Metarhizium* sp. and *Purpureocillium lilacinum*, were isolated from 40 samples of soil and big bud mites collected from hazelnut plantations in western Georgia. Generally, four species of fungi, *B. bassiana, M. brunneum, Metarhizium* sp. and *P. lilacinum,* dominated in the soil samples (Table 1). Various combinations of isolates of entomopathogenic fungi were found in different soil types, as follow. From the fluvisols of the Samegrelo region, the entomopathogenic fungus *M. brunneum* was isolated. From the cambisols of the Guria region, *B. bassiana* was isolated, and from the nitisols of Adjara, *P. lilacinum* and *Metarhizium* sp., were isolated. In addition, *H. thompsonii* and *L. lecanii* were isolated from the big bud mites collected from the hazelnut plantations of the surveyed regions (Table 1).

Sevim et al. [5] reported *B. bassiana* and *Metarhizium anisoplia* var. *anisoplia* from both agricultural and non-agricultural soils in the eastern Black Sea Region. *Beauveria bassiana* is normally associated with forested habitats but has also been reported from the Canadian Arctic [16]. We

Table 1. Fungal species isolated from the Black Sea Region of Georgia showing sampling date, spore size, microhabitat, soil types and locality

No	Sampling date	Isolates	Species	Size of spores (µm)	Micro-habitat	Soil type	Locality region
1	25.09.2020	IBB	*Beauveria bassiana*	1,5-3,0×1,5-2,5	soil	cambisols	Guria
2	05.05.2021	IM1	*Hirsutella thompsonii*	5,1-5,8×2,2-2,5	Big bud mites		Adjara
3	05.06.2021	IM2	*Hirsutella thompsonii*	5,2-5,7×2,3-2,5	Big bud mites		Guria
4	25.05.2020	IV1	*Lecanicillium lecanii*	5,0-6,1×0,3-2,2	Big bud mites		Guria
5	25.05.2020	IV2	*Lecanicillium lecanii*	5,0-5,9×0,4-2,1	Big bud mites		Adjara
6	25.09.2020	IH1	*Metarhizium* sp.	4,5-7,4×2,0-2,9	soil	nitisols	Adjara
7	05.09.2021	IH2	*Metarhizium brunneum*	4,3-8,8×1,3-2,9	soil	fluvisols	Samegrelo
8	05.05.2021	IP	*Purpureocillium lilacinum*	2,0-2,3×3,1-4,0	soil	nitisols	Adjara

isolated *B. bassiana* from the cultivated soil of young hazelnut plantations. *Metarhizium* sp. was isolated from the fluvisols and nitisols of hedgerow soils in hazelnut plantations. Sevim et al. [5] found that *M. anisoplia* var. *anisoplia* and *Evlachovaea* sp. had the highest insecticidal activity (86.6%) against *Melolantha melolantha* (L.). They considered that entomopathogenic fungi isolated from the soil of hazelnut plantations could be good biocontrol agents against *M. melolantha* which is one of the most serious pests of hazelnut. In the Adjara region, *P. lilacinum* was isolated from the soil of 10 years old hazelnut plantations. This fungus was previously known as a *Paecilomyces lilacinus*, although its current name is *Purpureocillium lilacinum* (Ophiocordycipitacea). It has been shown that different strains of *P. lilacinum* had entomopathogenic effects against some insects [17]; Amjad et al. [18] observed its development on the mite, *Tetranychus urticae* Koch.

From big bud mites, we also isolated *L. lecanii*. Ozman [6] found this species in the big buds of hazelnut in the Black Sea Region of Turkey. Ozman [6] and Ozman and Hatat [19] showed that this species of entomopathogenic fungus can kill the eggs, nymphs and adults of the big bud mites, *Phytoptus avellanae* Nal. and *Cecidophyopsis vermiformis* (Nal.), and that the efficacy was 99.5%. Therefore, this species is considered a potential biological control agent against hazelnut big bud mites.

BLAST sequence analysis demonstrated that the ITS region of rDNA generated 600 bp fragments and the mitochondrial nad1 (nad1A/nad1B) gene fragments of isolates had 95%, 97%, 99% or 100% similarity to *B. bassiana, H. thompsonii, L. lecanii*, *Metarhizium* sp., *M. brunneum* and *P. lilacinum* contained in the database (http://blast.ncbi.nlm.nih.gov/Blast) (Table 2).

The isolate of *H. thompsonii* from big bud mites in the Guria region was identical to the pathogen isolated from the infected big bud mites from the Adjara region. This entomopathogenic fungus is a strong pathogen for eriophyoid mites. It is currently used for the biological control of a mite – a formulation of *H. thompsonii* has been developed for the control of *Aceria guerreronis*, an extremely harmful eriophyoid mite, in coconut plantations [20].

The morphological and genetic analyses performed in this study showed that the pathogens isolated from infected big bud mites were *L. lecanii* and *H. thompsonii.* Moreover, they were 99% and 100% identical to the isolates MN588136 and KM652192.1, respectively, in GenBank. *Lecanicillium lecanii* and *H. thompsonii* have the potential to be used against big bud mites in biological control. *Beauveria bassiana, M. brunneum, Metarhizium* sp. and *P. lilacinum* were isolated from the soil and require in-depth study to determine their efficacy against big bud mites.

Entomopathogenic fungi have the ability to grow at different temperatures and levels of UV

Table 2. Fungal species isolated from hazelnut plantations in the Black Sea Region of Georgia showing gene accession numbers

No	Isolate	Species	532-600 bp Identity (%)	ITS GenBank	Identity (%)	nad1 GenBank
1	IBB	*Beauveria bassiana*	100	MT528685.1	100%	EU295631.1
2	IM1	*Hirsutella thompsonii*	100	KM652192.1	-	-
3	IM2	*Hirsutella thompsonii*	100	KM652192.1		-
4	IV1	*Lecanicillium lecanii*	99	MN588136	99%	EF512938.1
5	IV2	*Lecanicillium lecanii*	99	MN588136	99%	EF512938.1
6	IH1	*Metarhizium* sp.	99	MF872428	95%	LR792747.1
7	IH2	*Metarhizium brunneum*	100	KM371261	100%	LR792747.1
8	IP	*Purpureocillium lilacinum*	100	MH191215	97%	NC_056378.1

exposure [16, 21]. Their resilience gives them considerable potential as biological control agents of big bud mites in hazelnut plantations. Future areas of our work will include testing these entomopathogenic fungi against big bud mites to identify the more pathogenic species and strains and to determine the most appropriate periods and conditions for their use.

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ენტომოლოგია

საქართველოს შავიზღვისპირეთში თხილის ბაღებში გავრცელებული ენტომოპათოგენური სოკოების მრავალფეროვნება

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**ონდოკუს მეის უნივერსიტეტი, სოფლის მეურნეობის ფაკულტეტი, მცენარეთა დაცვის დეპარტამენტი, სამსუნი, თურქეთი
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(წარმოდგენილია აკადემიის წევრის გ. ალექსიძის მიერ)

თხილი საქართველოსთვის ერთ-ერთი ტრადიციული და საექსპორტო კულტურაა, მას ქვეყნისთვის დიდი ეკონომიკური მნიშვნელობა აქვს. თხილის კომერციული წარმოებისთვის აუცილებელია მისი მავნე ორგანიზმების კონტროლი. ადამიანის ჯანმრთელობისა და გარემოს დაცვითი პრობლემების გათვალისწინებით მათი რიცხოვნობის მარეგულირებლად შესაძლებელია გამოყენებულ იქნეს ენტომოპათოგენური სოკოები. ნაშრომში კვლევის მიზანს წარმოადგენდა საქართველოს შავიზღვისპირეთში თხილის ბაღებიდან ენტომოპათოგენური სოკოების გამოყოფა და სახეობების იდენტიფიკაცია. კვლევისთვის შეირჩა დასავლეთ საქართველოს რეგიონებში გურიის, სამეგრელოს და აჭარის თხილის ბაღები. ენტომოპათოგენური სოკოების სახეობების იდენტიფიკაცია მოხდა მათი მორფოლოგიური შესწავლით და ნუკლე-

არული რიბოსომული დეენემის ITS რეგიონის და მიტოქონდრიული nad1 გენის ფრაგმენტების სექვენირებით. იზოლატების სექვენსის BLASTN ანალიზმა გვაჩვენა ყველა იზოლატის 95-99% მსგავსება მონაცემთა ბაზაში (http://blast.ncbi.nlm.nih.gov/Blast) არსებულ ენტომოპათოგენური სოკოების იზოლატებთან. მორფოლოგიური და მოლეკულური კვლევის შედეგად გამოვლინდა ენტომოპათოგენური სოკოები *Beauveria bassiana, Hirsutella thompsonii, Lecanicillium lecanii, Metarhizium brunneum, Metarhizium* sp. და *Purpureocillium lilacinum.* პათოგენური სოკოები *H. thompsonii* and *L. lecanii* გამოყოფილ იქნა თხილის კვირტის ტკიპადან, ხოლო *B. bassiana, Metarhizium* sp., *M. brunneum, P. lilacinum* გამოიყო ნიადაგის სხვადასხვა ნიმუშებიდან: სამეგრელოს ალუვიური ნიადაგიდან – *Metarhizium* sp., *M. brunneum.* გურიის ყომრალი ნიადაგებიდან – *B. bassiana*, ხოლო აჭარის წითელმიწა ნიადაგებიდან გამოიყო *M. brunneum, P. lilacinum.* თანამედროვე ლიტერატურის მიმოხილვისა და ჩვენი კვლევების თანახმად აღნიშნულ სოკოებს ტკიპების ბიოკონტროლში გამოყენების დიდი პოტენციალი გააჩნია.

REFERENCES

1. Ozman-Sullivan S.K. (2018) Pest management in organic hazelnut growing. In: Vacante V. and Kreiter S. (eds.), Handbook of pest management in organic farming, pp. 297–327. CABI Publishing, Wallingford.
2. Kereselidze M., Beruashvili M. (2019) Hazelnut pests and diseases and their management in organic farming, 63 pp. Universal Press, Tbilisi (in Georgian).
3. Tavamaishvili L.E. (1990) The chief pests of hazelnut in subtropical western Georgia and their control. *Subtropicheskie Kul'tury*, 5: 119–125 (in Georgian).
4. Sharma V., Salwan R., Al-Ani L.K.T. (2020) Molecular aspects of plant beneficial microbes in agriculture, 438 pp. Elsevier, Academic Press.
5. Sevim A., Demir I., Höfte M., Humber R.A., Demirbag Z. (2010) Isolation and characterization of entomopathogenic fungi from hazelnut-growing region of Turkey. *BioControl,* 55: 279–297.
6. Ozman S.K. (1998) *Verticillium lecanii* (Zimm.) Viegas, a fungal pathogen of the big bud mites, *Phytoptus avellanae* Nal. and *Cecidophyopsis vermiformis* Nal. (Acarina: Eriophyoidea). Book of Abstracts, The VI European Congress of Entomology, p. 610. 23–29 August 1998, Ceske Budejovice, Czech Republic.
7. Del Guercio G. (1911) Prima contribuzione alla conoscenza degli eriofiidi delle gemme del nocciuolo e delle foglie del pero e le esperienze tentate per combatterli. *Redia*, 7: 1–64. Taveres (in Italian).
8. Pesante A. (1961) L'acariosi delle gemme del nocciolo. Estratto dal Bollettino del Laboratorio Sperimentale e Ossevatorio di Fitopatologia, 27–74. July-December, Torino (in Italian).
9. Narasa Reddy, Mahesh G., Priya M., Shailendra Singh R.U., Manjunatha L. (2020) *Hirsutella*. In: Amaresan N., Senthil Kumar M., Annapurna K., Kumar K. and Sankaranarayanan A. (eds.), Beneficial microbes in agro-ecology, bacteria and fungi, pp. 817–831. Elsevier, Academic Press.
10. Zimmermann G. (1986) The Galleria bait method for detection of entomopathogenic fungi in soil. *J. Appl. Entomol.,* 102: 213–215.
11. Shurtleff M.C., Averre C.W. (1999) The plant disease clinic and field diagnosis of abiotic diseases, 245 pp. APS Press.
12. White T.J., Bruns T., Lee S., Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J. and White T.J. (eds.), PCR protocols: a guide to methods and applications, pp. 315–322. Academic Press.
13. Kouvelis V.N., Ghikas D.V., Typas M.A. (2004) The analysis of the complete mitochondrial genome of *Lecanicillium muscarium* (synonym *Verticillium lecanii*) suggests a minimum common gene organization in mtDNAs of Sordariomycetes: phylogenetic implications. *Fungal Genet. Biol*., 41: 930–940.
14. Pantou M.P., Strunnikova O.K., Shakhnazarova V.Y., Vishnevskaya N.A., Papalouka V.G., Typas M.A. (2005) Molecular and immunochemical phylogeny of *Verticillium* species. *Mycol. Res*., 109: 889–902.
15. Sambrook J., Russell D.W. (2001) Molecular cloning: A Laboratory Manual. 3rd ed.: 2100 pp. Cold Spring Harbor Laboratory Press.
16. Bidochka M.J., Menzies F.V., Kamp A.M. (2002) Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. *Arch. Microbiol.*, 178: 531–537.
17. Demirci S.N., Altuntas H. (2019) Entomopathogenic potential of *Purpureocillium lilacinum* against the model insect *Galleria mellonella* (Lepidoptera: Pyralidae) *Environmental and Experimental Biology,* 17: 71–74.

18. Amjad M., Bashir M.H., Afzal M., Sabri M.A., Javed N. (2012) Synergistic effects of some entomopathogenic fungi and synthetic pesticides, against two spotted spider mite, *Tetranychus urticae* Koch (Acarina: Tetranychidae). *Pak. J. Zool.,* 44: 977–984.
19. Ozman S.K., Hatat G. (1999) Biological control of *Phytoptus avellanae* Nal. and *Cecidophyopsis vermiformis* Nal. (Acarina: Eriophyoidea) by *Verticillium lecanii* (Zimm.) Viegas. Proceedings of the Fourth Turkish National Congress of Biological Control, 189–200. 26-29 January 1999, Adana, Turkey (Abstract in English).
20. Sreerama Kumar P. (2010) *Hirsutella thompsonii* as a mycoacaricide for *Aceria guerreronis* on coconut in India: research, development, and other aspects. In: Sabelis M.W. and Bruin J. (eds.): Trends in Acarology. Proceedings of the 12th International Congress, pp. 441–444. Springer.
21. Bidochka M., Kamp A., Lavender T., Dekoning J., De Croos J. (2001) Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? *Appl. Environ. Microbiol.,* 67: 1335–1342.

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