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Nematophagous fungi species from Erzurum and Erzincan provinces in Türkiye

Türkiye'de Erzurum ve Erzincan illerinden elde edilen nematofag fungus türleri

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ABSTRACT

In Erzurum and Erzincan provinces, some nematophagous fungi were determined on roots of alfalfa (*Medicago sativa* L.), potato (*Solanum tuberosum* L.), and strawberry (*Fragaria x ananassa* Duchesne) plants during 2009-2011, and identification of fungi species was performed by classical and/or molecular techniques. Purified 5 isolates were determined as *Arthrobotrys cladodes Drechsler* 1937 (1 isolate), *Arthrobotrys conoides* Drechsler 1937 (1 isolate) and *Arthrobotrys superba* Corda 1839 (3 isolates). Identification of *Arthrobotrys* isolates was also confirmed by ribosomal DNA (rDNA)-ITS (internal transcribed spacer) sequence analysis. *Harposporium* genus was also determined considering the morphological characteristic of fungal spores growing on nematodes found in two samples, but these fungi could not be purified. *Harposporium* species were identified as *Harposporium anguillulae* Lohde emend. Zopf 1888 and *Harposporium crassum* A.M. Sheph 1955 according to the morphological characteristic. To our knowledge, all *Arthrobotrys* and *Harposporium* species identified in this study are reported for the first time in Türkiye.

INTRODUCTION

Plant-parasitic nematodes cause serious damage to many important crops such as alfalfa (*Medicago sativa* L.), potato (*Solanum tuberosum* L.), and strawberry (*Fragaria x ananassa* Duchesne). Fungi are a group of organisms that are highly abundant in soils, and some of these fungi species feed on various nematode species. In contrast, others infect many plant species or survive as saprophytic in the soil. More than 700 species of nematophagous (nematode-destroying) fungi can control plant-parasitic nematodes through antagonistic behavior (Li et al. 2015). Nematophagous fungi are potentially important for biological control, an indispensable component in sustainable agriculture, and play a major role in integrated pest management programs (Eken et al. 2023, Yadav et al. 2023).

Nematophagous fungi were divided into four main groups according to their mechanism of action: nematode-trapping, nematode egg and female parasites, endoparasitic, and toxin-producing fungi (Hyde et al. 2014). Species belonging to the genera *Arthrobotrys, Cystopage, Dactylella,*

Dactylellina, Drechslerella, Hohenbuehelia, Hyphoderma, Monacrosporium, Nematoctonus, Orbilia, Stylopage, Tridentaria, Tripospora and *Zoophagus* are nematode-trapping fungi; *Drechmeria, Harposporium, Hirsutella, Nematoctonus* and *Myzocytium* are endoparasitic fungi; *Lecanicillium, Nematophthora, Paecilomyces* and *Pochonia* are egg and cyst parasitizing fungi; *Coprinus* and *Pleurotus* are toxin-producing fungi (Yang and Zhang 2014, Zhang et al. 2011). These species belong to fungal taxa, including Oomycota, Chytridiomycota, Blastocladiomycota, Zygomycota, Ascomycota, and Basidiomycota (Li et al. 2015, Yang and Zhang 2014).

There are few studies on the isolation of *Arthrobotrys* species in Türkiye. *Arthrobotrys arthrobotryoides* (Berl.) Lindau 1905 was isolated from outdoor air (İmalı 2005, İmalı et al. 2011, Kalyoncu and Ekmekci 2008), *Arthrobotrys oligospora* Fresen. 1850 from egg masses and females of *Meloidogyne incognita* (Kofoid & White 1919) Chitwood 1949 (Tylenchida: Heteroderidae) nematode species (Karakas 2015), and *Arthrobotrys* sp. from wheat and barley seeds (Yurdakul 2019).

The study aims to identify nematophagous fungi by using morphological and/or molecular methods. These fungi were collected during the studies to determine the fungal plant pathogens that cause disease in the roots of alfalfa, potato and strawberry plants in Erzurum and Erzincan provinces.

MATERIALS AND METHODS

Isolation of nematophagous species

Nematophagous fungi were isolated during the studies to determine the fungal plant pathogens that cause disease in the roots of various plants. Diseased alfalfa, potato, and strawberry plants were collected from fields in Erzurum and Erzincan provinces during 2009-2011. The roots of the plants were washed in running tap water to eliminate the soil. Plant parts (1.5 cm long) were disinfected in 1% sodium hypochlorite (NaOCl) for 1 min, immersed with sterile distilled water, and placed on 1.5% Water Agar (WA) supplemented 50 mg/l streptomycin sulfate. After a 3-7 day incubation period at 25 °C in the dark, it was observed that nematodes growing in some Petri dishes were caught by various fungal structures. These plates were periodically examined for growth of nematophagous fungi for an additional 10-15 days. Nematophagous fungi were purified using the single spore isolation method, then transferred to a slant containing Potato Dextrose Agar (PDA) and stored at 5 °C.

Morphological and/or molecular identification of isolates

Nematophagous fungi species were determined using classical and/or molecular methods. In the classical method, fungi were characterized based on their morphological characteristics (Karling 1938, Shepherd 1955, Yu et al. 2014). Five purified isolates were grown on PDA in the dark at 25 °C for 7-14 days to determine differences in conidia size, colony morphology, and radial growth rate. A five mm diameter agar disk from the edge of the colony of the isolate was placed in the center of a 9 cm diameter Petri dish containing PDA, and colony diameter was determined after 7 days. Four Petri dishes were used for each isolate, and the average of the colony diameter measurements made at right angles to each other was taken to determine the measurement value in each replicate. Conidia size was determined for each isolate by measuring conidia (n= 50) at right angles longitudinally and transversely using a phase contrast microscope. Measurements values were recorded as minimum-maximum (average). The morphological features of the fungi in the two unpurified samples were examined only with the phase contrast microscope. All micromorphological features were photographed with an Olympus BH2 microscope.

Purified five isolates were also identified molecularly using ribosomal DNA (rDNA)-ITS (Internal Transcribed Spacer) regions (ITS1, 5.8, ITS2). Mycelia of the isolates to be used in DNA isolation were obtained as described by Genc Kesimci et al. (2022). Genomic DNA isolation, PCR amplification using ITS1 and ITS4 primers (White et al. 1990), and sequence analysis from these isolates were performed by REFGEN (Ankara University Technopolis, Ankara, Türkiye). The sequences of each isolate were edited using BioEdit software, version 7 (Hall 1999), and aligned using the Clustal W algorithm (Thompson et al. 1994). The sequences of isolates were performed by BLAST (Basic Local Alignment Search Tool) analysis and compared with the other sequences in the National Center for Biotechnology Information (NCBI) database. Sequence similarity of 97% or above was taken into account when determining the species. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) implemented in MEGA software, version 6 (Tamura et al. 2013) using reference isolates (GenBank MH857992.1, MH179686.1, and MZ427475.1) whose base sequences were obtained from the NCBI database, and *Dactylellina appendiculata* (Anastasiou) M. Scholler, Hagedorn & A. Rubner 1999 (GenBank MH858419.1) as the distant species, and 1000 bootstrap replicates. The sequences of 5 isolates were deposited in the NCBI database and accession numbers were obtained (Table 1).

Table 1. Isolate code, isolation source, sampling year, location, and GenBank accession number of isolates of *Arthrobotrys* species identified by classical and molecular techniques

Species	Isolate	Isolation Source	Year	Location	Genbank Accession Number
Arthrobotrys cladodes	S-KHB-14	Strawberry	2009	Yakutiye-Erzurum	OR149151
Arthrobotrys conoides	A-2	Potato	2011	Pasinler-Erzurum	OR149152
Arthrobotrys superba	NF-1	Strawberry	2009	Yakutiye-Erzurum	OR149153
Arthrobotrys superba	SS10-6	Strawberry	2009	Yakutiye-Erzurum	OR149154
Arthrobotrys superba	MTT-13	Alfalfa	2010	Pasinler-Erzurum	OR149155

RESULTS AND DISCUSSION

Five isolates were obtained from roots of alfalfa (1 isolate), potato (1 isolate), and strawberry (3 isolates) plants in Erzurum province (Table 1). These isolates were identified as *Arthrobotrys* genus by the classical identification techniques (Yu et al. 2014). Based on morphological descriptions, the 5 isolates were defined as *Arthrobotrys cladodes* Drechsler 1937 (teleomorph: *Orbilia cladodes* (Drechsler) E. Weber & Baral) (1 isolate), *Arthrobotrys conoides* Drechsler 1937 (teleomorph: unknown) (1 isolate) and *Arthrobotrys superba* Corda 1839 (teleomorph: *Orbilia auricolor* (A. Bloxam) Sacc.) (3 isolates). The *Arthrobotrys* isolates were examined for their morphological characteristics, including colony features (Figure 1 and Table 2) and conidia (Figure 2 and Table 2). Conidia size was given as minimum-maximum (average) measurements.

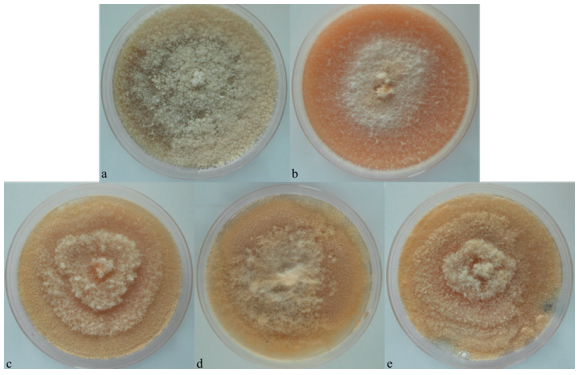

Figure 1. Colony of *Arthrobotrys* spp. on Potato Dextrose Agar media. a) *Arthrobotrys cladodes* (S-KHB-14); b) *Arthrobotrys conoides* (A-2); c-e) *Arthrobotrys superba* (NF-1, SS10-6 and MTT-13, respectively)

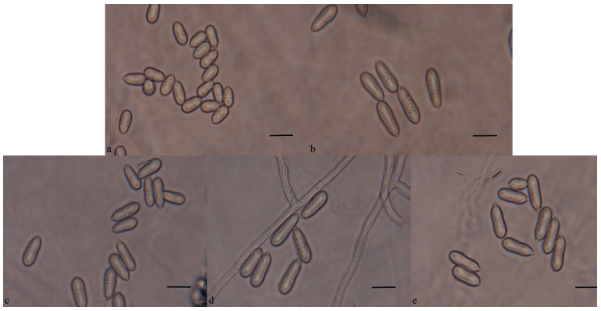

Figure 2. Conidia of *Arthrobotrys* spp. on Potato Dextrose Agar media. a) *Arthrobotrys cladodes* (S-KHB-14); b) *Arthrobotrys conoides* (A-2); c-e) *Arthrobotrys superba* (NF-1, SS10-6 and MTT-13, respectively). Scale bars: a-e= 20 µm

Arthrobotrys cladodes was isolated from strawberry root in Yakutiye-Erzurum, *A. conoides* from potato root in Pasinler-Erzurum, and *A. superba* from alfalfa and strawberry roots in Pasinler-Erzurum and Yakutiye-Erzurum, respectively (Table 1).

Arthrobotrys cladodes isolate (S-KHB-14) on PDA was whitish, then turned greenish-yellow and cottony (Figure 1a), growing rapidly to reach an 85 mm diameter after 7 days in the incubator at 25 °C (Table 2). Conidia ellipsoid, 1-septate at the center of the spore (Figure 2a), measuring 14.2-19.8 (16.6) x 6.6-11.2 (8.5) µm (Table 2).

Arthrobotrys conoides isolate (A-2) on PDA was initially whitish, then turned to reddish-orange, with white superficial hyphae towards the center (Figure 1b), growing rapidly to reach an 80 mm diameter after 7 days in the incubator at 25 °C (Table 2). Conidia elongate obconical, 1-septate about one-third from the basal end, and constricted at the septum (Figure 2b), measuring 21.1-38.4 (30.2) x 6.9-12.4 (9.5) µm (Table 2).

Table 2. Colony diameter, conidia length, and width of *Arthrobotrys* isolates

Species	Isolate	Colony Diameter (mm)	Conidia Length* (µm)	Conidia Width (µm)
Arthrobotrys cladodes	S-KHB-14	85	14.2-19.8 (16.6)	6.6-11.2 (8.5)
Arthrobotrys conoides	A-2	80	21.1-38.4 (30.2)	6.9-12.4 (9.5)
Arthrobotrys superba	NF-1	54	19.2-30.9 (24.2)	6.8-10.2 (8.4)
Arthrobotrys superba	SS10-6	54	21.8-33.8 (28.0)	7.0-10.0 (8.7)
Arthrobotrys superba	MTT-13	44	20.5-28.9 (24.5)	8.0-13.3 (10.1)

*: Minimum-Maximum (Average)

Arthrobotrys superba isolates (NF-1, SS10-6, and MTT-13) on PDA were initially whitish, then turned to yellowish-orange and cottony (Figure 1c-e), growing moderately to reach diameters of 54, 54, and 44 mm after 7 days in the incubator at 25 °C, respectively (Table 2). Conidia elliptical, 1-septate at the center of spore, slightly constricted at the septum (Figure 2c-e), measuring 19.2-33.8 (25.6) x 6.8-13.3 (9.1) µm (Table 2).

The *Arthrobotrys cladodes*, *A. conoides* and *A. superba* isolates obtained in this study are nematode-trapping fungi and capture nematodes using three-dimensional adhesive networks (Figure 3). According to our observations during the study, when *Arthrobotrys* species and nematodes were co-inoculated into Petri dishes containing 1.5% WA, abundant three-dimensional adhesive networks were formed in the entire colony, and nematode growth was 100% inhibited within two weeks. In the nematode-free Petri dishes, it has been observed that there is no adhesive network formation in the fungal colony or it was very few in the center of the colony, so three-dimensional adhesive network formation occurs in response to the presence of nematodes. The nematode species used in this study were not identified.

In addition to identification based on morphological characteristics, *Arthrobotrys* isolates were also identified based on sequence analysis of rDNA-ITS regions. A sequence similarity of 97% or above was considered for species determination. A neighbor-joining phylogenetic tree of the 5 *Arthrobotrys* isolates, reference isolates, and distant species is shown in Figure 4. Ultimately, *Arthrobotrys* isolates (Table 1) were confirmed as *A. cladodes* (1 isolate), *A. conoides* (1 isolate), and *A. superba* (3 isolates).

The *Harposporium* genus was determined on the morphological characteristic of fungal spores growing on nematodes in two samples from strawberry plants in 2009 (Figure 5), although these fungi could not be purified. *Harposporium* species were classified as *Harposporium anguillulae* Lohde emend. Zopf 1888 and *Harposporium crassum* A.M. Sheph. 1955 based on the morphological characteristics of the conidia and/or conidiophores (Karling

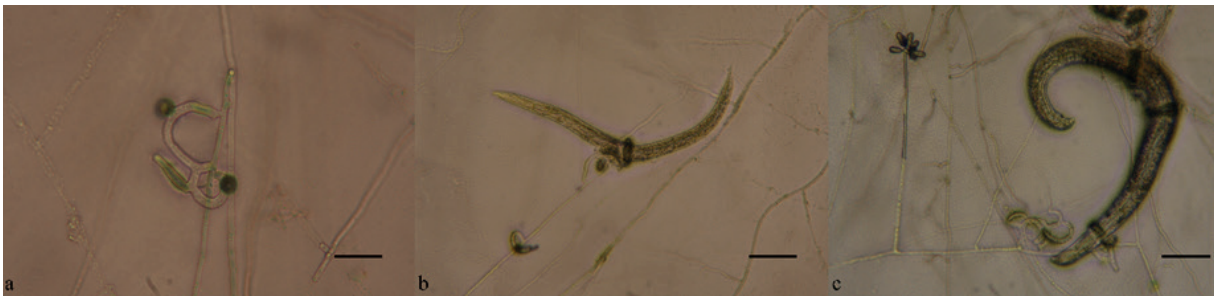

Figure 3. The trap formation process and interaction between the nematophagous fungi *Arthrobotrys* sp. and nematode on Water Agar surface. a) Three-dimensional adhesive network; b) Trapped nematode in three-dimensional adhesive network at a point; c) Conidia and conidiophores of *Arthrobotrys* sp., and nematode trapped in three-dimensional adhesive networks at various points. Scale bars: a= 40 µm; b, c= 80 µm

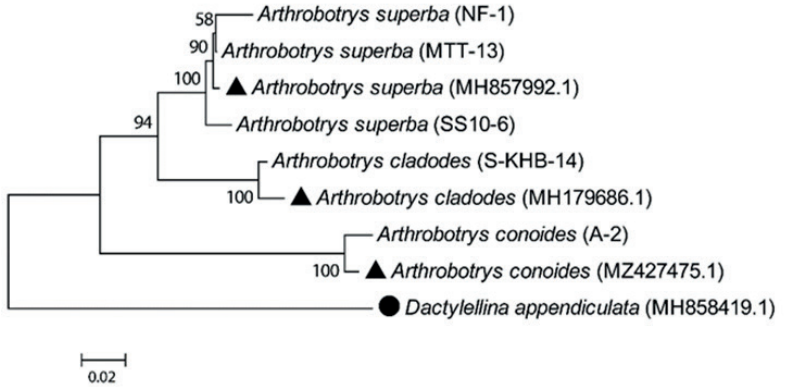

Figure 4. Phylogenetic tree based on rDNA-ITS sequences constructed using the neighbor-joining method for *Arthrobotrys* isolates. The numbers on the tree branches indicate the bootstrap values from 1000 replicates using Mega 6.0. The outgroup, *Dactylellina appendiculata* was used to root the tree. ▲: Reference isolates, ●: Outgroup

1938, Shepherd 1955, Wang et al. 2007). *Harposporium anguillulae* conidia were sickle-shaped, pointed at both ends, and non-septate (Figure 5a), measuring 8-12 (9.7) x 2 µm. *Harposporium crassum* conidia were arcuate, pointed at both ends, non-septate (Figure 5b), measuring 12-20 (14.5) x 2 µm. The spores of *H. anguillulae* are smaller than those of *H. crassum. Harposporium anguillulae* and *H. crassum* were determined in the samples collected from Yakutiye-Erzurum and Üzümlü-Erzincan, respectively.

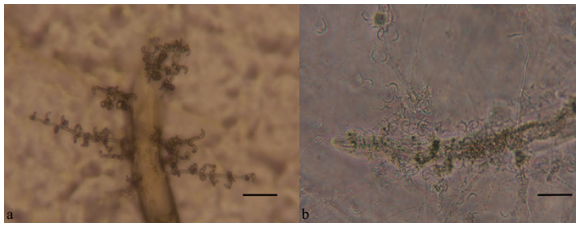

Figure 5. Conidia and/or conidiophores of *Harposporium* spp. on nematode body. a) *Harposporium anguillulae*; b) *Harposporium crassum*. Scale bars: a, b = 40 µm

In this study, *Arthrobotrys* and *Harposporium* species were determined from the roots of various plants or nematodes living on plant roots. These fungi were collected during the studies carried out for the detection of fungal pathogens that cause root rot diseases. In many studies, nematode–fungus disease complexes were reported in several host crops as they occupied the same ecological niche (Back et al. 2002). The fact that they are the most abundant organisms in the soil habitat causes multiple interactions as antagonistic and synergistic interactions between these two groups (Zhang et al. 2020). It is stated that nematophagous fungi are efficient in the biocontrol of parasitic nematodes in antagonistic interaction (De Freitas Soares et al. 2023).

Nematophagous fungi are natural enemies of nematodes and have cosmopolitan distribution (De Freitas Soares et al. 2018). *Arthrobotrys* is the most complex, wide, and dominant group genus of nematophagous fungi in most habitats representing 59 accepted species (Zhang et al. 2022). Also, *Harposporium* are commonly known nematode-trapping fungi genera (Yang and Zhang 2014). In Türkiye, *A. arthrobotryoides* was isolated from outdoor air samples in Çorum, Manisa and Van province (İmalı 2005, İmalı et al. 2011, Kalyoncu and Ekmekci 2008), *A. oligospora* from egg masses and females of *M. incognita* from tomato fields in Ankara province (Karakas 2015) and *Arthrobotrys* sp. from wheat and barley seeds in Konya province (Yurdakul 2019). It was reported that 3 *Arthrobotrys* species obtained in this study were isolated from the field and forest soil (Yu et al. 2014).

Nematode–trapping fungi form specific trapping structures on hypha, such as adhesive networks, adhesive knobs, and constricting rings (Rubner 1996). It is determined that *Arthrobotrys cladodes*, *A. conoides* and *A. superba* are nematode-trapping fungi and capture nematodes by three-dimensional adhesive networks in this study; however, *Harposporium* species are endoparasitic fungi and have specially shaped spores that are ingested by nematodes. Because of their shape, the spores get stuck in the esophagus of the nematodes and start the infection from there (Aschner and Kohn 1958). A nematode containing the species *H. anguillulae* or *H. crassum*, conidia and conidiophores form outside of the nematode cadaver.

Accurate identification of the biological agents is the most important condition for success in biological control. Morphological identification of fungi is a traditional method that has been used for many years. However, distinguishing morphologically similar species in this way

may lead to misconceptions for scientists who do not have sufficient experience. Therefore, in addition to the classical diagnostic method, ribosomal DNA-ITS regions are widely used for molecular identification of fungi species (Li et al. 2014, Wang et al. 2022). In this study, the identification of *Arthrobotrys* isolates was also confirmed by rDNA-ITS sequence analysis. Similar to the previous study, molecular techniques are used to identify fungi (Zhang et al. 2022). In this study, all *Arthrobotrys* and *Harposporium* species were determined for the first time from Türkiye. Studies on nematophagous fungi in Türkiye, which has a potential in terms of biological control, should be increased.

Author's Contributions

Authors declare the contribution of the authors is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

ÖZET

Erzurum ve Erzincan illerinde yonca (*Medicago sativa* L.), patates (*Solanum tuberosum* L.) ve çilek (*Fragaria* x ananassa Duchesne) bitkilerinin köklerinde 2009-2011 yıllarında bazı nematofag funguslar belirlenmiş ve fungus türlerinin tanılanması klasik ve/veya moleküler tekniklerle gerçekleştirilmiştir. Saflaştırılan 5 izolatın *Arthrobotrys cladodes* Drechsler 1937 (1 izolat), *Arthrobotrys conoides* Drechsler 1937 (1 izolat) ve *Arthrobotrys superba* Corda 1839 (3 izolat) türlerine ait olduğu belirlenmiştir. *Arthrobotrys* izolatlarının tanılanması ribozomal DNA (rDNA)-ITS (internal transcribed spacer) baz dizi analizi ile de doğrulanmıştır. İki örnekte bulunan nematodlar üzerinde gelişen sporların morfolojik özellikleri dikkate alınarak *Harposporium* cinsi de belirlenmiş, ancak bu funguslar saflaştırılamamıştır. *Harposporium* türleri morfolojik özelliklerine göre *Harposporium anguillulae* Lohde emend. Zopf 1888 ve *Harposporium crassum* A.M. Sheph 1955 olarak tanılanmıştır. Bildiğimiz kadarıyla bu çalışmada belirlenen tüm *Arthrobotrys* ve *Harposporium* türleri Türkiye'de ilk defa rapor edilmiştir.

Anahtar kelimeler: *Arthrobotrys, Harposporium*, nematofag fungus, rDNA-ITS bölgesi

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