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Investigation of pistachio saplings in Siirt province regarding soil fungal pathogens

Siirt ili Antep fıstığı fidanlıklarının fungal toprak patojenleri yönünden araştırılması

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ABSTRACT

Pistachio (*Pistacia vera* L.) is a significant agricultural product in the Southeastern Anatolia Region, particularly in the province of Siirt. A substantial portion of the region's demand for saplings is fulfilled by nurseries in this province. In this study, the objective was to identify fungal pathogens responsible for root, crown rot, and wilt symptoms, as well as to determine disease prevalence rates in pistachio nurseries located in Siirt province. The incidence of plants exhibiting disease symptoms in the examined nurseries was found to range between 1% and 6%. Since plants showing disease symptoms were observed in each nursery, the prevalence was calculated as 100%. As a result of the survey, 142 fungal isolates belonging to 12 species and 8 genera were obtained. These isolates were identified morphologically and molecularly. As a result of the pathogenicity studies, it was determined that several *Fusarium species* (*F. solani, F. oxysporum, F. verticillioides* (Syn. *F. moniliforme*), *F. equiseti, F. avenaceum, F. proliferatum, Fusarium* spp.) and *Neoscytalidium dimitatum*, *Rhizoctonia solani*, *Macrophomina phaseolina*, *Phoma* spp., *Cylindrocarpon* spp. colonized the pistachio branches, leading to tissue discoloration. According to the symptoms observed on the branches, the most pathogenic fungal isolates were identified as follows: *N. dimitatum* BŞR9.1, *N. dimitatum* AKT1.3, *F. solani* BŞR9.2, *F. oxysporum* BŞR5.4, *M. phaseolina* BŞR1.3, and *F. equiseti* BŞR2.3.1, respectively. Additionally, figures depicting the morphological characteristics and microscopic images of the pathogens are provided.

INTRODUCTION

Türkiye ranks third in global pistachio production, following the United States and Iran (Anonymous 2022). The cultivation and production of pistachios in Türkiye have a long-standing tradition, particularly in the Southeastern Anatolia Region, where the climatic conditions are well suited to meet the requirements of pistachio cultivation. Approximately 96% of Türkiye's pistachio production originates from this region, with Siirt province ranking

third with 320,600 acres, following Şanlıurfa and Gaziantep (Anonymous 2023). The tombul variety of pistachios is cultivated in Siirt and the surrounding regions. This variety is known for its large size, wide cracking interval, and white shell. Due to these characteristics, it enjoys strong demand in domestic and international markets, particularly as a snack. Furthermore, driven by its lucrative returns, production areas in Siirt and neighboring provinces such as Batman and Diyarbakır are continually expanding. The demand for saplings in these extensive cultivation areas is met by companies in the vicinity of Siirt province, many of which operate without licenses. In some newly established orchards with saplings obtained from certain nurseries, symptoms such as root rot and wilting have been reported, leading to the drying up of young trees (Aydın et al. 2023).

Pistachio yield and quality are influenced by various biotic and abiotic factors. The most significant among these factors are diseases, pests, fertilization, periodicity, drought, and temperature fluctuations. Fungal diseases tend to escalate depending on regional and climatic conditions, with pathogens such as *Armillaria mellea* (Vahl) P. Kumm., *Phymatotrichopsis omnivora* (Shear) Hennebert., *Fusarium* spp. Link, *Eutypa lata* (Pers. Fr.) Tul & C. Tul., *Cytospora terebinthi* (Bres., *Gibberella zeae* (Schwein.), *Rhizoctona solani* Kühn, *Phytophthora* spp. de Bary, *Sclerotinia sclerotiorum*, (Lib) de Bary and *S. minor* Jagger, *Verticillium dahliae* Kleb, *Macrophomina phaseolina* (Tassi) Goid., and *Neoscytalidum dimitatum* (Penz.) Crous & Slippers play a significant role in causing root, crown rot, and wilt in pistachios worldwide (Aydın 2019, Aydın et al. 2023, Chitzanidis 1995, Eskalen et al. 2001, Michailides et al. 1995, Teviotdale et al. 2002, Türkölmez et al. 2015). These pathogens cause symptoms on the roots and root collars of pistachio trees, ultimately resulting in their withering and drying. *Phytophthora* species, in particular, have been identified as the most significant and widespread disease pathogen in Iran, one of the world's leading pistachio producers (Banihashemi 1995, Sheibani 1995). A study conducted in California, a region known for intensive pistachio farming in the USA, reported the isolation of *Macrophomina phaseolina*, *Fusarium solani*, *F. equiseti*, *F. oxysporum* and *F. proliferatum* from diseased trees (Nouri et al. 2018b). *R. solani*, another significant polyphagous soil pathogen, has been reported to cause disease in young pistachio trees in both nurseries and orchards (Aydın and Ünal 2021, Holtz et al. 1996, Holtz and Teviotdale 2016). Additionally, it has been reported that certain *Fusarium species* (*F. solani, F. equiseti, F. proliferatum*) affect plants in pistachio orchards, particularly young trees (Aydın et al. 2023, Crespo Palomo et al. 2019, Nouri et al. 2018b, Triki et al. 2011). In studies conducted in Tunisia and Syria in 2011 and 2014-2015, it was reported for the first time that symptoms of drying in pistachio nurseries were caused by *Fusarium solani* (Triki et al. 2011, Walid and Abeer 2017). Furthermore, in studies conducted in California, USA, *F. solani*, *F. proliferatum*, *F. oxysporum* and *F. equiseti*, among other pathogens, were isolated. These fungi were found to induce discoloration and wilting in vascular bundles of plants (Nouri et al. 2018b).

In this study, fungal pathogens responsible for root and root collar rot, as well as wilt, were identified in nurseries located in Siirt province. Additionally, the disease rate within these nurseries and the prevalence rate across the province were determined, thus shedding light on the overall disease situation in the nurseries.

MATERIALS AND METHODS

Disease survey and collection of samples

The survey was conducted between May and November of the years 2021-2022. Information was obtained from the Siirt Provincial Directorate of Agriculture and Forestry to identify the nurseries included in the survey. The satellite image of the study areas is provided in Figure 1.

Figure 1. View of the areas where pistachio nurseries are located in Siirt province

General control of the sampled nursery was carried out in terms of disease symptoms and case of symptoms, sampling points were selected according to their size. If the nursery area is up to 1 da, a total of 300 plants from 3 points, if the nursery area is up to 1-5 da, a total of 600 plants from 6 points, if the nursery area is more than 5 da, a total of 900

plants from 9 points were examined (Bora and Karaca 1970). To determine the disease rate, the percentage of disease was calculated by considering whether the examined plants in each sampled nursery showed symptoms of the disease or not. If only one plant in the nursery production area showed symptoms of the disease, the production area under examination was considered infected, and the prevalence rate was determined (Table 1).

Isolation of fungi from diseased plants

The underground parts of plants exhibiting symptoms of wilting and root collar rot, collected from the study areas, underwent examination. Diseased plants were uprooted and transported to the laboratory in an icebox. Subsequently, the affected plant parts were washed under tap water, cut into 1-2 cm long segments, and subjected to superficial disinfection by soaking in 1% sodium hypochlorite (NaOCl) for 1-2 minutes. Following this, they were rinsed twice in sterile distilled water and dried between blotting papers. These tissue pieces were transferred onto various media including Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Oatmeal Agar (OA), Ethylene Water Agar (EWA), Corn Meal Agar (CMA), and Carrot Agar (CA), then incubated at 22-24 °C for 5-7 days. Mycelial tips from the periphery of fungal colonies developing from diseased parts were excised and transferred to suitable media, resulting in the isolation of pure cultures.

Identification of fungal isolates

The isolates were purified on suitable media conducive to the growth of each fungus, after which they were grouped based on color, growth characteristics, and spore structure observed under a microscope. Subsequently, they underwent morphological and molecular identification.

Morphological diagnosis

The fungi were identified both macroscopically and microscopically using diagnostic keys, considering colony color, growth rate, hyphae color and division, phialides shape, presence and size of macro and micro conidia, presence of chlamydospores, pycnidium, perithecium, and macro and micro sclerot formation (Booth 1971, Crespo Palomo et al. 2019, Hanlin 1998, Liu et al. 2015, Phillips et al. 2013,Samuels 2006, Seifert 1996). Images of the identified fungi are provided in the morphological characterization section.

Molecular diagnostics

Molecular studies were also carried out with some fungi found to be important as a result of morphological identification. For this purpose, isolation of fungal DNA and PCR tests were performed. The methods followed for molecular studies were as follows.

DNA extraction from fungus cultures

DNA isolation was performed based on the CTAB (cetyltrimethylammonium bromide) method developed by Doyle and Doyle (1987) and modified by Karaca et al. (2005). In the current study, DNA isolation was performed for a total of 21 fungal samples in the first step and then the samples were amplified by PCR with primers specific to ITS gene regions. For DNA isolation, the CTAB protocol was used, the fungal samples were placed at -80 °C and after the hyphae were frozen and hardened, the mycelia were placed in Eppendorf tubes with the help of a sterile scalpel, crushed thoroughly, 400 µl CTAB Buffer was added and vortexed. The mixture was incubated in a water bath (65 0C) for a total of half an hour and allowed to cool. An equal volume of 24:1 (Chloroform: Isomyl Alcohol) was added and mixed to dissolve. Centrifuged at 13000 rpm for 10 min and the supernatant was transferred to a new tube. Isoproponal was added as 2/3 of the obtained liquid. It was kept at room temperature for 20 min and centrifuged again at 13000 rpm for 20 min. The resulting pellet was washed with 1 ml 70% ethanol and the ethanol was poured over and left to dry for 20 min. The pellet was added 50 µl 0.1xTE Buffer and stored at -20 °C after thawing.

PCR study

Template DNA, Primer F, Primer R, dNTP, Taq, Taq Buffer, $MgCl_2$, BSA, ddH_2O components were used. PCR was performed in a volume of 25 µl. PCR was performed at 95 °C for 1 cycle 5 min, 94 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min, 72 °C for 1 min, and finally 4 °C for 35 cycles.

Sequence analysis and phylogenetic tree

Sequence readings of the samples were performed unidirectionally. The sequences were identified by using the NCBI BLAST programme with the most similar sequences in the DNA Data bank and a phylogenetic tree was constructed using the MEGA X software and Neighbour Joining (NJ) method.

Pathogenicity testing

Fungal isolates were tested on one-year woody shoots obtained from pistachio trees. Fresh vegetative shoots were collected from 10-15-year-old trees and cut into pieces approximately 30-35 cm in length and 5-7 mm in diameter. Surface sterilization was then carried out by treating the grafting area with 70% ethanol after removing the outer bark.

A piece of mycelium (4 mm in diameter and 3 mm thick) was taken from the edge of a one-week-old fungal colony growing on Potato Dextrose Agar (PDA) medium. This mycelial piece was then carefully inserted into a 1 cm hole, which was radially opened between two nodes using a drill. In the control shoots, only the PDA was inoculated. The inoculated areas were safeguarded by covering them with moist cotton and wrapping them with Parafilm to prevent drying and contamination. Four cut shoots were used for each fungal isolate. Following the wrapping of the inoculated shoot tips with Parafilm to preserve moisture, they were placed in glass Petri dishes containing moistened filter papers and then incubated at 24 ± 2 °C in an incubator. The experiment was arranged in a completely randomized design with four replications.

Evaluation

The evaluation was made after 90 days, taking into account the symptoms on the shoots. The Parafilm covering the shoots was removed, and the bark surrounding the infection site was peeled off to measure the length of color change occurring at the inoculation point. To fulfill Koch's postulates, tissue pieces were taken from the edges of necrotic lesions for re-isolation procedures. The significance of differences in mean lesion lengths was determined using analysis of variance (ANOVA), and the LSD test was employed to compare application means at a significance level of $P < 0.01$.

RESULTS

Determination of disease rate and prevalence in pistachio nurseries

A survey was carried out in the area where a total of 16 saplings were grown. The proportion and prevalence of plants showing disease symptoms in the nurseries studied are given in Table 1.

According to Table 1, plants exhibiting symptoms of root rot and wilt were detected in all nurseries at varying rates. Consequently, the prevalence rate of the disease was determined as 100% in nurseries across Siirt province. The highest disease rate in the nurseries was observed in Aktaş 3 nursery at 7%. Başur 5 ranked second with 6%, followed by Aktaş 2 with 5%. Overall, considering all nurseries, the disease rates ranged from 1% to 7%.

Identification of isolated fungi and their ratios according to nurseries

Following the isolation of fungi from plants exhibiting disease symptoms, the isolates were examined both

Table 1. The rate and prevalence of plants showing root rot and wilt symptoms in nurseries surveyed in Siirt province

Province	District	Nursery area	Disease rate (%)*	Prevalence rate (%)**
Siirt	Şirvan	Taşlı köyü	2	100
	Merkez	Aktaş 1	1	100
		Aktaş 2	5	
		Aktaş 3	7	
		Merkez 1	1	100
		Merkez 2	2	
		Başur 1	1	100
		Başur 2	2	
		Başur 3	3	
		Başur 4	3	
		Başur 5	6	
		Başur 6	3	
		Başur 7	3	
		Başur 8	2	
		Başur 9	1	
		Başur 10	2	

*h= z.100/x, h = disease rate, x = the total number of plants examined, z = the number of diseased plants

** y= c.100/a, y = the prevalence rate, a = the number of production areas surveyed, c = the number of production areas with disease detected

macroscopically and microscopically and morphological diagnosis were made based on species and genus using diagnostic keys. Subsequently, certain significant fungi were chosen and identified through molecular methods. Table 2 presents the number of fungi isolated and identified from each nursery.

According to Table 2, the highest number of fungal isolates was detected in nurseries AKT1 and BŞR1, while the lowest was found in nurseries MRK2 and BŞR8. Among the obtained fungi, *Fusarium* species were more prevalent compared to other fungi. *F. solani* was the most commonly isolated species with 41 isolates, followed by *F. oxysporum* with 23 isolates, *R. solani* with 16 isolates, *Fusarium* spp. with 14 isolates, *M. phaseolina* with 7 isolates, *F. verticillioides* (syn. *F. moniliforme*) with 6 isolates, *F. proliferatum* and *F. equiseti* with 4 isolates each, *Phoma* sp. with 3 isolates, and *F. avenaceum* with 2 isolates. Additionally, *N. dimitatum*, *Cylindrocarpon* spp., *Phytophthora* sp., *Bipolaris* sp., *Gliocladium* sp., *Alternaria* spp., *Aspergillus* spp., *Penicillium* spp., *R. endophytica*, and *Clonostachys rosea* (syn. *Gliocladium roseum*) were among the other important fungi isolated. The percentages of these fungi are given in Figure 2.

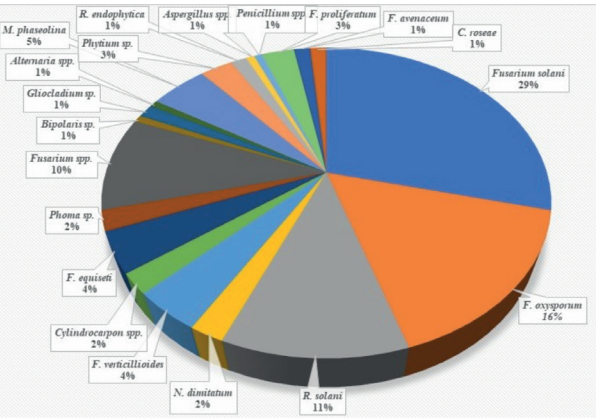

Figure 2. Rates of fungi isolated from nurseries in Siirt province (%)

According to Figure 2, *F. solani* was the most isolated fungus, accounting for 29% of the total fungi. It was followed by *F. oxysporum* at 16%, *R. solani* at 11%, *Fusarium* spp. at 10%, *M. phaseolina* at 5%, *F. verticillioides* and *F. equiseti* at 4% each, *F. proliferatum* and *Phytium* spp. at 3% each, and *Phoma* sp., *Cylindrocarpon* spp., and *N. dimitatum* at 2% each.

Pathogenicity studies were conducted with selected isolates from these fungi. The images of these selected fungal

Table 2. Fungi isolated from plants showing disease symptoms in nurseries and their quantitative evaluation

Fungi	Nurseries																Total
	ŞTYF	AKT1	AKT2	AKT3	MRK1	MRK2	BŞR1	BŞR2	BŞR3	BŞR4	BŞR5	BŞR6	BŞR7	BŞR8	BŞR9	BŞR10	
Fusarium solani	2	5	3	2	2	1	3	3	3	1	6	1	2	3	3	1	41
Fusarium oxysporum	1	2	2	1	0	0	2	1	1	3	2	3	0	2	2	1	23
Rhizoctonia solani	1	2	1	2	0	1	2	0	1	1	1	0	1	0	1	2	16
Neoscytalidium dimitatum	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Fusarium verticillioides	1	0	0	0	0	0	0	0	0	1	1	0	2	0	1	0	6
Cylindrocarpon spp.	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	3
Fusarium equiseti	0	1	0	0	0	1	0	1	0	0	1	0	1	0	1	0	6
Phoma sp.	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	3
Fusarium spp.	0	3	0	0	2	2	3	0	0	0	0	2	0	0	0	2	14
Bipolaris sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Gliocladium sp.	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Alternaria spp.	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Macrophomina phaseolina	0	0	0	0	1	0	2	1	0	1	0	1	0	0	0	1	7
Phytium sp.	0	0	0	0	0	0	1	0	0	0	0	2	0	0	1	0	4
Rhizoctonia endophytica	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2
Aspergillus spp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Penicillium spp.	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Fusarium proliferatum	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	4
Fusarium avenaceum	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2
Clonostachys roseae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2
Total	8	16	9	6	7	5	14	7	6	9	12	10	9	5	10	9	142

isolates on media and under a microscope, along with their characteristic features, are provided below under the title "Morphological Characterization."

Morphological characterisation of fungi

Fusarium species (*F. solani*, *F. oxysporum*, *F. verticillioides*, *F. poliferatum*, *F. equiseti*, *F. avenaceum*) and *Rhizoctonia solani*, *Macrophomina phaseoli*, *Cylindrocarpon* spp., *Neoscytalidium dimitatum*, *Phoma* spp., *R. endohytica* and *Cylonostachys rosea* were identified morphologically. Morphological characteristics, and macroscopic and microscopic images of these species are given below in detail.

Fusarium solani (Mart.) Sacc.; In this study, *Fusarium solani* emerged as the most prevalent fungus isolated from pistachio saplings. *Fusarium species*, including *F. solani*, are known to produce both micro and macroconidia, which primarily serve as asexual spores in their reproductive cycle. However, it's worth noting that certain *Fusarium* species have been documented to also produce ascospores (Cavinder et al. 2012). It produced initially white and cottony colonies which later turned slightly purplish on PDA medium (Figure 3A). The colonies completely covered the Petri dish after one week of incubation at 22-24 °C, albeit at a slower rate compared to *F. oxysporum*. Long monophialides were observed to develop on the conidiophores, bearing monoconidia (Figure 3B). Although macroconidia are typically three-septate, those with 4-5 septa were also observed (Figure 3C). Additionally, abundant chlamydospores were found to be produced singly or in pairs, characterized by round shapes, rough walls, and dimensions ranging from 6 to 11 µm (Figure 3D).

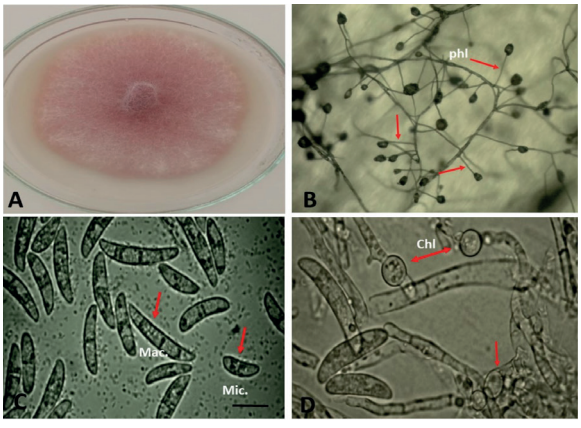

Figure 3. Image of *Fusarium solani* in PDA medium (A), under the microscope showing phialides (B), micro-macroconidia (C), and chlamydospores (D). Scala bar in C= 30 µm (magnification 400×); mac, macroconidia; mic, microconidia; chl, chlamydospore; phl, phialide.

Fusarium oxysporum (Schlecht.); The life cycle of this species resembles that of most *Fusarium species*. It can overwinter in soil and infected plant debris as spores or mycelium for many years. The hyphae developing on PDA medium were observed to be initially white and then turned purplish-pinkish from the center outwards (Figure 4A). It was observed that the phialides arranged on fungal hyphae were shorter and branched compared to *F. solani*, with microconidia present at the tip (Figure 4B). After one week on the medium, it was observed that chlamydospores were scattered irregularly, occurring singly or in pairs (Figure 4C), and abundant micro-macroconidia were formed, with microconidia being aseptate or one-septate, and macroconidia being 3-5 septate (Figure 4D).

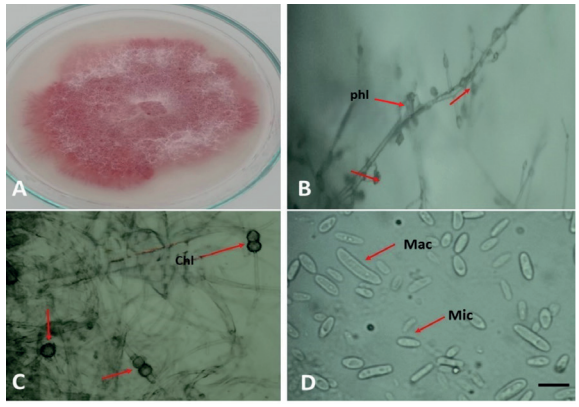

Figure 4. Images of *Fusarium oxysporum* on PDA (A), under the microscope showing phialides (B), chlamydiospores (C), and micro-macroconidia (D). Scala bar in D= 30 µm; (magnification 400×); mac, macroconidia; mic, microconidia; chl, chlamydospore; phl, phialide.

F. verticillioides (Sacc.) Nirenberg; It is also known as *Fusarium moniliforme*. Additionally, its teleomorph is *Fusarium fujikuroi* (*Gibberella fujikuroi*). This species produces potent mycotoxins that have a negative impact on the environment (Shephard 2011). It showed rapid growth on PDA and MEA medium. The colonies were white to pale salmon coloured and appeared powdery due to the production of mycelium and microconidia chains (Figure 5A). The phialides carried microconidia chains on hyphae and showed verticillate branching (Figure 5B).

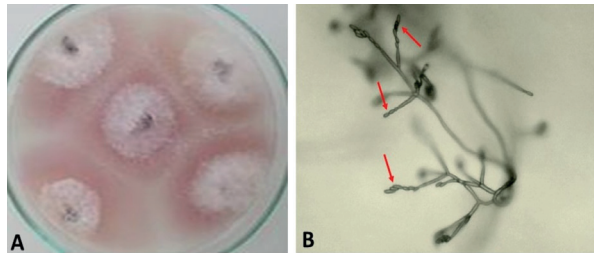

Figure 5. Images of *Fusarium verticillioides* on PDA (A), and the shape of verticillate branching with microconidial chains under the microscope (B)

Fusarium proliferatum (Matsush.) Nirenberg ex Gerlach & Nirenberg); The growth on PDA medium was slower compared to some other Fusarium species. It initially produced white pigments, which later turned light purplish on the medium (Figure 6A). Chlamydospores were observed either singly or in pairs, occasionally forming short chains. *F. proliferatum* was found to produce microconidial chains on the phialides (Figure 6B). Another *Fusarium species* with conidial chains had been previously mistaken for *F. verticillioides*. However, in *F. verticillioides*, the phialides typically exhibit verticillate branching.

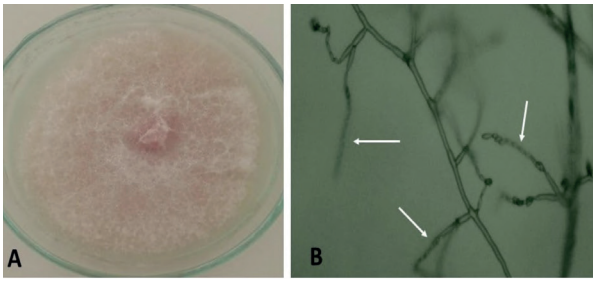

Figure 6. Image of *Fusarium proliferatum* on PDA medium (A) and microconidial chains under microscope (B)

Fusarium equiseti (Corda) Sacc.; In culture media, it exhibited light colors, initially white, then turning into a light orange shade (Figure 7A). Microconidia were observed to be oval, hyaline, and 0-1 septate, while macroconidia were conical with long apical pointed cells, hyaline, and 2-5 septate, with a curved shape. Abundant chlamydospores were produced, which were appendaged and often found in chains (Figure 7B).

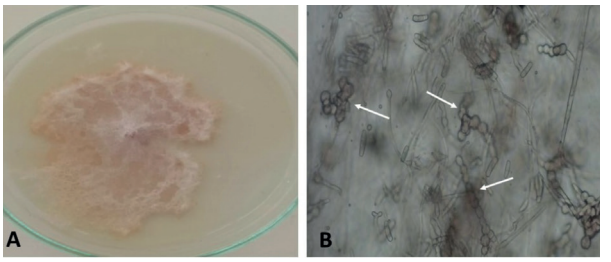

Figure 7. The development of *Fusarium equiseti* on PDA culture (A) and the appearance of chlamydospores under the microscope (B)

Fusarium avenaceaum (Fr.) Sacc.; Known as the teleomorph *Gibberella avenacea*, it exhibits a yellowish-red color on the upper part and a dark red color on the lower part of the PDA culture (Figure 8A). Macroconidia are typically 4-7 septate and needle-shaped, with the occurrence of microconidia being rare (Figure 8B). Furthermore, chlamydospore production was not observed.

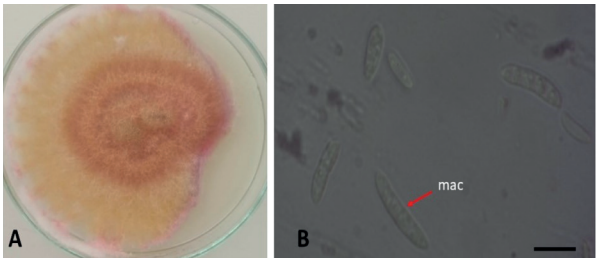

Figure 8. The appearance of *Fusarium avenaceum* on PDA (A) and the shape of conidia under the microscope (B). Scala bar in B= 25 µm; (magnification 400×); mac, macroconidia

Rhizoctonia solani Kühn.; It is a soil and seed-borne fungus belonging to the Basidiomycete group and is capable of living freely and saprophytically in soil (Boosalis and Scharen 1959). It is divided into 14 anastomosis groups (AGs), which are incompatible with each other in terms of reproduction and multiplication (Sneh et al. 1996). It has the potential to cause diseases in various annual and perennial fruit, vegetable, industrial, and cereal crops (Aydın 2022, Canpolat et al. 2023, Carling et al. 1994, Mohammadi et al. 2003). The colonies grown on nutrient medium in the laboratory initially appeared light brown, which later turned buff in color (Figure 9A). The hyphae were visibly septate, and as they aged, they changed from light to dark in color. When the hyphae branched, they formed a 90-degree angle, and a constriction was observed at the base of the hyphal branch (Figure 9B). The fungus persists through unfavorable conditions by forming resistant structures known as sclerotia and rhizomorphs.

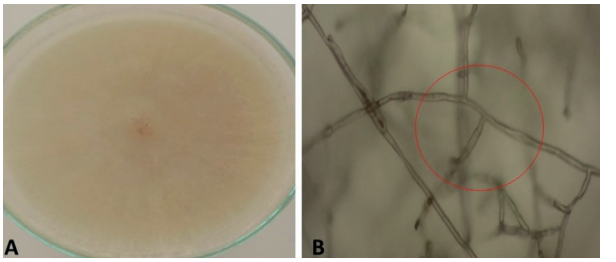

Figure 9. Images of *Rhizoctonia solani* on PDA (A), and under the microscope (B)

Neoscytalidium dimitatum (Penz.) Crous & Slippers; This fungus produces filamentous, dark-coloured, curved, and irregular hyphae. It is also characterized by overhead mycelium and rapidly growing colonies. In the early stages of incubation on PDA medium, colony growth exhibits a white color with a dark center (Figure 10A). In the later stages of development, the mycelium turns dark green and within a week becomes dark black (Figure 10B). Both chain-shaped arthroconidia and pycnidia are produced in the same culture (Figure 10C). These conidia appear undivided or with a single division, are thick-walled, brown, circular, and oval-shaped (Figure 10D).

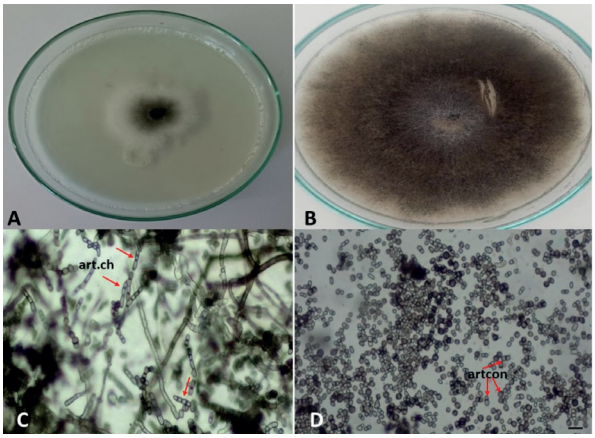

Figure 10. The initial growth stage of *Neoscytalidium dimitatum* on PDA medium (A), its development one week later (B), the microscopic view of arthroconidia (C), and the conidia (D). Scala bar in D= 10 µm; (magnification 400×); art.ch, arthric chains; artcon, arthroconidia

Cylindrocarpon spp. (Wollenw); *Cylindrocarpon* spp. (Wollenw); also known as Ilyonectria spp. The fungal colonies grew rapidly on PDA medium and appeared whitish in color, forming slimy masses (Figure 11A). Conidia were observed to be unicompartmental, hyaline, cylindrical, and flat-bottomed (Figure 11B).

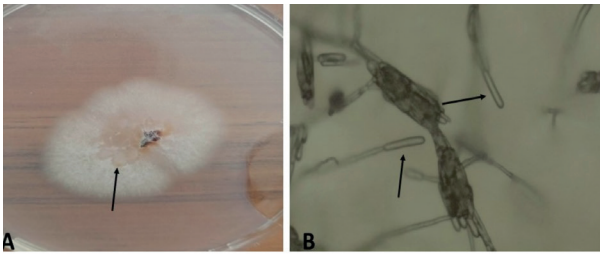

Figure 11. The appearance of *Cylindrocarpon* during its initial growth stage on PDA medium (A), and the shape of conidia under the microscope (B)

Phoma spp. (Saccardo); Development on PDA medium exhibited a dark brown color change toward the center and a lighter color change toward the outside (Figure 12A). One week later, it was observed that pycnidia were formed, and pycniospores emerged en masse. These spores were colorless and single-compartmented (Figure 12B).

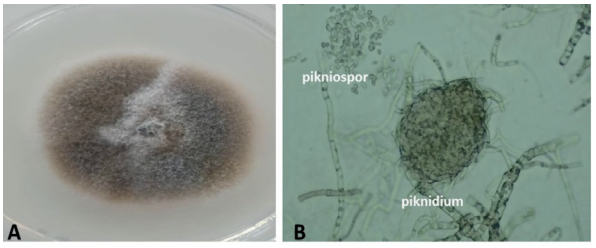

Figure 12. *Phoma* spp.'s development on PDA medium (A), and the microscopic view of pycnidia with pycnidiospores (B)

Macrophomina phaseolina (Tassi); is a soil-borne fungus that is widespread worldwide and causes charcoal-colored rot in the roots and root collars of both annual and perennial plants (Canpolat et al. 2022, Ghosh et al. 2018, Iqbal and Mukhtar 2014). The growth of this fungal pathogen on PDA and MEA media was similar, and it was observed that it covered all petri dishes after approximately one week of incubation. The color of mycelial hyphae was observed to change from dark grey to black (Figure 13A), and sclerotia were observed on top of these hyphae under the microscope (Figure 13B). It was detected in pistachio orchards in Turkey (Aydın et al. 2023).

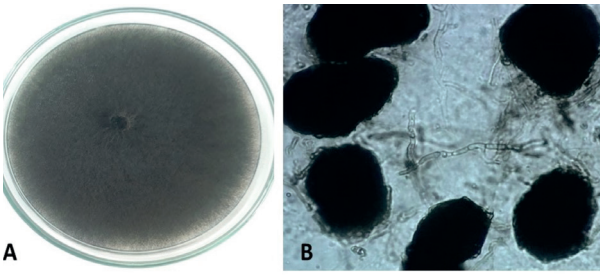

Figure 13. Image of *Macrophomina phaseolina* on PDA (A) and sclerotia under microscope

Cylonostachys rosea (Schroers); also known as *Gliocladium roseum*. As an endophyte, it colonizes living plants, and as a saprophyte, it can live on soil materials. It is known as a good biological control agent and has well-developed mycoparasitic properties (Roy 1989). Growth on PDA medium was slow, and it was only able to cover the petri dish completely in two weeks. Initial growth was light in color and white, cottony in appearance (Figure 14A). The mycelial color changed to light yellow with sporulation. Under the microscope, conidiophores were observed to be mostly branched in a penicillate or verticillate manner, with small clusters of spores forming at the tips of these branches (Figure 14B).

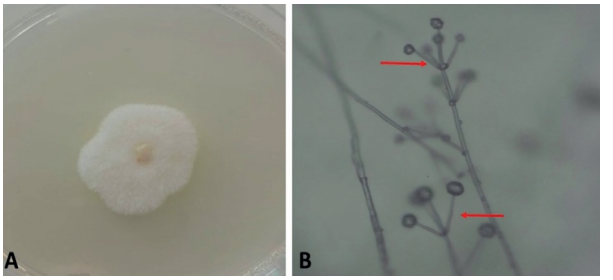

Figure 14. Development of *Cylonostachys rosea* on PDA medium and branching pattern of conidiophores under a microscope

Rhizoctonia endophytica (H. K. Saksena & Vaartaja); It is known as a non-pathogenic species among *Rhizoctonia species* and can be used in biological control. On PDA medium, unlike *R. solani*, it exhibited a light-colored appearance (Figure 15A). Microscopically, it appeared as thick hyphae with 45° and 90° angles (Figure 15B).

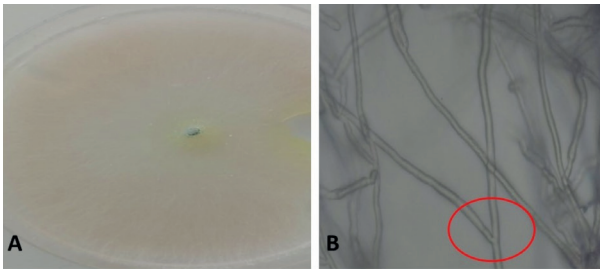

Figure 15. The appearance of *Rhizoctonia endophytica* on PDA medium (A) and the shape of hyphae under the microscope (B)

Molecular characterisation

Sequence analysis was carried out in one direction and reading was performed. The sequences were identified using the NCBI BLAST programme with the most similar sequences in the DNA Databank and the following phylogenetic tree was constructed using the MEGA X programme (Figure 16).

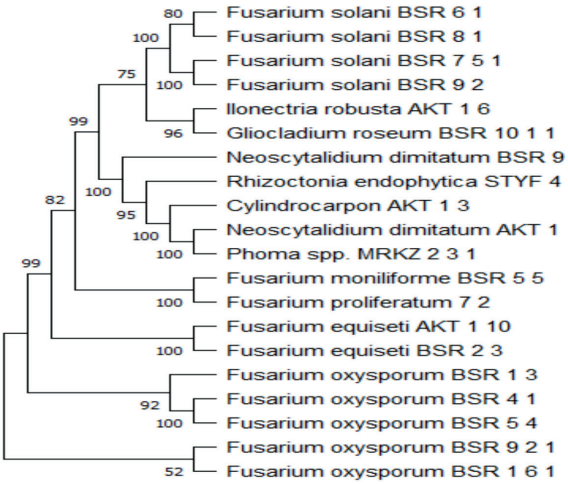

Figure 16. Neighbor-joining (NJ) phylogenetic tree generated for fungal isolates based on ITS sequences

The ITS gene sequence was used as the nuclear gene and the phylogenetic tree drawn using the Neighbour - Joining method is shown in Figure 16. Using the MEGA X software, Neighbour Joining was created with the bootstrap method with 500 repetitions. When the tree topology was analyzed, it was seen that the *Fusarium* samples collected from different geographical regions in the study were divided into 2 large groups. Among these two groups, the first group had a bootstrap value of 52 percent, while the second group had a bootstrap value of 99 percent. The second group was also divided into 5 subgroups with high bootstrap values. Within these groups, the majority of the samples collected from the same geographical regions were collected in one cluster. However, some specimens collected from the same region and morphologically identified as the same species were found to be in the same group with a different species as a result of molecular analyses (Figure 16). To confirm the accuracy of this situation more strongly, it is thought that the number of biological and technical sample replicates considered for molecular studies should be limited to at least three.

Results of pathogenicity studies

The selected fungal isolates were tested on one-year-old woody shoots collected from pistachio trees. The evaluation was conducted by peeling the bark surrounding the infection site and measuring the length of the discoloration at the point of inoculation. The significance of the differences in the lesion lengths of the treatments was determined using the JMP 5.0.1 statistical program, and the LSD test was utilized for the comparison of treatment means at $P < 0.01$. The differences were found to be significant in the comparison of treatment means. Thus, it was determined that the study was conducted within the appropriate statistical limits. The results obtained in the pathogenicity study are provided in Table 3.

According to Table 3, fungi caused lesions of different lengths on one-year-old plucked branches of the Siirt variety. On these shoots, a 10 mm area was opened for the placement of fungal discs, and measurements were made including this part. As a result of the measurements, *N. dimitatum* BŞR 9.1 and *N. dimitatum* AKT 1.3 isolates were found to have the highest degree of disease-causing lesion length of 58.18 mm and 57.43 mm, respectively. These isolates were followed by *Fusarium solani* BŞR 9.2, *F. proliferatum* BŞR 7.2 and *Macrophomina phaseolina* BŞR 1.3 with 55.2, 53.68, 50.10 mm, respectively. The lowest degree of disease was found in *Clonostachys rosea* (*G. roseum*) BŞR 10.1.1 and control isolates with 12.05 mm, and 10.58 mm, respectively. The appearance of the lesions on the branches as a result of the application is shown in Figure 17 and Figure 18.

Figure 17. Image of lesions on branches caused by some pathogens

Figure 18. Image of lesions on branches caused by some pathogens

Phoma spp. MRKZ 2.3.1, *Clonostachys rosea* BŞR 10.1.1, *Rhizoctonia endophytica* ŞTYF4 isolates and control treatment did not cause lesions on the branches (Figure 19).

Figure 19. Image of control and some fungus treatments on branches

After evaluations were made, pieces of plant tissue were transferred to culture media, and reisolations were performed. All fungi were reisolated except for the control, *Phoma* spp. MRKZ 2.3.1, *Clonostachys rosea* BŞR 10.1.1, and *Rhizoctonia endophytica* ŞTYF4. Thus, Koch's postulates were fulfilled.

DISCUSSION

The most important stage of pistachio production is to cultivate healthy saplings and provide them to farmers. Certified seedling production is crucial, particularly ensuring that the materials used in the growing environment (such as soil and seeds) are clean and free from disease sources. The nurseries in Siirt Province are typically situated approximately 10 kilometers outside the city center, near the Başur River. Despite official records indicating the presence of only four nurseries, in reality, there are dozens of nurseries operating in the area. Saplings cultivated in these nurseries are often transported in an unregulated manner and left by the roadside, where they are sold to producers. This has a negative impact on the cultivation of healthy saplings.

In the production areas, symptoms of root rot, root collar rot, and wilting were observed in saplings at rates ranging from 1 to 7 percent. It was also observed that nursery owners periodically removed seedlings showing signs of disease. In reality, the proportion of diseased saplings is likely to be higher. It was also discovered that a sapling carrying pathogen inoculum, although not showing any symptoms, dried up a few years after being planted in the pistachio orchard. Previous

Table 3. Mean degree of disease and groups formed by artificially infected fungi on detached one-year woody shoots

Seq. No.	İsolate Number	Fungi	Mean Disease Degree (mm)
1	BŞR 7.1.1	*Fusarium avenaceum*	37.38 HIJKL
2	AKT 1.6	*Cylindrocarpon* spp.	37.55 GHIJKL
3	BŞR 1.6.1	*Fusarium oxysporum*	41.80 EFGH
4	BŞR 6.1	*Fusarium oxysporum*	45.95 DE
5	BŞR 1.3	*Macrophomina phaseolina*	50.10 CD
6	BŞR 7.2	*Fusarium proliferatum*	53.68 ABC
7	AKT 1.10	*Fusarium equseti*	42.35 EFG
8	MRKZ 2.3.1	*Phoma* spp.	33.68 KL
9	BŞR 9.2	*Fusarium solani*	55.30 AB
10	AKT 1.2	*Didymella sinensis (Phoma* sp.*)*	43.95 EF
11	BŞR 9.2.1	*Fusarium oxysporum*	50.50 BCD
12	BŞR 2.3.1	*Fusarium equseti*	46.13 DE
13	BŞR 10.1	*Rhizoctonia* spp.	15.83 M
14	BŞR 7.5.1	*Fusarium solani*	40.43 FGHI
15	BŞR 5.4	*Fusarium oxysporum*	50.43 BCD
16	MRKZ 2.3	*Phoma* spp.	14.08 MN
17	BŞR 8.1	*Fusarium solani*	38.58 GHIJK
18	BŞR 9.1	*Neoscytalidium dimitatum*	58.18 A
19	AKT 1.3	*Neoscytalidium dimitatum*	57.43 A
20	AKT 2.4	*Fusarium oxysporum*	37.18 HIJKL
21	BŞR 4.1	*Fusarium oxysporum*	35.10 JKL
22	BŞR 1.3.1	*Rhizoctonia solani*	43.80 EF
23	BŞR 10.1.1	*Clonostachys rosea (G. roseum)*	12.05 MN
24	ŞTYF 4	*Rhizoctonia endophytica*	12.23 MN
25	BŞR 3.3	*Fusarium solani*	38.78 GHIJ
26	BŞR 5.4.2	*Fusarium* spp.	32.68 L
27	BŞR 5.5	*Fusarium verticillioides (F. moniliforme)*	35.65 IJKL
28	KONTROL	----------	10.58 N
OSH			1.74
p-değeri			<.0001
CV (%)			9.09

studies have identified that some soil pathogens, especially *M. phaseolina*, cause wilting in young trees aged 5-7 years (Aydın 2019, Nouri et al. 2018b).

The study isolated various *Fusarium* species, and it was determined that some of them are pathogens. *Fusarium* species cause symptoms of root and root collar rot as well as wilting in plants. Host plants include wheat, maize, barley, beans, cucurbits, strawberries, and anise. Additionally, *Fusarium* species cause damage to many fruit trees, including pistachios, as well as numerous forest plants (Arie 2019). It has been reported that the main species causing diseases in pistachios are *Fusarium solani*, *F. proliferatum*, *F. equiseti*, *F. oxysporum*, *F. moniliforme*, *F. redolens*, *F. brachygibbosum*, *F. chlamydosporum* and *Fusarium* spp. (Aydın et al. 2023, Crespo Palomo et al. 2019, Eskalen et al. 2001, Güldür et al. 2011, Nouri et al. 2018b, Swart and Blodgett 1998). Among the *Fusarium* species, *F. solani* was the most frequently isolated and identified as the pathogen. According to Triki et al. (2011), a study conducted in Tunisia reported *F. solani* as the primary pathogen causing root rot in pistachio trees. In another study by Naffaa and Rasheed (2017) in Syrian nurseries, significant mortality was observed in plants, and the cause was attributed to *F. solani*, highlighting the importance of this pathogen. It was reported that *F. moniliforme*, *Fusarium equiseti* and *F. oxysporum* fungi were pathogenic in pistachios in studies conducted in different years in the Southeastern Anatolia Region (Eskalen et al. 2001, Güldür et al. 2011, Tatlı 1996). Pistachio seedlings infected with *Fusarium* species showed symptoms of sudden drying on hot days, especially in July-August. *F. solani* causes root rot, while *F. oxysporum* causes both darkening of the roots and browning of the vascular bundles.

Neoscytalidium dimitatum was isolated from diseased plants in some nurseries and was found to be the most pathogenic isolate. This species belongs to the family Botryosphaeriaceae and has many hosts. In Turkey, it is reported to cause diseases on nutshell fruit trees such as almond, pistachio, and walnut (Dervis et al. 2019, Kurt et al. 2019, Ören et al. 2020). In these plants, it causes canker wounds on the roots, root collar, and branches (Nouri et al. 2018a).

Macrophomina phaseolina isolated from some nurseries was found to be one of the most pathogenic isolates. It is a soil-borne fungus that is widespread worldwide and causes charcoal-colored rot in the roots and root collars of both annual and perennial plants (Ghosh et al. 2018, Iqbal and Mukhtar 2014;). Nouri et al. (2020) reported that *M. phaseolina* is an important pathogen in pistachio trees, especially on young trees in orchards. It causes severe disease under high- temperature and low-humidity conditions in summer (Kaur et al. 2012).

Another important fungus obtained from the nurseries was *Rhizoctonia* sp. In the pathogenicity study, *R. solani* BŞR1.3.1 was found to be highly virulent. This pathogen has a wide host range and causes diseases in many cultivated plants, leading to significant economic losses globally (Sneh et al. 1996). In a study conducted by Aydın and Ünal (2021), it was reported that *R. solani*, AG-4 of the anastomosis group, is an important pathogen in pistachios in the Siirt region. Additionally, the virulence of two more *Rhizoctonia* isolates was investigated, and it was determined that they were not pathogenic. Among these, *R. endophytica* is reported to be endophytic and non-pathogenic.

Didymella sinensis and *Cylindrocarpon* spp. isolated from saplings were identified as pathogens, while *Phoma* sp. and *Clonostachys rosea* (syn. *Gliocladium roseum*) did not show any pathogenic symptoms. *Didymella* fungus is referred to as *Phoma*, Ascochyta-like in some literature (Woudenberg et al. 2009). These pathogens cause diseases in many plants, including root and root collar diseases. There is no literature evidence suggesting that this fungus causes root rot and wilting in pistachios. However, It was reported to cause leaf blight in pistachio (Moral et al. 2018).

One of the fungi isolated from diseased nursery tissues is *Clonostachys rosea*, which did not show any signs of disease on pistachio shoots. It is known that this fungus is an effective antagonist in biological control (Barnett and Lilly 1962).

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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

Antep fıstığı (*Pistacia vera* L.) Güneydoğu Anadolu Bölgesi'nde ve Siirt ili için önemli bir tarımsal üründür. Antep fıstığı fidan ihtiyacının önemli bir kısmı bu ildeki fidanlıklar tarafından karşılanmaktadır. Bu çalışmada, Siirt ilinde bulunan Antep fıstığı fidanlıklarında kök, kökboğazı çürüklüğü ve solgunluğa

neden olan fungal toprak patojenlerinin belirlenmesi ve hastalık yaygınlık oranlarının tespit edilmesi amaçlanmıştır. Hastalık belirtileri gösteren bitkilerin görülme sıklığı incelenen fidanlıklara göre %1 ila %6 arasında bulunmuştur. Her fidanlıkta hastalık belirtileri gösteren bitkiler görüldüğünden dolayı yaygınlık %100 olarak hesaplanmıştır. Sürvey sonucunda 12'si tür ve 8'i cins bazında olmak üzere 142 fungal izolat elde edilmiştir. Bu izolatlar morfolojik ve moleküler olarak da tanımlanmıştır. Patojenisite çalışmaları sonucunda çeşitli *Fusarium* türleri [*F. solani*, *F. oxysporum*, *F. verticillioides* (Syn. *F. moniliforme*), *F. equiseti*, *F. avenaceum*, *F. proliferatum* ve *Fusarium* spp.] ile *Neoscytalidium dimitatum*, *Rhizoctonia solani*, *Macrophomina phaseolina*, *Phoma* spp, ve *Cylindrocarpon* spp.'nın koparılmış Antep fıstığı dallarını kolonize ederek dokularda renk bozulmasına yol açtığı belirlenmiştir. En virulent fungal izolatlar sırasıyla, *N. dimitatum* BŞR9.1, *N. dimitatum* AKT1.3, *F. solani* BŞR9.2, *F. oxysporum* BŞR5.4, *M. phaseolina* BŞR1.3 ve *F. equiseti* BŞR2.3.1 olmuştur. Çalışmada ayrıca bu önemli patojenlerin morfolojik ve mikroskobik görüntüleri de verilmiştir.

Anahtar kelimeler: Antep fıstığı, fidanlık, toprak patojenleri, hastalık oranı

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