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Original article

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.

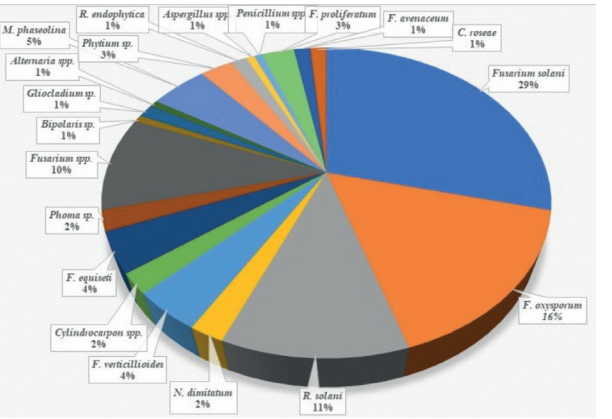

Figure 2. Rates of fungi isolated from nurseries in Siirt province (%)

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).

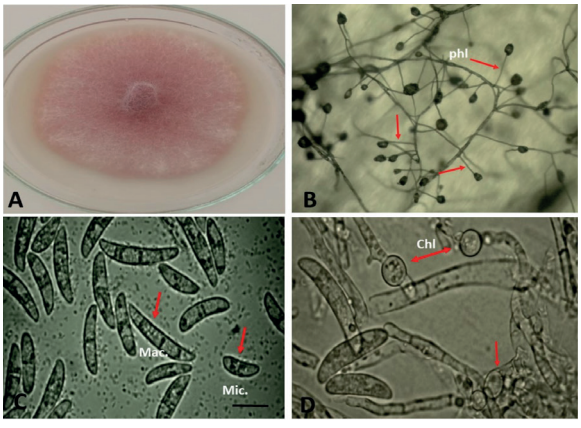

Figure 3. Image of *Fusarium solani* in PDA medium (A), under the microscope showing phialides (B), micro-macroconidia (C), and chlamydospores (D). Scala bar in C= 30 µm (magnification 400×); mac, macroconidia; mic, microconidia; chl, chlamydospore; phl, phialide.

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).

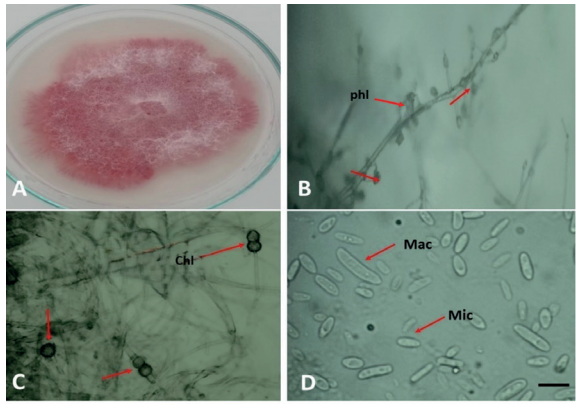

Figure 4. Images of *Fusarium oxysporum* on PDA (A), under the microscope showing phialides (B), chlamydiospores (C), and micro-macroconidia (D). Scala bar in D= 30 µm; (magnification 400×); mac, macroconidia; mic, microconidia; chl, chlamydospore; phl, phialide.

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).

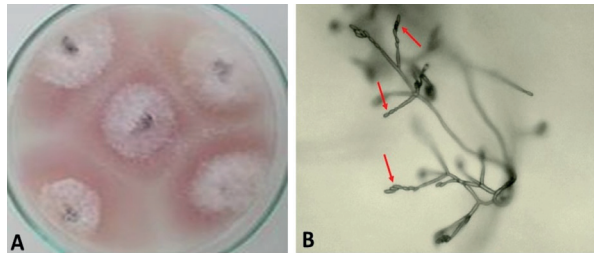

Figure 5. Images of *Fusarium verticillioides* on PDA (A), and the shape of verticillate branching with microconidial chains under the microscope (B)

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.

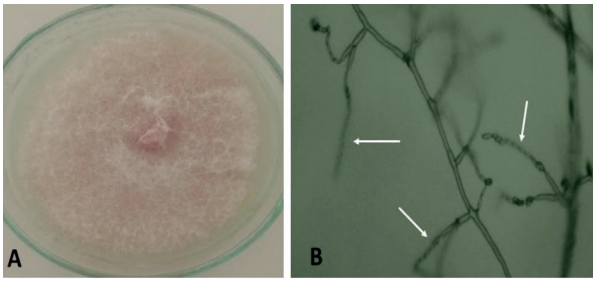

Figure 6. Image of *Fusarium proliferatum* on PDA medium (A) and microconidial chains under microscope (B)

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).

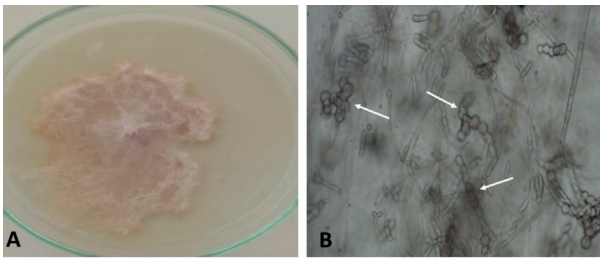

Figure 7. The development of *Fusarium equiseti* on PDA culture (A) and the appearance of chlamydospores under the microscope (B)

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.

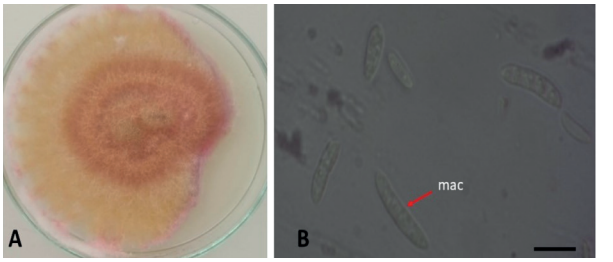

Figure 8. The appearance of *Fusarium avenaceum* on PDA (A) and the shape of conidia under the microscope (B). Scala bar in B= 25 µm; (magnification 400×); mac, macroconidia

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.

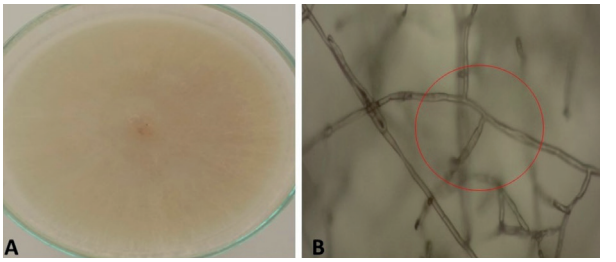

Figure 9. Images of *Rhizoctonia solani* on PDA (A), and under the microscope (B)

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).

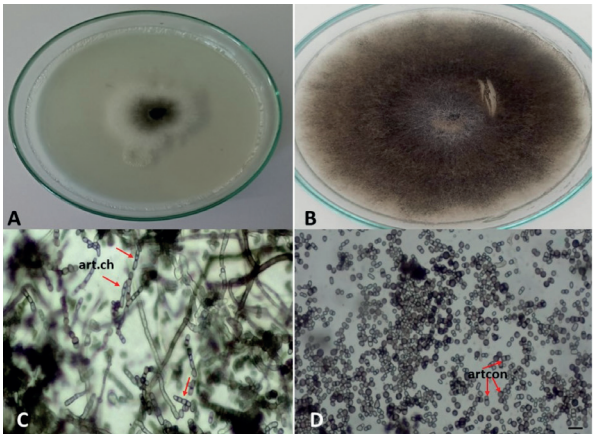

Figure 10. The initial growth stage of *Neoscytalidium dimitatum* on PDA medium (A), its development one week later (B), the microscopic view of arthroconidia (C), and the conidia (D). Scala bar in D= 10 µm; (magnification 400×); art.ch, arthric chains; artcon, arthroconidia

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).

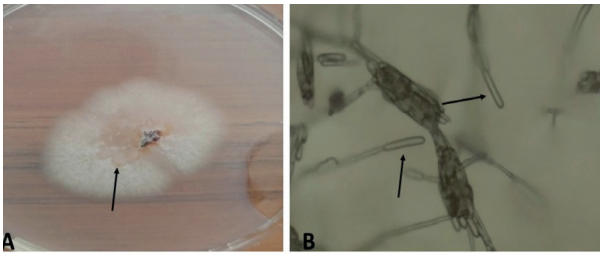

Figure 11. The appearance of *Cylindrocarpon* during its initial growth stage on PDA medium (A), and the shape of conidia under the microscope (B)

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).

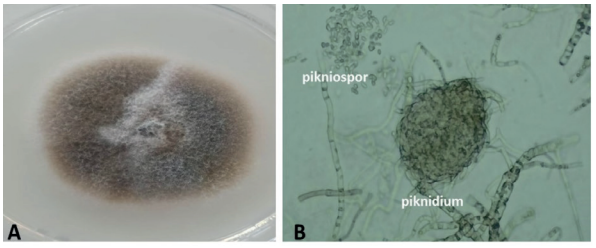

Figure 12. *Phoma* spp.'s development on PDA medium (A), and the microscopic view of pycnidia with pycnidiospores (B)

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).

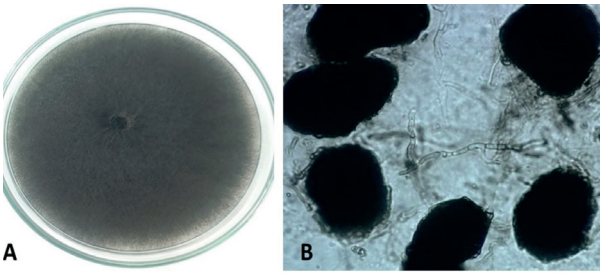

Figure 13. Image of *Macrophomina phaseolina* on PDA (A) and sclerotia under microscope

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).

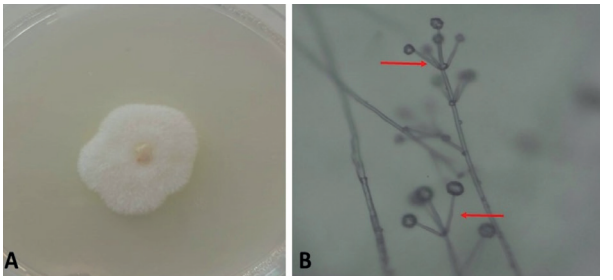

Figure 14. Development of *Cylonostachys rosea* on PDA medium and branching pattern of conidiophores under a microscope

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).

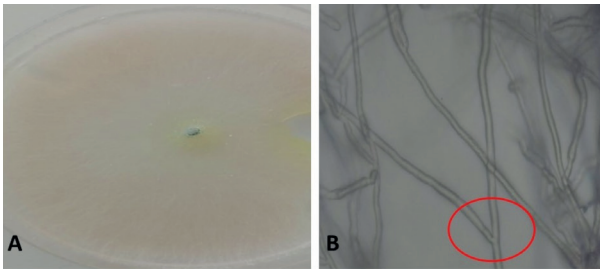

Figure 15. The appearance of *Rhizoctonia endophytica* on PDA medium (A) and the shape of hyphae under the microscope (B)

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).

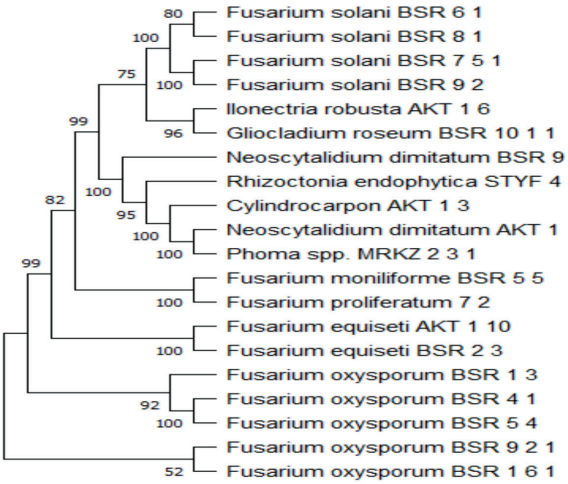

Figure 16. Neighbor-joining (NJ) phylogenetic tree generated for fungal isolates based on ITS sequences

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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Original article

Effect of peppermint leaf extract and peppermint oil on the flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae)

Nane yaprağı ekstresi ve nane yağının Kırmızı un böceği *Tribolium castaneum* (Coleoptera: Tenebrionidae) üzerindeki etkisi

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ABSTRACT

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), is among the global storage pests that lead to the deterioration of grains and stored products. A series of laboratory experiments were conducted to evaluate the efficiency of different concentrations of *Mentha piperita* L. (Lamiaceae) oil and leaf methanol extracts against the rust-red flour beetle in the different instars. The results of the present study showed that the mint, *M. piperita*, oil treatments at all concentrations used (8%, 10%, or 12%) were significantly effective, resulting in remarkable individual mortality of *T. castaneum* third instar larvae and adult mortality compared to the treatment of the peppermint methanol leaf extract at the highest concentration. Peppermint oil showed insecticidal effect against the third instar larvae of the red flour beetle, especially where sprayed at the highest concentration of 12%. The post-treatment results after seventh days showed 94% adult mortality and 82% mortality in the third instar of larvae. This study confirmed the possibility of using mint essential oil at relatively low concentrations as environmentally friendly product in controlling the rust-red flour beetle *T. castaneum*.

INTRODUCTION

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), is one of the most well-known agricultural pests in the field of food and grain storage (Pinto et al. 2020). It feeds on a variety of foodstuffs, such as grains, cereals, pulses, and other products, diminishing the quality of agricultural products and undermining food safety (Amoura et al. 2021, Uritu et al. 2018). Its presence can lead to significant economic losses, spoilage of stored goods, and a reduction in product quality and storability (Pavela 2015). Several methods have been developed to control this pest, including the use of traditional chemical pesticides (Aktar et al. 2009). However, concerns about the environmental and

health impacts of these pesticides, as well as the potential for the development of pesticide resistance, have reasonably increased the importance of seeking safe and effective alternatives to control this pest (Stejskal et al. 2015). *Mentha piperita*, commonly known as peppermint, is considered one of the most widespread garden plants (Mamadalieva et al. 2020). It is used in food and as a repellent for most types of insects and some other pests due to its active compounds and volatiles (Salehi et al. 2018, Uritu et al. 2018). Mint extract and essential oil combine an attractive aroma with potential effects on insects. Natural peppermint compounds are believed to have the ability to affect the sensing and communication systems of insects (Mishra et al. 2012), which may lead to reduced mating, egg production, and thus limit the insect's reproduction (Lu et al. 2012, Regnault-Roger et al. 2012). In addition, some compounds in mint have been reported to affect the hormonal and developmental systems of the insect, enhancing the chances of controlling their populations (Kumar et al. 2011). This study aims to evaluate the effect of mint *Mentha piperita* leaf extract and essential oil on different life stages of the red flour beetle *T. castaneum* and assess their attractiveness and repellent potential as a biological solution for controlling this pest.

MATERIALS AND METHODS

Insect rearing

Adult *T. castaneum* insects used in this experiment were collected from flour samples purchased from three different local retail markets. The culture was maintained in the laboratory in the Department of Plant Protection, Faculty of Agriculture, University of Kufa. Fifty pairs (males and females) of the insect were isolated and distributed into 2-liter glass breeding bottles, covered with pieces of milling cloth, and tied with rubber bands. The insects were fed on wheat flour free of insect infestation. The bottles were incubated at 26±2 °C and a relative humidity of 70±4%. The colony was constantly renewed, with careful renewal every 2-4 weeks, to obtain different larval stages and adults for subsequent experiments. The insect was identified as the rusty-red flour beetle *T. castaneum* according to the Natural History Museum and Research Center/University of Baghdad. Adults were starved for 24 hours before starting the experiments.

Isolating the insect at different life stages

The first larval instar was collected by placing pairs of adults in a plastic dish while monitoring the first instar larvae. Successive molts and the larval size were observed. Based on these observations, the different larval stages were isolated until the pupal stage and then adulthood. To control the movement of the adults during examination, they were immobilized by anesthetizing them through freezing for one minute (Arakane et al. 2004).

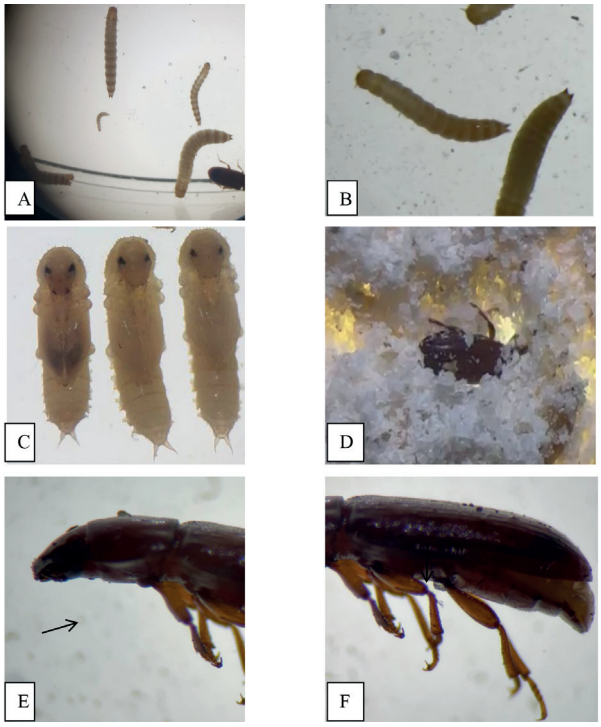

Figure 1. Different life stages of the red flour beetle *Tribolium castaneum* reared in the laboratory; A) Insect larvae at different instar ages, B) The 3rd instar larvae, C) Developed insect's nymphs, D) Adult insect digging and feeding in the flour, E and F) Adult insect's anterior.

Methanol leaf extract of peppermint (Mentha piperita)

Fresh mint plants were collected from a farm located in the south of Najaf province, Iraq, and then washed with running tap water. After washing, the mint leaves were left in the laboratory for seven days until completely dry. The dried leaves were cut, ground by hand, and stored in black polythene bags. A total of 100 g of the acquired powder was extracted by adding 0.5 liter of 70% methanol (MeOH) for 48 hours at room temperature. The methanol was then evaporated using a rotary evaporator at 30–40 °C to obtain the crude extract.

Essential oil of peppermint

A 100 g of dried plant material with 500 ml of water were subjected to hydro distillation for 4 hours using the Clevenger apparatus. The essential oil was separated and stored in a refrigerator at 4 °C for further analysis. To compare the effect of selected plant extracts and essential oils with conventional insecticides as positive control, Deltamethrin (Decis® 2.5EC) was chosen.

Bioassays

Spray method: Adults, pupae, and 3rd instar larvae were sprayed with 3 ml solutions of the mint extract (dissolved in methanol) or mint essential oil (dissolved in water) using a hand sprayer after calibration to ensure homogenous coverage. Insects were left for 15 minutes and then transferred to Petri dishes containing white flour supplemented with brewer`s yeast at 30 °C. Five biological replicates were used, each consisting of 10 insects. Survival was monitored daily for 7 days. To obtain 1% solution treatments, 0.5 ml of the tested materials and 1 ml of soap liquid were placed in a glass flask and filled to 50 ml.

Repellent / attractive effect

The repellent and attractive effects of *M. piperita* extract and oil were tested at different concentrations using a Chemotropometer (Figure 2). The test was conducted by placing a piece of cotton at the entrance to the opening of the tube containing the concentration of the extract or oil to be tested, while another piece of cotton containing only sterile distilled water was placed in the other hole. Ten adults were inserted for each replicate, with three replicates per concentration. The box was closed to prevent light from reaching the adults. The adults were allowed to move for 15 minutes, after which the box was opened, and the number of adults moving towards or away from the cotton treated with the extract or oil was recorded. The rate of attraction and repulsion was calculated as follows:

Attraction (%)=(No.insects directed to substance)/(Total No.of insects) x100

Repellence (%)=(No.insects opposit to substance)/(Total No.of insects) x100

Figure 2. Chemotropism device used in testing the repulsive and attractive properties of mint essential oil and mint methanol leaf extract

Experimental design and data analysis

All experiments were repeated twice, and data were subjected to Analysis of Variance (ANOVA) using GenStat package 2009, (12th edition) version 12.1.0.3278 (www.vsni.co.uk). Waller-Duncan's multiple range test was used for means separation wherever appropriate. The experiments were conducted using a Completely Randomized Design (CRD) with five replications. Means were compared for the least significant difference (LSD) using Duncan's multiple range test at 95% of confidence ($P \leq 0.05$).

RESULTS

The results of GC-MS analysis of peppermint leaf methanol extract

The results of the GC-MS analysis of alcoholic mint extract showed that there are ten active substances that recorded the highest estimated peaks for the compounds: Oxime-, methoxy-pheny ($C_8H_9NO_2$) with an area of 45.02 and a retention time of 4.169 min, Cyclotrisiloxane, hexamethyl ($C_6H_{18}O_3S$) with an area of 34.67 and a retention time of 6.566 min., Cyclotetrasiloxane, octamethyl ($C_8H_{24}O_4Si_4$) B with an area of 3.56 and a retention time of 9.249, Hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$) with an area of 1.72 and a retention time of 21.125 min, and Tetraacetyl-d-xylonic nitrile $C_{14}H_{17}NO_9$) with an area of 2.04 and a retention time of 21.713 min (Table 1).

The results of GC-MS analysis of peppermint essential oil

On the other hand, the results of the GC-MS analysis of peppermint oil showed that 15 compounds of active substances were recorded (Table 2). The most abundant compounds that recorded the highest estimated peaks were: 5-methyl-2-(1-methyl ethyl-trans) ($C_{10}H_{20}O$) with an area of 23.23 and retention time of 4.124 min, Cyclohexanol ($C_{20}H_{26}O$, and $C_{20}H_{26}O$) with an area of 47.50 and a retention time of 8.739 min, Menthyl acetate ($C_{11}H_{22}O$) with an area of 11.59 and a retention time of 10.678 min, beta-ylangene ($C_{15}H_{24}$) with an area of 3.61 and a retention time of 13.101 min, and Tetraacetyl-d-Caryophyllene oxide ($C_{15}H_{24}O$) with an area of 0.34 and 16.052 min retention time (Table 2).

Effect of spraying peppermint oil on flour beetle larvae

The results (Figure 3) indicate that peppermint oil showed a clear antibiotic effect against the third instar larvae of the red flour beetle, especially at the highest concentration of 12%, while the lower concentrations of 8%-10% of the oil were in most cases less than or equal to the effect of spraying with the control treatment (water and soap). It is noted that the higher concentration led to a mortality rate of more than 80% from the first day, compared to less than 10% mortality where using 10% oil spraying treatments, which generally recorded relatively higher mortality rates or close to those recorded when using oil at a higher concentration 8%.

Table 1. GC-MS analysis of the active compounds in peppermint leaf GC-MS analysis of peppermint leaf methanol extract

No.	R.t (min)	Nursery area	Molecular formula	M.W(g/mole)	Area %
1	3.710	Glycine-N,N-bis(methylenephosphonic acid) 113289 002439-99-8	$C_4H_{11}NO_8P_2$	263.08 g/mol	1.84
2	4.169	Oxime-, methoxy-phenyl-_ 24837 1000222-86-6	$C_8H_9NO_2$	151.16 g/mol	45.02
3	4.731	11H-Dibenzo[b,e][1,4]diazepin-11-one, 5,10-dihydro-5-[3-(-methylamino) propyl]- 128388 013450-70-9	$C_{13}H_{10}N_2O$	210.23 g/mol	2.88
4	6.566	Cyclotrisiloxane, hexamethyl- 79619 000541-05-9	$C_6H_{18}O_3Si_3$	222.46 g/mol	34.67
5	6.809	1H-Trindene, 2,3,4,5,6,7,8,9-octah ydro-1,1,4,4,9,9-hexamethyl- 129526 055682-87-6	$C_{21}H_{30}$	282.4629 g/mol	4.83
6	7.423	2-Aminononadecane 130243 031604-55-4	$C_{19}H_{41}N$	283.5 g/mol	1.92
7	9.249	Cyclotetrasiloxane, octamethyl- 141484 000556-67-2	$C_8H_{24}O_4Si_4$	296.61 g/mol	3.56
8	10.141	Arsenous acid, tris(trimethylsilyl) ester 178799 055429-29-3	$C_9H_{27}AsO_3Si_3$	342.49 g/mol	1.53
9	21.125	Hexadecanoic acid, methyl ester 119400 000112-39-0	$C_{17}H_{34}O_2$	270.5 g/mol	1.72
10	21.713	Tetraacetyl-d-xylonic nitrile 178849 1000130-04-4	$C_{14}H_{17}NO_9$	343.29 g/mol	2.04

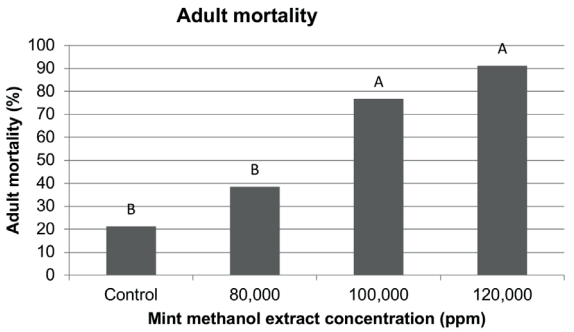

Figure 3. Effect of spraying with mint oil at different concentrations on the adults mortality red flour beetle *Tribolium castaneum*. Bars with different letter are significantly different according Fisher LSD grouping (P≤0.05)

Effect of spraying with mint oil on flour beetle adults

As for the effect of spraying with peppermint oil on adult insects (Figure 5), the results showed that the effect of the oil increased on insect mortality with increasing concentration used, as the concentrations differed significantly (P≤0.05). The concentration of 8% generally recorded the lowest mortality rate from the first day to the seventh day, which ranged from 35% -to 42%, while the 10% concentration led to a significantly higher mortality rate than the previous concentration, as it recorded a mortality rate that ranged from 68% -to 75%. This is compared to the higher concentration (12%), which recorded a mortality rate in insect adults on the first day, which exceeded 85% and ended on the seventh day with a percentage of 94%, which differed significantly (P≤0.05) from the effect of the two lower concentrations of 10% - 8% of the mint oil (Figure 4).

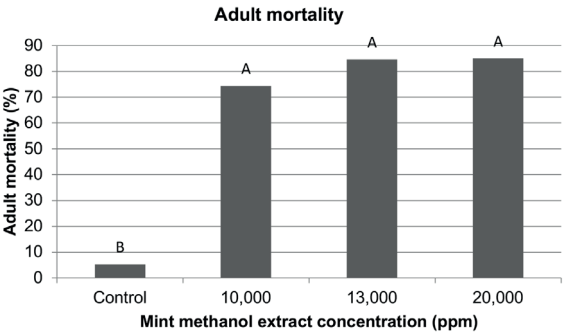

Figure 4. Effect of spraying with mint leaf methanol extract at different concentrations on the adults mortality red flour beetle *Tribolium castaneum*. Bars with different letter are significantly different according Fisher LSD grouping (P≤0.05)

Table 2. GC-MS analysis of the active compounds in peppermint essential oil

No.	R.t (min)	Compounds	Molecular formula	M.W(g/mole)	Area %
1	5.623	D-Limonene 15682 005989-27-5	$C_{10}H_{16}$	136.23 g/mol	1.12
2	6.999	Cyclohexanol, 5-methyl-2-(1-methy lethyl)-, (1.alpha.,2.alpha.,5.alph a.)- 28381 000491-02-1	$C_{10}H_{20}O$	156.26 g/mol	0.36
3	7.440	Butanal, 3-hydroxy- 2033 000107-89-1	$C_4H_8O_2$	88.11 g/mol	0.30
4	8.124	Cyclohexanone, 5-methyl-2-(1-methy lethyl)-, trans- 26912 000089-80-5	$C_{10}H_{18}O$	154.25 g/mol	23.23
5	8.349	Cyclohexanone, 5-methyl-2-(1-methy lethyl)-, trans- 26912 000089-80-5	$C_{10}H_{18}O$	154.25 g/mol	5.50
6	8.739	Cyclohexanol, 5-methyl-2-(1-methyl ethyl)- 28342 001490-04-6 Cyclohexanol, 5-methyl-2-(1-methyl ethyl)-, (1.alpha.,2.beta.,5.alpha .)-(.+/-.) 28387 015356-70-4	$C_{20}H_{26}O$ $C_{10}H_{20}O$	282.4 g/mol 156.26 g/mol	47.50
7	10.046	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethyl)- 25293 000089-81-6	$C_{10}H_{18}O$	154.25 g/mol	0.43
8	10.357	1-Decanol 29593 000112-30-1	$C_{10}H_{22}O$	158.28 g/mol	1.54
9	10.678	Menthyl acetate 59498 000089-48-5	$C_{12}H_{22}O_2$	198.30 g/mol	11.59
10	11.456	Cyclohexane, 1-ethenyl-1-methyl-2- (1-methylethenyl)-4-(1-methylethyl idene)- 64474 003242-08-8	$C_{15}H_{24}$	204.35 g/mol	0.17
11	12.495	(-)-.beta.-Bourbonene 64360 005208-59-3	$C_{15}H_{24}$	204.35 g/mol	1.25
12	12.824	photocitral B 25079 006040-45-5	$C_{10}H_{16}O$	152.23 g/mol	0.35
13	13.110	beta.-ylangene 64294 1000374-19-1	$C_{15}H_{24}$	204.35 g/mol	3.61
14	13.309	1,6-Cyclodecadiene, 1-methyl-5-met hylene-8-(1-methylethyl)-, [S-(E,E)]- 64463 023986-74-5	$C_{15}H_{24}$	204.35 g/mol	0.94
15	13.560	1,6-Cyclodecadiene, 1-methyl-5-met hylene-8-(1-methylethyl)-, [S-(E,E)]- 64463 023986-74-5	$C_{15}H_{24}$	204.35 g/mol	0.27
16	13.707	1H-Cyclopropa[a]naphthalene, 1a,2, 3,5,6,7,7a,7b-octahydro-1,1,7,7a-t etramethyl-, [1aR-(1a.alpha.,7.alp ha.,7a.alpha.,7b.alpha.)]- 64579 017334-55-3	$C_{15}H_{24}$	204.35 g/mol	0.62
17	14.140	Spiro[4.5]dec-7-ene, 1,8-dimethyl-4-(1-methylethenyl)-, [1S-(1.alpha .,4.beta.,5.alpha.)]- 64504 024048-44-0	$C_{15}H_{24}$	204.35 g/mol	0.25
18	14.572	1,6-Cyclodecadiene, 1-methyl-5-met hylene-8-(1-methylethyl)-, [S-(E,E)]- 64463 023986-74-5	$C_{15}H_{24}$	204.35 g/mol	0.38
19	16.052	Caryophyllene oxide 77539 001139-30-6	$C_{15}H_{24}O$	220.35 g/mol	0.34
20	21.116	Hexadecanoic acid, methyl ester 119400 000112-39-0	$C_{17}H_{34}O_2$	270.5 g/mol	0.26

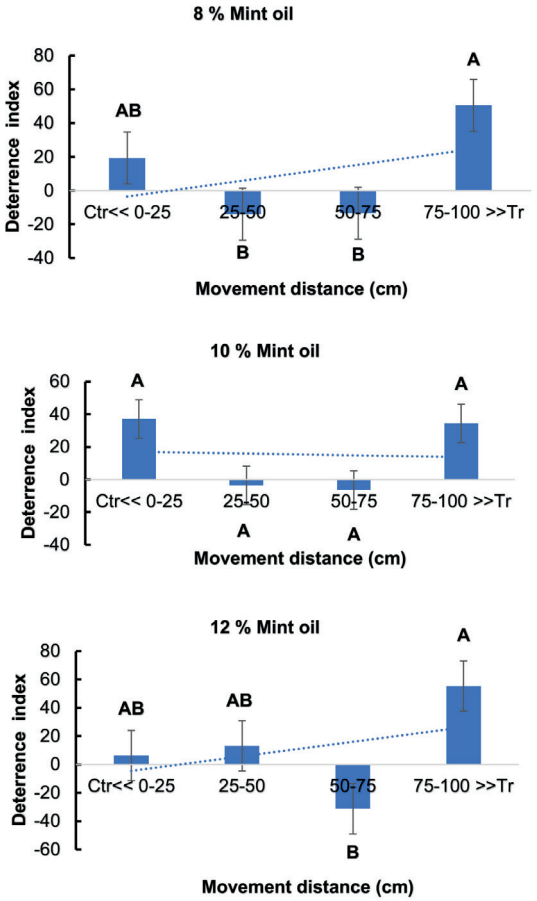

Figure 5. Peppermint *Mentha piperita* oil repellent/attractive effect on the red flour beetle *Tribolium castaneum* adults. The mint oil was used at three concentrations 8, 10, and 12% respectively

Peppermint oil repellent/attractive effect on the red flour beetle adults

In testing the repellent/attractive effect of peppermint oil on adult insects of the red rusty flour beetle, the results generally showed that peppermint oil exhibited a repellent effect towards the insect, especially at high concentrations. The repulsion rate of peppermint oil at the lowest concentration of 8% differed significantly from the attraction rate after 15 minutes of treatment. However, the results showed that sometimes the repellent/attractive effect of peppermint oil was not clear in the repulsion/attraction test, which did not differ between them at the concentration of 10% (Figure 5). On the other hand, the 12% concentration treatment recorded high significant differences between the rates of attraction/repulsion of the insect adults. Where the largest number was recorded in the direction opposite to the oil treatment with a percentage of expulsion (Figure 5).

The results showed that the effect of the methanolic mint leaf extract was somewhat similar in its repellent effect to that recorded in peppermint oil on the insect, especially at the higher concentration of the extract (15%). It was found that at a low concentration of the extract, the distribution of insects at a distance of 10-50 cm did not differ between them, while the insect concentration was large towards the control (Figure 6). The repellency rate also increased at the distance closest to the control 25-50 cm, and a significantly low repellency rate was recorded at distances of 50-100 cm, although the repulsion towards the treatment was mathematically higher than the average distance of 50-75 cm (Figure 6). However, it was found that the repellence to the adult insects was significant at the higher concentration of the 20% extract, which recorded a significant repellency at the end tube spot compared to all other distances (0 - 75 cm) of the scale (Figure 6).

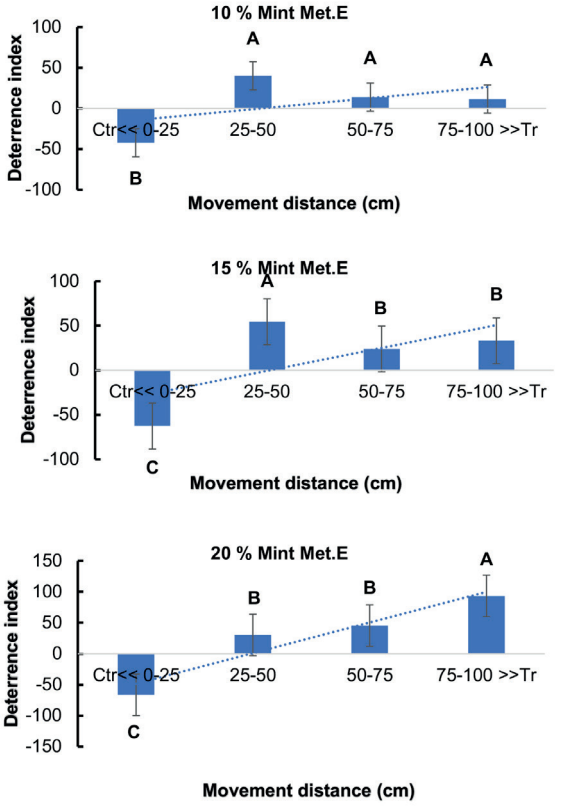

Figure 6. Peppermint *Mentha piperita* methanol extract (Mint Met.E) repellent/attractive effect on the red flour beetle *Tribolium castaneum* adults. The mint extracts were used at three concentrations 10, 15, and 20% respectively

DISCUSSION

The results obtained in this study agree with previous results in testing the efficacy of plant extracts from four plants: *Melissa officinalis*, *Thymus vulgaris*, *Mentha piperita* and *Rosmarinus officinalis*. Mint was the second-best treatment, leading to mortality in juveniles with a rate higher than 50% in *T. castaneum* juveniles and a lower rate in *T. granarium* juveniles, compared to higher levels of mortality using *R. officinalis*. Peppermint extract also recorded a similar effect on adult females with a mortality rate higher than 70%, but its effect was much lower on *T. granarium* adults, with a higher effect than *Thymus vulgaris* and *Melissa officinalis*, but a lower effect than *Rosmarinus officinalis* (Panezai et al. 2019). Damage to food products and stored grains can be considered an ongoing problem in all regions of the world and is more serious in developing countries (Stejskal et al. 2015). In general, stored grains are often affected by insect infestations that accompany crops stored in large warehouses or retail stores. The damage resulting from stored grain pests is estimated at approximately 10-40%, not only through the consumption of stored grains and materials, but also in reducing the quality of the stored product and making it unfit for human consumption or a significant reduction in its marketing value (Lefèvre and Fady 2016). The anti-inflammatory effect of peppermint oil on warehouse insects is often due to the nature of the oily fats, which interfere with their content of active substances to influence basic metabolism and physiological-biochemical processes, which may affect the behavior of the treated insects (Linsley 1944). In general, the anti-insect effect of peppermint oil has been the subject of many studies, most of which were directed towards combating insects of the order Coleoptera (Kumar et al. 2011). On the other hand, although vegetable oils are environmentally friendly and harmless to humans, the treatment of stored food products and grains must be accompanied by controls and consideration to remove the effect of the oil to a level that does not affect the stored food material and does not reduce the commercial properties of the material (Golob 1997). Sometimes, the presence of insect pests of stored materials, including rice weevils, corn weevils, red and similar rusty flour beetles, and other insects, is considered the determining factor for the survival of the crop with good economic quality (Keskin and Ozkaya 2013), since in these cases the specific damage to the stored material is more severe than the damage caused by quantitative loss (Kumar et al. 2011). Natural peppermint compounds have the ability to affect the sensing and communication systems of insects (Mishra et al. 2012), which may lead to reduced mating and egg production, and thus limit the reproduction of this pest (Regnault-Roger et al. 2012). It was also reported that some compounds in mint may affect the hormonal and developmental systems of insects, enhancing the chances of controlling them (Kumar et al. 2011). The study of Lee et al. (2002) indicated the effectiveness of *M. piperita* oil when used in relatively low concentrations (LD_{50}: 25.8 µl/l) against the red rusty flour beetle *T. castaneum*. This confirmed previous results in the study (Mishra et al. 2012). When using it on *S. oryzae*, it was found that mint oil used by evaporation or contact differed in its impact on the main warehouses insects *T. castaneum*, *Lasiooderma serricorne* and *Liposclis bostrychophiva* where a high mortality rate with low concentrations was recorded on *T. castaneum* by vaporization, which was LD_{50}: 18.1 µl/l (Pang et al. 2020). Mint is known for its active compounds such as menthol, methyl acetate, and menthone, possessing antibacterial (Fazal et al. 2023), antifungal (Abdelli et al. 2016), and insect-repellent properties (Kumar et al. 2011). The results of the study implicitly agreed on the effect of the *Mentha* spp. on *T. castaneum*, which was found to be relatively less sensitive compared to the *C. maculatus* insect, which showed a higher sensitivity to vegetable oil. Similar results recorded in the effect of methanolic mint extract of from peppermint, which recorded a death rate of 70% in insect food treatment after a period of 7 days of treatment in laboratory plastic vessels (Saljoqi et al. 2006). The study conducted by Negahban et al. (2007) was indicated that the insecticidal activity of vegetable oils varied with insect species, concentrations of the oil and exposure time. The results showed higher mortality rates in *C. maculatus* than in *S. oryzae* and *T. castaneum*. At 444 ml/L air the mortality was 100% after 12 h for *T. castaneum*. Based on their experimental study in a controlled laboratory environment, Atay et al. (2024) reported that the essential oil of *Mentha piperita* L. (Lamiaceae) has the potential to control two important stored product pests, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) and *Sitophilus granarius* (Linnaeus, 1758) (Coleoptera: Dryophthoridae).

The study included evaluating the effectiveness of peppermint oil and peppermint leaf extract (methanol) against different stages of the red rusty flour beetle. The study showed that treatments of *Mentha piperita* oil at all concentrations under study, when sprayed on the insect, were significantly effective in causing high mortality rate in *T. castaneum* adults. On the other hand, it was found that spraying with methanol mint leaf extract also led to a

clear mortality rate in insect adults, but with less efficiency than mint essential oil treatments. The peppermint oil and methanol extract of mint leaves showed a moderate to high repellent effect on insect adults, which fluctuated depending on the concentration used and the period post-treatment.

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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

Kırmızı un böceği *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) depolanan tahılların ve gıda ürünlerinin hem kalitesini hem de miktarını etkileyen olumsuz bir faktör olarak dikkate değer bir öneme sahiptir. Bu çalışmada, kırmızı un böceği zararlısını çevre dostu doğal materyaller kullanarak kontrol etme olasılığını araştırmak amacıyla, nane yağı ve alkollü (metanolik) nane ekstraktının Kırmızı un böceğinin farklı aşamalarına karşı etkinliğini değerlendirmek üzere bir dizi laboratuvar deneyi yapılmıştır. Bu çalışmanın sonuçlarına göre, %8, %10 ve %12'lik konsantrasyonlardaki nane *Mentha piperita* L. (Lamiaceae) yağının uygulamalarının, tüm dozlarda Kırmızı un böceği mortalitesi üzerine önemli bir etkiye sahip olduğu tespit edilmiştir. Sprey yöntemiyle yapılan uygulama sonucu ölüm oranı üçüncü larva döneminde %36'dan %80'e, ergin evrede ise %40'tan %94'e çıkmıştır. Nane yaprağı metanol ekstraktının en yüksek konsantrasyonda (13000 ppm) uygulanması, 7 günlük uygulama sonrası larva ölümlerinin %87'ye varan oranda artmasına neden olmuştur. Nane yağı, özellikle en yüksek konsantrasyonda (%12) uygulandığında, kırmızı un böceğinin üçüncü dönem larvalarına karşı önemli insektisidal etki göstermiştir. Ancak, nane uçucu yağının %8 ve %10'luk daha düşük konsantrasyonları kontrole göre önemli bir farklılık ortaya çıkarmamıştır. Bu çalışmanın sonuçları, Kırmızı un böceğine verilen yemde nane yağı arttıkça larva ölüm oranının arttığını, özellikle %12'lik en yüksek konsantrasyonda, 3. dönem larvalarda %82 ve yetişkinlerde %95 ölüm oranı kaydettiğini gösterdi. Bu çalışma, Kırmızı un böceğinin (*T. castaneum*) kontrolünde çevre dostu bir ürün olarak nane esansiyel yağının nispeten düşük konsantrasyonlarda kullanılmasının mümkün olabileceğini ortaya koymuştur.

Anahtar kelimeler: esansiyel yağı, nane, metanolik ekstraktlar, Tribolium castaneum, kırmızı un böceği

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Original article

Evaluation of pathogenic variation among *Rhizoctonia solani* isolates infecting different crops and potential biocontrol agents

Farklı ürün türlerini enfekte eden *Rhizoctonia solani* izolatları arasındaki patojenik varyasyonun ve potansiyel biyokontrol ajanlarının değerlendirilmesi

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ABSTRACT

Rhizoctonia solani is an important broad-spectrum fungal pathogen that infects over 200 plant species including tomato, melon, and watermelon. This study evaluated the pathogenicity of various *R. solani* isolates (Rs26, Rs94, Rs13, Rs57, and Rs123) and the efficacy of biological agents (*Trichoderma harzianum*, *T. viride*, *Metarhizium* sp., *Gliocladium* sp.) under laboratory and greenhouse conditions for eco-friendly disease management. The results of the pathogenicity assay confirmed the varying aggressiveness of the isolates, with Rs94 and Rs13 causing the most severe disease in watermelon (disease severity (DS) = 3.80 and 3.83, disease severity index (DSI) = 90.43% and 95.75%, respectively). Similarly, isolate Rs26 displayed the highest pathogenicity in tomatoes (DS = 3.84; DSI = 94.86%). Melon exhibited high susceptibility across all isolates, with consistently high DS and DSI values exceeding 2.59 and 80.97%, respectively. Subsequent *in vitro* and *in vivo* assays demonstrated the antifungal potential of all tested agents against *R. solani* isolates. Notably, *Trichoderma* spp. displayed the most consistent and significant inhibition (mycelial growth reduction 82.97%-94.67%), with *T. harzianum* demonstrating superior performance. Greenhouse trials confirmed the effectiveness of *T. harzianum* as a preventative treatment, enhancing plant enzyme activity [peroxidase = 4.97-5.29 units g^{-1} ml^{-1} min^{-1} for tomato and watermelon, respectively; catalase = 99.93-101.22 units g^{-1} ml^{-1} min^{-1} for watermelon and melon, respectively] and significantly reducing disease severity index (DSI < 12.43%). These findings highlight the potential of *T. harzianum* as a sustainable and eco-friendly strategy for managing *R. solani* damping-off disease in tomato, melon, and watermelon crops.

INTRODUCTION

Rhizoctonia solani JG Kühn (teleomorph: *Thanatephorus cucumeris* (AB Frank) Donk) is a complex of soil-borne fungal plant pathogens encompassing over 100 distinct species (Abbas et al. 2023, Li et al. 2021). This necrotrophic fungal pathogen thrives by deriving nutrients from dead or dying host tissues (Li et al. 2021). One of its key survival mechanisms is the formation of sclerotia, hardened structures that allow the fungus to persist in the soil during unfavorable conditions (Erper et al. 2021). This complex has a broad host range and global distribution, causing diseases in various economically important agricultural and horticultural crops and trees (Erper et al. 2021). The wide range of plants it can infect allows it to cause numerous diseases. For instance, it causes sheath blight in key field crops like corn and rice and it also causes root rot in vegetables and legumes (Canpolat and Tülek 2019, Canpolat et al. 2023, Dubey et al. 2012, Ozan and Aşkın 2006, Ozan and Maden 2004, Yücel and Çolak 2008). R. solani exhibits varying degrees of virulence towards different crops. It is particularly destructive to seedlings and seeds of vegetables like eggplant, pepper, lettuce, and zinnia (Abbas et al. 2023). This fungus causes stem canker and black scurf in potatoes, significantly reducing tuber yield and quality (Naqvi et al. 2024). However, one of the most critical diseases caused by *R. solani* is cotton root rot, posing a substantial threat to this economically vital crop. In greenhouses, it is a primary pathogen responsible for root and crown rot in tomatoes. This fungus extends its destructive reach to numerous vegetables, including cucurbits and tomatoes, causing various seedling diseases such as seed rot, root rot, pre-emergence damping-off, and post-emergence damping-off. The economic impact of *R. solani* is substantial, causing significant yield losses across more than a hundred crop and horticultural species annually. Moreover, its emergence as a significant problem is ongoing, with recent observations highlighting its ability to cause stem rot in sweet potatoes (Abbas et al. 2023, Naqvi et al. 2024).

Rhizoctonia solani is a ubiquitous and cosmopolitan soilborne fungal plant pathogen exhibiting a multifaceted lifestyle (Naqvi et al. 2024). This fungus can exist as both a saprophyte, decomposing dead organic matter in the soil, and a pathogen capable of infecting living plants (Li et al. 2021). The *R. solani* complex is further classified into fourteen genetically distinct anastomosis groups (AG1 to AG13 and AGBI) (Abbas et al. 2023). These groups exhibit host specificity, meaning they preferentially infect certain plant species and are unable to reproduce sexually with each other (Erper et al. 2021). As a whole, the complex has a very broad host range, encompassing numerous plant species crucial to agriculture, forestry, and the bioenergy sector (Yang et al. 2024). This includes prominent crops such as tomato, wheat, rice, barley, potato, melon, watermelon, and sugar beet. The extensive host range and diverse lifestyles within the *R. solani* complex highlight its significant role as a plant pathogen with a broad economic impact (Dubey et al. 2012). The increasing pathogenicity of *R. solani* strains underscores the urgent need for the development of effective control strategies to mitigate the widespread damage it causes (Abdelghany et al. 2022, Dubey et al. 2011).

Rhizoctonia solani is a major fungal pathogen responsible for significant pre- and post-emergence damping-off and root rot diseases in various vegetables (Yang et al. 2024). Unfortunately, effective fungicides for *Rhizoctonia* control are limited for many vegetables, with some chemicals like chlorothalonil, thyophanate methyl, and iprodione showing some efficacy but with growing concerns about their environmental impact (Agrios 1988, Hajji-Hedfi et al. 2023). This necessitates exploring alternative, more sustainable solutions. Biocontrol programs using fungal and bacterial mycoparasites offer a promising approach for managing soilborne pathogens like *R. solani* (Albastawisi and Kotan 2024, Mohamed et al. 2020, Ruiz-Cisneros et al. 2018). Among various biocontrol agents, *Trichoderma* spp. have emerged as particularly effective antagonists against *R. solani* (Behiry et al. 2023). *Trichoderma* spp. employ a multifaceted biocontrol strategy, including production of antibiotics and hydrolytic enzymes, direct mycoparasitism of *R. solani* hyphae, and hyphal disruption (Hajji-Hedfi et al. 2023). The specific mechanisms likely vary depending on the fungal strains involved, potentially involving a combination of these strategies acting independently or synergistically during microbial interactions (Almaghasla et al. 2023). Besides, *Trichoderma* spp. may influence the viability of *R. solani* sclerotia, offering an additional layer of control (Behiry et al. 2023). Overall, research on *Trichoderma* spp. and other biocontrol agents presents a promising and environmentally friendly approach for mitigating the detrimental effects of *R. solani* on vegetable crops (Shalaby et al. 2022).

The present study aimed to (i) investigate the pathogenicity of five *R. solani* isolates on tomato, melon, and watermelon, (ii) evaluate the *in vitro* and *in vivo* efficacy of *Trichoderma* spp., *Metarhizium* sp., and *Gliocladium* sp. as a potential biocontrol agent for managing *R. solani* infections.

MATERIALS AND METHODS

Pathogenicity test

A study investigated the virulence of five *R. solani* isolates (Rs26, Rs94, Rs13, Rs57, and Rs123) on three crops [tomato (cv. Firenze), melon (cv. Badii), and watermelon (cv. Crimson Sweet)]. The experiment aimed to identify variations in virulence among the isolates. Each isolate originated from a different soil source (watermelon, tomato, melon, tomato, and watermelon). Disinfested potting mix (clay:sand, 2:1 v/v) was added to sterilized pots (20 cm diameter) at 2.5 kg per pot. Inoculum for each fungal isolate was prepared by culturing them in sterilized sorghum grain medium for 15 days at 25 °C ± 2 °C. To infest the soil, the inoculum was mixed with the upper potting mix layer at a rate of 2% (w/w). The infested potting mix was thoroughly mixed and irrigated every other day for a week before planting to stimulate fungal growth and ensure proper distribution throughout the soil. Five healthy seeds from the Regional Centre of Agricultural Research of Sidi Bouzid, Tunisia were sown in each pot. Three replicate pots were used for each isolate-crop combination, with an additional three un-infested pots serving as controls (negative control). Plants were grown in a greenhouse chamber under a 16 h/8 h light/dark cycle at 23–25 °C with regular irrigation (Matrood and Rhouma 2021, Rhouma et al. 2024). Disease severity (DS) was evaluated after 60 days using a 0-4 scale adapted from Carling et al. (1999): 0 - no visible damage, 1 - minor hypocotyl discoloration, 2 - discoloration with small necrotic lesions (<1 mm diameter), 3 - discoloration with larger necrotic lesions (≥1 mm diameter), and 4 - plant death. These scores were then converted into Disease Severity Index (DSI) using McKinney's formula: DSI (%) = (Σvn)/(NV) × 100, where Σvn is the sum of all disease scores, N is the total number of plants, and V is the maximum possible score (Okon et al. 2023).

Antagonistic action of antagonistic fungi toward Rhizoctonia solani

A dual culture assay on potato dextrose agar (PDA) plates was conducted to evaluate the antagonistic interaction between four biocontrol agents (*T. harzianum*, *T. viride*, *Metarhizium* sp., and *Gliocladium* sp.) and *R. solani* isolates (Rs26, Rs94, Rs13, Rs57, and Rs123). Biocontrol agents were obtained from the Laboratory of Plant Protection's collection (CRRA, Sidi Bouzid, Tunisia) and were isolated from the rhizosphere soil of tomato plants collected in the Regueb agricultural fields of Sidi Bouzid. The assay employed 0.5 cm diameter agar plugs, one containing a four-day-old culture of the biocontrol agent and the other containing the target *R. solani* isolate. Following a standardized protocol, these plugs were placed on opposite sides of a single 9 cm PDA plate: the antagonist plug was positioned 2 cm from the edge towards the center, maintaining a 5 cm gap between the plugs. Control plates included only a blank PDA plug on one side and the *R. solani* isolate plug on the opposite side. Each treatment was replicated three times, with each replicate consisting of five plates. All plates were incubated for seven days at 28 °C ± 2 °C (Hajji-Hedfi et al. 2023, Rhouma et al. 2024). After incubation, the percentage inhibition of *R. solani* radial growth was determined using the formula established by Matrood and Rhouma (2021): I (%) = (1 - Cn/C0) × 100, where Cn represents the radial growth of the *R. solani* colony in the presence of the biocontrol agent and C0 represents the radial growth of the *R. solani* colony in the control plate without an antagonist.

In vivo evaluation of antagonistic fungi on tomato, melon, and watermelon plants inoculated with Rhizoctonia solani

The experiment employed a randomized complete block design with three replicate blocks, each containing 135 pots. Each pot held three seedlings of a single crop species: tomato (cv. Firenze), melon (cv. Badii), or watermelon (cv. Crimson Sweet). The potting mix consisted of a 1:1 (v/v) mixture of peat and vermiculite. Seedlings received designated treatments and inoculations after 15 days of growth (Hajji-Hedfi et al. 2023). Within each block, seedlings received five treatments: T1 (positive control) - inoculation with *R. solani*, T2 (negative control) - treatment with sterilized water only, T3 - dipping in *T. harzianum* conidial suspension for 30 min followed by *R. solani* inoculation (10 ml) 24 hours later, T4 - dipping in *T. viride* conidial suspension for 30 min followed by *R. solani* inoculation (10 ml) 24 hours later, and T5 - dipping in *Metarhizium* sp. conidial suspension for 30 min followed by *R. solani* inoculation (10 ml) 24 hours later. Specific *R. solani* isolates were used for each crop: Rs26 for tomato, Rs57 for melon, and Rs13 for watermelon. Following treatment, all pots were incubated in a growth chamber under controlled conditions for 60 days with an 8-hour dark/16-hour light photoperiod and a temperature range of 20-22 °C.

To prepare the fungi for the antagonism assays, each strain was grown individually on PDA media at a constant temperature of 25 °C for four days. This incubation period allows for sporulation. Following incubation, four colonized agar plugs of each fungal strain were used to inoculate separate flasks containing 50 ml of Potato Dextrose Broth (PDB) media. The use of liquid media in this step further promotes fungal growth and spore production. These flasks

were incubated on an orbital shaker for seven days to achieve even distribution and enhanced spore release. After this incubation period, spores were harvested from each fungal culture using a filtration technique. The concentration of spores in each fungal suspension was then quantified using a hemocytometer. This quantification process revealed a final spore density of 10^6 spores ml^{-1} (Matrood and Rhouma 2021).

A visual scoring system (0-4) was employed to assess DS according to Popoola et al. (2015). McKinney's formula then converted these scores into a DSI expressed as a percentage (Okon et al. 2023, Thakur and Tripathi 2015). Peroxidase (POX) and catalase (CAT) activity were assessed in plant root tissues following established protocols. Three root samples from each treatment and block were homogenized, and enzyme extracts were prepared. POX activity was determined spectrophotometrically at 470 nm. The reaction mixture contained 0.1 ml enzyme extract, 0.5 ml hydrogen peroxide, 0.9 ml distilled water, 1 ml phosphate buffer, and 0.5 ml guaiacol. CAT activity was also measured spectrophotometrically at 240 nm. The reaction mixture included 0.05 ml enzyme extract, 0.5 ml hydrogen peroxide, 0.95 ml distilled water, and 1.5 ml phosphate buffer (Rhouma et al. 2024).

Statistical analysis

The analysis employed a one-way ANOVA on the mean values of replicated data. This was performed using version 20.0 of the SPSS statistical software package (SPSS, SAS Institute, USA) to assess for significant differences between treatment groups. The homogeneity of variances and normality of the data were verified before the ANOVA. All statistical tests were conducted at a significance level of alpha = 0.01 ($P \leq 0.01$).

RESULTS AND DISCUSSION

Pathogenicity test

Analysis of DS scores revealed variations in the pathogenic potential of five *R. solani* isolates towards tomato, melon, and watermelon ($P < 0.01$). All isolates successfully infected all three crops compared to the control group, which exhibited no disease development. This confirmed the inherent pathogenicity of all tested *R. solani* isolates. Besides, a closer examination of the severity scores unveiled interesting patterns. Isolates Rs94 and Rs13 demonstrated the strongest virulence on watermelon (DS = 3.80 and 3.83, respectively), suggesting potential variations in isolate-specific virulence. However, isolate Rs26 exhibited the highest pathogenicity on tomato (DS = 3.84), further supporting the hypothesis of differential virulence among isolates. Interestingly, all isolates caused significant disease development on melon (DS > 2.72), suggesting a high level of susceptibility in this particular crop to all tested *R. solani* isolates (Table 1).

Table 2 investigated the impact of five *R. solani* isolates on the disease severity index in tomato, melon, and watermelon plants. Without any fungal introduction, the control group exhibited minimal disease in all three crops (DSI = 0%). All fungal isolates significantly increased disease severity compared to the control in each plant species ($P < 0.01$). However, the isolates' effect varied across crops. Rs57 caused the most severe disease in melon (97.52%), while Rs26 caused the most severe disease in tomato (94.86%). Interestingly, Rs13 caused the most severe disease in watermelon (95.75%) but ranked lower in tomato and melon disease severity (Table 2).

R. solani isolates exhibit intraspecific diversity in their virulence, as evidenced by this study's investigation into their pathogenicity on various crops (Abdelghany et al. 2022, Eken et al. 2024, Mustafa et al. 2021, Porto et al. 2019). All isolates tested caused damping-off and root rot diseases, albeit with varying degrees of severity. This finding aligns with prior research demonstrating the broad host range of *R. solani* isolates obtained from diverse environments (Abbas et al. 2023, Dubey et al. 2012, Erper et al. 2021, Porto et al. 2019, Yang et al. 2024). The extensive pathogenicity of *R. solani* is likely attributed to its production of polygalacturonase enzymes, which degrade plant cell wall pectate, as suggested by Naqvi et al. (2024). *R. solani* preferentially targets the hypocotyl region of seedlings at the soil line due to the heightened vulnerability of meristematic tissues to its cell wall degrading enzymes. As seedlings mature, they develop resistance mechanisms that counteract the fungus's virulence. These mechanisms include thickening the cuticle, which limits the amount of exudates the fungus needs to form infection cushions, and converting pectin into a form resistant to *R. solani*'s enzymes (calcium pectate) (Naqvi et al. 2024).

Antagonistic action of antagonistic fungi toward Rhizoctonia solani

This study employed a controlled laboratory setting to evaluate the potential application of antagonistic fungal isolates for managing the growth of *R. solani*. The experiment specifically focused on the impact of these antagonists on the mycelial development of five distinct *R. solani* isolates. The obtained results revealed promising antifungal activity from all four antagonists. Statistical analysis confirmed

Table 1. Effect of *Rhizoctonia solani* isolates (Rs26, Rs94, Rs13, Rs57, and Rs123) on disease severity in tomato, melon, and watermelon

Treatments	Disease severity		
	Tomato	Melon	Watermelon
Control	0±0d[a]	0±0c	0±0d
Rs26	3.84±0.07a	2.72±0.11b	1.91±0.06c
Rs94	2.59±0.04b	3.70±0.03a	3.80±0.04a
Rs13	2.02±0.01c	3.68±0.01a	3.83±0.05a
Rs57	1.90±0.02c	3.83±0.05a	3.55±0.14ab
Rs123	1.74±0.09c	3.59±0.06a	3.42±0.04b
P-value[b]	< 0.01	< 0.01	< 0.01

[a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at P≤0.01.
[b]Probabilities associated with individual F tests.

Table 2. Effect of *Rhizoctonia solani* isolates (Rs26, Rs94, Rs13, Rs57, and Rs123) on disease severity index in tomato, melon, and watermelon

Treatments	Disease severity index (%)		
	Tomato	Melon	Watermelon
Control	0±0e[a]	0±0d	0±0d
Rs26	94.86±1.51a	80.97±1.37c	63.65±1.18c
Rs94	76.61±1.27b	95.50±1.51ab	90.43±1.24b
Rs13	65.51±1.84c	94.35±1.68b	95.75±1.33a
Rs57	59.24±1.05d	97.52±1.54a	94.07±1.95ab
Rs123	56.43±1.87d	92.60±1.35b	90.96±1.07b
P-value[b]	< 0.01	< 0.01	< 0.01

[a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at P≤0.01.
[b]Probabilities associated with individual F tests.

significant inhibition of mycelial growth for most *R. solani* isolates compared to the negative control (P < 0.05). *T. harzianum* displayed the most broad-spectrum inhibition, ranging from 83% (Rs57) to 94.67% (Rs26). Interestingly, the effectiveness of the other antagonists varied. Thus, *T. viride* exhibited consistent inhibition across all *R. solani* isolates (P ≥ 0.05). *Metarhizium* sp. also demonstrated significant inhibition for most isolates, ranging from 65.57% (Rs26) to 75.57% (Rs94). *Gliocladium* sp., however, showed a more variable effect, with significant suppression observed for some isolates (51.35% for Rs13 and 66.75% for Rs26) but less consistent results for others. This observed variation in effectiveness among both the antagonistic fungi and the *Rhizoctonia* isolates suggests potential differences in how they interact (Table 3).

Multiple studies have demonstrated the efficacy of *T. harzianum* as a biological control agent against *R. solani*. In vitro dual culture experiments consistently reported significant reductions in *R. solani* linear growth following incubation with *T. harzianum*, with inhibition rates ranging from 55.55% to 65.18% (Abd-El-Khair et al. 2011, Ban et al. 2022, Brindhadevi et al. 2023, Elsheshtawi et al. 2012, Naeimi et al. 2010). These findings suggest that *T. harzianum* exerts its antagonistic effect through the secretion of diffusible non-volatile inhibitory compounds before hyphal contact, potentially including exochitinases, as reported by Brunner et al. (2005). Furthermore, Abbas et al. (2017) and Paula Junior et al. (2007) proved that *T. harzianum* can promote plant growth, diminish disease severity, and protect seedlings from *R. solani*-induced pre-emergence damping-off.

Table 3. Evaluation of mycelial growth inhibition in *Rhizoctonia solani* isolates (Rs26, Rs94, Rs13, Rs57, Rs123) by four antagonistic fungal isolates under *in vitro* conditions

Treatments	*T. harzianum*	*T. viride*	*Metarhizium* sp.	*Gliocladium* sp.
Rs26	94.67±±0.92a[a]	89.55±1.01a	65.57±0.62b	66.75±1.86a
Rs94	90.67±0.67ab	86.46±0.96a	75.57±0.54a	53.51±1.19b
Rs13	91.80±0.21a	82.97±0.72a	75.17±0.38a	51.35±1.27b
Rs57	83±0.76b	83.60±1.17a	71.75±1.51ab	51.90±1.35b
Rs123	86.34±0.75ab	89.48±0.86a	74.11±0.69ab	59.78±1.73ab
P-value[b]	< 0.05	< 0.05	< 0.05	< 0.05

[a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at P≤0.01.
[b]Probabilities associated with individual F tests.

In vivo evaluation of antagonistic fungi on tomato, melon, and watermelon plants inoculated with Rhizoctonia solani

Table 4 evaluated the potential of antagonistic fungal isolates to control damping-off disease caused by *R. solani* in tomato, melon, and watermelon plants. The experiment was conducted under controlled conditions. Table 4 presents the disease severity index, a numerical measure of disease intensity, for each crop-fungus combination. The positive control group represents plants infected only with *R. solani*. As expected, this group suffered the most severe symptoms in all three crops, with disease severity indexes close to 100%, indicating extensive disease development. In contrast, the negative control group, where no fungi were introduced, showed minimal to no disease (DSI = 0%). The data reveals a significant reduction in disease severity for all three beneficial fungi compared to the positive control in each plant type (P < 0.01). This confirmed that these fungi could effectively control *R. solani* infection. However, the extent of protection varies depending on the specific beneficial fungus and the crop. *T. harzianum* emerges as the most effective agent, significantly reducing disease severity across all crops. Tomato plants treated with *T. harzianum* showed the lowest disease severity index (10.26%), followed by melon (10.32%) and watermelon (12.43%). While all fungi bring benefits, *T. viride* offers a moderate level of protection, followed by *Metarhizium* sp. (Table 4).

The study examined the impact of fungal treatments on two enzyme activities within the root systems of tomato, melon, and watermelon plants (Tables 5 and 6). Peroxidase activity, an indicator of a plant's defense response against pathogens, was significantly enhanced (P <0.01) across all three plant species when treated with the fungal strains *T. harzianum*, *T. viride* and *Metarhizium* sp. compared to the control groups. Notably, *T. harzianum* (4.97, 5.29, and 5.27 units' g^{-1} ml^{-1} min^{-1}, respectively) consistently induced the highest level of peroxidase activity in all three plant roots, followed by *T. viride* and *Metarhizium* sp. (Table 5).

Table 4. *In vivo* evaluation of antagonistic fungal isolates on disease severity index (%) in roots in the presence of *Rhizoctonia solani* under controlled conditions

Treatments	Disease severity index (%)		
	Tomato	Melon	Watermelon
Positive control	93.58±1.05a[a]	97.7±0.91a	96.7±0.87a
Negative control	00±00e	00±00e	00±00d
T. harzianum + R. solani	10.26±0.85d	10.32±0.27d	12.43±0.54c
T. viride + R. solani	26.62±0.54c	18.13±0.18c	13.58±0.33c
Metarhizium sp. + R. solani	36.39±0.47b	23.47±0.67b	23.68±0.77b
P-value[b]	< 0.01	< 0.01	< 0.01

[a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at P≤0.01.
[b]Probabilities associated with individual F tests.

Table 5. *In vivo* evaluation of antagonistic fungal isolates on peroxidase activity in roots in the presence of *Rhizoctonia solani* under controlled conditions

Treatments	Peroxidase activity (units g^{-1} ml^{-1} min^{-1})		
	Tomato	Melon	Watermelon
Positive control	1.96±0.07d[a]	2.1±0.14c	1.98±0.05c
Negative control	0.89±0.01e	0.81±0.02d	0.88±0.06d
T. harzianum + *R. solani*	4.97±0.08a	5.29±0.11a	5.27±0.14a
T. viride + *R. solani*	3.72±0.11b	3.35±0.43b	3.2±0.03b
Metarhizium sp. + *R. solani*	2.92±0.13c	2.97±0.05b	2.92±0.02b
P-value[b]	< 0.01	< 0.01	< 0.01

[a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at P≤0.01.
[b]Probabilities associated with individual F tests.

Table 6. *In vivo* evaluation of antagonistic fungal isolates on catalase activity in roots in the presence of *Rhizoctonia solani* under controlled conditions

Treatments	Catalase activity (units g^{-1} ml^{-1} min^{-1})		
	Tomato	Melon	Watermelon
Positive control	15.52±1.71e[a]	14.64±1.32e	15.13±1.37e
Negative control	20.19±1.42d	18.99±1.24d	19.9±1.44d
T. harzianum + *R. solani*	100.15±1.85a	101.22±1.54a	99.93±1.72a
T. viride + *R. solani*	68.51±1.56b	66.89±1.89b	68.12±1.28b
Metarhizium sp. + *R. solani*	48.29±0.92c	47.44±1.67c	44.08±1.69c
P-value[b]	< 0.01	< 0.01	< 0.01

[a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at P≤0.01.
[b]Probabilities associated with individual F tests.

Similarly, all three plant species showed a significant increase (P<0.01) in catalase activity, another enzyme implicated in plant defense responses, when treated with the fungal strains as compared to the controls. Interestingly, *T. harzianum* (100.15, 101.22, and 99.93 units' g^{-1} ml^{-1} min^{-1}, respectively) caused the most substantial increase in catalase activity in all plants, followed by *T. viride* and *Metarhizium* sp. These findings suggested that the tested fungi might stimulate defense mechanisms against *R. solani* infection (Table 6).

This research investigated the efficacy of *T. harzianum*, *T. viride* and *Metarhizium* sp. in controlling *R. solani* infection in tomato, melon, and watermelon. The results revealed that *T. harzianum* and *T. viride* significantly reduced the DSI caused by *R. solani* across the tested crops. Additionally, the application of these strains was associated with an increase in plant enzyme activity. *T. harzianum* shows the most significant disease protection effect. This information is valuable for developing biocontrol strategies using these beneficial fungi to manage *R. solani* infection in various crops. These results aligned with prior greenhouse studies by Ali and Taha (2016), Devi et al. (2017), and Huang et al. (2011) who demonstrated the effectiveness of *T. harzianum* in controlling *R. solani*-induced tomato damping-off disease. Furthermore, Ban et al. (2022) reported that pre-seeding application of *T. harzianum* (five days before planting) in tomato and bean crops yielded significantly better disease control compared to simultaneous application. Additionally, Sreenivasaprasad and Manibhushanrao (1990) reported that *T. virens* were successfully used as a biocontrol agent against groundnut root rot and *R. solani*-induced damping-off in cotton and tomato.

Studies have explored various formulations and applications of *Trichoderma* spp. for disease control and plant growth promotion. Rehman et al. (2011) reported improved protection against damping-off disease and enhanced cauliflower seedling growth using a combination of farm

yard manure, *T. harzianum* and *T. viride* as seed treatments. Lewis and Lumsden (2001) demonstrated the effectiveness of a biocontrol formulation containing vermiculite, powdered wheat bran, and *Trichoderma* / *Gliocladium* biomass in controlling pepper and cucumber damping-off in greenhouse settings. Smolinska et al. (2007) further confirmed the efficacy of four *Trichoderma* strains against *R. solani* in lettuce and cucumber, with *T. harzianum* strain PBG notably increasing plant mass in both crops. Beyond disease control, *Trichoderma* has also been linked to improved and faster seed germination in various plant species, including silverweed (Oyarbide et al. 2001), cotton (Hanson 2000), rice (Mishra and Sinha 2000), chili (Asaduzzaman et al. 2010), and muskmelon (Kaveh et al. 2011).

Studies have shown that plant colonization by *Trichoderma* spp. is associated with reduced disease development in both roots and aboveground tissues. This phenomenon is likely attributed to the interactions between *Trichoderma* and the plant itself (Amer and Abou-El-Seoud 2008, Biam and Majumder 2019, Cai et al. 2013, Gajera et al. 2016).

Beyond its antagonistic interactions with plant pathogens, *Trichoderma* spp. also participated in competitive interactions with other soil microorganisms. This competition primarily revolves around securing essential resources like nutrients and space within the soil environment (Baghani et al. 2012, Bailey et al. 2008, Motesharrei and Salimi 2014, Segarra et al. 2010). Notably, *Trichoderma* spp. can compete for root exudates released by seeds. These exudates, while beneficial to plant growth, can also inhibit the germination of fungal propagules belonging to plant pathogens present in the soil (Howell 2003).

Research by Abd-El-Khair et al. (2011), Hajji-Hedfi et al. (2023), Kobori et al. (2015) and Rhouma et al. (2024) investigated the impact of *Trichoderma* application on enzyme activity in various plants. Their findings revealed a significant increase in the activity of several key enzymes, including polyphenol oxidase, chitinase, catalase, and peroxidase, in plants treated with *Trichoderma* compared to the untreated control group. These specific enzymes are essential for strengthening plant defense mechanisms against the invasion of pathogens.

The many advantages of *T. harzianum* have made it a leading biocontrol and biostimulant. This fungus exhibits remarkable antifungal activity against a broad spectrum of plant pathogens, including *R. solani*. Previous research has unequivocally demonstrated that *T. harzianum* can effectively suppress the growth of isolates of *R. solani*. The prevailing hypothesis suggests that this inhibitory effect stems from the production of antibiotic secondary metabolites. These bioactive compounds are believed to disrupt the growth and function of the target fungal pathogens. By unraveling the intricate mechanisms employed by *T. harzianum*, this study aims to provide a more comprehensive understanding of how microbial biocontrol agents and biostimulants operate. This knowledge is paramount to improving the effective and sustainable integrated plant disease management strategies that reduce reliance on chemical fungicides.

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Author's Contributions

Authors declare that each author's contribution is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

ÖZET

Rhizoctonia solani, domates, kavun ve karpuz dahil olmak üzere 200'den fazla bitki türünü enfekte eden önemli ve geniş spektrumlu bir fungal patojendir. Bu çalışmada, farklı *R. solani* izolatlarının (Rs26, Rs94, Rs13, Rs57 ve Rs123) patojenitesi ve çevre dostu hastalık yönetimi için *Trichoderma harzianum*, *T. viride*, *Metarhizium* sp., *Gliocladium* sp. gibi biyolojik ajanların etkinliği laboratuvar ve sera koşullarında değerlendirilmiştir. Patojenite testlerinin sonuçları, izolatların değişen virülensini doğrulamıştır. Rs94 ve Rs13 izolatları karpuzda en ciddi hastalığa neden olmuştur (hastalık şiddeti (HS)= 3.80 ve 3.83, hastalık şiddeti indeksi (HSİ)= %90.43 ve %95.75). Benzer şekilde, Rs26 izolatı domateste en yüksek patojenisiteyi sergilemiştir (HS= 3.84; HSİ= %94.86). Kavun, tüm izolatlara karşı yüksek hassasiyet göstermiş olup, sürekli olarak 2.59'dan yüksek HS ve %80.97'yi aşan HSİ değerleri kaydedilmiştir. Daha sonra yapılan *in vitro* ve *in vivo* denemeler, test edilen tüm ajanların *R. solani* izolatlarına karşı antifungal potansiyelini ortaya koymuştur. Özellikle *Trichoderma* spp., en tutarlı ve anlamlı inhibisyonu göstermiştir (miselyal büyüme azalması %82.97-%94.67). Bu konuda en iyi performansı ise *T. harzianum* göstermiştir. Sera denemeleri, *T. harzianum*'un önleyici bir tedavi olarak etkinliğini doğrulamış, bitki enzim aktivitesini artırmış (peroksidaz = domates ve karpuz için sırasıyla 4.97-5.29 birim g^{-1} ml^{-1} dk^{-1}; katalaz = karpuz ve kavun için sırasıyla 99.93-101.22 birim g^{-1} ml^{-1} dk^{-1}) ve hastalık şiddeti indeksini önemli ölçüde azaltmıştır (HSİ < %12.43). Bu bulgular, *T. harzianum*'un domates, kavun ve karpuz bitkilerinde *R. solani* fide yanıklığı hastalığının yönetimi için sürdürülebilir

ve çevre dostu bir strateji olarak kullanım potansiyelini vurgulamaktadır.

Anahtar kelimeler: Fide yanıklık hastalığı, *Trichoderma* spp., domates, kavun, patojenisite, karpuz

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Original article

Effects of some treatments on seed dormancy in branched asphodel (*Asphodelus ramosus* L.)

Bazı uygulamaların çirişağusu (*Asphodelus ramosus* L.) dormansisi üzerine etkisi

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ABSTRACT

Branched asphodel (*Asphodelus ramosus* L.) is an unpalatable geophyte that has been increasing spreading in the pastures of Aegean region of Türkiye. To formulate effective management strategies at various growth phases, it is necessary to initiate the germination of dormant seeds. Preliminary experiments indicated that branched asphodel seeds germinated better in darkness than in light. Several dormancy-release techniques were tested to develop a rapid, uniform and better germination protocol for branched asphodel seeds including cold stratification at +4 and -18 °C, mechanical scarification with sandpaper, chemical scarification with sulfuric acid, ethanol or hydrogen peroxide and application of chemicals, i.e., gibberellic acid or potassium nitrate. Chemical scarification with 95% sulfuric acid (for 1 min) or 20 mM or 40 mM hydrogen peroxide (for 24 hours) resulted in the highest germination percentages (over 81.3%) and adequately reduced Mean Germination Time (MGT). While chemical scarification with sulfuric acid for 5, 15, and 30 min also reduced MGT, extending scarification duration beyond one-minute decreased germination rates. Lower germination rates with gibberellic acid treatment and increased germination with scarification methods suggest that the seeds have physical dormancy rather than physiological dormancy. Manual scarification with sandpaper achieved germination rates over 75% and reduced MGT by more than 57%. In conclusion, manual sandpaper application considered to be preferable for avoiding the adverse effects of chemical treatments while providing an acceptable germination rate.

INTRODUCTION

Branched asphodel (*Asphodelus ramosus* L.) is a member of Asphodelaceae family and has perennial tuberous roots, which protect the plant from extreme heat and drought by enabling its survival under adverse effects (Anonymous 2022). This plant that is the subject of various studies due to the presence of numerous beneficial compounds (Apaydin and Arabaci 2017, Reynaud et al. 1997). However, the increasing density of this species in the pastures is causing significant problems, as both large and small ruminants tend to avoid it.

Unpalatable geophytes, such *Asphodelus*, are predominant life forms in the areas of the Mediterranean region that have been degraded by overgrazing and fire (Noy-Meir and Oron 2001, Terzi 2023). *Asphodelus* species are widely distributed across the Aegean, Marmara, Mediterranean, and Southeastern Anatolia regions of Türkiye, where Mediterranean climate prevails (Alatürk and Gökkuş 2019).

It has been reported that the density of *Asphodelus* in the pastures of the Aegean region can reach up to 10 units/m^2 (Eltez 1995). This species is commonly found in meadow and pasture soils, along roadsides, and especially in the hilly areas between agricultural fields in the Aydin district of the Aegean region. Its persistence is associated with the development of secondary chemical compounds and it is lower dependence on seed production compared to annual species (Sternberg et al. 2000).

Summer drought is one of the environmental constraints faced by branched asphodel in the western part of Türkiye, as is the case with summer asphodel (*Asphodelus aestivus* Brot.) in the Mediterranean region. Both species withstands summer drought by drying out their aboveground parts (Pantis et al. 1994). While biomass reduction is observed in the tuberous roots during the prolonged dry periods, as in *A. aestivus* (Sawidis et al. 2005), branched asphodel can still spread by its usually dormant seeds upon dispersal.

Branched asphodel is becoming one of the most troublesome weeds in pastures in Aydin province, Türkiye. Although mowing, with or without the removal of cut material, has shown promising results in restoring grassland biodiversity in *Asphodel*-dominant communities (Tesei et al. 2018), this method is limited due to the lack of funds and labor and the difficulty using equipment on sloping pastures in the region. A study conducted in Aydin province found that the lowest population of *A. aestivus* was achieved through paraquat application and hand weeding (Sürmen and Kara 2022). However, these methods are not applicable for branched asphodel recently, since paraquat (in addition to not having systemic effect in plants) is banned in Türkiye and hand weeding requires a lot of labor and financial resources.

No chemical control methods are currently being implemented in the pastures of Aydin province, Türkiye, which has contributed to the increasing density of branched asphodel. Due to the varying sizes of its tuberous roots, it has been difficult to assess the efficacy of chemical management strategies for this plant in trials. Initiating the germination process of dormant seeds is essential for formulating management strategies for the plant's various growth phases. Seed dormancy in branched asphodel may result either from an inhibitory action of the seed coat or from the characteristics of the embryo itself. This study aims to break seed dormancy and improve the germination of branched asphodel using different methods. The results of this study may also benefit researchers seeking to extract various chemicals from this plant.

MATERIALS AND METHODS

Seed collection and photoperiod treatments

Mature seeds of branched asphodel were collected from the pastures in Cine (37°37' N, 27° 57' E, 148 m above sea level), Efeler (37°47' N, 27° 56' E, 122 m above sea level), and Kocarli (37°45' N, 27°45' E, 44 m above sea level) districts of Aydin province, Türkiye, during June 2021. The seed collection sites characterized by a dry climate, with a mean temperature of 17.7 °C and mean annual rainfall of 659.9 mm. Seeds were stored in paper bags at room temperature (approximately 25 °C) in darkness until the experiments began in July 2021.

Seed dormancy was tested before the experiments using 20 seeds in four replicates with two repeats. Seeds were surface-sterilized with a 5% sodium hypochlorite solution for 15 minutes and then rinsed three times with sterile distilled water for 5 minutes each. The seeds were placed in glass Petri dishes containing sterile filter paper and moistened with 5 ml sterile distilled water. Potential seed germination was tested under optimal conditions: a temperature of 15 °C and 50% humidity. The photoperiod treatments were tested under 8 h light (10.000 lux)/16 h dark and complete darkness in climatic test chamber (Mikrotest/MIT-600).

Seeds were considered germinated when the radicle emerged to length of at least 2 mm. The experiments were conducted over 28 days, and seed germination was checked on day's 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 10^{th}, 14^{th}, 21^{st}, and 28^{th} day after the initiation of the experiments. All germinated seeds were removed from Petri dishes upon observation.

Experiments for breaking seed dormancy

Seeds of branched asphodel were exposed to incubation in low temperatures, mechanical scarification (with sandpaper), and chemical scarifications [with sulfuric acid (H_2SO_4), ethanol or hydrogen peroxide (H_2O_2)] as well as chemical treatments [with gibberellic acid (GA_3) or potassium nitrate (KNO_3)]. Sterile distilled water applied (5 ml in each Petri dish) was applied to seeds evaluated as controls. All seeds were surface sterilized as described in the pretreatment section before applications, except for seeds in

cold storage (seed surfaces were sterilized after incubation at low temperatures).

Cold stratification

To evaluate the effect of low temperatures on seed dormancy, seeds were placed in paper bags covered with aluminum foil, stored in refrigerator at 4 °C and -18 °C, and kept for 30 days. Afterwards, they were surface sterilized before starting the experiments.

Mechanical scarification

Mechanical scarification was investigated in two different ways. Seeds were placed in sandpaper-coated plastic containers and shaken at 800 rpm for two hours in an orbital shaker (Miprolab MLS 3535). In other way, seeds were gently and manually rubbed between two sheets of sandpaper for 10 seconds until the seeds coats were thinned.

Chemical scarification

Different chemicals at different concentrations or at different durations were used for chemical scarification. Dry seeds were soaked in 95% H_2SO_4 (Tekkim Chemistry) for 1, 5, 15, 30, 60, and 120 minutes; in 95% ethanol for 30 and 60 minutes; or in H_2O_2 (30%, Tekkim Chemistry) at concentrations of 20 and 40 mM for 24 hours. All seeds were washed with sterile distilled water three times for 5 minutes each before being placed them in Petri dishes.

Treatments with gibberellic acid and potassium nitrate

To evaluate effectiveness of chemical treatments on seed dormancy, two different concentrations (500 and 1000 ppm) of GA_3 (Aldrich Chemistry) and two concentrations of KNO_3 (0.1% and 0.2%; Sigma-Aldrich) were applied at 5 ml to the Petri dishes containing intact seeds.

Germination tests

Twenty seeds were placed in each glass Petri dish (90 mm), and all the experiments were conducted with four replications and two experimental runs. Petri dishes were placed in a climatic test chamber adjusted to 15 °C, 50% relative humidity, under complete darkness. Seed germination was monitored on the 1st, 3rd, 5th, 7th, 10th, 12th, 14th, 17th, 19th, 21st, 24th, 26th, and 28th day after the initiation of the experiments. Germinated seeds (>2 mm radicle) were removed from Petri dishes.

Statistical analysis

For all the experiments, the cumulative germination percentage (mean ± standard error) and mean germination time (MGT, mean in days ± standard error) were calculated. The seed germination percentage was calculated by multiplying the ratio of germinated seeds to the total number of viable seeds in a single Petri dish. MGT represented the average length of time in days it took seeds to germinate and was calculated by the following formula:

MGT: $\Sigma DN/\Sigma N$

Where D is the number of days counted from the date of sowing, and N is the number of seeds germinated on day D.

The data from the experimental runs for the light:dark experiment were combined for analysis since the interaction between runs was found non-significant in pretreatments. Then two different photoperiods were compared by independent t-test (P=0.05).

To compare the effects of dormancy-release treatments on germination percentage (%), the data were analyzed using General Linear Model/Univariate procedure and the treatment means were separated Tukey's HSD test at P≤0.05. Since H2SO4 applications at 60 and 120 minutes caused losses in seed viability, the results obtained from these applications were eliminated from statistical analysis. To satisfy normality and homogeneity assumptions, Lg10 transformed data of MGT were used. Statistical analysis was performed using IBM SPSS Statistics 21.

RESULTS

Effects of photoperiod on seed germination

Seed germination percentage varied among photoperiod conditions, while MGT remained unaffected (Table 1).

As seen in Figure 1 cumulative germination percentage was higher in darkness (45.63%) than 8-h photoperiod (40.63%). Therefore, the treatments for breaking dormancy were carried out in darkness.

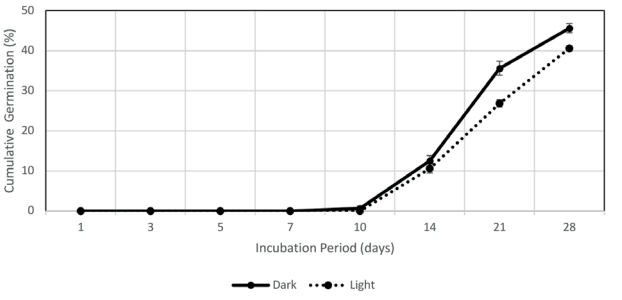

Figure 1. Cumulative germination percentage of branched asphodel seeds incubated under dark or light (8 h light/16 h dark) conditions (Vertical lines indicate the standard error.)

Table 1. Independent T-test results of the germination percentage and mean germination time (MGT) of branched asphodel seeds incubated in darkness or 8-h light conditions

	t	df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Germination percentage	3.864	14	.002	5.00000	2.22492	7.77508
MGT[a]	-2.046	14	.060	-.95375	-1.95365	.04615

[a]Abbreviation: MGT, mean germination time

Effects of treatments for breaking dormancy

Germination percentage and MGT varied significantly among 17 different treatments. Differences in germination percentage (%) and MGT in the first experimental run, tested by Tukey's HSD test, are shown in Table 2.

As seen in Table 2, treatments in the first repeat significantly increased germination percentage compared to the control (38.8%) were H_2SO_4 (1 min) and H_2O_2 applications at 20 mM and 40 mM concentrations. These same treatments also shortened the MGT, along with other H_2SO_4 durations and manual sandpaper applications.

Tukey's HSD test results of germination percentage and MGT of the second experimental run are shown in Table 3.

The treatments that significantly increased germination percentages of branched asphodel seeds in the second repeat were H_2SO_4 (1 and 5 min), 20 and 40 mM H_2O_2 and manual sandpaper. Addition to these treatments, other H_2SO_4 durations and sandpaper usage at 800 RPM for two hours significantly decreased MGT.

Table 2. Germination percentages and mean germination time (MGT) of branched asphodel seeds subjected to different treatments under darkness (1st experimental run)

Treatments	Germination (%)		MGT (days)	
H_2SO_4[a] (1 min)	86.3 ± 5.5[b]	ef	8.5 ± 0.1	bc
H_2SO_4 (5 min)	62.5 ± 8.3	cdef	7.4 ± 0.2	ab
H_2SO_4 (15 min)	53.8 ± 5.5	cdef	6.3 ± 0.6	a
H_2SO_4 (30 min)	12.5 ± 2.5	ab	7.9 ± 0.4	ab
0.1% KNO_3	51.3 ± 11.3	cde	20.2 ± 1.1	de
0.2% KNO_3	35.0 ± 10.8	abc	19.1 ± 0.9	de
500 ppm GA_3	61.3 ± 9.4	cdef	18.0 ± 0.5	d
1000 ppm GA_3	50.0 ± 6.1	bcde	18.5 ± 0.4	de
20 mM H_2O_2	90.0 ± 3.5	f	10.4 ± 0.4	c
40 mM H_20_2	81.3 ± 9.4	ef	8.9 ± 0.9	bc
+ 4 C° (1 month)	62.5 ± 6.0	cdef	18.7 ± 0.7	de
-18 C° (1 month)	53.8 ± 13.9	cdef	19.9 ± 1.3	de
95% ethanol (30 min.)	55.0 ± 2.9	cdef	18.3 ± 0.7	d
95% ethanol (60 min.)	27.5 ± 2.5	abc	17.0 ± 1.1	d
Sandpaper (800 RPM/2 hours)	7.5 ± 2.5	a	24.0 ± 1.1	e
Manual sandpaper	75.0 ± 6.1	def	7.4 ± 0.4	ab
Distilled water (Control)	38.8 ± 3.8	abcd	20.9 ± 0.9	ab

[a]Abbreviations: H_2SO_4, sulfuric acid; KNO_3, potassium nitrate; GA_3, gibberellic acid; H_2O_2, hydrogen peroxide; RPM, round per minute; MGT, mean germination time

[b]Values are represented by mean ± standard error. Different lowercase letters indicate significant differences among treatments in the same column

Table 3. Germination percentages and mean germination time (MGT) of branched asphodel seeds subjected to different treatments under darkness (2nd experimental run)

Treatments	Germination (%)		MGT (days)	
H_2SO_4[a] (1 min)	88.8 ± 4.7[b]	f	8.2 ± 0.3	c
H_2SO_4 (5 min)	77.5 ± 5.2	cdef	6.8 ± 0.2	b
H_2SO_4 (15 min)	37.5 ± 4.8	ab	6.4 ± 0.1	b
H_2SO_4 (30 min)	20.0 ± 3.5	a	8.0 ± 0.3	c
0.1% KNO_3	62.5 ± 8.3	bcdef	20.0 ± 0.5	fgh
0.2% KNO_3	57.5 ± 2.5	bcde	19.5 ± 0.3	fgh
500 ppm GA_3	62.5 ± 6.3	bcdef	18.3 ± 0.4	fg
1000 ppm GA_3	53.8 ± 8.3	bcd	17.6 ± 0.1	f
20 mM H_2O_2	87.5 ± 6.6	ef	9.6 ± 0.4	d
40 mM H_20_2	91.3 ± 5.5	f	5.2 ± 0.1	a
+ 4 C° (1 month)	66.3 ± 6.6	bcdef	19.3 ± 0.4	fgh
-18 C° (1 month)	53.8 ± 9.0	bcd	17.4 ± 0.5	f
95% ethanol (30 min.)	51.3 ± 8.0	bc	21.1 ± 0.2	gh
95% ethanol (60 min.)	37.5 ± 1.4	ab	21.4 ± 0.7	h
Sandpaper (800 RPM/2 hours)	61.3 ± 4.7	bcdef	14.2 ± 0.2	e
Manual sandpaper	82.5 ± 6.6	def	7.9 ± 0.3	c
Distilled water (Control)	45.0 ± 2.0	ab	18.6 ± 0.8	fg

[a]Abbreviations: H_2SO_4, sulfuric acid; KNO_3, potassium nitrate; GA_3, gibberellic acid; H_2O_2, hydrogen peroxide; RPM, round per minute; MGT, mean germination time

[b]Values are represented by mean ± standard error. Different lowercase letters indicate significant differences among treatments in the same column

DISCUSSION

The branched asphodel seeds germinated better in darkness during the current study, in contrast to germination studies of onionweed (*Asphodelus tenuifolius* Cav.), which were conducted in darkness and a 10-h photoperiod (Tanveer et al. 2014). Özturk and Pirdal (1986) also reported summer asphodel (*A. aestivus* Brot.) light sensitive based on studies carried out in different photoperiods and darkness conditions. Although we found a significant difference in light requirements, germination percentages (45.63% in darkness, 40.63% in photoperiod) were close to each other, indicating that both dark and light periods can be used for germination studies of branched asphodel. Previous research shows that light dependence declines with increasing seed size (Milberg et al. 2000), so the light independence for germination of branched asphodel seeds can be attributed to that.

Sulfuric acid treatments are usually preferred to break dormancy in seeds, especially those with a hard seed coat (Wang et al. 2007). Based on hardness of seed coat, the time required for soaking in H_2SO_4 solutions varies. In our study, as the treatment time with H_2SO_4 exceeded one minute, the germination rate decreased, and no germination was observed in some cases of H_2SO_4 applications. The seed coat destroyed in H_2SO_4 applications at 60 and 120 min (results were not shown and not included in the statistical analysis) and the seeds lost their viability. However, the H_2SO_4 (1 min) application gave good results for increasing germination and shortening MGT.

At low concentrations, potassium nitrate can stimulate seed germination in a variety of plant species, whereas high levels decrease seedling growth (Hernandez et al. 2022). Seed treatment with potassium nitrate (KNO_3) has been reported in many studies to break dormancy and improve germination (Anosheh et al. 2011) by modulating ABA metabolism or ABA signaling in developing seeds (Chahtane et al. 2017, Matakiadis et al. 2009) However, nitrate stimulation of seed germination is often associated with plant species whose seeds require light for germination and seed age (Footitt et al. 2013, Henson 1970). Therefore,

the fact that potassium nitrate treatments (0.1 and 0.2%) did not increase the germination percentage and germination speed significantly was attributed to the fact that the young seeds of branched asphodel not needing light to germinate and the thickness of seed coat in our study.

Gibberellins (GAs) play an important role in the stimulation of seed germination (Bewley 1997), and GA_3 is a well-known germination stimulator that can fully or partially replace light, after-ripening, and cold requirements (da Silva et al. 2005). Contrary to many reports on the stimulatory effect of GA_3 during seed germination, GA_3 can inhibit radicle protrusion in some species (Olvera-Carrillo et al. 2003) as well. The addition of GA_3 (500 and 1000 ppm) did not enhance seed germination of branched asphodel seeds (<65%) significantly in our study, suggesting that the seeds do not have physiological dormancy. GA_3 applications also did not shorten MGT.

With the enhancement of H_2O_2 in the embryo, seeds lose their dormancy, and the imbibitions of seeds with hydrogen peroxide (H_2O_2) increases the germination as well as seedling growth in many studies (Debska et al. 2013). H_2O_2 acts as a signaling molecule in the beginning of seed germination, involving specific changes at proteomic, transcriptomic and hormonal levels (Barba-Espín et al. 2012). In some cases, seed coat-imposed dormancy can be alleviated with oxidants such as H_2O_2, which can oxidize the phenolic compounds present in the seed envelopes and may allow improved oxygenation of the embryo during seed imbibitions (Ogawa and Iwabushi 2001). The imbibition of seed for 24 hours at two different concentrations of H_2O_2 (20 and 40 mM) improved seed germination and significantly shortened the MGT in this study.

In most weed species investigates, the main factors in the natural environment that lead to release from dormancy are light, two attributes of temperature—chilling (or stratification) and alternating temperature—and nitrate ions (Vincent and Roberts 1977). The effects of temperature on seed dormancy during chilling differ depending on whether the seed is wet or dry (Roberts and Totterdell 1981), and pre-chilling time is also important for including morphological changes in seed coat and increasing germination (Jordan and Jordan 1982). The effect of chilling on germination differs among species (Rezvani et al. 2014) There was an increase in germination percentage of branched asphodel seeds under dry cold storage (better in +4 °C than -18 °C), but it was not significant, and the MGT of seed under these treatments was similar to the untreated control. Increasing the cold storage period may contribute to raising the germination percentage. Wet pre-chilling can also be studied to improve germination and shorten the MGT.

Ethanol promotes germination in some species, such as oat and rice (Adkins et al. 1984, Miyoshi and Sato 1997) by inducing gibberellin, increasing oxygen uptake, or perturbing membranes (Taylorson and Hendricks 1979). It may play a role in breaking dormancy by promoting the Krebs cycle and/or glycolysis and may also stimulate germination as a respiratory substrate (Adkins et al. 1984, Corbineau et al. 1991). Germination percentages and MGT results obtained from ethanol treatments of branched asphodel seeds were similar to or below the control in this study. Therefore, it was thought that ethanol concentrations used in experiments were too high to promote germination. Since ethanol concentrations and the duration of ethanol effect the stimulation of germination, and failure to reduce ethanol concentration inhibits germination (Taylorson and Hendricks 1979), lower concentrations and durations should be studied in further.

Mechanical scarification methods are successful in stimulating germination of seeds, especially those with seed-coat imposed dormancy (Eisvand et al. 2006). It is thought that dormancy in branched asphodel seeds is based on seed coat permeability, so overcoming dormancy in these seeds was expected with mechanical scarification methods. However, placing the seeds in sandpaper-coated container and shaking them for two hours at 800 rpm in an orbital shaker did not provide sufficient scouring of the seed coat, while rubbing them between sandpaper for 10 seconds increased seed germination and shorted MGT.

When all the results are evaluated, scarification treatments of H_2O_2 (20 mM and 40 mM) and H_2SO_4 (1 min) significantly increased the germination percentage of branched asphodel seeds in both experiments. The increase in germination percentage observed with H_2SO_4 (5 min) and manual sandpaper applications in one repeat was not significant in another. Since increased H_2SO_4 duration reduced germination rate and seed viability, the one-minute duration in H_2SO_4 is preferable for breaking dormancy. Manual sandpaper application also showed promise for increasing germination percentage, with values exceeding 75% in both experiments.

As with increasing germination percentage, H_2O_2 (20 mM and 40 mM) and H_2SO_4 (1 min) applications significantly shortened the MGT compared to control. Other H_2SO_4 durations and manual sandpaper also decreased MGT significantly in both repeats, but they did not significantly increase germination in some cases.

In conclusion, H_2O_2 (20 mM and 40 mM) and H_2SO_4 (1 min) applications were found sufficient to germinate branched asphodel seeds and accelerated the germination process. Although it was not significant in one repeat, manual sandpaper application also resulted in a germination percentage of over 75% and shortened MGT by over 57%. Considering the possible negative effects of H_2O_2 and H_2SO_4 on seedling growth, rubbing the seeds between sandpaper for 10 seconds was found to be more reasonable method for germinating branched asphodel seeds in further studies.

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Author's Contributions

Authors declare that each author's contribution is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

ÖZET

Çirişağusu (*Asphodelus ramosus* L.), Ege bölgesi meralarında giderek artan, yenmeyen bir geofittir. Farklı büyüme aşamalarında kontrol stratejileri geliştirebilmek için dormant tohumlarının çimlendirilmesine ihtiyaç duyulmuştur. Ön deneylerde çirişağusu tohumlarının karanlıkta ışıktan daha iyi çimlendiği görülmüştür. Tohumlarında hızlı, tekdüze ve daha iyi çimlenme sağlamak için, çeşitli dormansi kırma yöntemleri (+4 ve -18 °C'de soğutma, zımpara kağıdı, sülfürik asit, etanol veya hidrojen peroksit ile aşındırma ve gibberellik asit veya potasyum nitrat ile işlemler) uygulanmıştır. Tohumların %95 sülfürik asitte (1 dakika boyunca) veya 20 mM veya 40 mM hidrojen peroksitte (24 saat boyunca) bekletilmesi, en yüksek çimlenme yüzdelerini (%81.3'ün üzerinde) sağlamış ve ortalama çimlenme zamanını (OÇZ) kısaltmıştır. Sülfürik asit diğer sürelerde de (5, 15 ve 30 dakika) OÇZ'yi kısaltmış, ancak sürenin bir dakikadan fazla arttırılması çimlenme oranını azaltmıştır. Gibberellik asite duyarsızlık ve aşındırma yöntemleriyle çimlenmenin artması, tohumların fizyolojik bir dormansi yerine fiziksel dormanside olduğunu göstermiştir. Sonuç olarak, çimlenmeyi %75'in üzerinde sağlayan ve OÇZ'yi >%57 kısaltan zımpara kağıdı ile mekanik aşındırmanın, kimyasalların olumsuz etkilerinden kaçınmak ve kabul edilebilir çimlenmeyi sağlamak açısından tercih edilebilir olduğu düşünülmektedir.

Anahtar kelimeler: kimyasal işlemler, dormansi kırma, çimlenme, düşük sıcaklıklar, aşındırma

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Original article

Assessment of Pm41 gene frequency in Turkish bread wheat germplasm

Türk ekmeklik buğday germplazmında Pm41 gen frekansının değerlendirilmesi

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ABSTRACT

Powdery mildew, caused by *Blumeria graminis* f. sp. *tritici*, poses a high risk to worldwide wheat production, resulting in severe yield reductions. Resistance breeding provides a sustainable approach to managing this disease, with the *Pm41* gene being pivotal in providing all-stage resistance. This research examined 96 cultivars of Turkish bread wheat (*Triticum aestivum*) from several Turkish research institutions for the *Pm41* gene. PCR analysis indicated that 57% of the studied cultivars possessed the Pm41 gene. The highest detection rate of 89% was recorded in cultivars from Bahri Dağdaş International Agricultural Research Institute Directorate/Konya, whereas Field Crops Research Institute Directorate/Ankara exhibited a lesser frequency of 29%. The data demonstrate regional disparities in *Pm41* presence. The observed *Pm41* gene in over half of the cultivars suggests that Turkish wheat cultivars possess gene variants that might be important for resistance. This work underscores the significance of preserving genetic materials for finding novel variants of the resistance genes, which are essential for sustainable wheat cultivation and food security.

INTRODUCTION

Bread wheat (*Triticum aestivum*) is one of the most significant crops globally, serving as a primary source of calories and proteins in human diets. Its importance is underscored by its role in food security, as it constitutes a staple food for billions of people worldwide. The cultivation of bread wheat has evolved significantly, with modern varieties developed through selective breeding and genetic improvements aimed at enhancing yield, disease resistance, and adaptability to various environmental conditions (Cavalet-Giorsa et al. 2024, Merchuk-Ovnat et al. 2016). However, the genetic diversity of contemporary bread wheat has been compromised due to the processes of domestication and polyploidization, which have led to a reduction in the genetic variability that is crucial for resistance against biotic and abiotic stresses (Cavalet-Giorsa et al. 2024, Zhou et al. 2020).

Local cultivars possess unique genetic traits that have evolved in specific environmental conditions, making them well-suited to local climates and agricultural practices. These cultivars are vital reservoirs of genetic diversity, harboring

alleles that may confer advantages such as biotic and abiotic stress resistance and nutritional quality (Morgounov et al. 2016, Cheng et al. 2019, Yang et al. 2022).

Wheat production is significantly threatened by various fungal diseases, with powdery mildew (caused by *Blumeria graminis* f. sp. *tritici*) being one of the most destructive. This biotrophic pathogen can lead to substantial yield losses of up to 40% depending on environmental conditions and crop management practices (Morgounov et al. 2012). The prevalence of powdery mildew is particularly concerning in regions with high humidity and mild temperatures, which facilitate rapid disease spread and infection. The global incidence of wheat diseases, including powdery mildew, underscores the urgent need for effective management strategies to mitigate their impact on wheat production (Chai et al. 2022).

Türkiye, one of the world's largest wheat producers, is particularly vulnerable to diseases like powdery mildew. It is manifested as one of the most destructive diseases of wheat, appearing in different regions (Arslan et al. 2024, Özdemir et al. 2017, Sönmezoğlu et al. 2019). Mostly it is observed in the Thrace, Eastern Marmara, and Black Sea Regions and occasionally in the Aegean Region (Aydin et al. 2021, Tosun et al. 2011).

Protection against powdery mildew primarily involves the use of resistant wheat cultivars and the application of fungicides. Breeding programs have focused on identifying and incorporating resistance genes into wheat varieties, which has proven effective and environmentally sustainable (Wu et al. 2021, Zhang et al. 2017). Addressing the challenge of powdery mildew in wheat requires a multifaceted approach that leverages genetic resistance, advanced breeding techniques, and environmentally sound management practices. As the global demand for grain rises, developing resistant varieties and effective control measures against powdery mildew will be critical to ensuring food security and sustainable agricultural practices.

The study of Cheng et al. (2022) aimed to assess powdery mildew resistance at both the seedling and adult stages, and to identify the presence of *Pm* genes in 332 germplasms from an international wheat collection utilizing molecular markers. It was determined that only a few accessions were resistant to *Blumeria graminis* f. sp. *tritici* (Bgt) races E09, E15, and A13, while all evaluated accessions were fully susceptible to Bgt race A44 in the seedling stage. The gene *Pm41* was identified in wild emmer wheat (*Triticum turgidum* var. *dicoccoides*), specifically from accession IW2, collected from Mount Hermon, Israel, which represents a significant advancement in the fight against powdery mildew (Li et al. 2009). This gene is part of a broader family of over 130 known powdery mildew resistance genes, with 69 officially designated (*Pm1-Pm69*) across various wheat species and related genera (Chen et al. 2024).

The discovery of *Pm41* is particularly noteworthy as it confers all-stage resistance to powdery mildew, making it a valuable asset for wheat breeding programs to enhance disease resistance. The resistance gene was successfully introduced into common wheat through backcrossing and marker-assisted selection (Li et al. 2009). Research indicates that *Pm41* was mapped to a genetic interval on chromosome arm 3BL, utilizing bulked segregate analysis and simple sequence repeat (SSR) markers (Li et al. 2020).

The *Pm41* resistance gene stands out as a critical component in the ongoing efforts to control powdery mildew in wheat. Its identification and characterization pave the way for innovative breeding approaches that leverage genetic diversity to enhance disease resistance in agricultural systems. With the integration of *Pm41* into breeding programs, wheat varieties that resist powdery mildew could be developed in an environmentally friendly manner.

In the present study, a total of 96 Turkish bread wheat cultivars sourced from various agricultural research institutes of Türkiye were screened for the presence of the *Pm41* gene. The objective was to identify this gene as a long-term solution to powdery mildew disease.

MATERIALS AND METHODS

Plant material

Ninety-six bread wheat cultivars of Turkish origin were used in the present study (Table 1).

DNA isolation

Every one of the 96 bread wheat samples was cultivated in a growth room, and approximately 100 milligrams of freshly harvested leaves were obtained for extraction of DNA. Following the freezing of the leaves in liquid nitrogen, they were pulverized into fine powder using a porcelain mortar. Genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) technique, as described by Doyle (1991), with minor modifications. The powder was introduced into a heat-treated CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris at a temperature of 65 °C, with a pH of 8.0). After incubating the samples at 65 °C for 30 minutes, chloroform–isoamyl alcohol was added and thoroughly mixed. Next, the top phase (600 µl) was transferred to

a fresh tube after being centrifuged at 6000 g at room temperature (RT) for 20 minutes. Cold isopropanol (1200 µl) at a temperature of -20 °C was added to the 2 ml tubes. Following centrifugation at 6000 g at RT for 5 minutes, the supernatant was removed and 600 µl of the washer buffer (70% EtOH, 10 mM NaAc) was added. Following a 20-minute incubation period at RT, the mixture was subjected to centrifugation at 6000 g at RT for 10 minutes, and the supernatant was removed. The pellet was rinsed with cold ethanol and then subjected to centrifugation at 6000 g at RT for 10 minutes. The supernatant was discarded, and the pellet was then dissolved in 50 µl of molecular grade water. Quantification of DNA concentration was conducted using Nanodrop (DS11 FX, manufactured by DeNovix Inc. in Wilmington, DE, USA) and adjusted to 100 nanograms per microliter.

PCR assay

PCR assay for detection of *Pm41* gene was performed using Pm41-645F/Pm41-645R forward and reverse using 2 primers (Li et al. 2022, Table 2). PCR mixture was adjusted according to the manufacturer's protocol; 5 µl of 10X Taq Buffer, 2.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTP, 0.5 µl of 10 µM forward and reverse primer each, 0.3 µl of DNA polymerase (5U/µl), 1 µl of DNA template (100 ng/µl) was mixed and the volume was filled with ddH_2O to 25 µl (Thermo Fisher EP0402). Thermocycler conditions were adjusted as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 45 s, and final extension at 72°C for 10 min (Baloch et al. 2024).

PCR amplification results were subjected to a 1% (w/v) agarose gel using TBE buffer (0.54% Tris base, 0.027% boric acid, 0.0037% EDTA) at a voltage of 120 V for 60 minutes. An ethidium bromide stain was applied to the gel and then visualized using a UV Imager (Bio-Rad Laboratories, Inc., located in Hercules, CA, USA). For band length comparison, GeneRuler 100bp Plus DNA ladder was used.

Table 1. Catalogue of historical Turkish bread wheat cultivars: origins, historical records, and information about other valuable characteristics (1963-2014)

Serial No.	Genotypes	Collection locations	Wheat type	Date of registration	Grain color	Presence of awn
1	Ankara 093/44	TARM/ANK	*T. aestivum*	7.10.1963	White	Yes
2	Köse 220/39	TARM/ANK	*T. aestivum*	7.10.1963	White	No
3	Sivas 111/33	TARM/ANK	*T. aestivum*	7.10.1963	-	Yes
4	Sürak M. 1593/51	TARM/ANK	*T. aestivum*	7.10.1963	-	Yes
5	Haymana 79	TARM/ANK	*T. aestivum*	15.05.1979	Red	Yes
6	Gün-91	TARM/ANK	*T. aestivum*	26.04.1991	Red	Yes
7	İkizce 96	TARM/ANK	*T. aestivum*	16.04.1996	Red	Yes
8	Mızrak	TARM/ANK	*T. aestivum*	12.05.1998	White	Yes
9	Türkmen	TARM/ANK	*T. aestivum*	12.05.1998	White	Yes
10	Uzunyayla	TARM/ANK	*T. aestivum*	12.05.1998	White	yes
11	Yakar-99	TARM/ANK	*T. aestivum*	26.04.1999	White	Yes
12	Aksel 2000	TARM/ANK	*T. aestivum*	28.04.2000	Red	Yes
13	Bayraktar 2000	TARM/ANK	*T. aestivum*	28.04.2000	White	Yes
14	Demir 2000	TARM/ANK	*T. aestivum*	28.04.2000	Red	Yes
15	Atlı-2002	TARM/ANK	*T. aestivum*	2.05.2002	Red	Yes
16	Zencirci-2002	TARM/ANK	*T. aestivum*	2.05.2002	White	Yes
17	Eser	TARM/ANK	*T. aestivum*	2.05.2003	White	Yes
18	Seval	TARM/ANK	*T. aestivum*	1.04.2004	Red	Yes
19	Tosunbey	TARM/ANK	*T. aestivum*	1.04.2004	White	Yes
20	Kenanbey	TARM/ANK	*T. aestivum*	6.04.2009	White	Yes

21	Lütfibey	TARM/ANK	*T. aestivum*	30.03.2010	Red	Yes
22	4-11	ATAEM/ESK	*T. aestivum*	7.10.1963	-	No
23	4-22	ATAEM/ESK	*T. aestivum*	7.10.1963	-	No
24	P 8-6	ATAEM/ESK	*T. aestivum*	7.10.1963	-	Yes
25	P 8-8	ATAEM/ESK	*T. aestivum*	7.10.1963	-	No
26	Melez	ATAEM/ESK	*T. aestivum*	11.04.2014	-	No
27	Ak 702	ATAEM/ESK	*T. aestivum*	12.04.2014	-	Yes
28	Sertak	ATAEM/ESK	*T. aestivum*	13.04.2014	-	Yes
29	Yayla 305	ATAEM/ESK	*T. aestivum*	9.04.1966	-	Yes
30	Yektay 406	ATAEM/ESK	*T. aestivum*	18.03.1968	Red	Yes
31	Bolal 2973	ATAEM/ESK	*T. aestivum*	27.04.1970	Red	Yes
32	Kıraç 66	ATAEM/ESK	*T. aestivum*	27.04.1970	White	Yes
33	Porsuk-2800	ATAEM/ESK	*T. aestivum*	13.05.1976	White	Yes
34	Gerek 79	ATAEM/ESK	*T. aestivum*	15.05.1979	White	Yes
35	Atay-85	ATAEM/ESK	*T. aestivum*	25.04.1985	White	Yes
36	Kutluk 94	ATAEM/ESK	*T. aestivum*	17.05.1994	White	Yes
37	Kırgız 95	ATAEM/ESK	*T. aestivum*	20.04.1995	White	Yes
38	Sultan 95	ATAEM/ESK	*T. aestivum*	20.04.1995	White	Yes
39	Süzen 97	ATAEM/ESK	*T. aestivum*	6.05.1997	White	Yes
40	Aytın 98	ATAEM/ESK	*T. aestivum*	12.05.1998	White	Yes
41	Yıldız 98	ATAEM/ESK	*T. aestivum*	12.05.1998	White	Yes
42	Harmankaya-99	ATAEM/ESK	*T. aestivum*	26.04.1999	Red	Yes
43	Altay 2000	ATAEM/ESK	*T. aestivum*	28.04.2000	White	Yes
44	Çetinel 2000	ATAEM/ESK	*T. aestivum*	28.04.2000	White	Yes
45	Alpu 2001	ATAEM/ESK	*T. aestivum*	24.04.2001	White	Yes
46	İzgi 2001	ATAEM/ESK	*T. aestivum*	24.04.2001	White	Yes
47	Sönmez 2001	ATAEM/ESK	*T. aestivum*	24.04.2001	Red	No
48	Soyer02	ATAEM/ESK	*T. aestivum*	2.05.2002	White	Yes
49	Müfitbey	ATAEM/ESK	*T. aestivum*	14.04.2006	White	Yes
50	Nacibey	ATAEM/ESK	*T. aestivum*	2.04.2008	Red	Yes
51	ES 26	ATAEM/ESK	*T. aestivum*	30.03.2010	White	Yes
52	Yunus	ATAEM/ESK	*T. aestivum*	17.04.2012	Red	No
53	Mesut	ATAEM/ESK	*T. aestivum*	12.04.2013	-	Yes
54	Dağdaş 94	BDUAAEM/KNY	*T. aestivum*	17.05.1994	White	Yes
55	Kınacı-97	BDUAAEM/KNY	*T. aestivum*	6.05.1997	Red	Yes
56	Göksu-99	BDUAAEM/KNY	*T. aestivum*	26.04.1999	White	Yes
57	Karahan-99	BDUAAEM/KNY	*T. aestivum*	26.04.1999	White	Yes
58	Bağcı-2002	BDUAAEM/KNY	*T. aestivum*	2.05.2002	Red	Yes
59	Konya-2002	BDUAAEM/KNY	*T. aestivum*	2.05.2002	Red	Yes
60	Ahmetağa	BDUAAEM/KNY	*T. aestivum*	1.04.2004	Red	Yes

61	Ekiz	BDUAAEM/KNY	*T. aestivum*	1.04.2004	Red	Yes
62	Eraybey	BDUAAEM/KNY	*T. aestivum*	17.04.2012	Red	Yes
63	Kırkpınar 79	TTAEM/EDN	*T. aestivum*	15.05.1979	White	Yes
64	Murat-1	TTAEM/EDN	*T. aestivum*	26.04.1991	-	Yes
65	Kate A-1	TTAEM/EDN	*T. aestivum*	26.04.1988	Red	No
66	Pehlivan	TTAEM/EDN	*T. aestivum*	12.05.1998	Red	No
67	Prostor	TTAEM/EDN	*T. aestivum*	26.04.1999	Red	Yes
68	Saroz 95	TTAEM/EDN	*T. aestivum*	26.04.1999	White	Yes
69	Atilla-12	TTAEM/EDN	*T. aestivum*	24.04.2001	Red	No
70	Saraybosna	TTAEM/EDN	*T. aestivum*	24.04.2001	Red	No
71	Gelibolu	TTAEM/EDN	*T. aestivum*	30.03.2005	Red	Yes
72	Tekirdağ	TTAEM/EDN	*T. aestivum*	30.03.2005	Red	Yes
73	Aldane	TTAEM/EDN	*T. aestivum*	6.04.2009	Red	No
74	Selimiye	TTAEM/EDN	*T. aestivum*	6.04.2009	Red	No
75	Bereket	TTAEM/EDN	*T. aestivum*	30.03.2010	Red	No
76	Saban	TTAEM/EDN	*T. aestivum*	11.04.2014	Red	Yes
77	Lancer	DATAE/ERZ	*T. aestivum*	12.05.1977	Red	Yes
78	Doğu 88	DATAE/ERZ	*T. aestivum*	16.04.1990	Red	Yes
79	Karasu 90	DATAE/ERZ	*T. aestivum*	16.04.1990	Red	No
80	Palandöken 97	DATAE/ERZ	*T. aestivum*	6.05.1997	White	Yes
81	Alparslan	DATAE/ERZ	*T. aestivum*	24.04.2001	Red	Yes
82	Nenehatun	DATAE/ERZ	*T. aestivum*	24.04.2001	White	Yes
83	Daphan	DATAE/ERZ	*T. aestivum*	2.05.2002	White	Yes
84	Yıldırım	DATAE/ERZ	*T. aestivum*	2.05.2002	White	Yes
85	Ayyıldız	DATAE/ERZ	*T. aestivum*	8.04.2011	Red	Yes
86	Kırik	DATAE/ERZ	*T. aestivum*	30.03.2010	-	Yes
87	Karacadağ 98	GATAEM/DYB	*T. aestivum*	12.05.1998	Red	Yes
88	Nurkent	GATAEM/DYB	*T. aestivum*	24.04.2001	White	Yes
89	Cemre	GATAEM/DYB	*T. aestivum*	2.04.2008	White	Yes
90	Dinç	GATAEM/DYB	*T. aestivum*	12.04.2013	White	Yes
91	Tekin	GATAEM/DYB	*T. aestivum*	11.04.2014	White	Yes
92	Inia 66	STAEM/SKY	*T. aestivum*	11.04.2014	-	Yes
93	Bezostaja-1	STAEM/SKY	*T. aestivum*	19.03.1968	Red	No
94	Bandırma 97	STAEM/SKY	*T. aestivum*	6.05.1997	White	Yes
95	Karacabey 97	STAEM/SKY	*T. aestivum*	6.05.1997	Red	Yes
96	Pamukova 97	STAEM/SKY	*T. aestivum*	6.05.1997	Red	Yes

* Abbreviations: ATAEM/ESK: Transition Zone Agricultural Research Institute Directorate/Eskişehir, BDUAAEM/KNY: Bahri Dağdaş International Agricultural Research Institute Directorate/Konya, DATAE/ERZ: Eastern Anatolia Agricultural Research Institute Directorate/Erzurum, GATAEM/DYB: GAP Agricultural Research Institute Directorate/Diyarbakır, STAEM/SKY: Sakarya Agricultural Research Institute Directorate/Sakarya, TARM/ANK: Field Crops Research Institute Directorate/Ankara, TTAEM/EDN: Thrace Agricultural Research Institute Directorate/Edirne.

Table 2. *Pm41* gene specific primers (Li et al. 2022)

Primer name	5' – 3' sequence	Expected band length
Pm41-645F	TCGGGTACATCTGACTGTTCA	1690 bp
Pm41-645R	TGGCCAGAGTAATTATCGCCA	

RESULTS

The Turkish bread wheat cultivars used in this study were registered between 1963 and 2014. This historical range illustrates ongoing wheat breeding efforts across several decades to improve and diversify wheat varieties. The majority of the cultivars were registered in the 1990s and 2000s, highlighting intensified wheat research during these decades. There is a mix of red and white grain varieties. White grains were more prevalent (42 genotypes), though red varieties were also widely represented (40 genotypes), especially in the later registrations. In 14 cultivars the color of grain was not specified. Grain color can affect market preferences and processing qualities. Most cultivars have awns, but some are awnless, particularly the older ones. The presence or absence of awns can influence threshing and feeding qualities. Several wheat varieties were collected from various research institutions in Türkiye, allowing for a wide geographical spread in the selection and testing process.

The electrophoresis results are shown in Figure 1. The scoring methodology employed a system, with a value of "+" denoting the presence of the band and a value of "–" signifying its absence at 1690 bp.

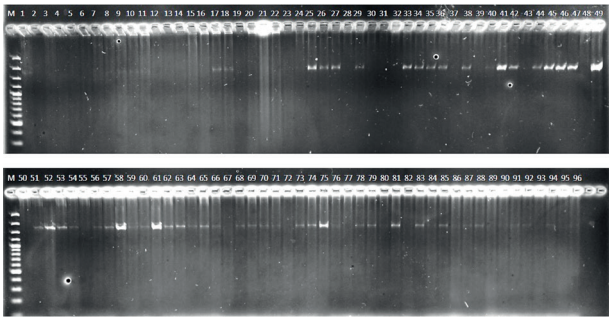

Figure 1. Electrophoresis results of *Pm41* gene in 96 Turkish bread wheat cultivars with an expected band at 1690 bp

The results of *Pm41* specific PCR are given in Table 3. Out of 96 samples, 55 were positive for the *Pm41* gene, indicating roughly 57% of the tested genotypes carried the gene. Samples from Field Crops Research Institute Directorate/Ankara displayed mixed results, with 6 out of 21 (29%) genotypes testing positive for *Pm41*. For Transition Zone Agricultural Research Institute Directorate/Eskişehir, 20 out of 32 (62%) genotypes were positive, suggesting a slightly higher detection rate in this location. In Bahri Dağdaş International Agricultural Research Institute Directorate/Konya, 8 out of 9 (89%) genotypes showed the presence of the gene, the highest proportion compared to other locations. Other locations, like Thrace Agricultural Research Institute Directorate/Edirne and Eastern Anatolia Agricultural Research Institute Directorate/Erzurum, also had a substantial number of positive results, with 11 out of 14 (79%) and 5 out of 10 (50%), respectively. GAP Agricultural Research Institute Directorate/Diyarbakır and STAEM/SKY: Sakarya Agricultural Research Institute Directorate/Sakarya comprised a low number of cultivars, five samples each, with 3 (60%) and 2 (40%) positives for *Pm41* respectively.

DISCUSSION

The existence of the *Pm41* gene alleles in Turkish wheat germplasm favours the global trends of searching for new forms of the gene for wheat breeding, where disease resistance is a primary focus due to the significant impact of fungal pathogens such as powdery mildew. The disease, whose causal agent is *Blumeria graminis* f. sp. *tritici*, remains a persistent threat in regions where climatic conditions, particularly high humidity, favour the rapid spread of the disease (Morgounov et al. 2012, Tosun et al. 2011).

The presence of the *Pm41* haplotypes in Turkish bread wheat cultivars is a significant resource in the country's efforts to control powdery mildew. The study's detection of the *Pm41* gene in 57% of the 96 tested cultivars indicates that Turkish wheat cultivars possess the gene, although the haplotype is currently unknown. Research by Li et al. (2020) demonstrated that *Pm41* confers broad-spectrum resistance and has been successfully mapped to chromosome arm 3BL, offering an effective genetic defence against powdery mildew. Despite these successes, there remain challenges in ensuring the long-term sustainability of powdery mildew resistance. Pathogens are highly adaptable and can overcome resistance genes through mutation and genetic variation (Cavalet-Giorsa et al. 2024). According to Li et al. (2020), among 31 common wheat accessions from China, only 3% have the *Pm41* gene absent from the genome (haplotype 3) whereas

Table 3. PCR screening results of *Pm41* gene

Serial No.	Genotypes	Detection of *Pm41* gene	Serial No.	Genotypes	Detection of *Pm41* gene
1	Ankara 093/44	+	49	Müfitbey	+
2	Köse 220/39	-	50	Nacibey	-
3	Sivas 111/33	-	51	ES 26	+
4	Sürak M. 1593/51	-	52	Yunus	+
5	Haymana 79	-	53	Mesut	+
6	Gün-91	-	54	Dağdaş 94	+
7	İkizce 96	-	55	Kınacı-97	-
8	Mızrak	-	56	Göksu-99	+
9	Türkmen	+	57	Karahan-99	+
10	Uzunyayla	+	58	Bağcı-2002	+
11	Yakar-99	+	59	Konya-2002	+
12	Aksel 2000	-	60	Ahmetağa	+
13	Bayraktar 2000	-	61	Ekiz	+
14	Demir 2000	-	62	Eraybey	+
15	Atlı-2002	-	63	Kırkpınar 79	+
16	Zencirci-2002	-	64	Murat-1	+
17	Eser	+	65	Kate A-1	+
18	Seval	+	66	Pehlivan	+
19	Tosunbey	-	67	Prostor	-
20	Kenanbey	-	68	Saroz 95	+
21	Lütfibey	-	69	Atilla-12	+
22	4-11	-	70	Saraybosna	+
23	4-22	-	71	Gelibolu	+
24	P 8-6	-	72	Tekirdağ	-
25	P 8-8	+	73	Aldane	+
26	Melez	+	74	Selimiye	+
27	Ak 702	+	75	Bereket	+
28	Sertak	-	76	Saban	-
29	Yayla 305	+	77	Lancer	-
30	Yektay 406	-	78	Doğu 88	+
31	Bolal 2973	-	79	Karasu 90	+
32	Kıraç 66	-	80	Palandöken 97	-
33	Porsuk-2800	+	81	Alparslan	+
34	Gerek 79	+	82	Nenehatun	-
35	Atay-85	+	83	Daphan	+
36	Kutluk 94	+	84	Yıldırım	-
37	Kırgız 95	-	85	Ayyıldız	+
38	Sultan 95	+	86	Kırik	-
39	Süzen 97	-	87	Karacadağ 98	+
40	Aytın 98	+	88	Nurkent	+

41	Yıldız 98	+	89	Cemre	-
42	Harmankaya-99	+	90	Dinç	-
43	Altay 2000	-	91	Tekin	+
44	Çetinel 2000	+	92	İnia 66	+
45	Alpu 2001	+	93	Bezostaja-1	-
46	İzgi 2001	+	94	Bandırma 97	+
47	Sönmez 2001	+	95	Karacabey 97	-
48	Soyer02	-	96	Pamukova 97	-

97% of them had *Pm41* gene (haplotype 2 and haplotype 7), which have some SNP and in/del mutations compared to the active form of haplotype 1. Seven haplotypes were observed in Durum wheat (*T. turgidum* subsp. *durum*) and wild emmer wheat (*T. turgidum* ssp. *dicoccoides*).

Previous studies have emphasized the need for incorporating multiple resistance genes to control evolving pathogens (Laroche et al. 2019). The results of this study have significant implications for wheat breeding and sustainable agricultural practices in the country.

In Türkiye, there have been studies conducted regarding the resistance of powdery mildew. Research by Aydın et al. (2021) shows that more than one gene controls the Tahirova-2000 variety's resistance to powdery mildew. In another study, two genotypes from CIMMYT were bred with susceptible varieties, including one of Turkish origin to find resistance through monogenic and digenic modes of inheritance (Ilker et al. 2009, Tosun et al. 2011).

As the increasing global demand for wheat, it is imperative to develop wheat varieties that are not only high yielding but also resistant to diseases like powdery mildew. Enhancing powdery mildew resistance in wheat can be effectively achieved by the identification and application of specific resistance genes. For instance, the gene *Pm21*, derived from the wild relative *Haynaldia villosa*, has been demonstrated to offer broad-spectrum resistance against several strains of the pathogen. This gene has been effectively introduced into common wheat, indicating its potential for lasting resistance (Cao et al. 2011, Wu et al. 2019). Similarly, the *Pm3* gene, one of the earliest found resistance loci, contains many alleles that have been extensively used in breeding programs, displaying its efficiency in different environments (Wu et al. 2021).

The use of wild relatives of wheat as sources of resistance genes has been a longstanding strategy in wheat breeding. Genetic studies have revealed that several wild species harbor valuable resistance genes that can be introduced into cultivated wheat. For example, the gene *Pm60*, derived from *Triticum urartu*, has been successfully transferred to common wheat, demonstrating its potential to enhance resistance (Zhang et al. 2021). The exploration of genetic diversity in wheat germplasm collections has also revealed novel resistance sources, which can be utilized to develop new resistant cultivars (Cheng et al. 2020, Li et al. 2016).

This study suggests that Turkish cultivars may possess novel *Pm41* genes, with silenced variants which can be utilized against emerging strains of powdery mildew.

One of the most noteworthy findings of this study is the significant regional variation in the detection of the *Pm41* gene. In the Bahri Dağdaş International Agricultural Research Institute Directorate/Konya, 89% of the tested cultivars were positive for *Pm41*, the highest detection rate observed in the study. In contrast, the Field Crops Research Institute Directorate/Ankara exhibited a much lower detection rate, with only 29% of cultivars testing positive for *Pm41*.

The findings from this study contribute to the broader global discourse on wheat disease resistance. The identification of *Pm41* in 55 Turkish cultivars is consistent with efforts in other wheat-producing regions to find novel genetic resistance genes against powdery mildew. The discovery and utilization of genes like *Pm41*, which originated from wild emmer wheat, demonstrate the importance of different alleles of the gene as a genetic diversity resource for wheat improvement.

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Author's Contributions

Authors declare that each author's contribution is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

ÖZET

Blumeria graminis f. sp. *tritici*'nin neden olduğu külleme hastalığı, dünya çapında buğday üretimi için yüksek risk taşır ve ciddi verim düşüşlerine neden olmaktadır. Direnç ıslahı, *Pm41* geninin tüm aşamalarda direnç sağlamada önemli rol oynamasıyla bu hastalığın yönetimi için sürdürülebilir bir yaklaşım sağlamaktadır. Bu araştırmada, *Pm41* geni için çeşitli Türk araştırma kurumlarından 96 Türk ekmeklik buğday (*Triticum aestivum*) çeşidi incelenmiştir. PCR analizi, incelenen çeşitlerin %57'sinin *Pm41* genine sahip olduğunu göstermiştir. En yüksek tespit oranı %89 ile Bahri Dağdaş Uluslararası Tarımsal Araştırma Enstitüsü Müdürlüğü/Konya'dan alınan çeşitlerde kaydedilirken, Tarla Bitkileri Araştırma Enstitüsü Müdürlüğü/Ankara'da %29 ile daha düşük bir frekans görülmüstür. Veriler, *Pm41* varlığında bölgesel farklılıklar olduğunu göstermektedir. Çeşitlerin yarısından fazlasında gözlemlenen *Pm41* geni, Türk buğday çeşitlerinin direnç için önemli olabilecek gen varyantlarına sahip olduğunu düşündürmektedir. Bu çalışma, sürdürülebilir buğday yetiştiriciliği ve gıda güvenliği için olmazsa olmaz olan direnç genlerinin yeni varyantlarını bulmak için genetik materyallerin korunmasının önemini vurgulamaktadır.

Anahtar kelimeler: külleme, direnç geni, buğday ıslahı, haplotip çeşitliliği

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