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Screening of Lentil Fields for Presence of Fusarium Wilt and Root Rot in Türkiye under Terrestrial Climate

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Abstract

Lentil is an important self-pollinated legume crop plant. Its production is limited by various biotic factors, especially fungal agents causing the wilt and root rot complex. The study aimed to understand the regional epidemiology and etiology of phytopathogenic fungal agents to develop control strategies against soilborne *Fusarium* spp. This study investigated 83 lentil sowing localities in Yozgat province for wilt, root and crown rot diseases caused by common *Fusarium* species during 2022 and 2023. Symptomatic lentil plants were collected for fungal isolation and identification. The *Fusarium* isolates were grouped according to colony morphology and cultured on PDA medium. Moreover, genomic DNAs obtained from *Fusarium* isolates were analyzed using PCR and compared with other *Fusarium* isolates registered in the NCBI GenBank. Genetic relationships among *Fusarium* isolates were determined using the Maximum Parsimony (MP) method in the Mega 11 program. The results, mean incidence and disease severity rate of wilt and root rot diseases in Yozgat province were determined to be 16.9% and 38.6%, respectively. *Fusarium* isolates were found in 95.4% of the samples. There was 99.5% to 100% nucleotide sequence homogeneity among *F. oxysporum*, *F. culmorum, F. graminearum, F. acuminatum* and *F. solani* isolates, and the most isolated species was *F. oxysporum*. The MP dendrogram of *Fusarium* isolates was divided into two main branches, the first branch included all *F. solani* isolates. The second main branch included other *Fusarium* species isolated in the present study and in NCBI GenBank. The study suggests periodic local surveys to determine the frequency of *Fusarium* wilt for suppression in lentils. Timely suppression of *Fusarium*-based damages is strongly suggested to control the disease and conserve the lentil production system.

Introduction

Lentil (*Lens culinaris* Medik.), a small edible grain legume belonging to the *Fabaceae* family, is a self-pollinating, cool-season crop with needle-like leaves and white to pale purple or dark purple flowers[1]. It was domesticated by humans about 10,000 years ago in the Mesopotamian part of the Fertile Crescent and quickly spread to the New World, including the Mediterranean Basin and Central Asia, and later it was naturalized to the Americas[2]. The world lentil cultivation area is about 5.5 million hectares with production of 6.6 million tons[3]. Türkiye ranks 4th in lentil production after Canada, India, and Australia. Lentil cultivation in Türkiye is very important and accounts for 6.7% of world production. Türkiye's total lentil production is 474,000 tons and is produced in at least 40 provinces[4]. About 89.5% of Türkiye's lentil production constitutes red and green lentils, which constitute 10.5% of the winter crop in the Southeastern Anatolia Region. The rest of the crop is grown as summer crops. Yozgat (39.5%), Konya (23.7%), Kırşehir (16.3%), Çorum (7.6%), and Ankara (2.9%) provinces largely contribute to the green lentil production[4]. Lentil production can be limited by biotic and abiotic stress factors. Frost and drought are the most common abiotic stress factors in summer green lentil production[5]. Fungal diseases like wilt, root, and crown rot complex caused by *Ascochyta lentis*, *Rhizoctonia solani*, *R. bataticola, Aphanomyces euteiche, Pythium,* and *Fusarium* species are the most important fungal diseases, which cause a combination of diseases including damping-off, seedling blight, wilt, and root rot, depending on the timing of infection, host susceptibility, and meteorological conditions[6,7,8].

Fusarium is a filamentous imperfect fungus found in soil, plants, and organic substrates and is a cosmopolitan genus among these pathogens[9]. It causes various diseases such as *Fusarium* wilt, root, and root collar rot, as well as Fusarium head blight in wheat, Fusarium wilt in cucurbits, and root rot in most legumes, including lentils[10,11,12]. Vascular wilt, root, and root collar rot caused by *Fusarium* spp. is the most important disease of lentils in many lentil cultivation areas globally[10]. *Fusarium oxysporum* is the most common *Fusarium* species associated with wilt, root, and root collar rot in lentils. Globally, wilt, root, and crown rot diseases are caused by *F. graminearum, F. sporotrichioides, F. equity, F. acuminatum, F. redolent, F. avenaceum, F. culmorum, F. solani,* and *F. verticillioides* in lentil planting areas[7]. Wilt, root and crown rot diseases caused by *Fusarium* spp. occur in both seedling and adult stages and cause sudden wilting, drying, and eventual death of the leaves. Symptoms of the disease include seed rot, root rot, wilting upper leaflets, stunting, shrinkage, and curling of leaves. In the middle and late pod-filling stages, seeds are usually shrunken, and root symptoms include stunted growth, brown discoloration, damaged taproot tips, and proliferation of secondary roots. Discoloration of the vascular tissue may not be seen in all cases[13].

In the Central Anatolia Region, studies on the status of wilt and root rot diseases in lentils have been conducted in limited numbers. Yozgat has a mild and moderate climate with abundant rainfall in winter when compared to summer and is classified as Dsb (Warm, humid terrestrial climate) by Köppen and Geiger[14]. The mean temperature is 9.6 °C with an average precipitation of 512 mm. Yozgat is located in the northern hemisphere. Summer occurs in June, July, August, and September. It is very important to have information about the regional epidemiology and etiology of the phytopathogenic fungal agents that cause the disease for developing different control strategies against soil-borne *Fusarium* spp., to control disease[15]. In this context, the objectives of the present study are to determine and identify -

the disease parameters (disease prevalence, incidence, and severity) of wilt, root, and crown rot diseases in lentils by conducting a survey in Yozgat province, where approximately 40% of the total green lentil production is done singly, the pathogenic *Fusarium* species that cause wilt and root rot in lentils by morphological and molecular analyses, and to determine the individual virulence levels of the *Fusarium* species by carrying out pathogenicity tests.

Protocol

NOTE: The details of the reagents and the equipment used in the study are listed in the Table of Materials.

1. Field survey, sampling, and fungal isolation

NOTE: Survey work was carried out in 2022 and 2023, according to Endes[16]. A total of 83 lentil planting areas covering nine districts in Yozgat province were observed for wilt, root, and root collar rot disease (**Figure 1**).

1. Select lentil fields with over 1000 m^2 area as a sampling area. Collect each sample by walking randomly from the border to the center or middle of the field by zigzags walking along the diagonals using a 1 m^2 frame, placing it randomly at a minimum of three randomly selected different points. Collect lentil plants showing disease symptoms from each point, put them in paper bags, and transfer them to the laboratory for use in fungal isolation and identification studies.

   NOTE: The roots and root collars of diseased lentil plants transferred to the laboratory were first washed with tap water to get rid of coarse residues; later, they were subjected to surface disinfection for the isolation of the fungal pathogens as described by Endes[16].

2. Soak diseased plant tissues in 70% ethanol for 10-15 s, and then rinse 3x for 3 min each in sterile water. Hold all of them in 1% sodium hypochlorite (NaOCl) for 5 min, and rinse 3x for 5 min each again with sterile water.

3. Dry the wet plant tissues on filter papers in a sterile cabinet to finish the surface disinfection process. Following this, cut the disinfected plant tissues into 5-10 mm long pieces and place 4-5 pieces on a PDA medium containing 0.01% streptomycin within Petri dishes (90 mm diameter). Put the Petri dishes in an incubator in the dark at 25 ± 1 °C for 4-7 days and observe the fungal growth.

   NOTE: The developing fungal isolates were purified through the single spore isolation method. For this purpose, the study conducted by Choi et al.[17] on obtaining single spore cultures of fungi belonging to Ascomycetes, Basidiomycetes, Coelomycetes, and Hyphomycetes were modified and used as described below.

4. To purify fungal isolates, keep the fungal isolates obtained at the end of the isolation studies in an incubator at 25 ± 1 °C for 12 h of fluorescent light / 12 h of darkness for 15 days to encourage the formation of anamorphic reproduction structures on PDA.

5. Weigh about 100 mg of fungus mycelium from 15-day-old cultures with a spatula, transfer it to a 1.5 mL sterile microcentrifuge tube, and then grind it thoroughly with sterile plastic pestles for homogenization.

6. Add 1 mL of sterile water and vortex for 1 min to ensure the transfer of spores into the water. To adjust the number of spores transferring into the water, draw 20 µL of this mixture with a pipette and check the number of spores under 10x magnification of the light microscope.

7. When the amount of spores is more than the desired amount, dilute the spore-water mixture at ratios such as 1/10, 1/100, and 1/1000. Provide a mixture containing 4-6 spores in the microscope field of view.

8. Take 100 µL of the prepared spore suspension and transfer it to 90 mm diameter Petri dishes containing PDA medium supplemented with 0.1% streptomycin. Then, spread the transferred suspension on the PDA with a Drigalski spatula.

9. Incubate the prepared Petri dishes in the dark at 25 ± 1 °C for 12-24 h. At the end of this period, transfer small pieces of hyphae developed from a single conidia with an inoculation loop to a new Petri dish containing PDA medium. Each culture obtained from each spore is a single spore culture. Store these to be used in pathogenicity tests and morphological and molecular identification.

   NOTE: The main purpose is to keep fungal isolates alive for a long time without changing their morphology, genetics, and virulence. The storage process was carried out using two different methods[18,19]. All the storage methods used in this study are explained in detail below.

   1. The first method to store samples is as follows. Grow fungal isolates on PDA medium at 25 ± 1 °C with a 12:12 h dark: light for 15 days to obtain 4 mm diameter mycelium disks that could be stored in sterile water.

   2. Cut mycelium discs (4 mm diameter, 10 discs) with a cork borer from fungal cultures that grew under the aforementioned conditions. Transfer the mycelium disks to microcentrifuge tubes containing 1 mL of sterile water. Keep the samples in microcentrifuge tubes in the refrigerator at -20 °C for 6 months.

   3. The second method to store samples is as follows. Firstly, take a pinch of the pure fungal disc from the single spore cultures obtained with an inoculation loop and transfer it to Petri dishes containing PDA supplemented with chloramphenicol, lactic acid, ampicillin, rifampicin, tetracycline, streptomycin, etc.

   4. Grow for 5-10 days prior to the storage process at 25 ± 1 °C with a 12 h: 12 h dark: light condition. Cut and sterilize 1 cm x 1 cm general-purpose filter papers in an autoclave at 121 °C, 15 psi for 60 min.

   5. Place the filter papers in new Petri dishes with the same or selective medium. Cut colonies/spores from pure fungal culture and place them on the top of each piece.

   6. Seal the Petri dishes and place them in an incubator at appropriate growth conditions (as mentioned above). The fungal isolates grow slowly on filter paper. Incubate for approximately 15 days to ensure complete colonization.

   7. After sporulation or complete colony formation on filter papers, transfer the individual pieces of paper to a new petri dish without a culture medium. Later, put the Petri dishes in the incubator until the filter paper and fungus are completely dry (approximately 20-30 days).

   8. After drying, put 10 pieces of filter paper in each sterile paper envelope, label each envelope, put these envelopes in plastic bags, and store the plastic bags containing the envelopes in a plastic and transparent container at -20 °C.

10. Calculate the prevalence rate of lentil wilt, root, and crown rot disease in Yozgat according to the formula given below, considering the prevalence of the disease

in each lentil field, followed by the name of the district of the province.

Disease prevalence rate (%) = (a / b) x 100

where a indicates the number of diseased fields; b indicates the total number of surveyed fields in a district.

11. Calculate the incidence of the disease according to the weighted average method reported by Bora and Karaca[20]. Count the plants in each square in the field. Separate them into diseased and non-diseased plants in each square and calculate disease prevalence according to the formula given below.

Disease prevalence rate (%) = (x / y) x 100

where x indicates the number of diseased plants; y indicates the total number of plants surveyed.

12. Calculate disease severity according to the scale 0-4 of Öğüt[21], where 0 = showed no symptoms, 1 = mild level symptoms in 25% of leaves; 2 = moderate level symptoms in 26%-50% of leaves; 3= severe chlorosis and wilting in 51%-75% of leaves; and 4 = very severe chlorosis and wilting symptoms or drying, shedding, growth retardation or dead leaves on more than 75% plants in the same order (**Figure 2**).

13. Calculate disease severity using the following formula with the obtained scale values[16].

Disease Severity (%) = [∑ [i (ni x vi) / (V) x N)] x 100

where ni number of plants in the scale value; vi scale value; V highest scale value; N total number of plants observed; i indicates the number of classes.

2. Meteorological data

1. Conduct survey studies. For this protocol, surveys were conducted during the periods of May and June 2022 and 2023. Obtain the temperature, relative humidity, and total rainfall values for the March-July period in 2022, 2023, and long years from the Directorate of Provincial Meteorology at Yozgat (**Table 1**).

3. Morphological identification

1. Compare the cultural (colony color, aerial mycelium, mycelia growth rate) and conidial (conidial dimensions, shape, color, and number of septum) characteristics of the *Fusarium* isolates to those of earlier studies and tentatively identify fungal species.

2. Select representative samples from the groups to be used in morphological identification. Purify these samples according to the single spore isolation method as mentioned above. During this period, observe the colony pigmentation characteristics of *Fusarium* isolates as well as micro/macroconidia and chlamydospore structures.

3. To promote the formation of micromorphological structures such as micro/macroconidia and chlamydospores in the obtained pure fungal cultures, incubate on PDA at 25 ± 1 °C and 12 h: 12 h dark: light for 25-30 days in an incubator. Measure the length and width of conidia for each *Fusarium* isolate by light microscopy. In addition, document the structure, shape, color, and septa or without septa of the conidia using a light microscope supplied with a digital camera.

4. Based on the above observations, group the *Fusarium* isolates according to species level, as described in Leslie and Suerell[22].

4. Molecular identification

NOTE: The total genomic DNA of the *Fusarium* isolates was extracted using the following method, which was slightly

modified from the protocol of Cenis[23]. PCR analyses and electrophoresis of Fusarium isolates were performed using the protocol described by Aras and Endes[24].

1. Fungal genomic DNA extraction
    1. From a fresh culture (10 days old) of *Fusarium* isolates grown on PDA, scrape 100 mg of mycelium with a sterile scalpel and then transfer it into a 2 mL microcentrifuge tube. Incubate the tubes at -20 °C overnight.
    2. After overnight incubation, add 500 µL of DNA extraction buffer (200 mM Tris-HCl pH: 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% Sodium dodecyl sulfate) into the tubes and crush with a sterile plastic pestle.
    3. Subsequently, add 150 µL of 3M sodium acetate (NaOAc) pH 5.2 to the tubes and incubate at -20 °C for 30 min. After this stage, centrifuge the tubes at 4,000 x *g* for 10 min.
    4. After centrifugation, transfer 400 µL of the supernatant at the top of the tube to new tubes (1.5 mL) and add an equal volume of isopropanol (2-propanol). Incubate the new tubes at -20 °C for 30 min. During this time, gently mix the tubes 5x or 6x.
    5. Centrifuge at 4,000 x *g* for 10 min to precipitate the genomic DNA and discard all the liquid remaining in the tubes.
    6. Add 1 mL of 70% ethanol to the genomic DNA pellet as a white or cream-colored sediment at the bottom of the tube. Gently mix the tube up and down 4x-5x for approximately 1 min, discarding all the ethanol in the tubes. Then, open the tubes in laminar air flow for 30 min to completely evaporate the ethanol on the DNA pellet.
    7. To dissolve the genomic DNA and store it for a long time, add 50 µL of TE (1M Tris-HCl pH: 8.0; 0.5M EDTA pH 8.0) buffer solution to the tubes and incubate the tubes in a water bath at 65 °C for 1 h. During this time, gently mix the tube up and down 8x-10x. Store the genomic DNA dissolved in TE buffer solution in a -20 °C deep freezer for use in molecular studies.
2. For PCR studies, use oligonucleotide primers ITS5 F (5′GGAAGTAAAGTCGTAACAAGG3') / ITS4 R (5'TCCTCCGCTTATTGATATGC3') to amplify the ITS region of rDNA[25]. Perform each PCR reaction with 2.5 µL of 10x PCR buffer, 2.5 µL of $MgCl_2$ (25 mM), 2.5 µL of dNTP (2 mM), 0.5 µL of each primer (10 µM), 2 µL of template DNA, 0.5 µL of Taq DNA polymerase (1 U/µL, Fermantas) and 14 µL of sQH2O in a total volume of 25 µL.
3. For the 25 µL PCR reaction, run the following PCR program 95 °C for 2 min (initial denaturation), followed by 40 cycles; 95 °C for 30 s (denaturation), 55 °C for 45 s (annealing), 72 °C for 90 s (extension) and 72 °C for 5 min (final extension). Electrophorese PCR products for 1.5 h at 90 V in 1.5% agarose gel prepared in 1x TAE (Tris Base - Glacial Acetic Acid - EDTA) buffer solution.
    1. To prepare TAE buffer for gel electrophoresis, dissolve 242 g Tris base in 700 mL of sterile water; add the 57.1 mL of Glacial acetic acid; add the 100 mL of 0.5 M EDTA solution, and adjust the volume to 1 L by adding sterile water. Adjust the final pH of the 1 L 50x TAE buffer to 8.5. To make the 1x TAE working buffer, add 49 parts of sterile water to 1 part of the 50x TAE buffer.

4. Stain the gels with 0.5 µg/mL ethidium bromide and visually inspect them by making them visible on a UV transilluminator.

5. In order to examine the phylogenetic relationship between root and crown rot agent isolates, obtain the ITS gene base sequences by PCR, which were synthesized bidirectionally (5′-3′ and 3′- 5′) through a vendor. Compare the base sequences with the gene data from the NCBI (National Center of Biotechnology Information) website and the base sequences of the ITS gene of other *Fusarium* isolates in the world using the Blast program. Use this to identify the isolates at the species level as described below.
    1. Go to https://www.ncbi.nlm.nih.gov website. Click on **BLAST** tab in the Popular Resources section.
    2. Click on the **Nucleotide BLAST** tab in the new window. In the Enter Query Sequence section in the new window, enter the base sequences in Fasta format, and write the name of the study in the Job Title section.
    3. Subsequently, check **Standard databases (nr etc.)** in the Database tab in the Choose Search Set section at the bottom.
    4. Check **Highly similar sequences (megablast)** in the Optimize for tab in the Program Selection section and click on the **BLAST** tab at the bottom of the page.

6. Use the MEGA 11 phylogenetic analysis program to determine the phylogenetic relationship between *Fusarium* isolates. Align the base sequences using the ClustalW program and create the genetic family trees of the isolates according to the maximum parsimony for the ITS gene[26].
    1. To download Mega software, go to the website https://www.megasoftware.net, and Install Mega software. MEGA software is provided FREE for use in research and education.
    2. First, save the sequences on the desktop as a Notepad file (.txt) in the FASTA format. Run the Mega software program and click on **ALIGN** tab. Click on **Edit/Build Alignment** in the window. Subsequently, check **Create a New Alignment** in the new window and click **OK** to confirm.
    3. Click on **DNA** tab. Delete the 1. Sequence that automatically appears in the window, and go to the Edit file, then click on **Insert Sequence from File** tab. Open the Notepad (.txt) file that contains the sequences and is located on the desktop.
    4. At this stage, all sequences appear on the screen. First, click on **Any Sequence** that appears on the screen, then mark all sequences with **CTRL + A**. Open the Alignment file and click **Align by Clustal W** from there, and click **OK** to run the program in default settings.
    5. After examining the differences in the alignment of the sequences, go to the Data file, click on **Phylogenetic Analysis** and then click on **No** in the checkbox in the window for whether the sequences synthesize proteins or not. Our sequences do not synthesize proteins because they belong to the ITS region.
    6. Return to the main window of Mega software. Click on **Phylogeny** and select **Construct/Test Maximum Parsimony Tree(s)**. In the new window, select **Bootstrap Method** for the Phylogeny test and enter bootstrap values 1,000 to test Branch

strength. In the Gaps/missing Data Treatment tab, select **Partial Deletion**, select **Subtree-pruning-Regrafting (SPR)** as the MP search method, and click **OK** to confirm the operations.

7. Wait for analysis result to show the Phylogenetic tree.

5. Pathogenicity test

1. Use four isolates for pathogenicity studies for each representative species from the *Fusarium* species that were identified by molecular methods. Perform pathogenicity studies at 24 °C, 16 h of fluorescent light/8 h dark photoperiod, with 65% humidity in an air-conditioned room.
2. Sow lentil seeds in black plastic vials with 45 holes of 5 cm diameter. Keep each vial in 1% sodium hypochlorite for 3 min and then rinse with sterile distilled water 3x. Dry the seeds in a sterile cabinet for 24 h and sow with one seed in each hole.
   NOTE: The lentil seeds of the Kayı 91 variety, sensitive to the disease, were used in all pathogenicity tests[6]. The seedling immersion technique was used as the inoculation method[27].
3. Incubate pure cultures of each isolate in PDA at 24 °C for 7-10 days. Scrape the colonies cultivated from the stock culture from the surface of the medium with a spatula and prepare the spore/mycelium suspension using sterile distilled water.
4. Remove large residues from the suspension by filtration through a 4-layer cheesecloth and adjust the spore/mycelium concentration to 1 x 10^6 spores/mL with the help of a hemocytometer.
5. After this stage, uproot the roots of the seedlings previously grown in the vials when they have 2-3 true leaves. Wash in tap water and slightly injure roots with a sterile needle. Immerse these seedlings in the prepared spore/mycelium suspension for 3 min and then transplant them into plastic vials containing sterile soil/peat (2:1; v/v) mixture.
6. For the seedlings used as controls, uproot their roots, injure them, and then plant them by immersing them only in sterile water. Make pathogenicity test evaluations 3 weeks after the inoculation process according to the 0-4 scale.
7. After the calculated disease severity values were subjected to angle transformation, subject the values obtained to variance analysis and evaluate the differences between the means according to Tukey's HSD ($p = 0.05$) test. Disease severity: Isolates with 0%-15% were evaluated as having very low virulence (LV), isolates with 16%-35% were evaluated as low virulence (LV), isolates with 36%-50% were evaluated as moderate virulence (O), isolates with 51%-70% were evaluated as high virulence (VV), isolates with 71%-100% were evaluated as very high virulence (VV) and isolates without disease symptoms were evaluated as saprophytic or epiphytic isolates.

Representative Results

Determination of disease parameters

A total of 83 lentil sowing areas covering nine different regions of Yozgat were evaluated in terms of wilt, root, and crown rot disease symptoms were surveyed, extending over an area of 1.1984 x 10^6 m^2 (**Table 2**). Wilt or root rot disease symptoms were encountered in all fields. However, the incidence of wilt and root rot disease in Yozgat was determined as 16.9%, with disease severity of 38.6% in the Sorgun and Sarıkaya districts. Considerable incidence of the disease was also determined in Şefaatli (26.4%), Boğazlayan (23.0%), and Sorgun (20.1%) districts. Paradoxically, the highest disease severity percentage was determined in the Sorgun district at 45.2%, followed by Boğazlayan at 36.0% and Sarıkaya at 35.4%. Furthermore, 679 plants showed disease symptoms in samples collected from the lentil fields of the marked areas examined (**Table 3**). It was followed by the morphological identification of the isolated fungal agents distributed in two groups. *Fusarium* isolates classified in the first group included pathogens or saprophytes such as Alternaria sp, Ascochyta sp., and *Rhizoctonia* sp. The isolation rate percentage of *Fusarium* isolates was 95.4%. *F. oxysporum* was determined as the most isolated fungus species from lentil plants showing disease symptoms in Yozgat with an isolation rate percentage of 59.5%. This pathogenic *Fusarium* species was followed by *F. graminearum* (15.8%) and *F. culmorum* (10.2%). Paradoxically, *F. solani* (4.4%) and *F. acuminatum* (5.5%) were isolated at lower levels from plants showing disease symptoms. *F. oxysporum* was obtained from all districts where the survey study was conducted, and the isolation rate percentage according to the districts was distributed between 45.3% and 72.2%. *F. oxysporum* was isolated the most in the Central (72.2%), Şefaatli (71.9%), Akdağmadeni (68.3%), and Sorgun (62.2%) districts of Yozgat. In contrast, *F. solani* and *F. acuminatum* were the least isolated *Fusarium* species in Yozgat. *F. solani* was not isolated from Çekerek and Central districts; *F. acuminatum* was also not isolated from Akdağmadeni, Central and Şefaatli districts (**Table 3**).

Morphological identification

Pure cultures of *Fusarium* isolates were identified morphologically according to their colony characters as well as micro-conidia, macro-conidia, and chlamydospore structures. Identification studies were carried out at the species level, according to Leslie and Summerell[22]. All fungal isolates obtained from plants showing wilt, root, and root collar rot symptoms on lentils were collected in six groups according to their colony and micro-morphology (**Table 3**). While the first five groups included isolates belonging to *Fusarium* species, the other group included pathogenic fungal isolates other than *the Fusarium* genus, such as *Alternaria, Rhizoctonia,* and *Ascochyta*.

F. oxysporum isolates with the highest isolation rate have white to yellow colonies and lilac-purple pigmentation. Macroconia are short to medium length, slightly curved, and usually have 3-5 septa. The spore structure of some isolates is slightly hooked with macroconidia dimensions determined as 33.8 to 71.5 µm x 3.1 to 4.5 µm. Microconidia are generally unseptated, oval, elliptical, or kidney-shaped. Chlamydospore formation occurred slowly (4-6 weeks). It was observed in double clusters and a short chain structure[16].

The second species with the highest rate of isolation, *F. graminearum* isolates, have white-pink aerial mycelium and dark red pigmentation. Macroconidia are thin, straight, or slightly curved, with five to seven septa. The septa are quite distinct. It has a pointed apical cell and a distinctly foot-shaped basal cell. The dimensions are 25.7 to 97.3 µm x 3.5 to 5.5 µm. Microconidia and chlamydospore formation were not observed[28].

Colonies of *F. culmorum* isolates in the third group were initially white, but with age, light pink to dark pink mycelial structure was observed. Macroconidia are short, 4-6 septate and slightly curved. Macrospore dimensions; 15.8 - 60.0 µm x 3.2 - 5.1 µm. They are numerous. Microspores were not observed. Chlamydospore formation is rapid compared to other species (3-5 weeks). They are found singly or in clusters of two[29].

The colonies of the fourth species, *F. acuminatum* isolates, are pale orange, orange, and light burgundy. It is a relatively slow-growing species. Its macroconidia are thin, have a distinct curvature, and have a 3-5 septate structure. Macroconidia dimensions were determined as 31.0-65.5 × 4.3-6.6 µm. Microconidia have 0 and 1 septate. It was observed rarely in microscopic images. Chlamydospore formation is very slow (more than 6 weeks). It forms in chains and clusters[22].

The colony color of the last pathogenic species identified, *F. solani* isolate, is white and cream-colored. Its macroconia is wide, flat, and slightly curved. Hyphae are 3-7 septate and abundant. Macrospore dimensions are determined as 20.2 to 50.6 µm x 3.1 to 6.2 µm. Microconidia are undivided or 1-divided. They are oval and ellipsoid in structure. Chlamydospores are found terminally in short chains in CLA medium within 2-4 weeks[30].

Molecular identification

PCR was performed using ITS4/ITS5 primers with total genomic DNA obtained from *Fusarium* isolates. The bidirectional (5´-3´ and 3´-5´) base sequences were registered and compared with other NCBI GenBank using the Blast program (**Table 4** and **Table 5**). *F. oxysporum* isolates showed 99.5% to 100% nucleotide sequence homogeneity with isolates from India (MT740398), Lithuania (KF646094) and Germany (MT453296). *F. culmorum* isolates showed 100% nucleotide sequence homogeneity with isolates from Canada (AY147290), France (OW983123), and Czechia (MT453296). *F. graminearum* isolates showed 99.5% to 100% nucleotide sequence homogeneity with isolates from Columbia (MT598163) and China (ON527490). *F. acuminatum* isolates showed 99.5% to 100% nucleotide sequence homogeneity with isolates from Uzbekistan (OR975902) and China (MZ424810, PP336554). *F. solani* isolates showed 100% nucleotide sequence homogeneity with isolates from Egypt (OR713084), China (PQ482231), and India (OP848138). Then, the genetic relationship between *Fusarium* isolates was determined by the phylogenetic tree obtained according to the Maximum Parsimony (MP) method using the Mega 11 program (**Figure 3**). In the MP phylogenetic tree, 638 nucleotide characters were used, including gaps, and 145 of these nucleotides were determined as parsimony-providing informative regions. MP analyses yielded one of the most parsimonious trees (**Figure 3**; Tree Length: 172; Consistency Index (ConI): 0.974; Retention Index (RI): 0.961; Composite Index (ComI): 0.7600). When this MP dendrogram of *Fusarium* isolates was examined, the family tree was first divided into two main branches. The first of these corresponded to all *F. solani* isolates supported by 100% bootstrap value. The other main branch was gathered within itself with a 78% bootstrap value and four subgroups *F. acuminatum*, *F. redolens*, *F. proliferatum* and *F. acutatum*. The other main branch was divided into five subgroups *F. oxysporum*, *F. culmorum*, *F. graminarum*, *F. pseudograminearum* and *F. equiseti,* with a bootstrap value of 97%.

Determination of virulence levels of *Fusarium* isolates

The results of pathogenicity studies of *Fusarium* spp. isolated from lentils showing wilt and root rot, for which morphological and molecular characterization studies were completed, are summarized in **Table 6** and **Figure 4**. In general, all *Fusarium* isolates showed differences in the severity levels of the diseases they caused in Kayı 91 lentil variety $F_{(19\text{-}60;\ 0.05)}$ = 43.06; p< *0.0001). The most virulent isolates were F. oxysporum* (YBUFol4), *F. culmorum* (YBUFc1, YBUFc2) and *F. graminearum* (YBUFg1, YBUFg3). These five isolates were found to have a very high virulence level. On the other hand, one isolate each of *F. oxysporum* (YBUFol2), *F. solani* (YBUFs1), and *F. graminearum* (YBUFg2) were found to have moderate virulence. In addition, *F. acuminatum* (YBUFa1, YBUFa2, YBUFa3, and YBUFa4) isolates were found to have weak or low virulence. All isolates used in pathogenicity studies ranged from 42.5% to 97.5% and were obtained after inoculation of lentil seedlings.

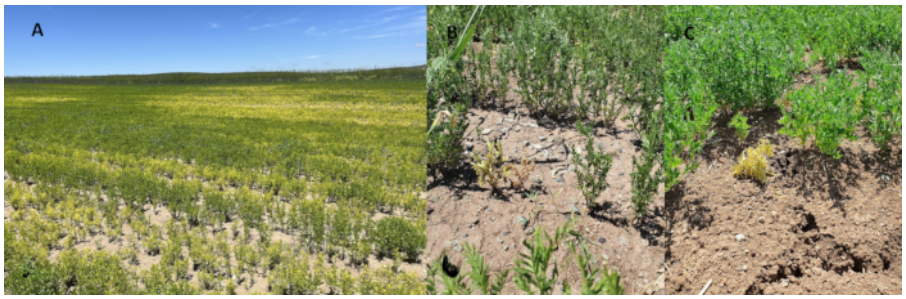

Figure 1: Lentil plants affected by *Fusarium* species. (**A**) General view of localized diseased areas in a lentil field with high disease severity. (**B**) View of plants with disease severity 4. (**C**) View of plants with disease severity 3. Please click here to view a larger version of this figure.

Figure 2: View of lentil plants at disease severity levels 0 - 4. Level 0 = showed no symptoms, 1 = the leaves symptoms in 25%; 2 = 26%-50%; 3 = 51%-75% of leaves; 4 = more than 75% in the same order. Please click here to view a larger version of this figure.

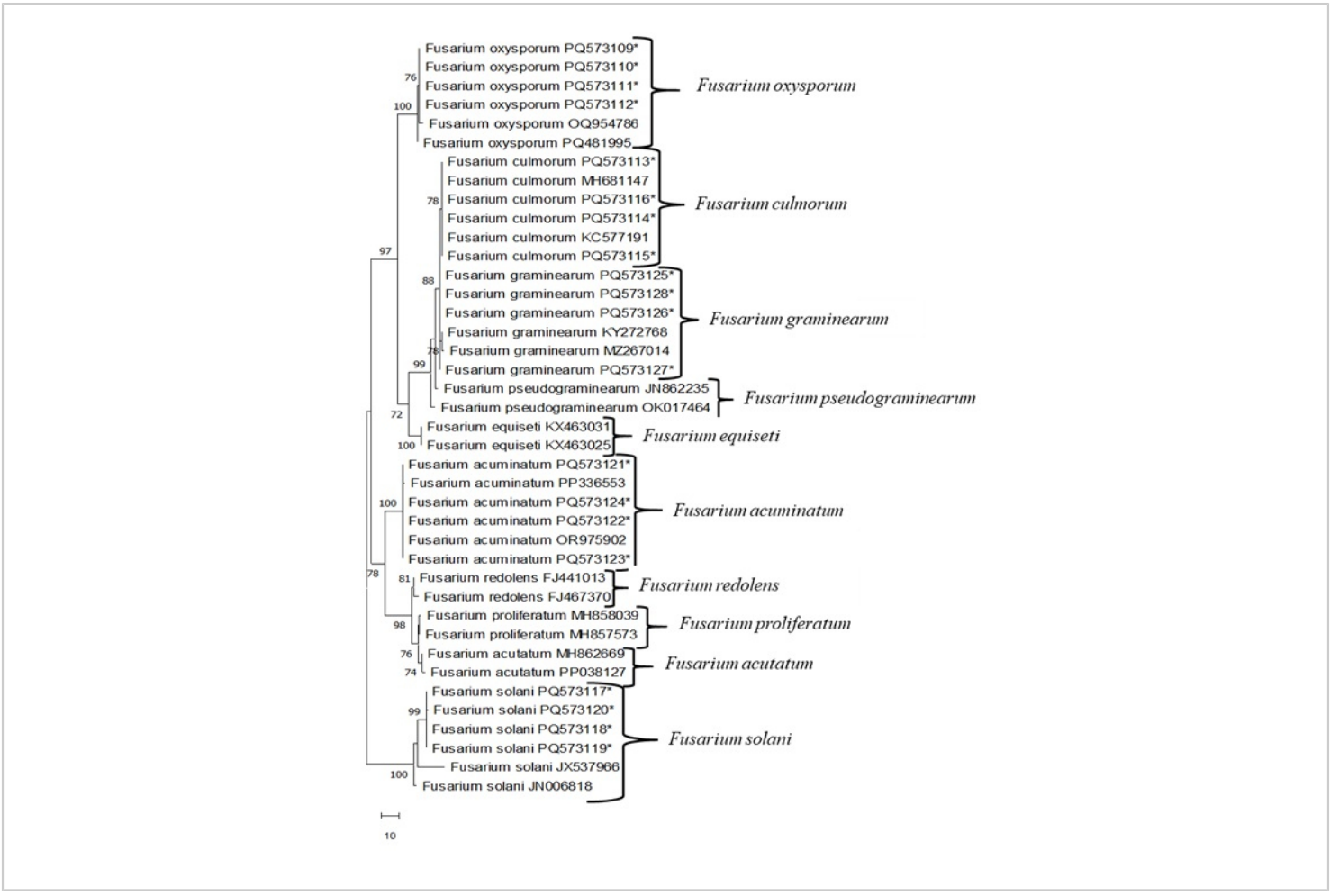

Figure 3: Most parsimonious unrooted tree based on ITS region of *Fusarium* sp. using MEGA 11. The isolates are indicated by asterisks. The rest are taken from GenBank. Please click here to view a larger version of this figure.

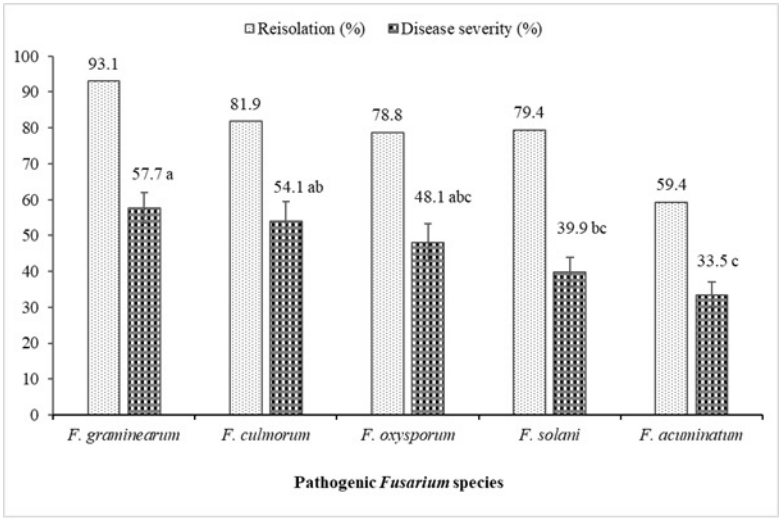

Figure 4: Reisolation (%) and disease severity rates (%) of *Fusarium* spp. isolated from lentil plants. Vertical lines represent the standard error on the bar (n = 16). Please click here to view a larger version of this figure.

	Temperature (°C)			Relative humidity (%)			Precipitation (mm)		
Month	2022	2023	Long-term	2022	2023	Long-term	2022	2023	Long-term
Akdağmadeni									
March	11.1	7.4	3.8	45.1	68.8	71.1	21.4	78.7	60.6
April	11.1	11.4	8.8	59.2	67	61.5	56.5	109.8	42.6
May	16.3	15.1	12.9	64.3	73.9	64.8	48.5	83.6	74.1
June	16.5	18.4	16.2	63.4	53.2	69.1	0.3	20.1	57.2
July	22	22.2	18.7	48	40.5	60.5	1.4	0	7
Boğazlıyan									
March	12.3	9.9	5.2	47.8	65.5	66	13	64	40.1
April	12.8	13.5	10.2	62.6	66.6	59.3	20.2	24.2	24.4
May	18.7	17.7	14.6	62.5	67.4	61.2	65.4	20	35.1
June	19.7	20.6	18.6	52.9	49.9	59.9	0	2.4	35.5
July	24.3	24	21.7	42.5	39.5	50	0	0	3.9
Çekerek									
March	13.5	10.8	6.6	52.8	70.4	69	24.3	111.3	48.7
April	14.1	13.9	11.5	63.9	72.3	61	48.1	73.4	32
May	19.3	18.6	15.8	69.1	72.7	65.4	80.3	69	59.3
June	20.1	21.8	19.4	61.5	57.5	67.8	0	16.4	62.8
July	24.6	24.7	22	57.8	52.4	57.5	33.2	0	10.9
Yozgat									
March	11.5	8.4	4	47.3	68.7	67.5	14.6	84.8	87.3
April	12.4	12.7	9.3	60.1	65.2	58.7	47.4	54.4	41.9
May	17.5	16.8	13.7	64.2	68	60.2	92.6	61.6	72.2
June	18.3	20.3	17.4	57.9	50.7	61.2	1	4.8	63.6
July	22.9	23.6	20.5	50.9	44.3	52.7	7	0.2	8.8
Saraykent									

March	14.8	9	6.2	50.9	71.1	71	11.1	77.3	55.3
April	12.5	12.3	10.5	64.6	73.2	60.7	35.3	36.1	29
May	17.6	16.4	14.2	69.1	76.7	65.9	47.8	72.4	57
June	18.2	19.2	18.2	63.8	61	69.6	0	28.6	56.1
July	23.2	23.1	21.6	54.2	51.7	57.9	0	0	7.4
Sarıkaya									
March	12.9	9.6	5.6	47	68.2	66.5	18.4	75.5	53.6
April	13.2	13.1	10.7	59	68.8	56.5	24.8	60.6	27.2
May	18.5	17.3	14.7	63.2	71.6	60.6	57.6	64.5	47.2
June	18.9	20.5	18.4	59	53.9	62	0	16.1	50.6
July	24	24	21.3	48.6	45.4	51.5	0	0	6.4
Şefaatli									
March	13.1	10.6	6.2	48.2	61.1	67	12.6	71.5	50.2
April	11.2	14.3	11.1	61.6	60.9	58.6	48.4	50.5	26
May	19.9	18.6	15.4	66.7	61.9	62.3	65.2	95.4	53.7
June	20.4	22.2	19.7	55.8	45.5	61.2	1.2	2.5	46.8
July	25	25.3	23.2	47.9	39.5	47.1	0	0	4.1
Sorgun									
March	12.2	9.4	4.8	48.4	68.2	67	21.6	104.2	49.8
April	13	13.3	10.1	59.7	66.1	58.9	53.2	33.8	31.7
May	18.3	17.4	14.5	63.7	69.1	60.9	56.6	89.6	44.2
June	18.9	20.3	18.3	56.8	53.4	61.5	0	16.6	55.2
July	23.7	23.7	21.2	50	44.9	52.9	4	0	7.5
Yerköy									
March	15	12.1	6	41.2	60.5	61.3	4.4	46.1	60.1
April	16.2	16.2	13.5	54.8	60.4	50.9	40.9	46.8	23
May	21.5	20.6	17.4	55.4	59.6	52.2	57	23.6	35.1

June	23	24.5	20.9	45.2	41.4	56.4	0.2	0	39.1
July	27.5	27.7	24.4	38.2	35.5	42.2	0.2	0	3.5

Table 1: Meteorological data of the location surveyed during the lentil production season by year.

County	Number of Field	Surveyed Sowing Area (Decare)	Disease Prevalence (%)	Disease Incidence (%)	Disease Severity (%)
Akdağmadeni	4	43.2	100	7	21.8
Boğazlıyan	4	86.4	100	23	36
Çekerek	1	2.7	100	1.6	14.4
Merkez	3	24.3	100	5.8	23
Saraykent	6	53.3	100	8.1	28.2
Sarıkaya	12	189.3	100	17	35.4
Sorgun	48	683.2	100	20.1	45.2
Şefaatli	2	73.7	100	26.4	23.4
Yerköy	3	42.3	100	9.1	32.9
Overall	**83**	**1198.4**	**100**	**16.9**	**38.6**

Table 2: Disease parameters, prevalence, incidence, and severity of wilt, root, and root collar rot in lentil fields.

County	Number of plants used for isolation	Isolation frequency (%)					
		F. oxysporum	*F. culmorum*	*F. solani*	*F. acuminatum*	*F. graminearum*	Other
Akdağmadeni	41	68.3	9.8	2.4	0	14.6	4.9
Boğazlıyan	57	54.4	1.8	12.3	7	15.8	8.8
Çekerek	21	52.4	4.8	0	23.8	9.5	9.5
Merkez	36	72.2	11.1	0	0	13.9	2.8
Saraykent	64	45.3	12.5	7.8	4.7	21.9	7.8
Sarıkaya	148	56.8	11.5	7.4	5.4	15.5	3.4
Sorgun	233	62.2	9.4	1.3	7.3	16.3	3.4
Şefaatli	32	71.9	15.6	3.1	0	9.4	0
Yerköy	47	57.4	14.9	4.3	2.1	14.9	6.4
Overall	**679**	**59.5**	**10.2**	**4.4**	**5.5**	**15.8**	**4.6**

Table 3: Information regarding lentils sampled for fungal isolation frequency from Yozgat province of Türkiye.

Fusarium species	Isolate	County	Isolation source	GeneBank accession number
F. oxysporum	YBUFol1	Sorgun	Root	PQ573109
	YBUFol2	Bogazlayan	Root collar	PQ573110
	YBUFol3	Sarıkaya	Root	PQ573111
	YBUFol4	Akdagmadeni	Root	PQ573112
F. culmorum	YBUFc1	Sorgun	Root	PQ573113
	YBUFc2	Yerkoy	Root	PQ573114
	YBUFc3	Sarıkaya	Root	PQ573115
	YBUFc4	Saraykent	Root collar	PQ573116
F. solani	YBUFs1	Yozgat	Root	PQ573117
	YBUFs2	Sorgun	Root	PQ573118
	YBUFs3	Sefaatli	Root	PQ573119
	YBUFs4	Sarıkaya	Root	PQ573120
F. acuminatum	YBUFa1	Cekerek	Root	PQ573121
	YBUFa2	Bogazlayan	Root collar	PQ573122
	YBUFa3	Sarıkaya	Root collar	PQ573123
	YBUFa4	Sorgun	Root	PQ573124
F. graminearum	YBUFg1	Yozgat	Root	PQ573125
	YBUFg2	Sorgun	Root	PQ573126
	YBUFg3	Saraykent	Root	PQ573127
	YBUFg4	Sarıkaya	Root collar	PQ573128

Table 4: *Fusarium* spp. isolates from lentil (*Lens culinaris*) from Yozgat Province, central Türkiye used in the phylogenetic study.

Fusarium species	Isolate	Country	Isolation source	GenBank accession number
F. acuminatum	AAG4	n/a	*Prunus persica*	OR975902
	WHWNSHJ1	China	*Malus domestica*	PP336553
F. acutatum	CBS 739.97	India	n/a	MH862669
	NSF1	Egypt	*Tetraena alba*	PP038127
F. culmorum	2090	India	n/a	KC577191
	G49	Poland	*Pisum sativum*	MH681147
F. equiseti	*Fusarium equiseti* A577	China	Patchouli	KX463031
	Fusarium equiseti A571	China	Patchouli	KX463025
F. graminearum	Wm-233	China	n/a	MZ267014
	16a	n/a	n/a	KY272768
F. oxysporum	YBUFoc4	Türkiye	*Cicer arietinum*	OQ954786
	LuC-8	China	*Chrysanthemum x morifolium*	PQ481995
F. proliferatum	CBS 246.61	Germany	n/a	MH858039
	CBS 186.56	n/a	n/a	MH857573
F. pseudograminearum	WZ-8A	China	Wheat	JN862235
	GAAET080	China	Maize	OK017464
F. redolens	M11	n/a	Mushroom	FJ441013
	2008	n/a	Mushroom	FJ467370
F. solani	S2-27	France	n/a	JX537966
	UENFCF279	Brazil	Guava	JN006818

Table 5: Sequences of *Fusarium* species used from GenBank in phylogenetic study.

Fusarium species	Isolate	Disease severity (%) [a] (Mean ± Standard Error)	Virulence level of isolate	Reisolation (%)
F. oxysporum	YBUFol1	20. 0 ± 1.1 i	Weak	62.5
	YBUFol2	56.9 ± 1.2 cde	Moderately	85
	YBUFol3	42.5 ± 1.0 efg	Less	77.5
	YBUFol4	73.1 ± 3.9 ab	Highly	90
F. culmorum	YBUFc1	72.5 ± 1.0 ab	Highly	95
	YBUFc2	72.5 ± 2.7 ab	Highly	95
	YBUFc3	23.6 ± 1.6 hi	Weak	60
	YBUFc4	48.1 ± 2.6 def	Less	77.5
F. solani	YBUFs1	58.8 ± 1.6 bcd	Moderately	97.5
	YBUFs2	46.9 ± 2.1 def	Less	75
	YBUFs3	17.5 ± 2.3 i	Weak	65
	YBUFs4	36.3 ± 1.6 fgh	Less	80
F. acuminatum	YBUFa1	42.5 ± 2.3 efg	Less	65
	YBUFa2	16.9 ± 2.8 i	Weak	42.5
	YBUFa3	30.6 ± 6.1 hi	Less	62.5
	YBUFa4	44.1 ± 7.1 defg	Less	67.5
F. graminearum	YBUFg1	76.3 ± 2.2 a	Highly	97.5
	YBUFg2	50.6 ± 3.3 def	Moderately	92.5
	YBUFg3	68.1 ± 2.1 abc	Moderately	95
	YBUFg4	35.6 ± 1.9 fgh	Less	87.5

Table 6: Pathogenicity tests of *Fusarium* species on lentil plants. Isolates mean rank <10% = non-aggressive; 11%-25% = weak aggressive; 26%-50% = less aggressive; 51%-70% = moderately aggressive; >70% = highly aggressive. [a] Statistical difference between isolates according to the Tukey HSD test ($p < 0.05$).

Discussion

Fusarium wilt is known to cause serious economic yield losses in some parts of the world[31]. The disease was first reported in Hungary[32] and later reported in many countries such as Egypt, India, Myanmar, Nepal, Pakistan, Türkiye, Syria, and the USA[33]. Kumar et al.[34] reported a wide distribution of lentil wilt, root, and root collar rot with reports of occurrence in at least 26 countries worldwide. In a recent study, 12 fungal species were isolated from diseased lentil plants collected from different states of India, and *F. oxysporum* f. sp. *lentis* was identified as the most important pathogen (30%), followed by *Rhizoctonia bataticola* (17.5%) and *Sclerotium rolfsii* (15.7%)[31]. Similarly, in the present study, the most frequently isolated fungal genus was *Fusarium* (95.4%). According to Zitnick-Anderson et al.[7], *Fusarium* (50%) was the dominant fungal genus causing wilt, root, and root collar rot in North Dakota lentil plantations. They also reported that *F. oxysporum, F. solani, F. culmorum, F. equiseti, F. acuminatum, F. graminearum, F. redolens* and *F. avenaceum* are pathogens within this genus.

The morphological characteristics of the five *Fusarium* species identified in this study were similar to those of recent studies[35]. According to Rathod et al.[36], *F. oxysporum* isolates reported that they showed septate, branched, initially white, and then raised or sunken colony development on PDA, which became colored in different pigmentations. Similarly, in the current study, *F. oxysporum* isolates initially formed white colonies, later yellow, violet, or pink. On the other hand, chlamydospores in the form of two or three chains, which were used as a morphological criterion of *F. oxysporum* isolates identification, were observed in colonies that were approximately 30 days old. As a matter of fact, Endes[15] reported that chlamydospores of *F. oxysporum* isolates held for >30 days obtained from had a higher tendency to infect plants in chickpea cultivation areas of Yozgat province were generally observed in old environments.

Fusarium root rot in chickpeas and lentils is caused by many *Fusarium* species, such as *F. solani, F. oxysporum,* and *F. graminearum*[37]. As reported by Dean et al.[38], *F. graminearum* and *F. oxysporum* are among the most commonly isolated plant fungal pathogens by plant mycologists worldwide. However, Aydın et al.[6] reported that *F. graminearum* is among the causative agents of wilt, root, and crown rot in lentil cultivation areas in the Southeastern Anatolia Region of Türkiye. In addition, *F. graminearum* is known to cause root and crown rot in lentils in the state of North Dakota, USA[7].

Fletcher et al.[39] reported that *F. culmorum* can rarely be isolated as a pathogen in lentil cultivation areas. In addition, in a recent study by Zitnick-Anderson et al.[7], it was reported that *F. culmorum* is associated with wilt, root, and root collar rot in lentil plants. However, Aydın et al.[6] identified many *Fusarium* species that cause wilt and root rot in lentil cultivation areas but did not report *F. culmorum* as a pathogen. This situation, which differs from the current study, may be due to the different number of areas surveyed or the support of morphological studies with molecular methods in the current study.

Zitnick-Anderson et al.[7] reported that *F. acuminatum* is among the *Fusarium* species that cause wilt and root and root collar rot in North Dakota lentil fields. On the other hand, Aydın et al.[6] documented that *F. solani* is among the causative agents of wilt and root rot in lentil cultivation areas. It is also known that *F. solani* is isolated from lentil cultivation areas as a weak pathogen[7].

Fusarium species are facultative parasites. Infections due to the genus *Fusarium* in lentils can cause complete destruction of plants, especially in extreme temperatures in late spring or early summer. Al Ahmad and Mouselli[40] reported that *F. oxysporum* and *F. solani* caused yellowing, defoliation and desiccation symptoms in lentil plantations in southern Syria. *F. solani* causes root rot and wilt diseases in Central Anatolia, including Yozgat[16]. In addition, some other *Fusarium* species have also been found to be pathogenic at certain rates. Zitnick-Anderson et al.[7] reported that *Fusarium* wilt disease is caused by *Fusarium oxysporum* f. sp *lentis*, but *F. culmorum, F. solani, F. graminearum* species can also cause wilt disease according to morphological, physiological and pathological characteristics. In addition, Fletcher et al.[39] reported that *F. acuminatum*, which causes wilt and root rot in lentils, has weak or low virulence.

The study revealed that 95.4% of the *Fusarium* isolates obtained from the examined lentil fields were *Fusarium* species and had an incidence of 1.6% - 26.4% and disease severity of 14.4% - 45.2% in lentil plants. Adverse climatic conditions such as hot and dry spring and early summer heat weaken plant growth and make plants susceptible to *Fusarium* species. Most *Fusarium* species are weak pathogens, and when environmental conditions weaken the host plant, they cause an increase in *Fusarium* wilt, especially in drought conditions.

Disclosures

The authors declare no conflicts of interest.

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