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Identification and pathogenicity studies of white clover (*Trifolium repens* L.) fungi on turfgrass areas in Turkey

Türkiye çim alanlarındaki ak üçgüllerdeki (*Trifolium repens* L.) fungusların belirlenmesi ve patojenisite çalışmaları

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ABSTRACT

White clover (*Trifolium repens* L.), commonly accepted as a forage crop, is a perennial legume plant. Environmentalists, park and garden designers pay attention to white clover due to its characteristics such as covering the ground, resistance to stepping, nice view, and short growing. Additionally, because of its tolerance to the shade, it is used as a cover plant in orchards to establish turfgrass areas. In 2015, surveys were conducted to turfgrass areas where parks and gardens, golf courses, recreation areas, stadiums, picnic areas, and refuges in İstanbul, Antalya, Ankara, İzmir, Kayseri, Bursa, Aydın, and Muğla. By examining the survey areas, a total of 60 white clover (*Trifolium repens* L.) samples, showing any symptoms such as stunting, wilting, yellowing on leaves and spots, blight, dry and lesions on root and crown, were collected. Totally of 222 fungal isolates were obtained from infected plants, then pathogenicity assays were conducted. *Rhizoctonia solani* AG 1, Binucleate *Rhizoctonia* AG G, AG I, AG K, *Macrophomina phaseolina*, *Fusarium chlamydosporum, F. oxysporum, F. equiseti, Myrothecium verrucaria, M. roridum, Curvularia spicifera, C. aeria, C. lunata, C. trifolii, Alternaria alternata, A. teniussima, A. rosae, A. infectoria, Colletotrichum destructivum, C. trifolii, C. truncatum, Phoma exigua, Epicoccum nigrum, Sordaria fimicola, S. macrospora, S. superba, Gnomoniopsis fructicola* species were identified according to morphological characteristics and DNA sequences analysis. Although, *A. alternata* on leaves and *F. oxysporum* on roots were common pathogens on White clover in Turkey, the most virulent leaf pathogen was *Curvularia spicifera* and the most virulent root pathogen was *R. solani* AG 1 in this study.

INTRODUCTION

Among the leguminous forage crops, white clover (*Trifolium repens* L.) is called as “tırfıl, tirfil” in Turkish. Fodder crop is

valuable due to its usage in the production of both grasses and cattle, for grazing grass, for making dry grass and for spreading animals. In addition, the usage of it in parks and gardens for greening is increasing day by day. Due to less water consumption and drought resistance, it can survive longer than other grass plants. When it is used for landscaping purposes, it can easily survive on the condition that irrigation with longer intervals comparing by the other grass plants. It is a perennial and evergreen plant. Although many grass plants in Gramineae turn into yellow by the effect of winter cold, white clover protects its greenery in all seasons. Moreover, it can survive up to -15 °C and preserves its health and greenness. As it is a leguminous plant, like other plants in this group, it binds the free nitrogen in the air by nodules in its roots and enriches the soil in nitrogen (Şener 2018).

Peronosproa trifoliorum, Pseudopeziza trifolii, Ascochyta trifolii, Kabatiella caulivora, Botrytis cinerea (Nadolnik 1981), *Uromyces trifolii, Mycosphaerella killianii, Leptosphaerulina trifolii, Cercospora zebrina, Stagonospora meliloti, Sclerotinia trifoliorum, Phoma* spp., (Skipp and Lambert 1984, Nelson and Campbell 1993), *Fusarium* spp. (Leath et al. 1971), *Fusarium chlamydosporum, F. equiseti, F. oxysporum* (Zahid et al. 2001), *Codinaea fertilis* (Menzies 1973; Campbell 1980; Zahid et al. 2001), *Rhizoctonia solani, Curvularia trifolii, Colletorichum trifolii, Politrincium trifolces* (Nelson and Campbell 1993) and *Macrophomina phaseoli (*Pratt et al. 1998; Zahid et al. 2001) were reported to cause disease in white clover. *Myrothecium roridum* and *M. verrucaria* cause root rot in red clover and alfalfa (Leath 1983).

This study was performed in 2015 to determine fungi and their virulences in white clovers which in turfgrass areas in İstanbul, Antalya, Ankara, İzmir, Kayseri, Bursa, Aydın, Muğla provinces. This study is important in terms of the first detection of the diseases that damage white clover plants used in turfgrass areas in Turkey.

MATERIAL AND METHOD

Survey and isolation

Symptomatic white clover samples were collected from parks, golf courses, stadiums, and recreation areas in turfgrass areas from İstanbul, Antalya, Ankara, İzmir, Kayseri, Bursa, Aydın, and Muğla provinces in May 2015. Fungi were isolated from leaves showing necrotic spots, blight and chlorotic lesions (Figure 1) and roots showing discoloration and necrosis. Infected plant explants were sterilized by 1% Sodium hypochlorite (NaClO) for the 30 s, then placed on filter paper for drying. Later on, they were cultured on Potato Dextrose Agar (PDA) (Difco, USA) amended with 100 μg^{-1} Streptomycin sulfate. Incubation was done in growth chamber under 25°C 16 h light and 8 h dark photoperiod, for 7 days long.

Molecular identification of fungal isolates

Isolations of fungal DNAs were carried out using Blood and Tissue Kit (QIAGEN Inc. Valencia, CA), as described by the manufacturer. The polymerase chain reactions (PCR) were performed using the ITS primers ITS-1 (5 'TCC GTA GGT GAA CCT GCGG 3') and ITS-4 (5 'TCC TCC GCT TAT TGA TATGC 3' (White et al. 1990). The PCR master mix prepared in a 50 µl reaction mixture containing 25 µl GoTaq® Hot Start Green Master mix (2×) (Promega, USA), 2 µl forward primer (10 mM), 2 µl reverse primer (10 mM), 13 µl sterile double-distilled water, 4 µl BSA, 4 µl template DNA. For the PCR reaction, initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 2 min, and a final elongation step of 72°C for 10 min. Sterile double-distilled water was used as a negative control.

PCR products were sequenced by GENOKS (Gene Research and Biotechnology Company, Ankara, Turkey). The nucleotide sequences were subjected to Basic Alignment Search Tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov) and compared to other sequences in the GenBank database.

Pathogenicity assay

All pathogenicity assays were conducted under greenhouse conditions. Root isolates were grown in sterile wheat brans and 4 g per kg of soil, composing fine sand, and cow manure mixture (2:1:1), from this inoculum were taken and were applied. There were three replicate pots (10 cm in diameter) for each treatment. Control pots did not contain any inoculum. All pots were covered by a sanitized polyethylene nylon and incubated for three days. At the end of the duration, thirty white clover seeds of turfgrass (cv. (*Trifolium repens* L.) were placed on the soil surface, coated with 1 cm of sterile natural soil, and watered with 9-10 ml of water (Zhang et al. 2014). The infected plants were examined after 3 weeks. Results were evaluated according to the scale of 0 to 5: 0= no disease, 1= 1-10% hypocotyl infected and / or shortening, 2= 11-30% hypocotyl infected and / or shortening, 3= 31-50% hypocotyl infected and / or shortening, 4= 51-80% hypocotyl infected and / or shortening and 5= the entire hypocotyl infected and / or shortening (Ichielevich Auster et al. 1985). Disease severity values were calculated by the Townsend–Heuberger Formula (Townsend and Heuberger 1943) using the disease scale that was given above.

Townsend–Heuberger formula = [Σ (*no. of plant in category* × *category value*)] × 100 / *Total no. of plants* x *max. category value*).

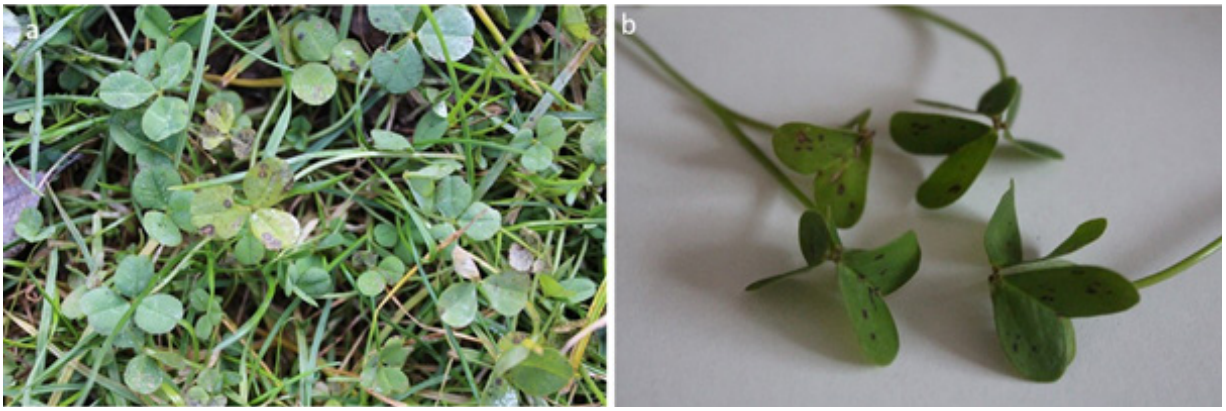

Figure 1. Simptoms on white clover leaves: *Colletotrichum trifolii* (a), *Alternaria alternata* (b).

Pathogenicity assays of leaf isolates were also performed under greenhouse conditions. Isolated fungi were activated on potato dextrose agar (PDA). After incubation for 7-10 days, spore suspensions (10^6 conidia/ml) were prepared for all fungi. The surfactant Tween-80 was added in the amount of 250 µl per liter of conidial suspension to aid in the dispersion of inoculum on the leaf surfaces. The conidial suspension was sprayed on the foliar surface of 8 wk post-emergent *Trifolium repens* plants using a hand atomizer. Control plants were sprayed with only 20 ml of sterile distilled water. After the inoculation, a polyethylene bag was placed over each inoculated pot to maintain high relative humidity. Pots remained inside the bags for the duration of the experiment of three days. Plants were placed into the greenhouse and temperature was adjusted to 25±3°C. After the incubation period, plants were removed from the moisture chamber and remained under the conditions of 12 h daylight 80% RH, 25±3°C until symptom development (~7 to 10 days). All plants were watered every other day during the experiment. The experiment was performed three replicates (Beirn et al. 2015).

For foliar inoculations, disease severity was rated 15 days after inoculations. On a 1 to 6 scale (Brecht et al. 2007), where 1 = no symptoms, 2 = 0 to 2 mm leaf tip die-back, 3 = 2 to 4 mm leaf tip die-back and/or less <1% chlorotic leaf lesions, 4 = leaf tip die-back plus < 5% chlorotic leaf lesions, 5 = leaf tip die-back plus 5 to 50% leaf lesions, and 6 = > 50% leaf necrosis and blighting of leaves. Disease severity values were calculated by Townsend–Heuberger formula. Data were analyzed by the analysis of variance method for the completely randomized experimental design using the JMP 7.0 Statistical Package Program. Means of disease severity for isolates were compared by Tukey multiple comparison test. Tests were conducted at a $p< 0,05$ significance level.

RESULTS AND DISCUSSION

White clover (*Trifolium repens* L.) is a plant found naturally in the meadows and pastures of Turkey. In addition to its importance in animal feed, apiculture, and soil protection, it is used for visuals especially in lawn mixtures in parks and gardens. Although it is affected by many pests and diseases, there are very few studies on these subjects of *Trifolium repens* in turfgrass areas around the world. In this study, white clover, present in the turfgrass areas, was examined for the first time in terms of the disease in Turkey. In 2015, surveys were carried out in 8 provinces. A total of 60 diseased white clover samples were collected from Istanbul (9), İzmir (7), Bursa (9), Ankara (10), Kayseri (6), Antalya (8), Aydın (4), and Muğla (7). As a result of DNA sequence analysis, a total of 51 isolates from roots and 171 isolates from leaves were determined to belong to 11 different genera and 27 different species (Table 1).

Fungi sequences obtained from amplification of conserved ribosomal ITS region were compared with sequences from National Center for Biotechnology Information (NCBI) database using BLAST 2.0. Identified species showed 98-100% similarity with the isolates belong to similar species in NCBI.

In this study, the most commonly isolated leaf pathogen was *Alternaria alternata* with 19 isolates. The most commonly isolated root pathogen was *Fusarium oxysporum* with 15 isolates. While *A. alternata* isolates were isolated from all surveyed provinces (Table 1), *F. oxysporum* isolates were isolated from Ankara, Antalya, Muğla, Bursa provinces (Table 2). In consequence of the pathogenicity tests, the most virulent root pathogen group in white clover was *Rhizoctonia solani* AG 1 with 90.06% followed by *Fusarium* spp. (Table 2). Disease severity values of *F. oxysporum* and *F. chlamydosporum* were determined as 89.76% and 89.48%, respectively (Table 2). *Curvularia spicifera* from leaf fungi

Table 1. Fungi, plant origin, location, number of isolates and disease severity values isolated from white clover leaves

Fungi	Plant origin	Location	Number of Isolates	*Disease Severity (%) and Standart Deviation (The lowest disease rate (%)- The highest disease rate (%)
Curvularia spicifera	Leaf	Bursa, İstanbul, İzmir, Aydın, Muğla, Antalya, Kayseri, Ankara	13	87.32 a ±0.51 (77.47-95.01)
Curvularia aeria	Leaf	İstanbul, Ankara, Bursa	7	86.76 a ±0.39 (82.40-90.01)
Curvularia lunata	Leaf	Ankara, Antalya, Muğla, İstanbul	8	83.18 abc ±1.65 (74.22- 90.00)
Curvularia trifolii	Leaf	İstanbul, Bursa, Muğla, Ankara	14	85.45 ab ±0,63 (77.12-92.75)
Alternaria alternata	Leaf	Bursa, İstanbul, İzmir, Aydın, Muğla, Antalya, Kayseri, Ankara	19	57.57 e ±1.15 (42.80-70.00)
Alternaria teniussima	Leaf	Bursa, İstanbul, İzmir, Antalya, Kayseri, Ankara	9	44.33 f ±0.43 (37.50-53.75)
Alternaria rosae	Leaf	Kayseri, İstanbul	5	27.88 g ±1.22 (20.45-32.60)
Alternaria infectoria	Leaf	İzmir, Aydın, Antalya, Ankara	7	20.57 g ±1.41 (14.90- 25.25)
Colletotrichum destructivum	Leaf	Ankara, Muğla, Bursa, Kayseri	11	83.68 abc ±0.85 (75.00-90.62)
Colletotrichum trifolii	Leaf	İstanbul, Ankara, Bursa, İzmir	12	79.02 c ±0.63 (70.50-86.87)
Colletotrichum truncatum	Leaf	Ankara, Bursa	4	76.68 c ±0.96 (70.25- 80.06)
Phoma exigua	Leaf	İstanbul, Ankara, Antalya, Muğla, İstanbul	12	49.01 f ±1.28 (42.80-58.80)
Myrothecium verrucaria	Leaf	Bursa, İstanbul, İzmir, Aydın, Muğla, Antalya, Kayseri, Ankara	12	67.58 d ±3.05 (58.64-80.00)
Myrothecium roridum	Leaf	İstanbul, Bursa, İzmir, Antalya	6	76.97 c ±1.33 (72.35-80.75)
Epicoccum nigrum	Leaf	Bursa, İstanbul, İzmir, Aydın, Muğla, Antalya, Kayseri, Ankara	16	Non-Pathogenic
Sordaria fimicola	Leaf	Bursa, İstanbul, İzmir, Muğla, Antalya, Ankara	8	Non-Pathogenic
S. macrospora	Leaf	Bursa, Antalya, Kayseri	3	Non-Pathogenic
S. superba	Leaf	Ankara	2	Non-Pathogenic
Gnomoniopsis fructicola	Leaf	Kayseri, İzmir	3	Non-Pathogenic

* Levels not connected by same letter are significantly different. (TUKEY HSD: 7,08) (P <0.05).

was found to be the most virulent species in white clover plants with 87.32% disease severity (Table 1). In this study, binucleate *Rhizoctonia* AG G, AG I, AG K, *Epicoccum nigrum, Sordaria fimicola, S. macrospora, S. superba, Gnomoniopsis fructicola* were founded as non-pathogen species (Table 1 and 2).

The studies conducted around the world indicated that several *Fusarium* spp. including *F. oxysporum*, *F. avenaceum*, *F. culmorum, F. chlamydosporum, F. equiseti*, *Codinaea fertilis Rhizoctonia* spp. cause root rots in white clover (Leath et al. 1971; Menzies 1973; Campbell 1980; Zahid et al. 2001). Similarly, *F. oxysporum, F. chlamydosporum, F. equiseti, R. solani* AG 1, and *M. phaseolina,* isolated from roots, were found high virulent in this study. *Bimuria novae-zelandiae, Ceratobasidium cornigerum* spp. as well as many *Phoma, Penicillium, Chrysosporium, Cylindrocarpon, Colletotrichum, Acremonium, Trichoderma, Periconia, Gliocladium, Phomopsis* spp., oomycetes and basidiomycetes fungi have been also isolated in white clover (Skipp and Christensen,1983). In a survey of naturally infected field of white clover grown for seed in Poland. In that report, the most prevalent leaf pathogens were *Peronosproa trifoliorum, Pseudopeziza trifolii,* and *Ascochyta trifolii*. It was detected that the most prevalent stem, peduncle and occasionally leaf pathogens were *Kabatiella caulivora* and *Botrytis cinerea* (Nadolnik 1981). In a study conducted in the turfgrass areas in North Carolina, *Rhizoctonia solani, Pseudomonas andropogonis, Staganospora meliloti, Cercospora zebrina, Curvularia trifolii, Colletorichum trifolii, Politrincium trifolces, Uromyces* sp., and *Politrincium trifolces* were reported as pathogen in white clover (Nelson and Cample 1993). To support these reports, in our study, *Curvularia trifolii, Colletotrichum trifolii* were found as pathogen in white clover. In addition to these fungi, *Alternaria alternata, A. teniussima, Colletotrichum destructivum, Colletotrichum truncatum, Curvularia spicifera, C. aeria, C. lunata* were determined as pathogenic in white clover in the present study. On the other hand, *Rhizoctonia* AG G, AG I, AG K, *Phoma exigua, A. rosae, A. infectoria, Epicoccum nigrum, Sordaria fimicola, S. macrospora, S. superba,* and *Gnomoniopsis fructicola* species were founded as non pathogenic in white clover in turfgrass araes. Leath (1983) reported that *Myrothecium roridum* and *M. verrucaria* caused root rot and leaf blight in red clover and alfalfa. In our study, *M. roridum* and *M. verrucaria* were also found to cause leaf blight on white clovers.

Further study is needed on how environmental factors such

Table 2. Fungi, plant origin, location, number of isolates and disease severity values isolated from white clover roots.

Fungi	Plant origin	Location	Number of Isolates	*Disease Severity (%) and Standart Deviation (The lowest disease rate (%)- The highest disease rate (%)
Rhizoctonia solani AG 1	Root	İzmir, Ankara, Antalya	5	90.06 ±1.30 (82.62-93.00)
Binükleat Rhizoctonia AG G	Root	Antalya, Ankara	3	Non-Pathogenic
Binükleat Rhizoctonia AG I	Root	Muğla, İstanbul	2	Non-Pathogenic
Binükleat Rhizoctonia AG K	Root	Ankara, Bursa	3	Non-Pathogenic
Macrophomina phaseolina	Root	İzmir, Antalya	2	87.01 ±0.96 (82.75- 89.00)
Fusarium chlamydosporum	Root	Ankara, Bursa, Antalya, Ankara	13	89.48 ±1.29 (77.12- 98.75)
Fusarium oxysporum	Root	Ankara, Antalya, Muğla, Bursa	15	89.76 ±0.35 (78.90- 98.00)
Fusarium equiseti	Root	Ankara, Antalya, Muğla, İstanbul	8	89.38 ±1.25 (85.90-93.75)

* Comparison of means of disease severity was statistically not significant

as temperature, relative humidity, soil moisture, and watering as cultural practices affecting infection of fungi to manage diseases in turfgrass areas.

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ÖZET

Ak üçgül (*Trifolium repens*) çok yıllık bir baklagil bitkisidir ve esas olarak bir yem bitkisidir. Aynı zamanda toprak yüzeyini çok iyi kaplayan, basmalara dayanıklı, kısa ve güzel görünümlü bir bitki olduğundan, çevrecilerin, park bahçe ve oyun alanları tasarımcılarının da en çok aradığı ve kullandığı bitkilerden biridir. Gölgeye toleransı nedeniyle, ağaçlarda ve meyve bahçelerinde veya çim alanlarının oluşturulmasında örtü bitkisi olarak kullanılmaktadır. 2015 yılında İstanbul, Antalya, Ankara, İzmir, Kayseri, Bursa, Aydın, Muğla illerinde bulunan parklar, bahçeler, golf sahaları, rekreasyon alanları, stadyumlar, piknik alanları ve refüjlerden oluşan çim alanlarına surveyler düzenlenmiştir. Survey alanları incelenerek, bodurlaşma, solgunluk, yaprak sararması, lekelenme, yanıklık, kök ve kökboğazı lezyonları gibi belirti gösteren toplam 60 adet ak üçgül (*Trifolium repens* L.) örneği toplanmıştır. Bu bitkilerden yapılan izolasyonlar ve DNA sekans analizleri sonucunda *Rhizoctonia solani* AG 1, Binükleat *Rhizoctonia* AG G, AG I, AG K, *Macrophomina phaseolina, Fusarium chlamydosporum, F. oxysporum, F. equiseti, Myrothecium verrucaria, M. roridum, Curvularia spicifera, C. aeria, C. lunata, C. trifolii, Alternaria alternata, A. teniussima, A. rosae, A. infectoria, Colletotrichum destructivum, C. trifolii, C. truncatum, Phoma exigua, Epicoccum nigrum, Sordaria fimicola, S. macrospora, S. superba, Gnomoniopsis fructicola* türlerine ait 222 adet fungus izolatı elde edilmiştir. İzole edilen tüm izolatların hastalık şiddeti değerleri sera koşullarında tespit edilmiştir. Çalışmada en çok izole edilen yaprak patojeni *A. alternata* iken en çok izole edilen kök patojeni *F. oxysporum* olmuştur. En virülent yaprak patojeninin *Curvularia spicifera* ve en virülent kök patojeninin ise *R. solani* AG 1 olduğu tespit edilmiştir.

Anahtar kelimeler: Fungus, *Trifolium repens* L., DNA sekans, patojenisite

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