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Identification, genetic diversity and biological control of dollar spot disease caused by *Sclerotinia homoeocarpa* on golf courses in Turkey

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Abstract

Dollar spot caused by *Sclerotinia homoeocarpa*, can create damage to warm and cool-season turfgrasses, is a significant disease. In the study, 13 golf courses in 5 provinces of Turkey were surveyed for dollar spot disease caused by *Sclerotinia homoeocarpa*. During the survey, samples were taken from leaves with cream spots, framed by a reddish-brown edge and from areas of light yellow small patches. Identifications of *Sclerotinia* isolates were performed by DNA sequencing analysis. Five bacterial strains were examined to detect their antifungal influences against the dollar spot by using the seed coating method in greenhouse conditions. As a result of isolations from infected plants from 13 golf courses, 7 *Sclerotinia homoeocarpa* (Sh) isolates were obtained. Pathogenicity tests performed in the greenhouse ranged from 81.03% to 90.75%. The consequence of biologic control studies, *Pseudonomas putida* 88cfp, *Pseudomonas putida* 166fp, and *Bacillus cereus* 44bac were found efficient the ratio of 92.99%, 88.71%, and 87.50% respectively. In further studies, effective bacterial strains (88cfp, 166fp, and 44bac) should be carried out in large golf courses.

Keywords: *Sclerotinia homoeocarpa*, turfgrass, virulence, biological control

Introduction

In recent years, Turkey has become a golf center by bringing together golf lovers around the world with international golf facilities and tournaments. There are golf courses in the provinces of Muğla, Istanbul, Ankara, Aydın, and Samsun, especially in Antalya. Particularly Belek Town of Antalya creates a unique golf tourism potential with its cultural, historical, and natural structure as well as qualified golf courses and facilities and hosts international tournaments. The establishment and protection of such areas around the world have become a million-dollar industry. Maintaining and ensuring the continuity of such turf areas requires great effort, cost, and expertise. Millions of dollars are spent annually in the world and in Turkey in recent years only for the maintenance of golf courses and the control against diseases. In addition, additional expenses are made to repair and renew heavily damaged grasses.

These efforts and expenses can only be paid for if the plants are healthy and the grass fields are long-lasting. For all these reasons, there is the excessive and indiscriminate use of fungicide in such areas, especially in Turkey. It is thought that these practices will increase problems such as soil and groundwater pollution.

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One of the diseases that has been a problem for some years in the golf courses of the south and west coasts of Turkey and requires struggle is the 'dollar spot' disease. However, a comprehensive survey of the disease in golf courses in Turkey was carried out for the first time with this study.

Dollar spot (*Sclerotinia homoeocarpa* F.T. Bennett) is a major disease that affects grass areas. The disease causes crucial damages, especially to highly preserved golf courses (Goodman and Burpee 1991, Viji et al. 2004; Liberti et al. 2012). The first symptoms of the disease are yellow-green blotches on the leaves. Later, it turns into cream-colored spots with a reddish-brown border and these spots get larger over time. Dollar spot symptoms vary with the types of grass and the nature of the turf. In short mown areas such as golf courses, the disease is manifested as coin-sized, round, cream-light yellow patches with a diameter of 2 to 8 cm. For this reason, the disease has been called the "Dollar Point" disease (Smiley 1992). As a result of recent technological advances, researchers have observed that there are subtle morphological and genetic differences between species and within the genus of *Sclerotinia* and there are subtle differences in reproductive structures and they agree that the identification and taxonomy of this species should be reconsidered (Jackson 1973; Kohn and Grenville 1989; Novak and Kohn 1991; Carbone and Kohn 1993; Holst-Jensen et al. 1997; Vargas and Powell 1997). In a study by Liberti et al. (2012) they separated *S. homoeocarpa* isolates into F-type and C-type based on the morphological characteristics of their fungus and they supported it molecularly Salgado-Salazar et al. (2018) reported that four different species that give rise to "dollar spot" disease in grass areas. These species that are in the genus *Clarireedia* were named *C. jacksonii, C. monteithiana, C. homoeocarpa,* and *C. bennettii*. The use of fungicides has increased in turfgrass areas, especially in golf courses in the world. This situation led to pollution of the environment, groundwater, and seas, deterioration of human health, and formation of pathogen endurance (Balcı and Gedikli 2012). For these reasons, alternative methods are needed which are less harmful to the environment. The use of beneficial microorganisms can be alternative or complementary practices to the existing harmful practices used in the control of the disease. The most prevalent beneficial microorganisms used in the control of plant pathogens are *Bacillus* and *Pseudomonas* species. This study, it was aimed to identify *S. homoeocarpa* isolates isolated from golf courses in Turkey, to determine their genetic differences and virulence, and to determine the effects of some local bacterial isolates against the disease under greenhouse conditions.

Materials and Methods

Survey and isolation of the pathogen

Sixty-two diseased turfgrass plants were picked up from the 13 golf courses in Antalya (9), İstanbul (1), Ankara (1), Aydın (1), and Muğla (1) Provinces in 2015. Infected plant samples were surface disinfested for 40-50 s in 1 % sodium hypochlorite (NaOCl), rinsed for 40s in sterile water, and put PDA (Potato Dextrose Agar) (Difco, USA) added 50 mg gentamicin per ml. They were then kept at 25°C for 4-5 days under fluorescent light conditions for 12 hours day and 12 hours night.

Pathogenicity Tests

The tests were carried out in a greenhouse with all isolates. Twenty creeping bentgrass (*Agrostis stolonifera* L.) seeds were sowed in the pots (10 cm in diameter) included the sterilized (two consecutive days at 121 ºC for 45 minutes) soil, sand, and burnt fertilizer mix (2:1:1). After emergence, the grass was cut to a height of 1,5-2 cm.. Isolates were incubated on PDA (Difco, USA) at 25°C for 1 week. Inoculums were prepared consisted of mixing 500 ml of rye grains with 100 ml of deionized water in heat-resisting bottles. Then, these bottles with rye grains were autoclaved twice at 90ºC for 90 min on each of 2 consecutive days. Each bottle was inoculated with 15 discs (5mm diameter), containing the fungi, cultured for 8-10 days at 25 ºC under fluorescent light at 12-h day and night cycles. After incubation, five infested rye grains have put on the surface of the tall fescues. Pots were covered with polyethylene bags after inoculation. The pots were placed under continuous at 27±1 °C and relative humidity of 92% to 95%. There were three replicate pots for treatment. The control consisted of pots without inoculum. One week after inoculation, the bags were removed, and severity of disease was evaluated on a scale of 0 to 5: 0= 100% healthy plants, 1= 1-10%, 2= 11-30%, 3= 31-50%, 4= 51-80% and 5= 100% diseased plants (Mocioni et al. 2011). Disease severity values were calculated using these scales and the following formula.

Disease Severity= Σ [(the number of samples in the scale with different disease grades x scale value) / highest scale value x total number of samples observed]×100 (Townsend and Heuberger 1943)

Bacterial isolates

The bacteria (44bac, 88bfp, 88cfp, 215b and166fp) used in this study were obtained from tomato and cucumber rhizosphere in a previous study (Aşkın 2008), and 44bac and 88bfp were found effective on *Sclerotium rolfsii*. Diagnosis of the five antagonistic bacteria used in the study was done by the molecular method in our previous study (Ünal et. al. 2019).

Molecular identifications of fungal isolates

Fungal DNA isolation was performed by using QIAGEN Blood and Tissue Kit, according to the manufacturer's procedure. The polymerase chain reaction was done using primers ITS-1 and ITS-4 (White et al. 1990). PCR (The polymerase chain reaction) was performed in a 50 µl reaction mixture containing 25 µl GoTaq® Hot Start Green Master mix (2×) (Promega, USA), 13 µl sterile double-distilled water, 4 µl BSA, 2 µl forward primer (10 mM), 2 µl reverse primer (10 mM), 4 µl template DNA. The PCR cycling protocol consisted of initial denaturation at 94 ºC for 4 min, followed by 30 cycles of 94 ºC for 45 s, 55 ºC for 45 s, and 72 ºC for 2 min, and a final elongation step of 72 ºC for 10 min.

Molecular identifications of bacteria (166fp, 215b, 44bac, 88bpf, 88cpf) were made in another study (Ünal et al. 2019).

Genetic diversity

In this study, the sequenced isolates' ITS regions composed of ITS1, ITS2, and 5.8S were aligned by using ClustalX, and also phylogenetic tree was constituted by using the Maximum Likelihood method and Jukes-Cantor model (Jukes and Cantor 1969) in MEGA 7 (Kumar et al. 2016). The tree was constituted to scale, which was based on branch lengths measured in the number of substitutions per site. This analysis involved 8 nucleotide sequences. *Sclerotinia sclerotium* isolate was used as an external isolate. Complete deletion option and bootstrap test with 1000 replications were used in this program (ie. all positions containing gaps and missing data were eliminated).

Bacterial inoculum

Suspensions of 1×10^8 cfu/mL concentration were prepared from each bacterial strain developed in PDW (Potato dextrose water) and their measurements were made with a spectrophotometer. After sterilizing the seed surfaces, they were immersed in bacterial solutions and kept for 12 hours (Aşkın 2008).

Biocontrol assays

Biocontrol assays were performed using turfgrass seeds mixture containing cv. *Lolium perenne, Festuca arundinacea, Cynodon dactylon* and the virulent *S. homoeocarpa* species (Sh 4). Sterilized garden soil: burnt manure: river sand (2: 1: 1) mixture was used in the experiments. The fungal inoculum was prepared by developing in rye grains as in pathogenicity study. Bacteria were applied by coating to the seeds. Studies were conducted on both sterilized and non-sterilized soils in three applications: (1): Planting uncoated grass seeds in inoculated soils (positive control) (2): Planting uncoated grass seeds in non-inoculated soils (negative control), (3): Planting coated grass seeds in inoculated soils.

Coated with the antagonist bacteria and uncoated grass seeds were sowed at a depth of 2 cm as 30 seeds per 10 cm in diameter pots. The pots were put in greenhouse conditions containing 12 hours of light, 12 hours of darkness, and 24 ± 1 ºC temperature. When the plants germinated and reached 1 cm, five infested rye grains were placed on the surface of the plants. Pots were covered with a polyethylene bag after inoculation. The pots were placed under continuous at 25°C and relative humidity of about 95%. After the inoculation, 0-5 scale was used after 25 days according to disease development status (Mocioni et al. 2011). These scale values were converted to disease severity values using the diseases severity formula.

Statistical analysis

Variance analyzes were carried out using SPSS GLM statistical program to determine the differences in both virulence level of isolates and disease rates in biocontrol assay. Disease ratios obtained according to scales were applied to the Towsend-Heuberger formula to calculate disease severity, and the activity of bacterial isolates with the Abbott formula was determined from disease severity values. Disease severity was compared by Tukey multiple comparison test on these ratios.

Results

During the survey phase of the study, a total of 62 samples were collected from 13 golf courses in 5 provinces in Turkey. As a result of isolations from leaves that have cream color blotches surrounded by dark red color (Fig. 1a) and from plants on small yellowed circular patches (Fig. 1b), seven *S. homoeocarpa* isolates were obtained that was based on both colony morphologies and rDNA internal transcribed spacer (ITS) region sequences (Table 1). The colony color of all isolates on PDA was initially white but after 7-10 days became black-white color (Fig. 1 c). 'Y-shaped branching ' in fungus hyphae was observed (Fig. 1d) on a light microscope.

Figure 1. *Sclerotinia homoeocarpa*: symptoms on leaves on 'Rough' area (a) 'dollar spot' symptoms on 'Green' area (b) in a golf course, colony appearance on PDA (c), 'Y-shaped branching' on hypha (d)

As a result of the study, amplicons displayed by gel transilluminator were found to be approximately 550 bp which is specific to *S. homoeocarpa.* The sequences of all isolates were 99-100% similar to those of *S. homoeocarpa* deposited in the NCBI database. Average disease severity values of *S. homoeocarpa* isolates in pathogenicity tests carried out ranged from 81.03 to 90.75%. The most virulent isolate was Sh 4 isolated from Antalya province with 90.75% disease severity (Table 1).

Table 1. Origin, turf composition, and disease severity values of *Sclerotinia homoeocarpa* (Sh) isolates isolated from golf courses

Isolate numbers	Origin	Turfgrass Composition	*Disease severity (%)
Sh1	Antalya	*Cynodon dactylon* *Lolium perenne* *Poa trivialis*	90,66±2,54a
Sh2	İstanbul	*Festuca arundinacea* *Lolium perenne* *Poa pratensis* *Agrostis stolonifera*	86,18±2,34ab
Sh3	Antalya	*Agrostis stolonifera* *Cynodon dactylon*	81,03±1,84b
Sh4	Antalya	*Agrostis stolonifera* *Cynodon dactylon*	90,75±2,02a
Sh5	Muğla	*Cynodon dactylon* *Lolium perenne* *Poa trivialis*	81,10±3,03b
Sh6	Antalya	*Cynodon dactylon* *Lolium perenne* *Poa trivialis*	86,70±1,41ab
Sh7	Antalya	*Cynodon dactylon* *Lolium perenne* *Agrostis stolonifera*	90,70±0,87a

*There is no difference between the values expressed in the same letter, P<0.0001

Data obtained with ITS 1 and ITS4 primers on the 7 isolates of *Sclerotinia homoeocarpa* were used to produce the dendrogram shown in Fig. 2. *S. homoeocarpa* species took part in the same cluster on the tree.

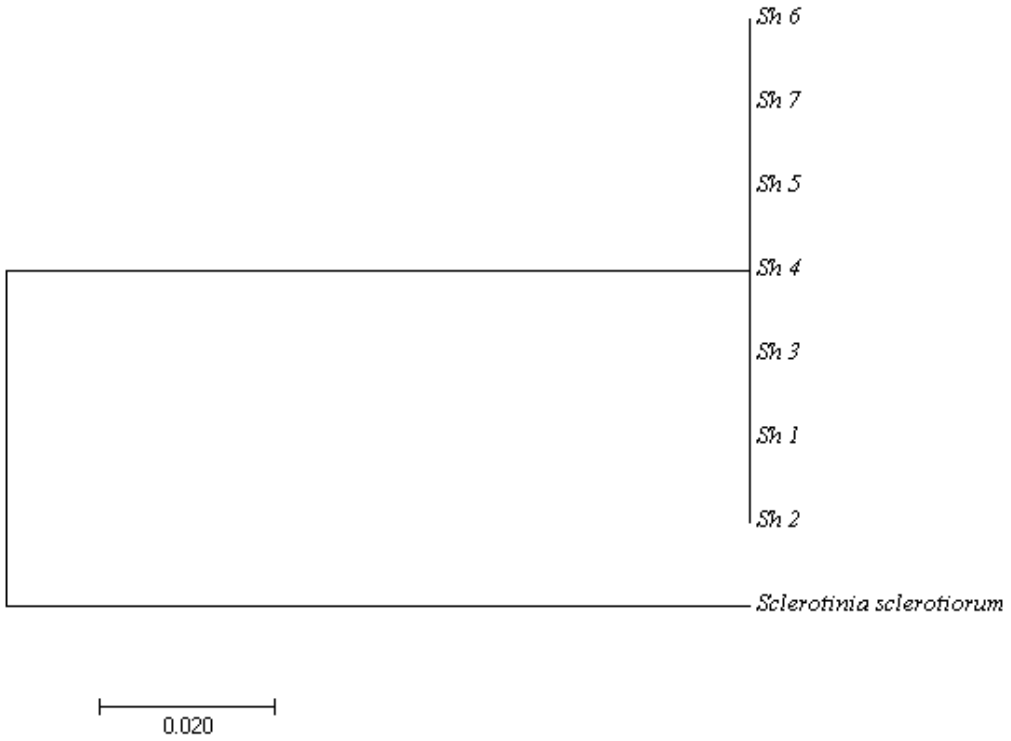

Figure 2 Maximum likelihood tree showing the relationship among *Scleratinia homoeocarpa* (*Sh*) isolates obtained from turfgrasses in golf courses

In greenhouse experiments, all bacterial isolates were found to be effective when compared to the severity of the disease in control. The lowest disease severities were measured (P<0.0001) in the treatment of *Pseudomonas putida* 88cpf, *Pseudomonas putida* 166fp, and *Bacillus cereus* 44bac as 6.13%, 9.87%, and 10.93% respectively. The highest disease severity was measured in the treatment of *Paenibacillus* sp. 215b as 37.33% when compared to the disease severity value in control. The highest protection effect was observed on isolate *Pseudomonas putida* 88cpf (92.99%). The isolates *Pseudomonas putida* 166fp (88.71) and *Bacillus cereus* 44bac (87.50) followed it (Fig. 3, Table 2). During the evaluations, it has been observed that antagonist bacteria have a positive effect on plant growth and quality.

Table 2. Effects of bacterial strains against the disease of *Sclerotinia homoeocarpa* on turfgrass

Treatments	**Disease severity *(%)**	**Efficacy (%)**
Pseudomonas putida 88cpf	6.13 ± 1.578^{e}	92.99
Pseudomonas putida 166fp	$9.87 \pm 1.578^{c\,d}$	88.71
Bacillus cereus 44bac	10.93 ± 1.578^{cd}	87.50
Stenotrophomonas rhizophila 88bpf	17.07 ± 1.578^{c}	80.48
Paenibacillus sp.215b	37.33 ± 1.578^{b}	57,32
(+) Control	87.47 ± 1.578^{a}	-
(-) Control	0.00	

*There is no difference between the values expressed in the same letter, P<0.0001

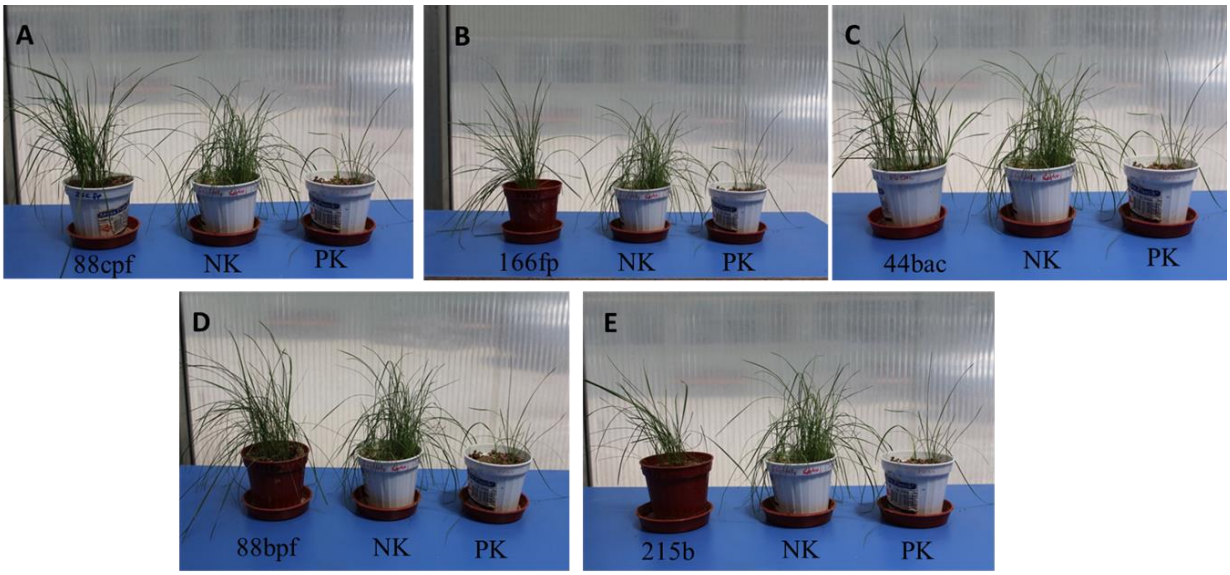

Figure 3. Effects of bacterial strains against *Sclerotinia homoeocarpa* in greenhouse experiments: **A**. *P. putida* 88cpf, **B.** *P. putida* 166fp; **C.** *B. cereus* 44bac, **D.** *Stenotrophomonas rhizophila* 88bpf, **E.** *Paenibacillus* sp. 215b

Discussion

In the studies on the control of the 'Dollar spot' disease in the world, various biological control strategies have been investigated besides chemical control. These studies are mostly in the form of practice of organic materials and nutrients to alert as living naturally microorganisms in the phyllosphere or direct application of bacteria and fungi that are known to suppress the disease. Although most biological control strategies evaluated to date are less efficient than fungicides, some of them deserve further research. In some of these studies, some commercial organic materials such as organic manure, compost, and mud have been investigated for the suppression of dollar spot diseases, and some compost, organic fertilizers, and mud have been observed to decrease the severity of dollar spot disease, but it has been reported by the researchers that further investigation is needed to clarify the effects of these materials in reducing the *S. homoeocarpa* and to determine the mechanisms of action. (Landschoot and McNitt 1997; Liu et al. 1995; Nelson and Craft 1991a; Landschoot and McNitt 1997). The effects of many fungal and

bacterial microorganisms have also been investigated for the control of the disease and different results have been obtained. *Trichoderma* species were mostly used in biological control studies of dollar spot disease as fungal microorganisms. *Trichoderma harzianum* and *Fusarium heterosporium* were detected as the most effective fungi. In a study conducted in Turkey, on the biological control of the disease, *Trichoderma harzianum* (TRIC8) isolate was found to be 65.60% effective against dollar spot disease in field conditions (Aşkın et al. 2019). In studies with antagonist bacteria, the highest effect was detected in *Pseudomonas* species. *Pseudomonas fluorescens* Migula and *P. lindbergii* ATCC 31099 strains were found effective to dollar spot disease on Kentucky bluegrass (*Poa pratensis* L.) under controlled conditions (Hodges et al. 1994; Rodriguez and Pfender 1997). In this study, it was concluded that pyrrolnitrin, an antibiotic produced by *P. fluorescens*, inhibited *S. homoeocarpa*. (Rodriguez and Pfender 1997). Similarly, in this study, *Pseudonas putida* (88cfp) and *Pseudomonas putida* (166fp) were among the most effective bacterial species. In this study, apart from *Pseudomonas* spp., a *Bacillus cereus* isolate (44 Bac) also showed high efficacy against the disease. In some studies on the biological control of the disease, *Enterobacter cloacae* (EcCT-501) was effective only in young grass areas (Nelson and Craft 1991b), while *Streptomyces* sp (Schumann and Reuter 1993). The use of "transferable hypovirulence" to suppress the disease has been reported (Zhou and Boland 1997, 1998).

Conclusions

With this study, *S. homoecarpa* fungi causing "Dollar spot" disease was isolated and identified in large golf areas of Turkey for the first time. In addition to these, virulences of these isolates, their phylogenetic analyses, and biologic control opportunities with some domestic bacterial isolates were investigated. In further studies, biological control studies of the disease using these bacterial strains should be carried out in large golf courses. Formulation studies of effective isolates in the field should also be carried out.

Authors' contributions

FU survey, isolation, identification, pathogenicity, phylogenetic analysis, biological control studies of *S. homoeocarpa* isolates. Designed the study and wrote the manuscript with input from all authors, AA and EK provision of bacterial isolates and biological control studies, MY analyzed the data (Statistic analyses), read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests

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