J. Turk. Phytopath., Vol. 48 No. 1-3, 21-29, 2019 ISSN 0378 - 8024

Determination of the Biocontrol Efficiency of Native *Bacillus* and Fluorescent *Pseudomonas* Isolates Against *Rhizoctonia solani* Causing Brown Patch Disease on Turfgrass Areas*

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Received: 10 September 2019 Accepted for publication: 14 February 2020

ABSTRACT

Rhizoctonia solani is a pathogenic fungus found world-wide which attacks a great number of plants. The agent also causes significant damages on turfgrass. *R. solani* anastomosis groups cause leaf blight, large patch and brown patch diseases on turfgrasses areas. Brown patch disease caused by *R. solani* causes considerable damage especially in golf courses and sport fields. In this study, seven antagonist bacterial isolates were evaluated for their effect on protect to *Rhizoctonia solani* on turfgrass in greenhouse conditions. The bacteria were inoculated into seed of turgrass at the rate of 10^8 cfu/ml. Results from the assay showed that turfgrass from the inoculated treatment have less susceptible to *R. solani* than the uninoculated treatment. The disease severity observed in the inoculated treatment was significantly suppressed in comparison to the uninoculated treatment ($P < 0.001$). The highest protection effect was observed on isolate 253e (91.43%). The isolates 187c (87.62%) and 166fp (81.59%) followed it. Identifications of bacterial isolates were performed by DNA sequencing analysis. These isolates were identified as *Bacillus cereus*, *Bacillus* sp., *Paenibacillus* sp., *Pseudomonas putida, Stenotrophomanas rhizophila*. Consequently the study, It has been detected that *B. cereus* 253e, *Bacillus* sp. 187c and *P. putida* 166fp strains are potential isolates that can be used in the biological control of brown patch disease in turfgrass areas.

Keywords: Turfgrass, *Rhizoctonia solani*, biological control, plant growth promoting

ÖZ

Çim Alanlarında Kahverengi Yama Hastalığına Neden Olan *Rhizoctonia solani*'ye Karşı Yerli *Bacillus* ve Florosan *Pseudomonas* İzolatlarının Biyokontrol Etkinliğinin Belirlenmesi

Rhizoctonia solani, çok sayıda bitkide hastalık oluşturan tüm dünyada yaygın bir patojen fungustur. Etmen ayrıca çim üzerinde önemli zararlara neden olmaktadır. *R. solani* anastomosis grupları, çim alanlarında yaprak yanıklığı, geniş yamalar ve kahverengi yama hastalıklarına neden olmaktadır. *R. solani*'nin neden olduğu kahverengi yama hastalığı, özellikle golf sahalarında ve spor alanlarında ciddi hasara neden olmaktadır. Bu çalışmada, yedi antagonist bakteriyel izolat sera koşullarında çim bitkilerinde *Rhizoctonia solani*'ye karşı etkileri açısından değerlendirilmiştir. Çalışmada bakteri izolatları, 10^8 cfu/ml oranında çim tohumlarına bulaştırılmıştır. Çalışma sonuçları, tohuma muamele yapılarak elde edilen çim tohumlarının, muamele edilmeyenlere kıyasla *R. solani*'ye daha az duyarlı olduğunu göstermiştir. Bakteri inoküle edilen uygulamalarda gözlenen hastalık şiddeti, uygulanmamış olanlara kıyasla anlamlı ölçüde azalmıştır ($P < 0.001$). En yüksek koruma etkisi 253e izolatında (%91.43) gözlenmiştir. Bunu 187c (%87.62) ve 166fp (% 81.59) izolatları izlemiştir. Bakteri izolatlarının tanımlanması, DNA sekans analizi ile yapılmıştır. Bu izolatlar *Bacillus cereus, Bacillus sp., Paenibacillus sp., Pseudomonas putida, Stenotrophomanas rhizophila* olarak tanımlanmıştır. Çalışma sonucunda, *B. cereus* 253e, *Bacillus* sp. 187c ve *P. putida* 166fp strainlerinin, çim alanlarında kahverengi yama hastalığının biyolojik kontrolünde kullanılabilecek potansiyel izolatlar olduğu belirlenmiştir.

Anahtar Sözcükler: Çim, *Rhizoctonia solani*, biyolojik mücadele, bitki gelişiminin teşvik edilmesi

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* This study was presented at the International Congress of Agricultural Sciences (Van) (2018) and published as abstract in the proceedings book.

INTRODUCTION

Rhizoctonia solani Kuhn (teleomorph: *Thanatephorus cucumeris* [Frank] Donk) is a devastating pathogen on gramineae. It is the causal agent of brown patch (blight) disease on *Agrostis* spp., *Lolium* spp., *Festuca* spp., *Cynodon* spp. and *Zoysia* spp. (Burpee and Martin, 1992; Couch, 1995; Clarke, 2017). Until today, fourteen Anastomosis groups (AGs) and twelve subgroups were identified in *R. solani* (Sneh, 1994, Hsiang and Dean, 2001). Martin and Lucas (1983) first reported that *R. solani* cause brown patch on turfgrass areas. Six *R. solani* AGs or subgroups were detected from turfgrasses in the world. *R. solani* AG 1, AG 2, AG 4, AG 5 and AG 7 were identified on turfgrass areas in Turkey (Ünal et al., 2016 and 2019). Diseases symptoms come in to view under high temperature and humidity conditions. The syptoms start on plant leaves and then spread to crowns and roots. The first symtoms in long-cut turfgrass areas are many irregular and circular-shaped brown patches with a diameter of 2 to 13 cm. These patches can expand up to 55- 60 cm in diameter. The fungi survive in thatches in winter (Tani and Beard, 1996). These patches reduce the quality and beauty of the turfgrass areas. Especially in golf courses, many fungicides are applied to control this disease. Excessive use of fungicide in these areas creates excessive fungicide pollution in soil and groundwater. In addition, these practices threaten the health of both players and field workers (Jeon et al., 2015).

Biological control is an alternative way used of control grass diseases. Although the biological control of grass diseases are still in the evolvement stage, made researches on the apply of biofungicides in turf diseases control has been promising. Result of performed related to this subject laboratory and greenhouse studies have shown that antagonist fungi and bacteria can suppress brown patch and many the other grass diseases (Nelson, 1992; Lo *et al.,* 1996).

Some bacterial strains isolated from the turfgrass plants have been found to be effective in controlling some leaf, root and crown diseases on turfgrass through systemic resistance. For example, *Lysobacter enzymogenes* C3 strain, was found effective caused a brown patch disease in *R. solani* and leaf spot disease *Bipolaris sorokiniana* on *Festuca aurundinacea* (Giesler and Yuen, 1998; Kilic-Ekici and Yuen, 2003). *Entorobacter coloasae* was effective in suppressing brown patch in greenhouse trials (Nelson, 1992). *Bacillus* spp. are stick-shaped gram positive bacteria that form a protective endospore that can tolerate extreme environmental conditions. They are most commenly used as biopesticides to control a lot of plant diseases. These bacteria control fungal diseases with nutritional and place competition, antibiosis, and induced resistance mechanisms. Competitive colonisation of the rhizosphere and successful emplacement in the root zone is very important for effective biocontrol. In antagonism a lot of microbial metabolites play major role (Weller, 1988).

Fluorescent *Pseudomonas* are gram-negative, polar-flagged bacteria that give fluorescence glow under UV light on King B medium. These bacteria protect against diseases by promoting plant growth, inducing tolerance, producing antibiotics or creating physical barriers against the pathogen (Mondal and Battacharyya, 2003; Tang *et al.,* 2005; Li and Jiang, 2006).

Commercial preparations of biopesticides containing microorganisms in the world are successfully used against turfgrass diseases. Bio-Trek (*Trichoderma harzianum* strain 1295-22 also known as KRL-AG2). Rhapsody (*Bacillus subtilis*), Guard TM (*B. licheniformis*), Botrycid (*Pseudomonas aureofaciens*) and Actinovate SP (*Sypretomyces lydicus*) are microbial pesticides applicated on turfgrass diseases (Cawoy *et al.,* 2011). There is no registered biopesticide against turf diseases in Turkey.

The aim of this research is to investigate the biological control possibilities of *R. solani*, which causes brown patch disease in grass fields by using some native bacterial isolates.

MATERIALS and METHODS

Microorganisms

Rhizoctonia solani was isolated from turfgrass and was found 85% in the greenhouse as a virulent to turfgrass in a previous work (Ünal *et al.*, 2016). Fresh fungal culture was kept on PDA and stored at 4 °C.

The seven antagonistic bacterial isolates (253e, 215b, 187c, 44Ba, 88cfp, 166fp and 88bfp) were isolated from tomato rhizosphere and cucumber leaves in Turkey. 88cfp, 166fp and 88bfp have been previously reported to protect damping off (*Pythium deliense*) and root rot (*Sclerotinia minor*) and early blight (*Alternaria solani*) in tomato in field conditions. These bacteria also promise planth growth (Aşkın and Katırcıoğlu, 2009). 253e, 215b, 187c and 44 were suppressed cucumber downy mildew in previous work (Aşkın and Ozan, 2013). Species diagnosis of all bacterial isolates in this study was determined for the first time with this study.

Pathogen Inoculum

For *R. solani* inoculum, the wheat bran was filled in bottles of 500 ml and these bottles were autoclaved for 60 min at 121 °C for 48 hours in a row. 10-mm diameter *R. solani* discs, developed on the PDA (Difco Laboratories, Detroit, MI), were placed in 10 bottles and was incubated for 15-20 days at 23 ± 2 °C (Papavizas and Davey, 1962; Singleton *et al.,* 1991; Hatat, 1995; Aşkın and Katırcıoğlu, 2008).

Bacterial Inoculum

One day after bacteria were cultured in PDB (Potato dextrose broth) medium, their colony-forming units were counted by spectrophotometry and their concentrations were adjusted to 1×0^{8} cfu/ml. Grass seeds, disinfected with 1% sodium hypochlorite (NaClO) for 0,5 minutes, were inoculated with bacterial solutions by soaking and shaking for 12 hours.

Moleculer Identifications of Bacterial Isolates

Molecular identifications of bacteria were performed using the Blood and Tissue DNA isolation Kit (QIAGEN Inc. Valencia, CA). The 16S rDNA gene fragments were amplified by PCR (polymerase chain reaction) using the universal primers 27F and 1492R as described previously (Lane, 1991). The modified PCR was executed in a 50 µl reaction mixture containing 5 µl template DNA, 2 µl forward primer, 2 µl reverse primer, 0.4 µl $MgCI_2$, 5 µl reaction buffer (10x), 1 µl dNTP, 0.5 µl Taq DNA Polymerase, and 34.1 µl sterile doubledistilled water (Ozturk *et al.,* 2019) DNA replication were performed in the ABI Veriti (Applied Biosystem) thermal cycler using the following cycles. The initial denaturation consists of 5 min at 94 °C, 35 cycles of amplification step consisting of denaturation of 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 120 sec and final extension of 10 min at 72 °C. In consequence of the study, amplicons viewed by gel transilluminator were detected to be between 1500-1550 bp (Lane, 1991 Sanger sequence analyzes of PCR products were done in a special Arge Laboratory

The PCR products were subjected to sanger sequence analyze in a special Arge Laboratory (BM Laboratory Systems, Ankara, Turkey). Bipartite raw sequence electropherograms were compared to the isolate sequences in Gen Bank after BLAST screening in NCBI.

Biocontrol Assays

The biocontrol assay studies were carried out using mixture of grass seeds (cv. *F. arundinacea, F. rubra, Lolium perenne* and *Poa pratensis*). The soil was prepared in the form of a mixture garden soil, stream sand and burnt manure (2: 1: 1). Soil was autoclaved three times at 121 °C for 45 min on two consecutive days. Studies were made in sterilized and non-sterilized soils, where 3 aplications were carried out: 1. Negative control (uncoated grass seeds in non-inoculated soils), 2. Positive control (uncoated grass seeds in inoculated with *R. solani* soils to evaluate the varietal sensitivity), 3. Sowing of coated grass seeds in inoculated soil (evaluation of effects of the antagonistic isolates on the biocontrol of the pathogen). Soils were filled in plastic pots. Inoculums were mixed into sterile soil in pots, which was 50 g to 1 kg of soil. After 4-5 days, turfgrass seeds subjected to superficial disinfection for 2

minutes in 1% NaOCI were sown at a depth of 2 cm as 30 seeds per pot. The plants were grown in greenhouse at conditions of 12 hours of light, 12 hours of darkness and 23 ± 1 °C temperature. All experiments were carried out with three replicates. After the inoculations, observations were done at 10-day intervals and the disease rating was evaluated with 0-5 scale as follow after 30 days. The modified 0-5 scale is; 0= no disease, 1= 1-10%, 2= 11-30%, 3=31-50%, 4= 51-80% hypocotyl infection and / or shortening of plant height, 5= Dead plant and / or ungerminated seed (Ichielevich-Auster *et al.,* 1985, Ünal, 2013).

Disease severity values were calculated the scale values and the following formula (Karman, 1971).

Disease Severity % = [∑ (no. of plant in category × category value) × 100 / Total no. of plants × max. category value].

Statistical Analysis

To determine the differences between the applications, the variance analyzes were carried out using SPSS GLM statistical program. The values obtained according to scales were applied to the Towsend-Heuberger formula to calculate disease severity and the biocontrol activity of bacterial isolates with Abbott formula was applied to data from disease severity values. The disease severity was compared by Tukey multiple comparison test on these values.

RESULTS and DISCUSSION

R. solani is an important fungi which causes diseases in at least 12 turfgrass species. It was determined four anastomosis groups as pathogen in turfgrass in Turkey (Ünal *et al.*, 2016). Antagonistic bacterial isolates (253e, 215b, 187c, 44Ba, 88cfp, 166fp and 88bfp) used in this study were isolated from tomato rhizosphere and cucumber leaves in Turkey (Aşkın and Katırcıoğlu, 2009). 88cfp, 166fp and 88bfp were found efficient *Pytium deliense* on tomato, tomato root rot (*Sclerotinia minor*) and early blight (*Alternaria solani*) disease in field conditions in a previous work (Aşkın and Katırcıoğlu, 2009). 253e, 215b, 187c and 44Ba suppressed cucumber downy mildew in Aşkın and Ozan (2013) study.

It was founded that all antagonist isolates were effective compared to control trials in greenhouse conditions. The lowest disease severity was measured in treatment of *B. cereus* 253e as 7.20%. In treatments *Bacillus* sp. 187c and *Pseudomonas putida* 166fp, diseases severities values were 10.40% and 15.47% respectively. Three applications were statistically included in the same group. The highest disease severity was measured in treatment of *Stenotrophomanas rhizophila* 88bpf as 51.20% when compared to the severity of the disease in control (Table 1 and Figure 1).

Table 1. Effect of bacterial strains against disease of *Rhizoctonia solani* on turfgrass

Treatments	Disease severity *(%)	Efficacy (%)
Pseudomonas putida 166fp	15.47 ± 2.9 d	81.59
Bacillus sp.187c	10.40 ± 2.9 d	87.62
Paenibacillus sp. 215b	47.27 ± 2.9 bc	43.73
Bacillus cereus 253e	7.20 ± 2.9 d	91.43
Bacillus cereus 44Ba	44.00 ± 2.9 bc	47.62
Stenotrophomanas rhizophila 88bpf	51.20 ± 2.9 b	39.05
Pseudomonas putida 88cpf	34.67 ± 2.9 c	58.73
(+) Control	84.00 ± 2.9 a	-
(-) Control	0.00	-

*There is no difference between the mean values followed by same letter. According to TUKEY test ($P < 0.001$)

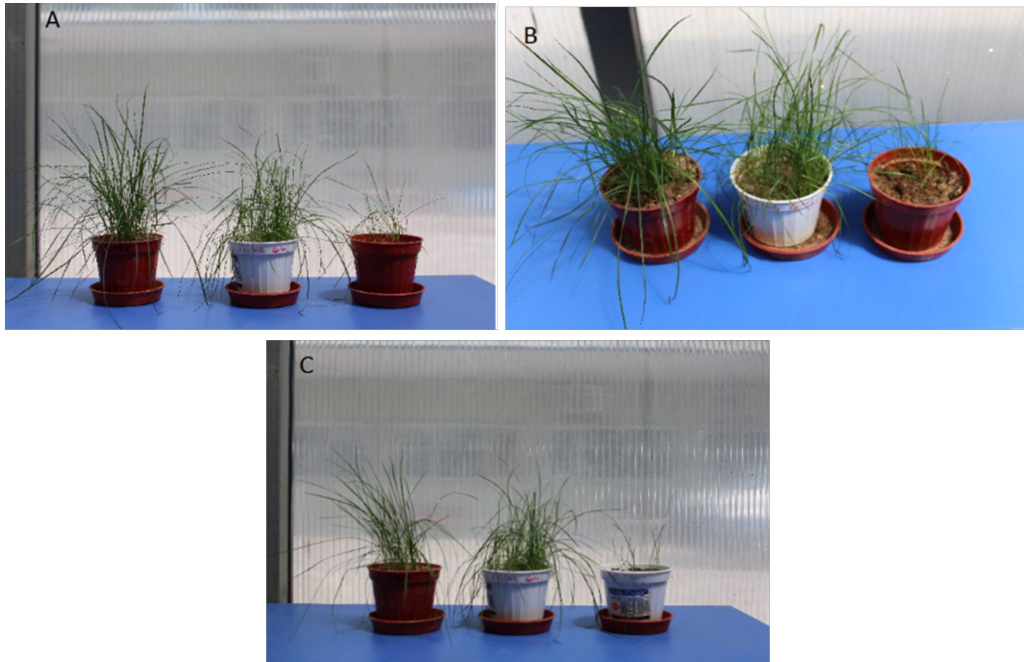

Figure 1. Effects of bacterial isolates against brown patch causing *Rhizoctonia solani* in greenhouse experiments. A*:* *B. cereus* 235e, B: *Bacillus* sp. 187c, C: *Pseudomonas putida* 166fp

According to result given in Table 1, the highest protection effect was shown by *B. cereus* isolate 253e (91.43%). This isolate was followed by the isolates *Bacillus* sp. 187c (87.62%) and *Pseudomonas putida* 166fp (81.59%) (Figure 2). In this study, it was also observed that *P. putida* 166fp strain increased plant growth in grass plants (Figure 3).

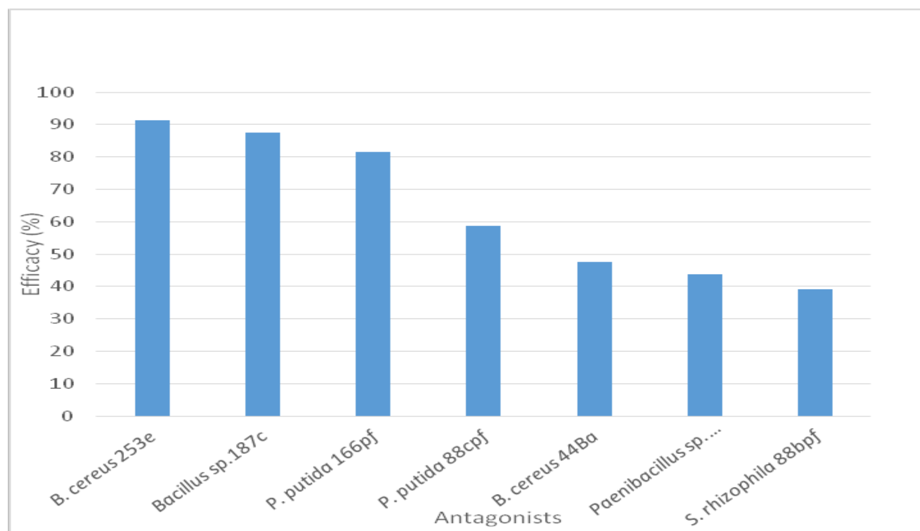

Figure 2. Efficacy of bacterial isolates against brown patch causing *R. solani* in greenhouse experiments

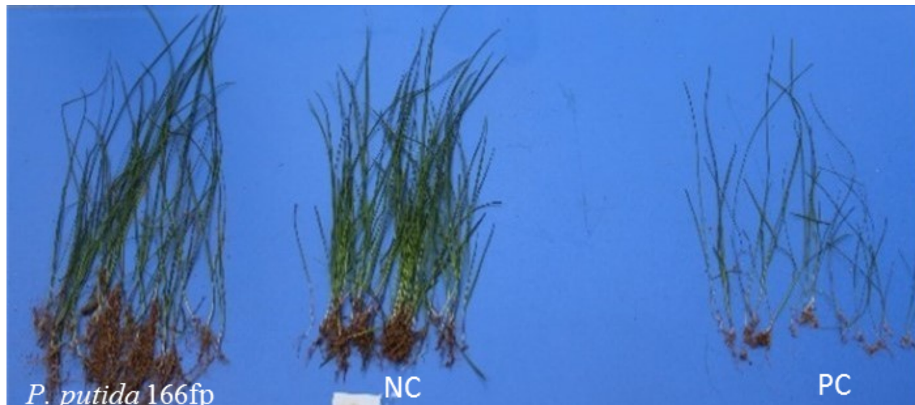

Figure 3. After seed covering *Pseudomonas putida* 166fp isolate, growth of turfgrass with *Rhizoctonia solani*

Following molecular studies, sequences of bacterial isolates indicated 99% -100% similarity to those isolates sequences deposited in GenBank. In consequence of the BLAST analyses of the bacterial isolates, it was determined that 88bfp was *Stenotrophomonas rhizophila*,166fp and 88cfp were *Pseudomonas putida*, 215b was *Paenibacillus* sp., 253e and 44Ba were *Bacillus cereus* and 187c was *Bacillus* sp. (Table 2).

Table 2. Molecular identifications of bacterial isolates and their similarity rates with Genbank isolates

Isolata Number	Bacteria	Similarity rates with GenBank isolates (%)
253e	*Bacillus cereus*	100
215b	*Paenibacillus* sp.	99.79
187c	*Bacillus* sp.	95.52
44Ba	*Bacillus cereus*	96.97
166fp	*Pseudomonas putida*	100
88cfp	*Pseudomonas putida*	99.64
88bfp	*Stenotrophomanas rhizophila*	100

In this study, biological control possibilities were investigated against brown catch caused by *R. solani* on turfgrass using indigenous rhizobacterial isolates of *Pseudomonas* and *Bacillus* species. *Pseudomonas* spp. used in this study have been previously reported to possess plant growth promoting features (Aşkın, 2008). Biocontrol agents are effective against pathogens by such forms of antagonism as competition, antibiosis and parasitism. Plant growth-promoting rhizobacteria (PGPR) colonize plant roots, and stimulate plant growth and/or reduce severity of plant diseases (Kleopper *et al.*, 1978). These PGPR largely consist of *Pseudomonas* and *Bacillus* bacteria which are antagonists of important soilborne root pathogens such as *Sclerotinia* spp., *Rhizotonia* spp., *Fusarium* spp., and *Macrophomina phaseolina* (Haas and Defago, 2005; Soylu *et al.,* 2005).

In bacterial antogonist studies, the mode of application, time and incubation period are the factors affecting the success of the application. Clemente *et al.* (2000) found that 12-hour seed bacterial growth increased the number of bacteria entering the seed when compared to the 2-hour seed coating of fluorescent *Pseudomonas* under the control of *R. solani.* In the study, at the begining, *P. fluorescens* P190 containing 1×10^9 cells / ml was measured. After 12 hours spore density was measured as 5.55×10^5. The same incubation period (12 hours) was used in this study.

In studies on the biological control of pathogens with rhizosphere bacteria, bacteria can be applied in different forms. These methods include; blending the bacterial suspension into sterile soil, immersing the seedlings in suspension of bacteria or coating seeds with a high number of bacteria prior to sowing (Kluepfel, 1993). The plant induced systemic resistance depends on the colonization of PGPR in root system. This adequate colonization is achieved by coating the seed with a high number of bacteria or by adding the bacterial suspension to the soil before planting (Kloepper, 1996). Rizobacteria on; Although they are applied to the seed or soil, they reach as far as above-ground failure (Kluepfel, 1993). In our study, bacteria were applied in the form of seed coat.

Various bacterial and fungal antagonists were found to prevent turfgrass pathogens including *R. solani* (Sutker and Lucus, 1987; Yuen *et al.*, 1994; Lo *et al.*, 1996; Thompson *et al.,* 1996; Zhang and Yuen, 1997). However, report on the growth promotion of turfgrass by plant growth promoting rhizobacteria (PGPR) is very few. The plant growth promotion along with control of disease by *Paenibacillus* spp. has been reported in some plants (Khan *et al.,* 2008; Larsen *et al.,* 2009; Naing *et al.,* 2014). The plant growth promotion by *Paenibacillus* spp. includes mechanisms, such as nitrogen fixation, production of plant hormones, soil phosphorus solutionizing, the suppression of phytopathogens (Timmusk *et al.*, 1999; Coelho *et al.,* 2003; Raza *et al.,* 2011; Khan *et al.,* 2012).

Researches have shown that in the genera *Bacillus*, *Enterobacter, Pseudomonas* and *Erwinia*. microorganisms can hinder pathogen populations in a number of ways. Particularly *Pseudomonas, Bacillus* and *Erwinia* produce antibiotics that inhibit pathogen growth and development. Some *Pseudomonas* and *Enterobacter* species outcompate pathogens for essential nutrients and other growth factors, reducing their germination, growth and pathogenisity (Nelson, 1992). We found *Bacillus cereus* 253e, *Bacillus* sp. 187c and *Pseudomonas putida* 166fp were promising to be used in the biological control of brown patch caused by *R. solani*. It is considered especially *P. putida* 166fp suppress *R. solani*.

The members of *Bacillus* and *Pseudomonas* genus are among the beneficial bacteria that are mostly developed and registered as microbiyal fungicide exploited against turfgrass disease in the world, but according to our knowledge, this is the first report that the native antagonist bacteria were used against brown patch disease in turfgrass in Turkey.

Our result will be important to carry out open field experiments with these strains and to determine their mechanisms of action against these diseases.

Biological control possibilities of *Rhizoctonia solani* on turfgrass has investigated with this study. Strains of *B. cereus* 253e, *Bacillus* sp.187c and *P. putida* 166pf were found to be efficient on *R. solani* under greenhouse conditions. However, these studies need to be repeated under field conditions.

ACKNOWLEDGEMENTS

We would like to acknowledge Ankara Plant Protection Central Research Institute for their support.

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