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***Orijinal araştırma* (*Original article*)**

The Use of Turkish Bacterial Strains for the Biological Control of *Fusarium cerealis* which Causes Root and Crown Rot in Turfgrass

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Çimlerde Kök ve Kökboğazı Çürüklüğüne Neden Olan *Fusarium cerealis*'in Biyolojik Mücadelesinde Yerli Bakteriyel izolatların Kullanımı

Öz: Çimlerde *Fusarium cerealis*'in neden olduğu kök ve kökboğazı çürüklüğü Türkiye'nin birçok bölgesindeki çim alanlarındaki önemli hastalıklardan biridir. Bu çalışmada, *F. cerealis*'in biyolojik mücadelesinde, domates rizosferinden ve hıyar yapraklarından elde edilen beş bakteri izolatının (*Stenotrophomonas rhizophila* 88bfp, *Pseudomonas putida* 166fp, *Pseudomonas putida* 88cfp, *Paenibacillus* sp. 215b ve *Bacillus cereus* 44) etkinliği iklim odası koşullarında tohum inokulasyonu (10^8 hücre/ml) yoluyla araştırılmıştır. Sonuçlar, *S. hizophila* 88bfp ve *Paenibacillus* sp. 215b bakteri izolatlarının çimde kök ve kökboğazı çürüklüğü simptom gelişimini, kontrol bitkilerine kıyasla sırasıyla % 85,25 ve % 75,77 oranında azalttığını göstermiştir. *P. putida* 88cfp ve *P. putida* 166fp uygulanan denemelerde bu değer sırasıyla % 58.69 ve% 56,72 olarak tespit edilmiştir. En düşük etki *B. cereus* 44 uygulamasında, kontrolle kıyaslandığında % 32.13 olarak hesaplanmıştır. Bu çalışma sonucunda, iki yerli antagonistik bakteri *S. rhizophila* 88bfp ve *Paenibacillus*. sp. 215b'in Türkiye'de çimlerdeki *F. cerealis*'in neden olduğu kök ve kökboğazı çürüğünün biyolojik mücadelesinde ümit var olduğu bulunmuştur.

Anahtar sözcükler: Çim, *Fusarium cerealis*, Biyolojik mücadele

Abstract: Root and crown rot of turfgrass caused by *Fusarium cerealis* is an important disease in many parts of Turkey. The ability of five bacterial strains, namely *Stenotrophomonas rhizophila* 88bfp, *Pseudomonas putida* 166fp, *Pseudomonas putida* 88cfp, *Paenibacillus* sp. 215b and *Bacillus cereus* 253e, isolated from the tomato rhizosphere and cucumber leaves to control *F. cerealis*, was studied with turf grass seed treatment (10^8 cfu/mL) under growth chamber conditions. *S. rhizophila* 88bfp and *Paenibacillus* sp. 215b reduced root and crown rot symptom development in turf grass by up to 85.25% and 75.77%, respectively, when compared to the untreated control plants. In treatments with *P. putida*

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88cfp and *P. putida* 166fp, these values were 58.69% and 56.72%, respectively. *Bacillus cereus* 44 had the lowest efficacy of 32.13 %. Two local antagonistic bacteria *S. rhizophila* 88bfp and *Paenibacillus.* sp. 215b were found promising candidates for the biocontrol of root and crown rot caused by *F. cerealis* in turf grass in Turkey.

Key words: Turfgrass, *Fusarium cerealis,* Biological control

Introduction

The areas of picnic and parks, football fields, gardens and golf courses are increasing daily as the amount of leisure time increases for many people. The planting of turfgrass is used to create these areas. However, many fungal pathogens cause diseases in turfgrass, including, species of *Bipolaris, Fusarium, Magnaporthe, Pythium* and *Rhizoctonia* (Nelson & Craft, 1991). Among them, *Fusarium* is considered the most dangerous. *Fusarium* species have frequently been found to play a role in seedling diseases (Smiley et al, 1992), with *Fusarium* spp. having been reported as the causal agents of leaf spot, leaf blight and crown and root rot (Smiley and Thompson, 1985). The intensive management of turfgrass, e.g. the frequent use of nitrogenous fertilizers and regular mowing provide favorable conditions for infection by *Fusarium* spp. (Smith et al, 1989). *Fusarium* species isolated from diseased plants, such as *F. culmorum, F. graminearum, F. avenaceum* and *F. equiseti*, are among the main causal agents of crown and root rot (Smiley and Thompson, 1985). The fungus, *Fusarium cerealis* (Cooke) Sacc. (1886) (syn. *F. crookwellense* L.W. Burgess, P.E. Nelson&Toussoun 1982), is a pathogen of many plant species.

The role of *Fusarium* species in root and crown rot and the appearance of other symptoms in turfgrass areas was studied in Qom Province, Iran. In that study, sampling was done in all turfgrass areas exhibiting damage and suspected to have fungal contamination (Poor & Riahinia, 2017). The highest pathogenicity and frequency were recorded for *F. cerealis* and *F. solani*, respectively. Many *Fusarium* spp. were detected in turfgrass in research carried out between 2014 and 2018 years in Turkey, with root and crown rot caused by *F. cerealis* determined to be one of the important diseases caused by *Fusarium* species in different regions of Turkey (Ünal et al, 2016). The routine spraying of synthetic fungicides for the control of turfgrass diseases causes contamination of groundwater and threatens the health of both players and workers (Chai et al, 2002). For this reason, microbial antagonists have been used to protect turfgrass areas (Ippolito & Nigro, 2000; Droby, 2006; Cuppels et al, 2013).

Biological control is an effective alternative control strategy for the suppression of turfgrass diseases. Biocontrol agents are effective against pathogens through forms of antagonism such as antibiosis, competition and parasitism. Plant growth-promoting rhizobacteria (PGPR) competitively colonize plant roots and stimulate plant growth and/or reduce the incidence of diseases (Kloepper & Schroth 1978). PGPR plays an important role in enhancing plant growth through the stimulation of a wide variety of mechanisms that include (i) abiotic stress tolerance in plants; (ii) nutrient fixation for easy uptake by plant; (iii) plant growth regulators; and the

production of: (iv) siderophores, (v) volatile organic compounds, and (vi) protective enzymes such as chitinase, glucanase, and ACC-deaminase for the inhibition of plant diseases (Yuen et al, 1994; Ippolito & Nigro, 2000).

Despite the biological control of turfgrass disease still being in its developmental stages, research results on the use of microbial fungicides for turfgrass disease control have been promising. The results of laboratory and greenhouse studies have shown that antagonistic fungi and bacteria are effective in turfgrass diseases. Some bacterial strains isolated from turfgrass have shown promise for the control of some root, leaf and crown diseases of turfgrass through the enhancement of systemic resistance. For example, the C3 strain of *Lysobacter enzymogenes* was effective against brown patch disease in *Rhizoctonia solani* (Giesler & Yuen 1998) and leaf spot disease *Bipolaris sorokiniana* on *Festuca aurundinacea* (Kilic-Ekici & Yuen, 2003). In addition, *Entorobacter coloasae* was effective in suppressing brown patch in greenhouse trials (Nelson 1992).

Commercial preparations of bio-pesticides containing microorganisms are being successfully used against diseases of turfgrass. Bio-Trek (*Trichoderma harzianum* strain 1295-22, also known as KRL-AG2) is the first Environmental Protection Agency (EPA). Guard TM (*Bacillus licheniformis*), Rhapsody® (*B. subtilis*), Act inovate® SP (*Sypretomyces lydicus*) and Spotless® (*Pseudomonas aureofaciens*) are other microbial fungicides used against *Pythium* spp., *Rhizoctonia* spp., *Fusarium* spp., *Colletotrichum graminicola* and *Sclerotinia homeocarpa* on turfgrasses (Cawoy et al, 2011). However, no microbial fungicides have been registered against turfgrass diseases in Turkey yet.

The objective of this research was to test bacterial strains from Turkey against *F. cerealis*, which causes root and crown rot turfgrass, in a greenhouse experiment and to proceed to biopesticide production if a promising bacterial isolate was found.

Materials and Methods

Microorganisms

A *Fusarium cerealis* strain (CFc1) isolated from diseased turfgrass plants and found to be 92% virulent on turfgrass in a greenhouse experiment (Ünal et al. 2016). Fresh *F. cerealis* culture was developed on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) and stored at 4 ^{o} C. Five antagonistic bacterial strains isolated from the tomato rhizosphere and cucumber leaves in Turkey (Aşkın & Katırcıoğlu 2009) were grown on nutrient agar (NA).

Identification of bacteral strains

Molecular identification was based on the analysis of the 16S rrnA gene. Genomic DNAs of bacterial strains were obtained using a Blood and Tissue Kit (QIAGEN Inc. Valencia, CA). The 16S rDNA gene fragments were amplified by PCR by using the universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') (Lane 1991). The PCR reaction mixture and conditions (temperature and time) were modified to carry out the PCR reaction. DNA

replication was performed in an ABI Veriti (Applied Biosystem) thermal cycler by using the following cycles for the initial denaturation: 5 min at 94 °C; 35 cycles of amplification consisting of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 2 min.; and a final extension of 10 min at 72 °C. The amplicons visualized with gel translimunator were in the range of 1500-1550 bp reported by Lane (1991). The PCR products were subjected to Sanger sequence treatment in an Arge Laboratory (BM Gene Research and Biotechnology Company, Ankara, Turkey). Bipartite raw sequence electropherograms were compared to the isolate sequences in Gen Bank after BLAST screening in NCBI.

Pathogen Inoculation

Bottles of five hundred ml capacity containing wheat bran were autocalved for 1 hour at 121°C for two successive days. The *F. cerealis* isolates propagated in PDA medium were placed on 10 mm diameter discs, 10 in each bottle, and incubated at 23 ± 2 °C for 15-20 days (Papavizas & Davey 1962; Singleton et al. 1991).

Bacterial Inoculation

The bacterial strains were cultured in potato dextrose broth (PDB). After 24 hours, the bacterial concentration was verified through spectrophotometry at a wavelength (λ) of 600 nm at an absorbance between 0.9 and 1 which is equivalent to a concentration of $1x10^8$ cfu/ml, and also by counting the colony forming units cfu/ml via a total viability count. Turfgrass seeds surface-disinfected with sodium hypochlorite (1%) were inoculated with the bacterial solutions by soaking during shaking for 12 hours. Rhizobacterial stock cultures were maintained in nutrient agar (NA) amended with 15% glycerol and stored at -80°C. Before being used in the bioassays, the stock cultures were streaked onto NA plates and incubated at 28°C for 48 h (Aşkın & Katırcıoğlu 2009).

Growth chamber experiment

A mixture of turfgrass seeds containing four species, namely *Festuca rubra*, *Lolium perenne*, *Poa pratensis* and *Festuca arundinacea*, was used. The soil used was a 2: 1: 1 mixture of garden soil, river sand and burnt manure sterilized three times in an autoclave at 121 °C for 45 min on consecutive days. The tests were carried out in both sterilized and non-sterilized soils with three treatments: (1) negative control - uncoated turfgrass seeds in sterilized soil; (2) positive control - uncoated turfgrass seeds in infested soil to evaluate varietal sensitivity; and (3) coated turfgrass seeds in infested soils to evaluate the antagonistic capacity of each isolate against *F. cerealis*.

The mixture of inoculum (*F. cerealis*) and soil (5 g to 1 kg of soil) were filled in sterile plastic pots (10 cm in diameter). After 4–5 days, turfgrass seeds coated with the individual bacteria were planted at 1 cm depth 30 seeds per pot. The experiments were carried out in 3 replicates in a randomized plot design. After incubation for 30 days at 23±1°C under a photoperiod of 12 h, the disease level on the seedlings was rated on a modified 0-5 scale (Ichielevich-Auster et al. 1985), as follows

0. healthy plant

1. 1-10%, reductionof plant height

2. 11-30%, reduction of plant height

3. 31-50%, reduction of plant height

4. 51-80%, reduction of plant height

5. Dead plant and / or ungerminated seed

These scale values were converted to disease severity values by using the following formula of Karman (1971). Disease severity %= [∑ (no. of plants in category x category value)] x 100 / Total no. of plants x max. category value.

Statistical Analysis

Variance analyses were performed with the SPSS GLM statistics program to determine the differences between the treatments. The values obtained according to the 0-5 scale were applied to the Tawsend-Heuberger formula to calculate the severity of the disease and the activity of the bacterial isolates was determined with the Abbott formula from the disease severity values. Disease severity values were compared with the Tukey multiple comparison test.

Result and Discussion

Sequence data of the bacterial isolates used in this study showed 99-100% similarity with isolates in GenBank. The identified bacterial isolates are as follow, with the similarity percentage in brackets: one isolate - *Stenotrophomonas rhizophila* (100%), one isolate - *Pseudomonas putida* (100%), one isolate - *Pseudomonas putida* (99.64 %), one isolate - *Paenibacillus* sp. (99.79 %) and one isolate - *Bacillus cereus* (100%) (Table 1).

Table 1. Bacterial isolates used against *Fusarium cerealis* on turfgrass

Strain Code	Bacteria
166fp	*Pseudomonas putida*
215b	*Paenibacillus* sp.
44	*Bacillus cereus*
88bfp	*Stenotrophomanas rhizophila*
88cfp	*Pseudomonas putida*

In the growth chamber experiments, the five tested bacterial strains had efficacies ranging from 32.13% to 85.25% higher than the control. The lowest and highest disease severities of 12.00% and 55.20%, respectively, were measured in the treatments with *S. rhizophila* 88bfp and *B. cereus* 44, respectively. The highest

protection effect was observed on isolate *S. rhizophila* 88bfp (85.25%). The isolate *Paenibacillus* sp.215b (75.77%) followed it (Table 2) (Figure 1).

Table 2. The effects of Turkish bacterial strains on the turfgrass pathogen, *Fusarium cerealis*

Treatments	**Disease severity(%)***		**Efficacy (%)**
Pseudomonas putida 166fp	35.20 ± 3.922	c	56.72
Paenibacillus sp.215b	19.73 ± 3.922	cd	75.77
Bacillus cereus 44	55.20 ± 3.922	b	32.13
Stenotrophomanas rhizophila 88bfp	12.00 ± 3.922	d	85.25
P. putida 88cfp	33.60 ± 3.922	c	58.69
(+) Control	81.33 ± 3.922	a	
(-) Control	**0.00**		

*****There is no difference between the mean values followed by the same letter (P<0.0001).

In this greenhouse study, the suppression of root rot caused by *F. cerealis* on turfgrass with strains of bacterial species collected in Turkey was investigated. Fungi, actinomycetes and bacteria have been identified as useful microorganisms for the biocontrol of soil-borne pathogens and insects, and they provide a promising alternative to the use of chemical pesticides in plant protection (Becker & Schwinn, 1993; Cook, 1993; Cronin et al, 1997; Dunne et al, 1996, 1997; Keel & Defago, 1997). An important feature of effective biocontrol agents is their ability to remain in the soil and aggressively colonize the rhizosphere. For this reason, it is suggested that indigenous biocontrol microorganisms may provide more effective plant protection in similar environments to those in which they normallyfunction (Cook 1993). In addition, several bacterial and fungal antagonists were found to inhibit turfgrass pathogens *Rhizoctonia solani*, *Sclerotinia homoeocarpa, Pythium graminicola* (Yuen et al, 1994, Lo et al, 1996, Zhang & Yuen, 1997), *Magnaporthe poae* (Thompson et al, 1996), *Bipolaris sorokiniana* and *Pythium ultimum* (Zhang & Yuen, 1997).

The use of bacteria as biological control agents is one of the fastest growing areas of research in disease management. The bacterium *Stenotrophomonas maltophilia* (=*P. maltophilia* or *Xanthomonas maltophilia*) has been isolated from the rhizospheres and phyllospheres of grasses (Juhnke & Des Jardin, 1989; Palleroni & Bradbury, 1993; Kobayashi et al, 1995; Behrendt et al, 1997). There are reports of rhizosphere strains being used as effective antagonists of fungal root pathogens (Dunne et al. 1997). Growth chamber studies indicate that the repeated administration of *Stenotrophomonas maltophilia* has improved disease suppression in comparison to standard practice. *S. maltophilia* populations re-established above 10^7 cfu/g of rhizosphere sample following each repeated application, indicating that

these high populations are critical for disease suppression. Field work suggest that *S. maltophilia* populations can be established in turfgrass at this level and disease management can be achieved (Kobayashi et al, 1999). *Stenotrophomonas maltophilia* strain W81, isolated from the rhizosphere of field-grown sugar beet, produced the extracellular enzymes chitinase and protease. *Stenotrophomonas maltophilia* is also an emerging human pathogen responsible for fatal infections. Unlike *S. maltophilia*, *S. rhizophila* has no human pathogenic properties (Ribbeck-Busch et al, 2005; Hagemann et al, 2006). *Stenotrophomonas rhizophila* has the potential to directly improve plant growth as well as inhibit plant pathogens (Wolf et al, 2002) and produce antifungal volatiles (Kai et al, 2007). *Stenotrophomonas rhizophila* and *S. maltophilia* produce active antibiotics against certain fungi and bacteria (Minkwitz & Berg, 2001; Wolf et al, 2002; Romanenko et al, 2008). *S. Stenotrophomonas maltophilia* R3089 and *S. rhizophila* P69 also produce small, volatile compounds that negatively affect the mycelial growth of the soil-borne phytopathogenic fungus, *Rhizoctonia solani* (Kai et al, 2007). In this study, *S. rhizophila* 215b isolate increased the plant growth, as well as inhibited *F. cerealis*.

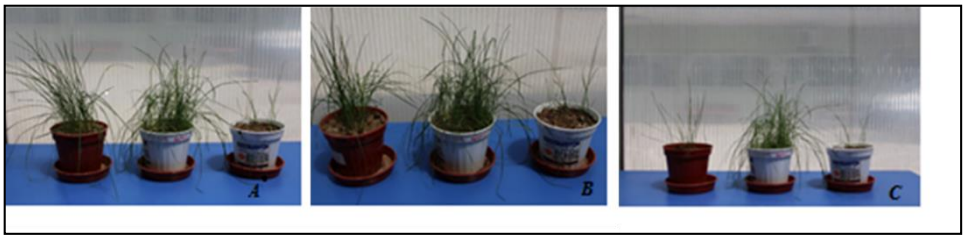

Figure 1. Effects on turfgrass seedlings of bacterial strains antagonistic to root rot causing *F. cerealis* in greenhouse experiments: A) *S. rhizophila* 88bfp; B) *Paenibacillus* sp. 215b; and C) *B. cereus* 44.

The promotion of plant growth through the simultaneous control of disease by several species of *Paenibacillus*, which has been reported in many plants (Larsen et al. 2009; Khan et al, 2012; Naing et al, 2014), involves indirect mechanisms such as the stimulation of plant hormone production, and direct mechanisms such as nitrogen fixation, soil phosphorus dissolution and the suppression of phytopathogens (Timmusk et al, 1999; Coelho, 2003; Khan et al, 2008; Raza et al, 2011).

The authors of the present study understand that this is the first time that the native Turkish *S. rhizophila* 88bfp strain and *Paenibacillus* sp. have been used against root and crown rot of turfgrass in Turkey. Open field experiments with the *S. rhizophila* 88bfp and *Paenibacillus* sp.215b strains to determine their mechanism(s) of action would complement the present study.

The future of biological control of turfgrass diseases appears promising. Resistance-inducing and antagonistic rhizobacteria may be useful in formulating new inoculants, thereby offering an attractive alternative for the environmentally friendly biological control of plant diseases and improving the profitability of

cropping systems in which they can be successfully applied. These PGPRs require the implementation of a systematic strategy designed to fully utilize all their potential benefits; the application of combinations that include different mechanisms of action will help maintain or even increase crop yields while the need for chemical treatments is reduced.

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