Antagonistic Effects of *Pseudomonas* spp. against Turfgrass Pathogenic Soil Fungi

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잔디 주요 토양 병해에 대한 토양세균 *Pseudomonas* spp.의 길항 효과

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ABSTRACT

Bacterial isolates collected from rhizosphere of turfgrass showed strong *in vitro* antagonistic activities against a number of turfgrass soilborne pathogens such as *Rhizoctonia cerealis*, *R. solani* AG-1(1B), *Sclerotinia homoeocarpa* and *Typhula incarnata*. *In vivo* study, four bacterial isolates selected have control values over 60% against one or more turfgrass pathogenic fungi. The antagonistic effects of the bacterial isolates varied depending on fungal species, host plant, and disease pressure, indicating that control effects of the antagonists could be variable depending on field conditions. They were classified as belonging to the genus *Pseudomonas* species, based on morphological and biochemical characteristics as well as 16S rRNA analysis. The four

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bacterial isolates are under a study for finding proper cultural conditions and determination formulation type.

Key words: Antagonistic activity, *Pseudomonas* spp., turfgrass diseases

INTRODUCTION

In sustainable agriculture, biological control of plant pathogens using antagonistic microorganisms is an attract management strategy as an alternative of chemical control (Handelsman and Stabb, 1996 Kim et al., 1997; Suh et al., 1999). Because of concerns such as environmental contamination and fungicide resistance etc., the total amount of biocontrol agents used in the agricultural system have been increased worldwide (Jo et al., 2008 Mathre et al., 1999 Schisler et al., 2004).

Pseudomonas spp. are gram-negative soil bacteria that have been widely used as biocontrol and growth-promoting agents (Stockwell and Stack, 2007; Weller, 2007). Weller (2007) reviewed that as agricultural materials the bacteria (i) grow rapidly *in vitro* and to be mass produced; (ii) rapidly utilize seed and root exudates; (iii) colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant; (iv) produce a wide spectrum of bioactive metabolites such as antibiotics, siderophores, volatiles, and growth-promoting substances, (v) compete aggressively with other microorganisms; and (vi) adapt to environmental stresses.

Above all, *Pseudomonas* spp. have been considered more rhizosphere-competent compared with different microorganisms including *Bacillus* species for reasons described above (Weller, 1988). Management of fungal disease in golf courses is highly dependent on the application of chemical fungicides (Jo et al., 2008). The development of resistance has promoted research into identification of novel chemical fungicides as well as biological control agents (Mathre et al., 1999; Powell et al., 2000).

As biocontrol candidates we have isolated four *Pseudomonas* spp. from golf course soils. They were responsible for the antifungal activities against many plant pathogens as well as turfgrass pathogenic fungi. The objectives of thisstudy were to identify the isolates and evaluate the antifungal activity of the isolates against turfgrass pathogenic fungi.

MATERIALS AND METHODS

Bacterial isolation

The bacteria were isolated from the rhizosphere of bentgrass root in golf courses in Kangwon province, Korea. Appropriate serial dilutions from soil suspensions in 0.01 M phosphate (pH 7.2) were plated on NA (nutrient agar) medium and plates were incubated at 30℃ for 48 hr. Four bacterial isolates were finally chosen based on preliminary *in vitro* antifungal activity against the selected fungal turfgrass pathogens (Table 1).

Table 1. Identification of antifungal bacterial isolates collected from rhizosphere of turfgrass plants

Isolate	Source isolated	Location	Nearest relative[z]	Acession no. of nearest relative [z]	Similarity (%)
YU-O5G-1-3	rhizosphere	Kangwon	*Pseudomonas* sp.	GBEV864269.1	99
YU-O5G-3-3	〃	〃	〃	GBEF126753.1	99
YU-F3R-3	〃	〃	〃	GBEV781733.1	99
YU-F6F-1	〃	〃	〃	GBEF427849.1	99

[z] Based on a Blast search of the NCBI database

Identification of selected bacteria

Sequencing of the 16S rRNA using universal primers (forward-27F, reverse-1492R) and a BLAST search of the National Center for Biotechnology Information (NCBI) database (http:www.ncbi.nlm.) were used for identification of the bacterial isolates. The isolates were also confirmed by using BIOLOG system by GN2 and GP2 plates (Biolog Inc, Hayward, CA. USA) (Praphailong et al., 1997). All isolates were maintained on a King's B medium (glycerol 10g/L, peptone 20g/L, K_2HPO_4 1.5g/L, $MgSO_4$ 1.5g/L, and agar 15g/L) at 10℃ and used after overnight culture in King's B broth (glycerol 10g/L, peptone 20g/L, K_2HPO_4 1.5g/L, and $MgSO_4$ 1.5g/L).

Plant pathogenic fungi

Rhizoctonia solani AG-1(1B) (KACC-40142) was obtained from Korea Agricultural Culture Collection (KACC), Rural Development Administration, Korea. *R. cerealis* van der Hoeven, *Sclerotinia homoeocarpa* F.T. Bennett, and *Typhula incarnata* Lasch ex Fr. were obtained from lesions of turfgrass leaves in golf courses in 2008 and 2009, respectively. All isolates were maintained on a Potato Dextrose Agar (PDA) medium at 10℃.

In vitro antifungal test

In vitro test was conducted to evaluate the ability to inhibit fungal growth. For the antifungal activity of the bacterial isolates, each overnight culture was streaked

across the center of agar plates. Fresh agar disks (5mm in diameter) of two target turfgrass pathogens grown on PDA media were placed on the opposite side of the plate streaked by an antifungal bacterium. Except plates of *R. cerealis* (20℃) and *T. incarnata* (10℃), all streaked plates were incubated at 25℃ and scored after two weeks or four weeks (*T. incarnata*) measuring the distance between the edges of the bacterial colony and fungal mycelia. There were two experiments with three replicates per experiment.

In vivo test

Creeping bentgrass cv. Penncross (*Agrostis palustris* L.) and Kentucky bluegrass cv. Prosperity (*Poa pratensis* L.) were used for *in vivo* test. Plant preparation and maintaining were conducted by following the procedures in Chang et al. (2007). The two cultivars (Penncross: 0.01g of seeds, Prosperity: 0.03g of seeds) were evenly sown into a plastic pot (5.3×5.3×5.1cm) containing commercial potting soil mixture (Metro Mix 366-P, Scott's company, Marysville, OH, USA). The plants were grown in the growth chamber at 18 to 28℃ with light and dark cycle of 16 and 8 hours, respectively. The plants were mowed weekly with scissors to a height of 0.6 cm (creeping bentgrass) and 2cm (Kentucky bluegrass) from the beginning 2 weeks. Fertilizer (SunGrow company, Austin, Texas, USA) was applied at 0.02-0.005-0.02g (N-P-K) per pot biweekly for 4 weeks after germination to 2 weeks prior to inoculation.

Inoculum preparation and inoculation of three pathogens (*R. cerealis*, *R. solani* AG-1(1B), and *T. incarnata*) were conducted by modifying the procedure in Chang and Jung (2009). In brief, three 5-mm diameter plugs were taken from the edge of colonies growing on PDA and transferred to 20ml potato dextrose broth in a plastic Petri dish (diameter 9cm) and grown for a week at 25℃±1 with no light. For each fungal isolate, four to six Petri dishes of mycelium were harvested, mixed, and then air-dried for 30 minutes under a laminar flow hood. Mycelia were weighed and homogenized in a blender with sterile, distilled water for 30 seconds. The chopped mycelial suspensions were then adjusted to 0.2g/mL using sterile, distilled water.

Sterile pipettes were used to deliver 1ml of the mycelium suspension directly to the soil surface in the center of each pot. Immediately after inoculation, distilled water was applied to the foliage with a hand sprayer until runoff. Inoculated pots were then placed in a plastic box (46×32×17cm, Komaz, Seoul, Korea)with approximately 30% of the total volume of the box filled with moist potting soil (1 soil : 1 distilled water in volume). The box was covered with a lid to maintain the high humidity required for proper adaptation of fungal isolates on soil surface and then

transferred to a controlled environment chamber maintained at 25℃ for 24 hours. Randomization of pots and boxes in each replicate were based on a randomized block plot design in ARM 6.1.12 (Gylling Data Management Inc., Brookings, SD, USA).

The bacterial suspension was prepared by bacterial growth in KBB for 18 hours at 25℃ with shaking at 200 rpm. The bacterial culture was then centrifuged at 10,000 rpm for 20 min and cells were resuspended in water. The desired concentration (approximately 1×10^7 cells/ml) was obtained by adjusting the suspension according to the standard curve with a UV/VIS spectrophotometer (Carry 100, Varian, Melbourne, Australia). Soil surface of each pot was covered with 10ml of bacterial suspension (10 ml of sterile distilled water for a check) prepared as described above. The infected plants were moved to greenhouse for assessing the disease severity. After a week, disease severity was assessed by a visual estimation of the percentage of diseased areas. This experiment was conducted twice using a randomized block plot design with three replications.

Statistic analysis

Analysis of variance of all data was performed and the least significant-difference (LSD, α=0.05) test was used for means separation,using PROC ANOVA (SAS Institute Inc., Cary, NC).

RESULTS

For the selection of potential antagonists which inhibit the growth of turfgrass pathogenic fungi, over 100 isolates of bacteria were initially obtained from rhizosphere soil in golf courses. From the results of *in vitro* screening, four candidates producing inhibition zones of 10mm or more against *R. solani* AG-1(1B) or *S. homoeocarpa* were selected among these isolates. They also showed antagonistic activities against four turfgrass pathogenic fungi, *R. cerealis*, *T. incarnata*, *R. solani* AG-1(1B) and *S. homoeocarpa* (Table 2 and Fig. 1). Isolate YDU-05G-3-3 usually showed highest antifungal activities against the fungal pathogens.

The antagonists were gram-negative bacteria with short rod features and identified as *Pseudomonas* spp. based on biochemical characteristics. The similarity test of these isolates using 16S rRNA sequencing confirmed the isolates as a same genus (Table 1).

In vivo trial, application of the antagonistic bacteria in potting soil was effective against *R. cerealis*, *R. solani* AG-1(1B) and *S. homoeocarpa*, which are turfgrass pathogens, causing yellow patch, brown patch, and dollar spot, respectively (Table 3).

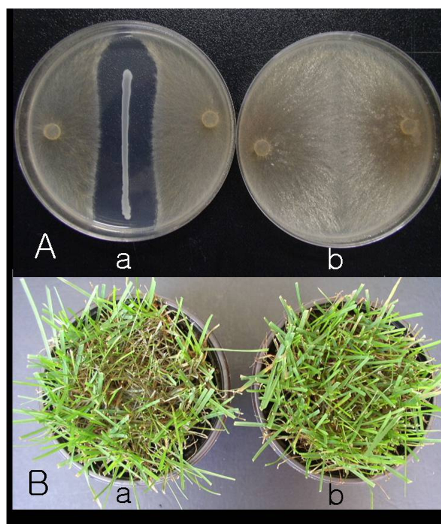
Antagonistic effects of the bacterial isolates were varied depending on fungal species, host plant, and disease pressure. For example, YU-F6F-1 was effective on brown patch, whereas YU-O5G-1-3 effectively controlled dollar spot and yellow patch. Disease severities on kentucky bluegrass were usually lower than those of creeping bentgrass. The control values of the bacteria tested were also low under higher disease pressures.

Table 2. Antifungal activity of four *Pseudomonas* spp. isolates collected from rhizosphere of turfgrass against four turfgrass pathogens

Isolates	Inhibition zone (mm) [z] against			
	Rhizoctonia cerealis	*Rhizoctonia solani* AG-1(1B)	*Sclerotinia homoeocarpa*	*Typhula incarnata*
YU-O5G-1-3	16.4a [y]	10.5bc	11.3b	23.2a
YU-O5G-3-3	15.9a	12.0a	15.3a	22.7a
YU-F3R-3	16.0a	9.8c	12.5b	22.3a
YU-F6F-1	16.4a	11.0b	11.3b	22.7a
$LSD_{0.05}$	1.1	0.9	1.7	2.5

[z] The inhibition zone was determined by the paired bioassay on potato dextrose agar (PDA) medium after inoculation. Distance between the edges of the bacterial agar pieces (about 5 mm square) and fungal mycelium on PDA plates after two weeks or four weeks (15℃ incubation for *Typhula incarnata*) incubation at 25℃

[y] Values in each column with different letters show significant difference at P=0.05 according to the Fisher's protected least significant difference (LSD) test. Data are means of two experiments.



Dual culture (A) and *in vivo* (B) tests for antagonistic evaluation of *Pseudomonas* spp. against a turfgrass pathogenic fungus, *Rhizoctonia solani* AG-1(1B). A, an inhibition zone of mycelial growth of *R. solani* AG-1(1B) by *Pseudomonas* spp. YU-F6F-1 (a) and check (b). B, diseased plants (cv. Prosperity) caused by *R. solani* AG-1(1B) (a) and suppressed plants by *Pseudomonas* spp. YU-F6F-1 (b).

Fig 1.

Table 3. Suppression effect on turfgrass diseases by four *Pseudomonas* spp. isolates collected from rhizosphere of turfgrass plants

Isolates	Brown patch				Dollar spot				Yellow patch			
	Creeping bentgrass		Kentuky bluegrass		Creeping bentgrass		Kentuky bluegrass		Creeping bentgrass		Kentuky bluegrass	
	DS [z]	CV [y]	DS	CV	DS	CV	DS	CV	DS	CV	DS	CV
YU-O5G-1-3	62.5c [x]	27.1	21.7c	66.7	36.7d	55.6	0.0e	100.0	32.5d	64.5	1.7b	85.7
YU-O5G-3-3	71,7b	16.5	58.3b	10.3	47.5c	42.4	7.0c	44.5	55.0c	40.0	4.2b	42.9
YU-F3R-3	62.5c	27.2	24.2c	62.8	62.5b	24.2	4.2 d	67.0	62.5b	31.8	4.2b	42.9
YU-F6F-1	52.5d	38.8	7.5d	88.5	58.3b	29.3	10.0b	19.6	55.8c	39.1	1.7b	85.7
Check	85.8a	-	65.0a	-	82.5a	-	12.5a	-	91.7a	-	8.2a	-
LSD $_{0.05}$	5.3	-	5.8	-	8.4	-	2.2	-	5.8		3.0	

[z] Disease severity (DS) was assessed by a visual estimation of the percentage of diseased areas.
[y] Control value (CV) was calculated from the equation (1-DS of treatment/DS of control) × 100.
[x] Values in each column with different letters show significant difference at *P*=0.05 according to the Fisher's protected least significant difference (LSD) test. Data are means of two experiments.

DISCUSSION

Since several *Pseudomonas* were approved as registered biopesticides in USA in the 1990s, commercial products originated from *Pseudomonas* spp. have been used as biological control agents of many plant diseases (Handelsman and Stabb, 1996; Stockwell and Stack, 2007 Weller, 2007).

In the present study, four *Pseudomonas* spp. isolates evaluated from *in vitro* and *in vivo* study had antagonistic effects against turfgrass soilborne pathogenic fungi, suggesting that the isolates can be a prospective candidate for biocontrol agent against the diseases.

The antagonistic effects of the *Pseudomonas* spp. isolates against turfgrass pathogenic fungi varied depending on fungal species. The fact can imply to be influenced by different mode of action, indicating that control effects of the antagonists could be variable depending on target fungal species in the field (Shim et al., 2006; Stockwell and Stack, 2007). Lee et al. (1998) reported that there are big differences in the antagonistic activity of soil microorganisms collected.

Usually, disease severities on kentucky bluegrass were lower than those of creeping bentgrass. A possible interpretation for the result may be the slower infection caused by higher plant height and fewer tiller in pots of kentucky bluegrass plants than in the pots of creeping bentgrass plants (Chang et al., 2007). The control value of the bacteria was usually low under high disease pressure, suggesting that variation in disease pressure can play a critical role in the success of disease management

strategies (Jung et al., 2008).

Interestingly, there are differential interactions between bacterial isolates and turfgrass pathogens, based on control values. For example, YU-F6F-1 was effective on brown patch, whereas YU-O5G-1-3 effectively controlled dollar spot and yellow patch, suggesting that the result may be a good indication to overcome the ineffectiveness of single biocontrol agent under diverse environmental conditions, applying them in mixture or treating more than a biocontrol agent at a given time (Guestsky et al., 2001). Guestsky et al. (2001) reported that introduction of two or more biocontrol agents to the rhizosphere with complicate ecological environment requirement result in reducing the variability and increasing the reliability of biological control.

Further research should focus on field evaluation to confirm the current results. Identification at the species level and knowing the mode of action of the candidates are very important for the next steps such as mass production and determination of formulation type (Schisler et al., 2004 Steddom and Menge, 2001). *Pseudomonas* spp. generally includes antibiosis, parasitism, competition, induced resistance, and production of hydrolases as mode of actions in the interaction with plant pathogen (Handelsman and Stabb, 1996 Weller, 2007). If the antifungal substances are identified and the mode of action is well elucidated, the compounds will have the potential to control different plant diseases.

국문 요약

본 연구에서는 골프장에서 문제가 되는 주요 병의 방제가능한 생물학적 제제를 개발하기 위하여 실시하였다. 강원도 소재 골프장의 잔디 근권토양으로부터 채취한 100여개의 미생물로부터 4개의 길항 미생물을 선발하였다. 배지를 이용한 저지원 실험에서 4개의 균주는 한국 골프장에서 문제가 되고 있는 옐로우패취(*Rhizoctonia cerealis*), 브래운패취(*R. solani* AG-1(1B)), 동전마름병(*Sclerotinia homoeocarpa*), 그리고 설부갈색소립균핵병원균 (*Typhula incarnata*)에 대하여 효과적이었다. 옐로우패취, 브래운패취, 동전마름병을 대상으로 온실실험 결과, 4개의 균주는 병의 종류, 기주 식물체 및 병 발생 정도에 따라 효과가 달랐지만 한개 혹은 그 이상의 병에 대해 60% 이상을 방제가를 기록하였다. 4개의 균주는 생화학적 특성과 16S rRNA 분석을 통해 *Pseudomonas* spp.로 밝혀졌다. 현재 4개의 균주는 적정 배양조건과 제제화를 위한 연구 중에 있다.

주요어 : 길항성, *Pseudomonas*속 세균, 잔디 병

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