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Serratia plymuthica Strain A21-4: A Potential Biocontrol Agent Against Phytophthora Blight of Pepper

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A promising biocontrol agent, A21-4, against Phytophthora blight of pepper was selected from 351 bacterial isolates collected from rhizosphere soils and roots of onion (*Allium fistulosum* L.). The isolate A21-4 was identified as *Serratia plymuthica* based on its 16S rRNA sequence and key characteristics as compared with that of an authentic culture of *S. plymuthica* (ATCC No. 6109D01). The isolate readily colonized on roots of various crops including pepper when inoculated on seed and root. Strain A21-4 showed narrow spectrum of antibiotic activity, as revealed in its strong inhibitory activity to the genera *Pythium* and *Phytophthora*, but not to *Fuasrium* and *Rhizoctonia*. In pot experiments, none of the pepper seedlings treated with A21-4 were infected by *Phytophthora capsici*, while 86% of the control plants were killed by the pathogen.

Keywords : biocontrol, pepper, root colonization, *Serratia plymuthica.*

The soil-borne pathogen *Phytophthora capsici* is widely spread throughout Korea and causes various diseases on pepper, tomato, cucumber, watermelon, and melon (Jee et al., 2000). The damages on these crops are frequently severe under conditions favorable to the pathogen. In particular, Phytophthora blight of pepper is the most destructive among the diseases. Hence, it is considered as the principal limiting factor in pepper cultivation in Korea (Jee et al., 2000; Hwang and Kim, 1995).

Fungicide treatment is the main control against the disease. However, chemical treatment is often not satisfactory and generates undesirable effects to the environments (Jee et al., 1988). Although numerous attempts have been made to control the disease by biological means, most of them were not practically feasible. The main reason for the failure was insufficient population of the biocontrol agents to suppress the pathogens in the soil throughout the growing season.

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Root colonizing ability of the biocontrol agent is the prime requirement to protect the root from soil-borne pathogens (Weller, 1988). A bacterial isolate designated as A21-4 that readily colonized on plant root and was antagonistic to *P. capsici* was selected. The isolate was identified and its potential as a biocontrol agent against Phytophthora blight of pepper was evaluated in this study.

Materials and Methods

Isolation of antagonistic microorganisms. To obtain bacterial isolates antagonistic to *P. capsici*, roots of welsh onion (*Allium fistulosum* L.) and onion (*Allium cepa* L.) were collected from 26 different locations in major cultivation areas in the southern parts of Korea. The roots were washed and macerated in a sterile mortar and pestle. The roots were suspended in 30 ml of 0.1M $MgSO_4$ solution in a test tube. Then, 0.1 ml of the preparation was plated on 1/10 Tryptic Soy Agar (TSA) for isolating a single colony. Bacterial colonies grown on 1/10 TSA after 3 days of incubation were selected randomly by using sterile toothpicks for further tests.

The isolates selected were dabbed on eight spots on a petri plate containing 20 ml of potato dextrose agar (PDA) with equal spacing around the perimeter. Six (6) mm diameter mycelial disk of *P. capsici* grown on PDA at 28°C for 3 days was placed at the center of the plate. Inhibition zones of mycelial growth of *P. capsici* were rated 5 days after incubation at 28°C. The bacterial isolates showing antifungal activity to *P. capsici* were selected and stored at -70°C in TSB with 20% glycerol. Rifampicin resistant clones were spontaneously obtained from cultures on TSA medium supplemented with 100 μl/l of rifampicin.

Identification of selected bacterial isolates. Taxonomic schemes and criteria for the identification of A21-4 followed Bergey's Manual of Systematic Bacteriology. Key characteristics of A21-4 were compared with that of an authentic isolate of *Serratia plymuthica* obtained from ATCC (ATCC No. 6109D01). For the confirmation, 16S rRNA sequence of A21-4 was analyzed and aligned with the reference 16S rRNA gene sequence using NCBI BLAST search analysis. Chromosomal DNA of A21-4 was extracted by using the method of Wilson (1987), while PCR was performed by using oligonucleotide primers. PCR denaturation, annealing, and extension temperatures were 92°C, 55°C, and

72°C, respectively. Sequencing vector was pBluescript II SK(+), and compliment bacterium was DH5. Identification of the present isolates was also tried using the fatty acid analysis procedures by Sherlock system (Microbial ID, Inc., Newark, Del) for fatty acid methyl-esters analysis.

***In Vitro* assay of root colonizing ability: Double Layer Filter (DLF) paper method.** Five seeds of various plants were sterilized with 1% NaOCl for 2 minutes, soaked in the suspension of A21-4, placed at the bottom layer of filter paper on a Petri plate, and then covered with another filter paper. After adding 4.5 ml sterile distilled water, the Petri plate was sealed with polypropylene wrap to maintain constant moisture level. The Petri plate was then placed perpendicularly in the cupboard box and incubated at 28°C for 5 days in the dark to allow straight growth of emerging root. After incubation, five 1-cm segments from the root tips were cut and placed into a test tube containing 10 ml of 0.1 M $MgSO_4$. Then, the tube was vigorously stirred on a vortex mixer. The colony forming units (CFU) of each root segment were determined by dilation plate method on the 1/10 TSA.

Root colonization in soil medium. Polypropylene tubes (10×3 cm) were cut into half and filled with sandy loam soil with 17% (w/w) moisture content. Inoculated seeds prepared as described above were placed on the half-cut tube 1 cm below the rim, covered completely, and randomized in a plastic box (12×12×10 cm) containing equal amounts of sandy loam soil and water. The box was covered with the same size of plastic box to maintain constant matric potential, placed in a growth chamber, and incubated for 14 h with light at 25°C and for 10 h in the dark at 15°C for 7 days. After 7 days of incubation, the tubes were carefully separated. Root tips were cut and transferred into a test tube containing 9 ml sterile water. The number of colony forming units (CFU) of A21-4 on the root tip of each segment was determined by plating a series of 10-fold dilution on TSA media containing 10 μl/l rifampicin.

Assessment of disease suppression and enumeration of population density in pot experiment. The roots of 50-day-old pepper seedlings (variety Nok-kwang) obtained from a commercial nursery were inoculated with the bacterial suspension (10^9 cfu/ml) for 1 h by submerging and transplanting into the pots. Pot mix soil (Tosilee Sinangro Co., Korea) was previously inoculated with 10 ml of zoospore suspension (10^4 cfu/ml) of *P. capsici* per 90 g of Tosilee. After transplanting, infected plants showing typical Phytophthora blight symptom were carefully examined. Changes in population density of A21-4 in the root and soil were examined at 7-day interval up to 21 days after transplanting. One (1) gram rhizosphere soil or roots was macerated in a sterile mortar and pestle suspended with 9 ml of 0.1 M $MgSO_4$ and placed on a vortex mixer for 1 minute to take out the bacteria from the soil and root. The population density was determined by dilution plate counting on 1/10-strength TSA containing rifampicin 50 μl/ml.

Results

Isolation of antagonistic microorganisms. A total of 351 isolates was selected based on size and morphology of colonies that appeared on the 1/10 TSA plate from 26 different field samples of onion and welsh onion roots. Among them, 49 isolates showed antifungal activity. The antifungal activity of the isolates with clear zone ranged from 2 mm to 15 mm.

Strain A21-4 seemed to exhibit a narrow spectrum of antifungal activity. It showed much stronger inhibitory activity to genera *Pythium* and *Phytophthora* compared with that of other biocontrol agents which have been previously selected in the laboratory. However, A21-4 did not show any inhibitory effect on the growth of *Fusarium* species and *Rhizoctonia solani* groups (Table 1).

Identification of strain A21-4. The isolate strain A21-4 was identified as *Serratia plymuthica* based on the 16S rRNA sequence using NCBI BLAST search analysis data with 98% identification and physiological characteristics. The physiological and morphological characteristics of strain A21-4 were compared with that of the authentic strain, *S. plymuthica* 6109D01 (ATCC number 53858). Two strains of *S. plymuthica* tested in this experiment showed completely identical characteristics as that of the authentic strain (Table 2).

Root colonization of strain A21-4. The root colonization of strain A21-4 on cucumber, watermelon, barley, rice, Chinese cabbage, tomato, and pepper was determined by using the double-layered filter paper method and the soil medium. A21-4 readily colonized the roots of all the plants tested. The population density of A21-4 analyzed through the filter paper method was over 10^4 cfu/cm root tip. In the

Table 1. Antifungal activity of *S. plymuthica* A21-4 and previously selected biocontrol agents against fungal root pathogens

Strains		Inhibition zone (mm)			
		Pythium	*Phytophthora*	*Rhizoctonia*	*Fusarium*
Serratia plymuthica	A21-4	14.0	15.3	1.1	1.0
Pseudomonas fluorescens	MC07[a]	6.5	7.2	7.0	6.8
Pseudomonas fluorescens	M45[a]	4.0	7.1	6.5	6.5
Paenibacillus polymyxa	E681[a]	7.8	7.9	10.5	11.0
Paenibacillus polymyxa	G157[a]	7.6	7.8	10.6	10.9

[a]Biocontrol agents which have been previously selected in the authors' laboratory.

Table 2. Comparison of the characteristics of antagonistic bacterial isolate A21-4 with an authentic isolate, *Serratia plymuthica* 6109D01

	S. plymuthica[a]	A21-4	6109D01
Gram reaction	–	–	–
Cell morphology	Straight rod,	Straight rod	Straight rod
Flagella	Peritrichous flagella	Peritrichous flagella	Peritrichous flagella
Cell size	0.6-1 μm, 1.2-3 μm	0.7-1 μm, 1.4-2.5 μm	0.7-1 μm, 1.5-2.5 μm
Anaerobic growth	+	+	+
Pigment	–	–	–
Catalase	+	+	+
Methyl Red	–	–	–
Gelatinase	+	+	+
Urease	–	–	–
Arginine dihydrolase	–	–	–
Starch hydrolysis		+	+
Chitinase	+	+	+

[a] As described in Bergey's Manual of Systematic Bacteriology.

Table 3. Population density of *Serratia plymuthica* A21-4 on the root segment of various crops analyzed by double layered filter paper method and soil medium

Plants tested	Population Density	
	Filter paper (Log cfu/cm root)	Soil medium (Log cfu/g roots)
Cucumber	4.00	5.60
Watermelon	4.89	5.00
Barley	4.00	6.26
Rice	4.00	5.89
Chinese cabbage	4.92	5.02
Tomato	4.37	5.25
Pepper	4.56	5.95

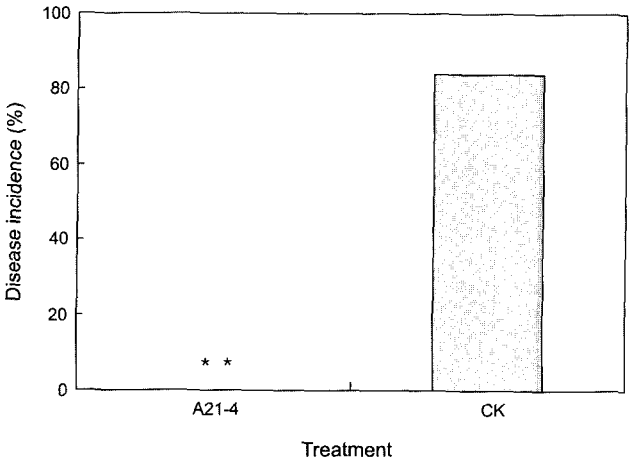

Fig. 1. Suppression of Phytophthora blight of pepper treated with *Serratia plymuthica* A21-4. The diseased plants were recorded 10 days after inoculation in the pot. The asterisks on top of the bars indicate significant difference by Duncan's multiple range test ($p = 0.01$).

soil medium method, population density of A21-4 reached up to 10^5 cfu/g roots in all the plants tested (Table 3).

Disease suppression and population density in pot experiment. In the control plots, 83.7% of pepper plants were infected with the disease 10 days after transplanting. Meanwhile, none of the plants in the plot treated with strain A21-4 were infected (Fig. 1). Population density of A21-4 in the pepper growing soil was maintained at over 10^6 cfu/g soil after 21 days of bacterial treatment. Population density of A21-4 on pepper root decreased slightly 14 days after transplanting. However, it increased afterwards and sustained over 10^6 cfu/g root (Fig. 2).

Discussion

Antagonistic root-associated bacteria are an important functional group of beneficial bacteria responsible for the control of soil-borne pathogens (Weller, 1988). In general, root colonizing antagonistic bacteria show a wide range of antifungal activity to major soil-borne pathogens such as *Pythium*, *Phytophthora*, *Fusarium*, and *Rhizoctonia* (Yeom and Park, 1995). In this study, however, *Serratia plymuthica* strain A21-4 strongly inhibited *Pythium* and *Phytophthora* but not other fungal pathogens such as *Fusarium* and *Rhizoctonia*. The isolate also did not inhibit bacterial plant pathogens (data not shown). This suggests that strain A21-4 is different from other root colonizing antagonistic strains in terms of its spectrum of antagonistic activity.

Control of soil-borne diseases and increased plant yields by biocontrol agents are well-documented (Park et al., 2000). Effective agents for the control of root-infecting pathogens may be introduced either on seeds or as soil treatment if the agent is rhizosphere competent and is able

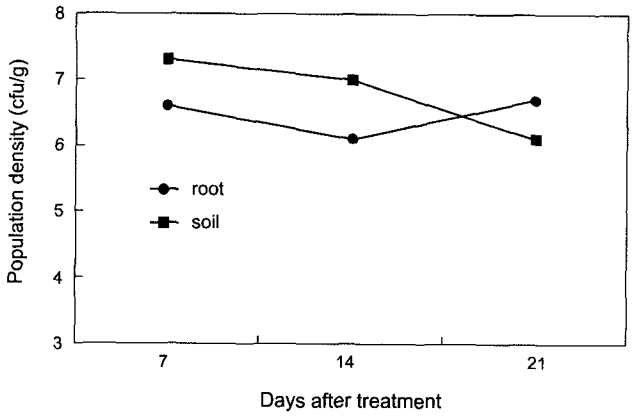

Fig. 2. Population changes of *Serratia plymuthica* A21-4 in the rhizosphere soil and root of pepper inoculated with *Phytophthora capsici*.

to effectively colonize roots. Both bacterial and fungal biocontrol agents may be rhizosphere competent, but it is believed that the long-term efficacy of individual biocontrol strains is unlikely. Strain A21-4 maintained the population densities in rhizosphere soil up to 10^6 cfu/g soil after 21 days. It also increased the population in the rhizoplane by the end of the experiment.

Many promising biocontrol agents such as *Burkholderia cepacia*, *Pseucomonas fluorescens*, *Trichoderma harzianum*, *Bacillus* sp., and *Enterobacter agllomerans* have been reported previously for the control of Phytophthora blight of pepper (Park and Kim, 1989; Hwang and Kim, 1995). However, it was proven in this study that *S. plymuthica* A21-4 could be a promising biocontrol agent for Phytophtora blight of pepper since it showed strong antibiotic activity and high root affinity to pepper. Further studies on the utilization and improvement of the bacterial activity are in progress.

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