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Induction of Systemic Resistance in Watermelon to Gummy Stem Rot by Plant Growth-Promoting Rhizobacteria

Yong Hoon Lee*, Wang Hyu Lee[1], Hyeong Kwon Shim and Du Ku Lee

Division of Plant Environment, National Honam Agricultural Experiment Station, Iksan 570-080, Korea

[1]Faculty of Biological Resources and Science, Chonbuk National University, Chonju 560-756, Korea

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The selected five plant growth-promoting rhizobacteria (PGPR) strains, WR8-3 (*Pseudomonas fluorescens*), WR8-6 (*P. putida*), WR9-9 (*P. fluorescens*), WR9-11 (*Pseudomonas* sp.), and WR9-16 (*P. putida*) isolated in the rhizosphere of watermelon plants were tested on their growth promotion and control effect against gummy stem rot of watermelon. Strains, WR8-3 and WR9-16 significantly increased stem length of watermelon, and there was a little increase in leaf area, fresh weight and root length when strains, WR8-3, WR9-9 and WR9-16 were treated. Generally, seed treatment was better for plant growth promotion than the soil drench, but there was no significant difference. Seed treatment and soil drench of each bacterial strain also significantly reduced the mean lesion area (MLA) by gummy stem rot, but there was no significant difference between the two treatments. At initial inoculum densities of each strain ranging from 10^6 to 10^{15} cfu/g seed, approximately the same level of disease resistance was induced. But resistance induction was not induced at the initial inoculum density of 10^3 cfu/g seed. Resistance was induced by treating the strains, WR9-9, WR9-11 and WR9-16, on all of four watermelon varieties tested, and there was no significant difference in the decrease of gummy stem rot among varieties. Populations of the strains treated initially at log 9-10 cfu/g seed, followed with a rapid decrease from planting day to 1 week after planting, but the population density was maintained above log 5.0 cfu/g soil until 4 weeks after planting. Generally no or very weak *in vitro* antagonism was observed at the strains treated excepting WR9-11. Rifampicin-resistant bacteria which had been inoculated were not detected in the stems or leaves, which suggesting that the bacterium and the pathogens remained spatially separated during the experiment. This is the first report of resistance induction in watermelon to gummy stem rot by PGPR strains.

Keywords **:** *D. bryoniae,* ISR, PGPR, rhizobacteria, watermelon.

*Corresponding author.
Phone) +82-63-840-2293, FAX) +82-63-840-2112
E-mail) Leeyh0@rda.go.kr

Plants are equipped with a variety of mechanisms that enable them to defend themselves against pathogens (Van Loon et al., 1998; Keen, 1992). As one of those mechanisms, disease resistance can be induced by many ways : by colonization of the rhizosphere with selected plant growth-promoting rhizobacteria (PGPR; Alstrom, 1991, 1995; Van Peer et al., 1991; Wei et al., 1991); by infection with a pathogen (Ross, 1961; Kuc and Richmond, 1997); by treatment with chemicals (Kessmann et al., 1994), or inactive forms and cell constituent of the same or different pathogens (Waspi et al., 1998; Enkel et al., 1999; Schweizer et al., 2000). Generally, induced resistance is systemic, because the defensive capacity increased not only in the primary infected plant parts, but also in non-infected, spatially separated parts (Ross, 1961; Ryals et al., 1996; Sticher et al., 1997). And induced resistance can even be transmitted to regenerants from tissue cultures of induced plants (Tuzun and Kuc, 1987).

Prospective biological control agents have been expected to comprise a major part of the natural microflora. Nevertheless, because only a small percentage of the total bacteria may be beneficial, a large number of bacteria may need to be screened (Xu and Gross, 1986). Among thess beneficial microorganisms, fluorescent pseudomonads which characterized by the production of yellow-green pigments that fluoresce under UV light and function as siderophores are one of the most widely investigated bacteria. There are many reports in recent years that plant growth-promoting *Pseudomonas* spp. induce systemic resistance to bacterial (Alstrom, 1991, 1995), fungal (Van Peer et al., 1991; Van Peer and Schippers, 1992; Zhou et al., 1992; Zhou and Paulitz, 1994), and viral pathogens (Maurhofer et al., 1994; Raupach et al., 1996).

In response to environmental and health concerns about extended use of chemicals, there is considerable interest in finding alternative control methods for use in integrated disease management strategies. Recently, the potential of PGPR-mediated induced systemic resistance (ISR) as a tool in integrated disease management is becoming more widely recognized, especially the information regarding the occurrence of the phenomenon for an increasing number of

plant-pathogen interactions and knowledge of the underlying mechanisms of the resistance responses become more abundant, and the necessity of these kinds of research also has been increasing.

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) is one of the most extensively planted fruit crops in greenhouses and fields of Korea. The gummy stem rot caused by the soilborne fungus *Didymella bryoniae* is one of the most serious diseases of watermelon and is a major factor that limits production in many areas.

The objective of the present study was to evaluate the ability of selected PGPR strains isolated in the rhizosphere of watermelon on plant growth promotion of watermelon and control effect against watermelon gummy stem rot caused by *D. bryoniae*.

Materials and Methods

Isolation and inoculum preparation of PGPR strains. The selected 5 PGPR strains, WR8-3 (*P. fluorescens*), WR8-6 (*P. putida*), WR9-9 (*P. fluorescens*), WR9-11 (*Pseudomonas* sp.), and WR9-16 (*P. putida*) were used.

To isolate bacteria, roots (2 g per sample) and soils (10 g per sample) in the rhizosphere of watermelon plants grown in a field suppressive or non-suppressive to gummy stem rot caused by *D. bryoniae* were washed in a 10-fold (w/v) quantity of sterile distilled water by agitation for 30 min on a rotary shaker (150 rpm). Aliquots (0.1 ml) from appropriate serial dilutions were plated on King's medium B (KB) agar. Plates were incubated for 48 hr at ambient temperature (approximately 25°C), and then UV-fluorescent colonies of *Pseudomonas* spp. were isolated, and tested, and then finally above five PGPR strains selected for more study.

For long-term storage, the strains were maintained at -70°C in tryptic soy broth (TSB, Difco Laboratories, Detroit) with 20% glycerol or at 5°C on KB agar.

Inoculation on seed and soil. For experimental use, bacteria were incubated in TSB with shaking at 150 rpm at 28°C for 24 hr. Cultures were centrifuged at 2,000 g for 10 min, and then the supernatant was discarded and the final concentration was adjusted to $10^{9\text{-}10}$ cfu/g seed of watermelon.

For seed treatments, watermelon seeds, pregerminated on moist filter paper for 48 hr, were added to the bacterial suspension in 1% sodium alginate and incubated for 3 hr with slow stirring. Inoculated seedlings with primary roots 3 to 5 mm long were each planted in a pot containing approximately 100 g of agricultural soil (Baroker^{R}, Seoul Agricultural Materials Co.).

For soil inoculation, single colonies grown overnight in TSB as described above, centrifuged, the pellet was resuspended in 50 ml of sterile distilled water, and then the bacterial suspension was poured in the pregerminated watermelon seed planted pot.

Plants were maintained in the greenhouse at temperatures of 22-35°C, at a relative humidity of approximately 70%.

Biocontrol assay by PGPR strains. Three weeks after planting, the first and second leaves of watermelon seedlings were inoculated by placing ten 10 μl droplets of a suspension of *D. bryoniae* (10^5 propagule/ml). Inoculated plants were placed in 100% humidity at 25°C for 24 hr and then moved to a greenhouse. The size of lesions was determined 7 days after challenge inoculation by measuring their length and width, and calculating the mean lesion area (MLA) in square millimeter.

Watermelon varieties. Watermelon variety Geumchon was used in all of this experiment. And, to investigate effects of varieties on PGPR-mediated ISR, four watermelon varieties, namely Dalgona, Sambok, and Festival including Geumchon were tested.

Effect of inoculum density. To investigate the threshold population size and effect of inoculum density on the control of gummy stem rot, each PGPR strain inoculated on the seed at the concentration of log 3-15 cfu/g seed as described above.

Rhizosphere colonization. Rifampicin-resistant mutants of strains were selected on TSA with 100 μg of rifampicin per ml. For inoculation, the mutants were incubated in TSB with 100 μg of rifampicin per ml with shaking at 150 rpm at 28°C for 24 hr and were treated on seeds of watermelon as described above.

Colonization of the rhizosphere of plants by rifampicin-resistant PGPR strains was examined 1, 2, 3, 4 weeks after inoculation. Three pots were randomly picked on selected sampling days. Unattached soil was gently removed from the roots, weighed, and shaken vigorously for 1 min in 100 ml of 10 mM phosphate buffer. Appropriate dilutions were plated onto KB agar supplemented with cycloheximide (100 mg/l), ampicillin (50 mg/l), chloramphenicol (13 mg/l), and rifampicin (150 mg/l), which is selective for rifampicin-resistant, fluorescent *Pseudomonas* spp. (Geels and Schippers, 1983). After overnight incubation at 28 °C, the number of rifampicin-resistant cfu/g of root fresh weight was investigated. Root colonization was analyzed by analysis of varience on logarithmically transformed data after checking normally.

***In vitro* antagonism assay.** To investigate the possibility that the observed protection was caused by a direct effect on the pathogen, the strains were tested on various media for their ability to inhibit *D. bryoniae* in potato dextrose agar (PDA), oat meal agar (OMA), KB, corn meal agar (CMA), V-8 juice agar (V-8) plates.

Mycelial plugs (5 mm) of *D. bryoniae* were deposited in the center of the plates and incubated for 1 day, and then each of the strains were inoculated approximately 3.5 cm apart from the fungi. Plates were sealed with parafilm, incubated at 28°C and inhibition zones were measured 5 days after incubation. Five replicate plates were used for each strain on each medium. The mycelial growth rate was compared with the control without bacteria.

Possible systemic colonization of the plants. To determine possible spreading of strains, stems and leaves of watermelon that had been assessed for rhizosphere colonization were examined. Each plants were surface disinfected first with 70% ethanol for 30 seconds, then rinsed three times with sterile distilled water. Afterward, stems and leaves were ground with a sterile mortar and pestle in 5 ml of a 0.1 M phosphate buffer (pH 7.0). Appropriate serial dilutions were plated on KB agar supplemented with cycloheximide (100 mg/l), ampicillin (50 mg/l), chloramphenicol (13 mg/l), and rifampicin (150 mg/l).

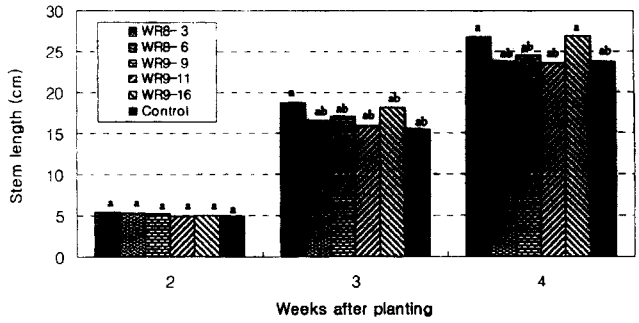

Fig. 1. Time course effect of selected five PGPR strains on the growth of stem of watermelon. Seeds of watermelon variety Geumchon, pregerminated on moist filter paper were treated with each PGPR strain (10^{9-10} cfu/g seeds) in 1% sodium alginate. Data are means of single representative trial consists of 10 replicate plants, obtained 2, 3, and 4 weeks after planting. Values followed by common letter are not significantly different at the 5% level according to Duncan's multiple range test (DMRT). LSD at 5% level was 0.94, 2.69 and 2.58 after 2, 3 and 4 weeks of planting, respectively.

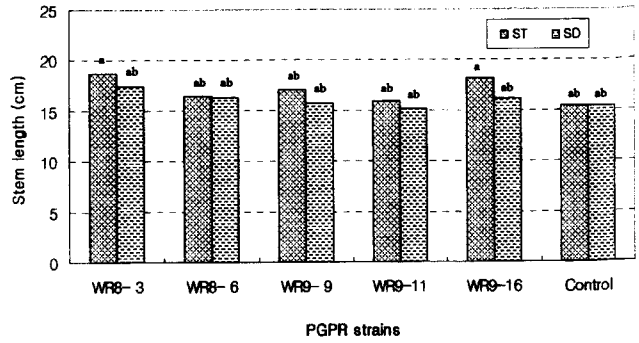

Fig. 2. Plant growth promotion according to the treatment methods. For seed treatment (ST), watermelon seeds, pregerminated on moist filter paper for 48 hr, were added to the bacterial suspension in 1% sodium alginate and incubated for 3 hr with slow agitation. For soil drench (SD), bacterial suspensions were poured (80 ml per pot) in the watermelon seeds planted pot. Data are means of single representative trial consists of 10 replicate plants, and stem length was investigated 3 weeks after planting. Values with the same letter are not significantly different according to DMRT (P=0.05).

Results

Watermelon growth promotion by selected PGPR strains. The strain WR8-3 and WR9-16 significantly increased stem length of watermelon 3 and 4 weeks after planting (Fig. 1). The strain WR9-9 also increased the growth a little, but there was no significant difference when compared with non inoculated control. The other two strains, WR8-6 and WR9-16, showed similar stem length as control. The other experiments which were not represented here showed similar results with a few exceptions.

There was a little increase in leaf area, fresh weight and root length, when the strains, WR8-3, WR9-9 and WR9-16 were treated. But the significant increase up to 10% was observed only in fresh weight when the strains, WR8-3 and WR9-16 were treated (Table 1).

Generally, the seed treatment showed a little increase than the soil drench. As shown in Fig. 2. The significant increase was observed only when the strains, WR8-3 and WR9-16 were treated on seed. But there was not much difference in plant growth promotion between the two treatments.

Effect of bacterial strains on disease reduction. The incidence of gummy stem rot was significantly reduced when the plants had been bacterized with selected PGPR strains (Fig. 3). Three strains, WR9-9, WR9-11, and WR9-16 significantly reduced lesion diameter in all trials. In general, seed treatment with the PGPR strains reduced the MLA

Table 1. Effects of selected PGPR strains on fresh weight, leaf area, and root length of watermelon 3 weeks after planting[a]

Strains	Leaf area (cm^2)	Fresh weight (g)	Root length (mm)
WR8-3	167.7 (106)[b] a[c]	14.5 (109) a	129.0 (104) a
WR8-6	158.0 (100) a	13.6 (102) b	125.6 (102) a
WR9-9	161.8 (102) a	13.9 (105) ab	126.6 (102) a
WR9-11	157.2 (99) a	13.4 (101) b	123.6 (100) a
WR9-16	166.0 (105) a	14.6 (110) a	128.3 (104) a
Control	158.0 (100) a	13.3 (100) b	123.6 (100) a
LSD (5%)	11.02	7.08	7.26

[a] Data are means of single trial consists of 10 replicate plants, and investigated 3 weeks after planting. PGPR strains are treated on seeds.
[b] Relative percentage to that of control.
[c] Values with the same letter are not significantly different according to DMRT (P=0.05).

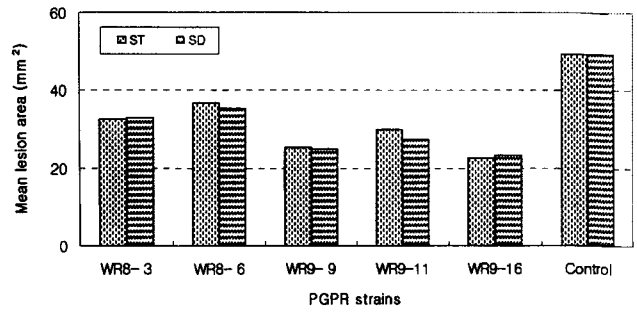

Fig. 3. Reduction of incidence of gummy stem rot caused by *D. bryoniae* by seed treatment and soil drench with each PGPR strains. ST and SD means seed treatment and soil drench, respectively. Three weeks after planting, the first and second true leaves of watermelon seedlings were inoculated with placing ten 10 μl droplets of a suspension of *D. bryoniae* (10^5 propagule/ml), and disease severity was determined 7 days after inoculation. Data are means of single representative trial consists of 10 replicate plants. LSD at 5% level was 12.5.

caused by *D. bryoniae* than soil drench. But, there was not much difference in resistance induction between the two treatments. The other experiments which were not represented here showed similar results with a few exceptions.

When the disease suppression by strains expressed either as a reduction relative to the control, 60 to 70% disease reduction was observed when compared with the non-induced control.

Effect of inoculum density on PGPR-mediated ISR. The relationship between population densities of PGPR strains and disease suppression was investigated by introducing the strains, WR9-9, WR9-11 and WR9-16 on the seed at different initial densities.

Increasing the rhizosphere population density of strains up to approximately 10^6 cfu/g seed resulted in a decrease of MLA. At initial inoculum densities of each strains ranging from 10^6 to 10^{15} cfu/g seed, approximately the same level of disease resistance was induced. There was no significant reduction of disease at initial density of 10^3 cfu/g seed. As shown in Fig. 4, regression analysis demonstrated that significant relationships between the rhizosphere population density of treated strains and the MLA of diseased plants ($Y=0.27X^2-0.80X+69.51$, $R^2=0.90$).

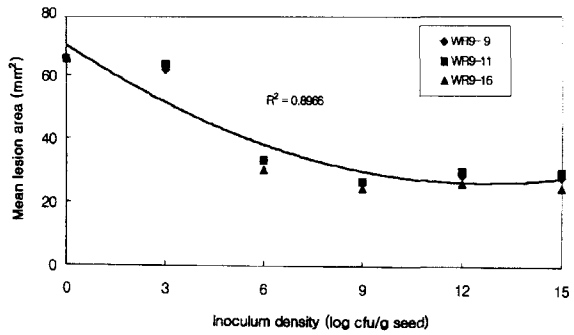

Fig. 4. Relationship between the inoculum density and MLA by strains, WR9-9, WR9-11, and WR9-16. Regression analysis demonstrated a significant relationship ($Y=0.27X^2-0.80X+69.51$). Mean values of single representative result are presented.

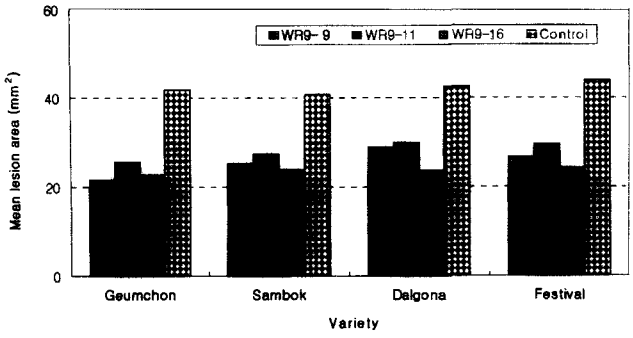

Fig. 5. Disease incidence of different varieties of watermelon which treated with selected PGPR strains, WR9-9, WR9-11, and WR9-16. The PGPR strains were treated on seed. A suspension of *D. bryoniae* (10^5 propagule/ml) was treated 3 weeks after planting and disease severity was determined 7 days after inoculation. Data are means of single representative trial consists of 10 replicate plants. LSD at 5% level was 17.8, 12.2, 10.7 and 12.0 at Geumchon Sambok, Dalgona, and Festival, respectively.

Effects of varieties on PGPR-mediated ISR. Resistance could be induced by treating the strains, WR9-9, WR9-11 and WR9-16 in all of four watermelon varieties tested. And, there was no significant difference in the decrease of gummy stem rot between varieties (Fig. 5). This result means that there was no variety specificity in PGPR-mediated ISR.

Population dynamics of PGPR strains. Efficient root colonization by PGPR strains is critical to the suppression of soilborne plant pathogens. The population dynamics of the PGPR strains from planting to 4 weeks after planting are shown in Fig. 6. Populations of the strains followed a similar pattern with a rapid decrease from planting day to 1 week after planting. But all of the introduced PGPR strains colonized the roots of watermelon resulting in population densities between log 5 and 8 cfu/g of root to 4 weeks after planting. There was no significant difference in colonization capacity between strains. It appears that qualitative rather than quantitative properties can account for their activity.

***In vitro* antagonism and systemic colonization.** *In vitro* antagonism was variable depending on the test medium, but generally no or very weak antagonism was observed when all strains were treated on each agar excepting WR9-11. Growth of *D. bryoniae* was inhibited by WR9-11 in all of the media tested, predominantly on PDA, KB and CMA (Table 2). WR8-6 and WR9-9 showed no *in vitro* antagonism in all of the media. And there was no relationship between *in vitro* antagonism and effectivity in control of gummy stem rot in this test.

No rifampicin-resistant bacteria were detected in the stems or leaves, which indicated that the bacterium and the challenged pathogen remained spatially separated during

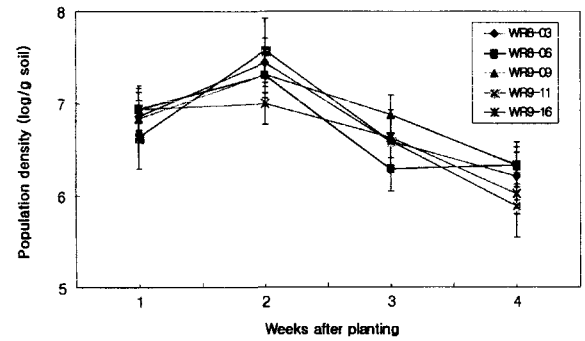

Fig. 6. Time course fluctuations of population density of selected PGPR strains on the root surfaces of watermelon variety Geumchon. Colonization was investigated by using rifampicin-resistant mutants.

Table 2. *In vitro* antagonism of selected PGPR strains against *D. bryoniae* on different culture media

PGPR strain	Zone of inhibition (mm)[a]				
	PDA	V-8	KB	CMA	OMA
WR8- 3	1.0	+	+	1.6	−
WR8- 6	−	−	−	−	−
WR9- 9	−	−	−	+	−
WR9-11	5.4	4.6	7.4	8.6	3.8
WR9-16	0.6	+	0.6	+	−

[a] Antagonism was investigated by zone of inhibition measured in millimeter from bacterial colony to the margine of fungal colony 5 days after inoculation. +; no distinct zone of inhibition but a visible decrease in density of fungal colony near to the bacterial inoculum. −; no visible antagonism. Each value represents the average of 5 replications.

the experiment. Because PGPR strains and the pathogen were spatially separated, control of the disease was not due to antagonism and niche competition. Consequently, the protection resulted from plant-mediated induction of systemic resistance by PGPR strains applied.

Discussion

Induction of resistance is usually accomplished by adding bacterial suspensions to the soil before sowing or at transplanting, or by coating seeds with high numbers of bacteria (Kloepper, 1996). In this test, effects of PGPR application methods were conducted to compare the level of plant growth and ISR resulting from PGPR application by seed treatment and soil drench. The present result shows that seed treatment increased watermelon growth a little more than the soil drench, but there was no significant difference between the two treatments.

ISR-inducing rhizobacteria show variety specificity in regard to eliciting resistance (Van Wees et al., 1997), which indicates that specific recognition is a prerequisite for the activation of the signaling leading to ISR. In carnation, *P. fluorescens* WCS417 reduced incidence of fusarium wilt in the moderately resistant carnation cultivar and less consistently in the susceptible cultivar. In contrast, no genetic differences in inducibility were observed in radish (Leeman et al., 1995), and also reported as strain that has been shown to trigger an ISR response in several plant species, including carnation (Van Peer et al., 1991), tomato (Duijff et al., 1997), and *Arabidopsis* (Pieterse et al., 1996). *P. fluorescens* WCS374 induced a similar level of resistance against fusarium wilt in six cultivars ranging from highly susceptible to relatively resistant. The present result shows that the treated strains showed a similar effect among the varieties, which means that there was no variety specifcity (Fig. 5).

Resistance is commonly induced only when plants are inoculated with a dosage of bacteria that exceeds a threshold population size. No increase in resistance is evident when the dosage of bacteria is increased further (Leeman et al., 1995; Raaijmakers et al., 1995). In this test, as shown in Fig. 4 increasing the rhizosphere population density of PGPR strains on seed up to approximately 10^6 cfu/g seed, plant growth was promoted, and significant increase was observed from the inoculum density of 10^9 cfu/g seed. At least 10^5 cfu/g root appears to be required for induction of systemic resistance in radish by *Pseudomonas* spp. (Raaijmakers et al., 1995), and no disease suppression was occurred when rhizosphere colonization by WCS374 or WCS417 of the roots did not reach this level (Leeman et al., 1995). And also 10^5 cfu/g root of *P. putida* WCS358 is required and this population must be maintained for sustained disease suppression. The present result indicated that threshold density effect was necessary to promote plant growth and induce resistance. But, in this test the significant disease suppression by bacterial treatment was generally observed when MLA in the non-bacterized control treatment was ranged from approximately 25 to 60 mm^2. So, it can be stated that the efficacy of the suppression of gummy stem rot depends on the level of disease incidence and the rhizosphere population density of the strains.

Although total bacterial populations in the rhizosphere can exceed such levels by far, bacterial diversity is enormous and any inducing strain that may be present naturally unlikely to exceed this threshold (Van Loon et al., 1998). So, in addition to the inoculum density, maintenance of rhizosphere population density in the rhizosphere and on roots is considered important aspects of biological control. The present result shows that populations of bacterial strains followed generally a similar pattern with a steady decrease from planting day to 4 weeks after planting. But the population density for these period maintained from log 5.0 to log 8.0, which was relatively high compared with total rhizosphere population and threshold level (Fig. 6). However, in this study no relationship was obtained between disease incidence at later stages and the remaining rhizosphere population densities. So we still need to understand the factors governing the distribution and activity patterns of the inoculated strains. Anyway, the present result shows that the initial rhizosphere population density of strains, which leading to colonization more than threshold level is an important determinant of its efficacy to suppress gummy stem rot.

The best evidence for PGPR-mediated ISR is obtained when the rhizobacterium does not antagonize the pathogen in culture. Thus, in order to prove that resistance is systemically induced, it must be shown that inducing rhizobacteria are absent from the infection site of the pathogen, and that the inducing bacterium and the pathogen remain spatially

separated. (Van Loon et al., 1998). The PGPR strains used in this study showed no or weak *in vitro* antagonism excepting WR9-11. And systemic colonization was not occurred. So, the other mechanisms which still remain to be elucidated might be involved. But the above results meant that the ISR by the PGPR strains must have been plant mediated, which agrees with that of Van Peer et al. (1991) and Wei et al. (1991) because the pathogen was spatially separated.

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