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Factors Relating to Induced Systemic Resistance in Watermelon by Plant Growth-Promoting *Pseudomonas* spp.

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The plant growth-promoting *Pseudomonas* strains, WR8-3 (*Pseudomonas fluorescens*), WR9-11 (*Pseudomonas* sp.) and WR9-16 (*P. putida*), which induced resistance systematically in watermelon to gummy stem rot were investigated on their induced systemic resistance (ISR)-related characteristics. The pyoverdine production was repressed in the standard succinate medium by increasing the concentration of $FeCl_3$. But the iron-binding ability on chrome azurol S agar media (CAS) was observed only in the strains, WR8-3 and WR9-16. When the two strains were mutated, the resulting iron-binding siderophore-negative mutants, WR8-3m and WR9-16m, failed to promote the growth of watermelon and to induce resistance. The strains, WR8-3 and WR9-16, slightly inhibited the growth of *Didymella bryoniae* at a low concentration of $FeCl_3$ on King's medium B, but not to exert control effect. The strain WR9-11 showed antagonism in the concentration of $FeCl_3$ from 0 to 1,000 μM. When the crude lipopolysaccharide of each strain was treated in the rhizosphere of watermelon, mean lesion area was similar to that of the untreated control. The strains, WR9-11 and WR9-16 produced some level of hydrogen cyanide (HCN). Salicylic acid production was not detected in all of the strains.

Keywords **:** *Didymella bryoniae,* ISR, mechanism, PGPR, watermelon .

Bacterial inoculation of seeds or roots of a plant sometimes leads to the growth promotion of the plant and biological control of several kinds of plant pathogens. Mechanisms of these kinds of induced systemic resistance (ISR) are complex and often cannot simply be explained by a single factor. Fluorescent *Pseudomonas* spp. have been reported to control some soilborne pathogens through siderophore-mediated competition for iron, competition for nutrients, or antibiosis (Baker et al., 1986, 1987; Buysens et al., 1996; Schippers, 1992; Van Loon et al., 1998).

Among them, siderophore-mediated activity was thought as an important mechanism for the biocontrol activity of plant growth-promoting rhizobacteria. The fluorescent pseudomonads produce siderophores with high affinities for iron, so deleterious microorganisms were deprived of the iron necessary for their growth (Xu and Gross, 1986). This hypothesis was supported by the observations that treatment with purified pyoverdines had a disease-suppressive effect similar to that of treatment with the producing strain (Kloepper et al., 1980), and that siderophore-negative mutants had no growth-promoting activity (Baker et al., 1986). Fusarium wilt of carnation and radish was controlled by *P. putida* stain WCS358, which secretes a pyoverdine-type siderophore that chelates the scarcely available ferric ion as a ferric (Baker et al., 1986; Geels and Schippers, 1983; Raaijmakers et al., 1995). *P. fluorescens* strain WCS417 was also reported to suppress fusarium wilt in carnation by competition for iron (Duijff et al., 1993; Van Peer et al., 1990).

Bacterial lipopolysaccharide (LPS) which contributes to growth and survival of the bacteria, is also involved in bacterium-plant interactions. Van Peer and Schippers (1992) and Leeman et al. (1995) showed that the outer membrane LPS of strain WCS417r is the main determinant for the induction of ISR against fusarium wilt disease in both carnation and radish. In the systemic protection of carnation against fusarium wilt by *P. fluorescens* WCS417, heat-killed bacteria or the purified bacterial outer membrane LPS were as effective in inducing resistance as were live bacteria.

Hydrogen cyanide (HCN) was also reported as a factor of inducing resistance. For instance, *P. fluorescence* strain CHA0 stimulated plant growth and suppressed root rot, whereas a HCN-mutant did not (Defago et al., 1990).

Notwithstanding these kinds of many reports, the full relationships between disease protection with PGPR-mediated ISR and level of bacterial colonization, and mechanisms are still controversial. Considering the potential of

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ISR as a tool in integrated disease management, the necessity of this kind of research has been increasing. The objective of the present study was to elucidate the mechanisms of the *Pseudomonas* spp. strains in relation to their watermelon growth promotion and resistance induction against gummy stem rot caused by *Didymella bryoniae*. The potential role of siderophore and HCN production, antibiosis and other modes of action reported to confer activity to PGPR were investigated.

Materials and Methods

PGPR strains, watermelon cultivar and challenge inoculation. PGPR strains, WR8-3 (*P. fluorescens*), WR9-11 (*Pseudomonas* sp.) and WR9-16 (*P. putida*) which reported to promote growth and induce resistance in watermelon to gummy stem rot were used (Lee et al., 2000). For experimental use, the bacteria were incubated in trypic soy broth (TSB), cultures were centrifuged, and then the final concentration of bacteria was adjusted to $10^{9\text{-}10}$ cfu/g seed of watermelon. Watermelon seeds, pregerminated on moist filter paper for 48 h, were added to the bacterial suspension in 1% sodium alginate and incubated for 3 h with slow stirring. Inoculated seeds were each planted in a pot containing approximately 100 g of agricultural soil. Watermelon cultivar Geumchon was used in this experiment.

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

Assay on siderophore production and siderophore-mediated activity. PGPR strains were plated on chrome azurol S agar medium (CAS) and incubated at 30°C for 72 h. CAS allowed a rapid identification of siderophore producers and siderophore-negative mutants by the change of coloration (Schwyn and Neilands, 1986).

To obtain siderophore-negative mutants of the strains, WR8-3 and WR9-16 were inoculated in 30 ml of Luria-Bertani medium (LB) and grown for 16 h on a rotary shaker (150 rpm) at 28°C. Of this subculture 5 ml was transferred to the same volume of LB medium and incubated for 6 h under the same condition. A volume of 1 ml was diluted in 9 ml of peptone water (5 g/l NaCl, 1 g/l bactopeptone, 1 ml/l Tween 80) supplemented with 50 μl of saturated MNNG (N-methyl-N'-nitro-N-nitrosoguanidine, Sigma Co.) solution. Samples were incubated for 15 min under constant agitation and cells were removed by centrifugation (16,000 g) at room temperature for 10 min. Cells were washed three times in 9 ml of sterile peptone water to eliminate MNNG residues.

Following these manipulations, siderophore-negative mutants were selected out of 200 colonies, and growth and other phenotypical characteristics were compared with those of the wild type. The pyoverdine production level for the mutant was estimated by measuring the absorbance at 400 nm of the culture supernatant after growth in standard succinate medium (SSM; 6 g K_2HPO_4, 3 g KH_2PO_4, 4 g succinic acid, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 15 g agar in 1 *l* of distilled water, pH 7.0 with NaOH) for 30 h at 28°C. Antagonism in King's B medium (KB), growth promotion and resistance induction were compared with those of wild type.

Influence of iron concentration on inhibition of *D. bryoniae*. To investigate influence of iron concentration on inhibition of *D. bryoniae* by PGPR strains, $FeCl_3$ was added at 0, 1, 10, 100, and 1,000 μM to KB. Mycelial plugs (5 mm) of *D. bryoniae* were deposited in the center of the plates and incubated for one day, and then each strain was inoculated approximately 3.5 cm apart from the pathogen. Plates were sealed with parafilm, incubated at 28°C, and inhibition zones were measured from the margin of the bacterial colonies to the point of *D. bryoniae* 5 days after treatment.

Effect of lipopolysaccharide. Effect of crude LPS was tested as described by Leeman et al. (1995). Cell walls of PGPR strains were isolated from cultures grown overnight in TSB at 28°C. The bacteria were collected by centrifugation and resuspended in 50 mM Tris-HCl plus 2 mM EDTA (pH 8.5). The cells were then sonicated eight times for 15 seconds on ice. Intact cells were removed from the sonicated suspension by centrifugation at 600 g for 20 min, after centrifugation of the supernatant at 8,000 g for 60 min, the pellet of LPS-containing cell walls was resuspended in 10 mM phosphate-buffered saline (pH 7.2) plus 0.01% sodium azide and stored at -70°C until further use. The absence of living bacteria was verified by plating on KB agar plates.

For the bioassay, 15 μl of the crude LPS was diluted in 20 ml of distilled water, and the suspension was drenched on the root of one week old watermelon seedlings. *D. bryonaie* was inoculated 2 weeks after treatment and then disease severity was investigated as described above.

Production of pyoverdine and pyochelin siderophores, and salicylic acid by the PGPR strains. Fluorescence was observed under UV light (365 nm) after 24 h of growth on KB medium at 28°C, and the production level of pyoverdine was estimated by measuring the absorbance as described above.

TLC was performed to assess pyochelin and salicylic acid (SA) production (Buysens et al., 1996). Pyochelin and salicylic acid were isolated by ethyl acetate extractions of acidified supernatants obtained by centrifugation at 2,800 g for 15 min after 30 h culture in 30 ml of casamino acid broth (CAA; 5 g casanino acid, 0.9 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 15 g agar in 1 l of distilled water). After evaporation of the ethyl acetate phase, the dry residue was resuspended in a small volume of methanol and applied to TLC plates (20 × 20 cm, Merck silica gel 60 F_{254} plates, Merck, Darmstadt, Germany), and then developed in mixed solutions (chloroform:acetic acid:ethanol, 90:5:2.5, v/v/v).

Hydrogen cyanide production of the PGPR strains. For the determination of HCN production, the PGPR strains were grown on trypic soy agar (TSA) medium supplemented with 4.4 g/l of glycine, placing filter paper strips soaked in picric acid solution (2.5 g of picric acid, 12.5 g of Na_2CO_3, 1 l of distilled water) in the lid of each Petri dish. Dishes were sealed with parafilm and incubated at 28°C for 3 days. HCN production was indicated by the changes of color. Reactions were scored as none, weak (brown), or strong (reddish brown) for each of the PGPR strains.

Results

Siderophore production and siderophore-mediated activity. The percentage of pyoverdine was reversely related to the concentration of $FeCl_3$ (Fig. 1). The addition of 10 μM of $FeCl_3$ to the SSM repressed production of pyoverdine, and at the concentration of 100 μM, the production of pyoverdine was repressed greatly.

The siderophore-negative mutants, WR8-3m and WR9-16m, could not produce iron-binding siderophores, as revealed by a yellow zone on CAS surrounding the colonies. Pyoverdine production (absorbance at 400 nm) of mutant strains, WR8-3m and WR9-16m, were significantly repressed from 0.56 and 0.61 to 0.25 and 0.14, respectively (Table 1).

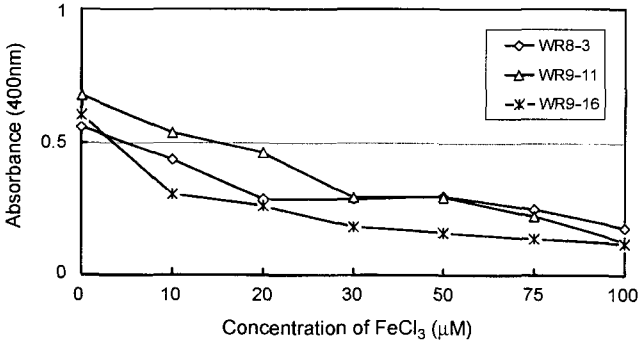

Fig. 1. Effect of $FeCl_3$ concentration on the production of pyoverdine by PGPR strains. The PGPR strains were grown for 30 h in a standard succinate broth at 28°C, and centrifuged at 3,500 rpm. The pyoverdine production level was estimated by measuring the absorbance at 400 nm of the culture supernatant. Data are means of 3 replications.

Table 1. Comparsion of the pyoverdine production, *in vitro* antagonism, plant growth promotion, and control effect between the wild type strains, WR8-3, WR9-16 and their siderophore-negative mutants

Characteristics	WR8-3		WR9-16	
	Wild	Mutant[a]	Wild	Mutant
Pyoverdine[b]	0.56	0.25	0.61	0.14
Antagonism on KB (mm)[c]	+	0	0.6	0
Stem length (cm)[d]	18.8	15.7	18.4	15.5
Mean lesion area (mm^2)[e]	23.7	45.2	20.3	45.9

[a] Mutants were isolated and selected after treatment of MNNG.
[b] Pyoverdine production level was estimated by measuring the absorbance at 400 nm of the SSM supernatant.
[c] Antifungal activity on KB was investigated as zone of inhibition measured in millimeter from bacterial colony to the fungal colony 5 days after inoculation.
[d] Stem length was measured 3 weeks after planting. PGPR strains were treated on seeds before planting. The control was 16.0 cm.
[e] The mean lesion area of control was 50.7 mm^2.

Table 2. Influence of iron concentration on the inhibition of *Didymella bryoniae* by the selected PGPR strains

PGPR Strains	Zone of inhibition in the presence of $FeCl_3$ at μM (mm)[a]				
	0	1	10	100	1,000
WR8- 3	+	+	–	–	–
WR9-11	7.4	7.7	3.3	2.6	2.4
WR9-16	0.6	+	–	–	–

[a] Inhibition zones on KB supplemented with $FeCl_3$ were measured from the margin of the PGPR colonies to the point of *D. bryoniae* 5 days after inoculation. Data represent the mean of 5 replications. +; no distinct zone of inhibition but a visible decrease in density of fungal colony near to the bacterial inoculum. –; no visible antagonism.

And siderophore-negative mutants did not inhibit mycelial growth on KB when compared to the wild types. The mutants also couldn't promote the watermelon growth and induce resistance. When considering all of the results, the ISR by these two strains resulted from iron-binding siderophore.

Effect of iron concentration on *in vitro* antagonism. WR8-3 and WR9-16 which produce siderophore on CAS, slightly, but not to exert control activity, inhibited the growth of *D. bryoniae* at a low concentration of $FeCl_3$. But, no antagonism against the pathogen was observed as iron concentration increased (Table 2). The strain WR9-11 showed antagonism from 0 to 1,000 μM of $FeCl_3$, which indicates that other mechanisms are involved in the antifungal activity.

Effect of Lipopolysaccharide. When the crude LPS of each strain was treated in the rhizosphere of watermelon, the MLA was similar to the noninoculated control (Fig. 2). This result means that the crude LPS of the strains did not induce resistance, and that the LPS of the strains used in this experiment was not the major determinants.

Other PGPR-related characteristics. All of the strains produced fluorescent pigments on KB media, and the level of pyoverdine production was similar in SSM (Table 3). But the iron-binding ability on CAS was observed only in the WR8-3 and WR9-16 strains, which meant that pyoverdine production didn't confirm the iron-chelating ability.

Two of the PGPR strains, WR9-11 and WR9-16, produced some level of HCN. The induced resistance by the strain WR9-11, which produced HCN and pyochelin, and showed strong antifungal activity, could be estimated as the production of HCN, but further studies in detail will be needed.

SA did not produced in all of the PGPR strains, indicating that SA was not involved in ISR of the strains.

Discussion

Fluorescent *Pseudomonas* spp. are among the most effec-

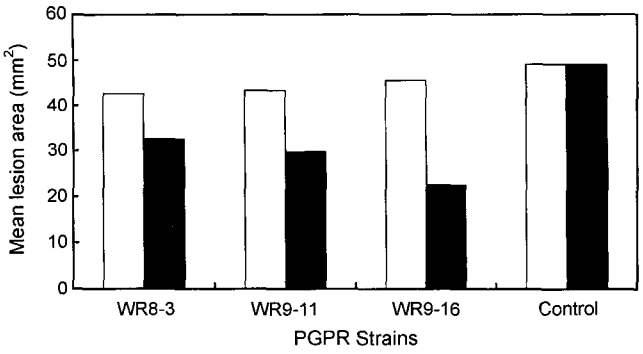

Fig. 2. Reduction of gummy stem rot caused by *Didymella bryoniae* by treatment with LPS of PGPR strains. Twenty millimeter of crude LPS was treated in the rhizosphere of each plant (left bar). PGPR strains were treated on the seeds of watermelon (right bar). Three weeks after planting, the first and second true leaves of watermelon seedlings were inoculated with placing ten 10 μl droplets of a suspension of *D. bryoniae* (10^5 propagule/ml). Data are means of single representative trial consisting 10 replicate plants, and disease severity was determined 7 days after inoculation.

tive rhizosphere bacteria in reducing soilborne diseases in disease-suppressive soils (Weller, 1988). In recent years, selected PGPR strains have been demonstrated to control plant diseases effectively by suppressing pathogens and deleterious microorganisms through siderophore-mediated competition for iron, antibiosis, competition for niche, and production of phytoalexin or extracellular enzymes (Kloepper et al., 1980; Van Loon et al., 1998; Van Peer et al., 1991). In spite of major advances in the understanding of the protective role of PGPR, their activity has remained controversial.

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 bacteria and the pathogens remain spatially separated for the duration of the experiment (Van Loon et al., 1998). The PGPR strains used in this study showed a slight *in vitro* antagonism which was maybe by iron-binding ability as shown in Table 2, but not to exert control activity excepting strain WR9-11. And no systemic colonization occurred (Lee et al., 2000).

Some of the rhizobacteria have been found to excrete siderophores, iron-chelating molecules, in the rhizosphere and under conditions of iron deficiency in culture (Baker et al., 1986, 1987; Leeman et al., 1996; Meyer, 1992). And, in a number of cases, plant growth promotion and biological control against plant pathogens has been attributed to siderophore-mediated iron deprivation of deleterious microorganisms. However, the exact role of siderophore in the

Table 3. PGPR-related characteristics of the PGPR strains

Characteristics	PGPR Strains		
	WR8-3	WR9-11	WR9-16
Fluorescence on KB[a]	+	+	+
Production of pyochelin[b]	−	+	-
Hydrogen cyanide production[c]	−	++	+
Salicylic acid production[d]	−	−	−

[a]Fluorescence was observed under UV one day after incubation on KB.
[b]The production of pyochelin was observed by TLC method.
[c]HCN production was observed by the color changes of filter papers soaked in picric acid solution. ++; strong (reddish brown), +; weak (brown), −; non.
[d]SA production was confirmed by TLC method.

observed reduction of disease incidence is still a matter of controversion in many cases (Weller, 1988). For instance *P. fluorescens* WCS417 was about twice as effective as *P. putida* WCS358 in suppressing fusarium wilt in carnation. However, the siderophore of WCS417 did not induce ISR (Duijff et al., 1993). This means that other mechanism excepting competition for iron was responsible for protection by WCS417.

Pseudomonads produce pyoverdines, yellow-green fluorescent, water-soluble pigment or pyochelin, nonfluorescent, composed of salicylic-substituted cyteinyl peptide. But the structural diversity of siderophores is quite considerable and depends on the producing microorganisms. And the role of pyoverdines in the suppression of soilborne plant diseases by fluorescent pseudomonads may vary with the strain of *Pseudomonas* evaluated.

All of the PGPR strains used in this study produced fluorescent pigment. Among them two strains, WR8-3 and WR9-16, which showed a powerful iron-chelating ability on CAS, were postulated to be a siderophore-mediated mechanism for the biocontrol activity. This was confirmed by siderophore-negative mutants (Table 1). But the production of pyoverdine did not confirm the disease resistance-inducing ability. Therefore, other mechanisms have been investigated to explain disease suppression.

Bacterial LPS which contributes to growth and survival of the bacteria in plants by aiding colonization, is the major bacterial trait implicated in the elicitation of resistance responses in plants. It is reported that the O-antigenic side chain of LPS was a major determinant of ISR under low-iron conditions. Purified LPS and LPS-containing cell wall preparations of *P. fluorescens* WCS417r were as effective as live bacteria in inducing ISR when applied in roots of carnation and radish (Leeman et al., 1995; Van Peer and Schippers, 1992). But the present study shows that the crude LPS of the strains were not involved in inducing resistance. This means that the LPS of the strains used in

this test was not the major determinants in ISR and the other mechanisms were involved in the induction of resistance.

Several resistance-inducing bacteria can produce SA as a siderophore under iron-limiting conditions in culture. *P. aeruginosa* 7NSK2 produced SA which contributes to the induction of systemic resistance (De Meyer and Hofte, 1977). *P. fluorescens* CHA0, which produces a pyoverdine siderophore and SA in culture (Meyer et al., 1992), also induced resistance to tobacco necrosis virus (TNV) (Maurhofer et al., 1994). In radish, induction of systemic resistance by WCS374 and WCS417 was clearly associated with the capacity of these strains to produce SA (Leeman et al., 1996). But the PGPR strains used in this test did not produced SA, which means that SA was not involved in resistance induction.

In conclusion, the present works support the concept that PGPR can protect plants against the pathogens by inducing defense mechanisms by iron-binding siderophore, HCN and other associates. LPS of the strains and SA production were not involved in the PGPR activities of this test. Collectively, this study may extend the known mechanisms for biological control of plant pathogens by PGPR. However, further research, including analysis of mutants lacking HCN production, is necessary to elucidate whether HCN production is associated with the strain WR9-11.

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