Induction of Systemic Resistance in Cucumber Against Bacterial Angular Leaf Spot by Plant Growth-Promoting Rhizobacteria

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ABSTRACT

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Two strains of plant growth-promoting rhizobacteria (PGPR) *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166, that previously induced systemic resistance (ISR) in cucumber against cucumber anthracnose induced resistance against bacterial angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans*. Treatment of seeds or cotyledons with both strains resulted in significant decreases in lesion number and size compared to those of a noninduced disease control. Pathogen populations (log CFU per cm^2 of leaf) in inoculated leaves also were significantly decreased with PGPR treatments, declining from log 8.0 CFU per cm^2 in noninduced controls to log 5.5 CFU per cm^2 with strain 89B-27 and log 6.2 CFU per cm^2 with strain 90-166 when cotyledon injections were used. There was no significant difference in the level of induced resistance between seed inoculation and cotyledon injection with both PGPR strains. Classic ISR treatments (either induced by introducing *Colletotrichum orbiculare* or *P. syringae* pv. *lachrymans* on the first true leaves) also significantly decreased lesion number and size and population densities of the pathogen in inoculated leaves compared to those of the noninduced disease control. Neither lesion size nor pathogen population densities was significantly different between PGPR-mediated ISR and classic ISR. Local necrotic lesions on cotyledons were observed 5 days after inoculation with the pathogen, and necrosis was required in the classic ISR system. However, no visible lesions developed on cotyledons after injections of PGPR. Populations of strains 89B-27 and 90-166 increased rapidly in the cotyledons after injection and were maintained above log 9 CFU per cotyledon up to 14 days after inoculation. The results indicate that PGPR-mediated ISR is similar to classic ISR in that multiple pathogens may be controlled. However, unlike classic inducing agents, PGPR do not cause visible localized necrosis.

Additional keywords: beneficial bacteria, biological control.

Beneficial bacteria have been widely investigated for use in agriculture. Early work involved investigations on physiological aspects of plant-bacteria interactions, including nitrogen-fixation, phosphate-solubilization, and production of antibiotics and plant growth regulators (4,21,28). During the 1970s, specific rhizosphere bacteria applied to seeds were reported to colonize roots and promote plant growth (11) and were termed "plant growth-promoting rhizobacteria" (PGPR). PGPR are plant root colonizers and belong to different genera and species; most reported strains are from *Pseudomonas* and *Bacillus* spp. (10). Studies of mechanisms indicated that PGPR promote plant growth directly by production of plant growth regulators or stimulating nutrient uptake (17) or indirectly by production of siderophores or antibiotics to protect plants from soilborne pathogens or deleterious rhizobacteria (7,22,29).

Recently, investigations on mechanisms of biological control by PGPR revealed that some PGPR strains protect plants from various pathogens by inducing systemic resistance (ISR) in plants. In 1991, three research groups demonstrated that PGPR could induce systemic resistance in different plant-pathogen systems. Van Peer et al. (26) reported ISR in carnation against Fusarium wilt by a strain of *Pseudomonas* sp. Wei et al. (27) reported ISR in cucumber leaves against anthracnose caused by *Colletotrichum orbiculare* (Berk. & Mont.) Arx after seed inoculation with selected PGPR strains. Alström (1) demonstrated ISR in bean against halo blight caused by *Pseudomonas syringae* pv. *phaseolicola* by seed treatment with *P. fluorescens* strain S97. More recently, Maurhofer et al. (18) in 1994 reported that a *P. fluorescens* strain, CHA0, when applied in soil induced resistance in tobacco against tobacco necrosis virus. Strains of *Pseudomonas* spp. were reported by Zhou and Paulitz (32) and Zhou et al. (33) to induce systemic resistance in cucumber against *Pythium aphanidermatum*. All of these are examples of PGPR-mediated ISR by root, soil, or seed treatments with PGPR. The potential of foliar application of PGPR to induce resistance has not been evaluated.

Classic ISR by prior inoculation of foliage with pathogens has been demonstrated for over two decades against multiple pathogens (14,15,23,24). Classic ISR in cucumber extends to bacterial pathogens, including *P. syringae* pv. *lachrymans*, which causes angular leaf spot (2). It is not known, however, if PGPR-mediated ISR also results in protection against multiple pathogens.

The main objective of this research was to determine if PGPR strains that previously induced systemic resistance against *C. orbiculare* (27) also could reduce severity of symptoms caused by *P. syringae* pv. *lachrymans*. A second objective was to determine the relationship between symptom amelioration and pathogen population densities. Finally, the level of protection resulting from applications of PGPR to seeds and from injection of PGPR into cotyledons was compared. A portion of this work was previously published in abstract form (16).

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MATERIALS AND METHODS

Microbial cultures. Cultures of *Pseudomonas syringae* pv. *lachrymans* were obtained from the Department of Horticulture, University of Wisconsin, Madison, and stored in tryptic soy broth (TSB; Difco Laboratories, Detroit) with 20% glycerol at –80°C. For experimental use, cultures were incubated in 25 ml of TSB at 25°C with continuous shaking at 150 to 200 rpm for 24 h. Cultures were centrifuged, bacterial pellets were resuspended in sterile 0.85% NaCl solution, and concentration was adjusted to log 9.5 CFU per ml for preinfection and challenge-inoculation.

PGPR strains 89B-27 (*Pseudomonas putida*) and 90-166 (*Serratia marcescens*), which have previously demonstrated ISR activity against cucumber anthracnose (27), were used. PGPR cultures were stored under the conditions described above. For seed treatment, bacterial pellets were mixed with seeds prior to planting. For cotyledon injection, centrifuged bacterial cells were resuspended in sterile water, adjusted to log 10 CFU per ml, and 100 µl of the bacterial suspension was injected into cotyledons (two cotyledons per plant) 10 days after planting using a 1-cc sterile syringe with a 27.5-gauge needle.

Strains 89B-27r3 and 90-166r2, spontaneous rifampicin-resistant mutants of 89B-27 and 90-166, respectively, were selected to monitor population dynamics of PGPR in inoculated cotyledons. Both rifampicin-resistant mutants were selected on tryptic soy agar (TSA) amended with 100 µl of rifampicin per ml. Cultures were stored, incubated, and introduced into cotyledons as described above.

C. orbiculare and *P. syringae* pv. *lachrymans* were used as inducers in classic ISR controls. Cultures of *C. orbiculare* were obtained from J. Kuć, Department of Plant Pathology, University of Kentucky, Lexington, and were maintained for long-term storage at –80°C on green bean (GB) agar (6) plates covered with 50% glycerol. For experimental use, *C. orbiculare* was incubated on GB agar at room temperature for 5 to 7 days. Spores of *C. orbiculare* were washed with sterile distilled water and adjusted to log 6 spores per ml for inoculation of first leaves in the classic ISR control. Suspensions of *P. syringae* pv. *lachrymans* were prepared in a 0.85% NaCl solution, and the concentration was adjusted to log 9 CFU per ml. First true leaves of cucumber seedlings were inoculated by placing 10 10-µl droplets of a suspension of either *C. orbiculare* or *P. syringae* pv. *lachrymans* on leaves 2 weeks after planting.

PGPR-mediated ISR against bacterial angular leaf spot. Cucumber (*Cucumis sativus* L.) cultivar Straight 8, which is susceptible to *P. syringae* pv. *lachrymans*, was used in all experiments. The experimental design was a randomized complete block design with five treatments and six plants in each treatment. Treatments included two PGPR strains, two classic ISR controls (induced with either *C. orbiculare* or *P. syringae* pv. *lachrymans*), and a disease control (noninduced and challenge-inoculated with *P. syringae* pv. *lachrymans*). Bacteria-treated seeds were planted in 10-cm^2 plastic pots containing Pro-Mix soilless mix (Premier Peat Ltd., Rivière-du-Loup, Québec). Three weeks after planting, the second leaves in all treatments were challenge-inoculated with *P. syringae* pv. *lachrymans* by swabbing a suspension (log 9 CFU per ml) onto the abaxial side of leaves. Inoculated plants were placed in a high-humidity chamber at 25°C for 24 h and then were moved to a greenhouse at 32/25°C day/night. Six days after challenge-inoculation, total lesion number (TLN) and total lesion area (TLA [square millimeters]) were recorded for each plant. Data from all experiments were pooled after confirming homogeneity of variances with Bartlett's test (5) and were analyzed using the general linear models procedure in PC-SAS (SAS Institute, Cary, NC). The experiment was conducted four times.

Effects of PGPR application methods on ISR. Experiments were conducted to compare the level of ISR resulting from PGPR application via seed treatment and cotyledon injection. The experimental design was a 2 × 4 factorial with three replications, each with one plant, arranged as a randomized complete block. Factors were two PGPR application methods and four treatments: two PGPR strains, one classic ISR control induced with *C. orbiculare*, and one noninduced disease control. For seed treatment, the method was the same as described above. For cotyledon injection, cucumber seeds were planted without PGPR seed inoculation. Ten days after planting, 0.1 ml of log 10 CFU per ml of PGPR suspension was injected into the cotyledons as described above. For the disease control, cotyledons were injected with only sterile water. Plants were challenge-inoculated onto second leaves with *P. syringae* pv. *lachrymans* 3 weeks after planting. TLN and TLA were recorded and analyzed as described above.

The relationship between symptom development and populations of the pathogen was assessed to determine if symptom expression was correlated with the presence and quantity of the pathogen. After recording TLN and TLA, all challenge-inoculated leaves were detached to detect the bacterial populations. The leaves were surface-disinfested with 1% sodium hypochlorite for 30 s. Six disks from each leaf were removed using a #5 cork borer (8 mm in diameter) and placed in 10 ml of 0.85% NaCl solution. Samples then were triturated with sterilized mortars and pestles, diluted, and incubated on Pseudomonas agar F (Difco) at 25°C for 24 h. Leaves from nonchallenged healthy plants were used as the control to determine background levels. All experiments were conducted in a greenhouse at 32/25°C day/night. The experiment was conducted four times. Data were transformed into mean log CFU per cm^2 and analyzed as described above. Correlation analysis of SAS was used to determine if pathogen population densities were correlated with lesion number and area.

Populations of PGPR in inoculated cotyledons and formation of local lesions in classic ISR treatments. Experiments were conducted to determine if PGPR survive and cause necrotic lesions after injection into cotyledons. Cucumber seeds were planted as described above. Two rifampicin-resistant mutants, 89B-27r3 and 90-166r2, were used. Experimental design was a randomized complete block with six treatments and three replications, each with one plant. Treatments included the two PGPR strains, the two classic ISR controls, a control injected with only sterile water, and a control without any treatment. Ten days after planting, suspensions of PGPR strains were injected into cotyledons as described above. For the classic ISR controls, 6 10-µl droplets of a suspension of *C. orbiculare* or *P. syringae* pv. *lachrymans* were inoculated on the adaxial side of cotyledons. Local lesions that formed on inoculated cotyledons were recorded over time.

PGPR were reisolated from cotyledons and stems (1 to 2 cm above and below the cotyledons) at 0, 3, 7, and 14 days after inoculation. Preliminary experiments on population dynamics with six replications indicated that population variance among replications was very low ($\pm\log_{10}$ 0.2), and therefore, this experiment was conducted with three replications. Samples of cotyledons and stems were surface-disinfested with 1% sodium hypochlorite for 30 s, rinsed with sterile distilled water three times, triturated with sterile mortars and pestles, diluted in 0.85% NaCl, and incubated on TSA with 100 µg of rifampicin per ml. Population densities of strains 89B-27r3 and 90-166r2 in samples were calculated after incubation for 24 h at 25°C. Data were transformed to log CFU per cotyledon and analyzed using the general linear models procedure in PC-SAS. The experiment was conducted twice.

RESULTS

PGPR-mediated ISR against bacterial angular leaf spot. Seed treatment with strains 89B-27 and 90-166 significantly decreased the number and size of angular leaf spot lesions compared to the disease controls (Table 1). The average reductions in TLA were 53.2% after treatment with strain 89B-27 and 60.0% after treat-

ment with strain 90-166. Both *C. orbiculare* and *P. syringae* pv. *lachrymans* when applied as inducers for the classic ISR controls decreased mean TLN and TLA significantly compared to the disease control. There was a significant difference between PGPR treatments and classic ISR control in TLN but not in TLA.

Effects of PGPR application method on ISR. Factorial analysis revealed no interactions between application method and treatments in number of lesions (Fig. 1), lesion area (Fig. 2), or pathogen population densities (Fig. 3). There were no significant differences between classic ISR controls and PGPR treatments for any of these parameters. With seed treatments, populations of the pathogen in leaves were log 7.8, 5.8, 5.3, and 6.1 CFU per cm^2 for the disease control, classic ISR control, strain 89B-27, and strain 90-166 treatments, respectively. With cotyledon injections, populations of the pathogen were log 8.1, 6.3, 5.3, and 6.5 CFU per cm^2 for the disease control, classic ISR control, 89B-27, and 90-166 treatments, respectively (Fig. 3). Treatment with 89B-27 resulted in populations 100-fold lower than those in the disease control. With strain 90-166, population densities of the pathogen also were significantly lower than those in the disease control. There were no significant differences between the classic ISR control and the two PGPR strains in bacterial populations in challenge-inoculated leaves. Correlation analysis revealed that pathogen population densities were correlated with TLA ($r = 0.73$, $P = 0.001$) and TLN ($r = 0.58$, $P = 0.005$).

Populations of PGPR in inoculated cotyledons and formation of local lesions in classic ISR treatments. Population densities of strains 89B-27r3 and 90-166r2 in inoculated cotyledons increased rapidly during the first 3 days (Fig. 4). With strain 89B-27r3, mean population peaked at log 10.83 CFU per cotyledon at 7 days after inoculation, whereas the population of strain 90-166r2 peaked at log 12.30 CFU per cotyledon at 3 days after inoculation. Neither bacterial strain was recovered from stems 1 to 2 cm above or below inoculated cotyledons at any sampling time.

Neither PGPR strain caused visible local lesions within 14 days after inoculation. Local lesions also did not develop in controls treated with sterile water and nontreated controls. Local lesions developed on cotyledons 5 days after inoculation with both *C. orbiculare* and *P. syringae* pv. *lachrymans*.

TABLE 1. Size and number of lesions of *Pseudomonas syringae* pv. *lachrymans* on cucumber cultivar Straight 8 with seed treatment with plant growth-promoting rhizobacteria (PGPR; *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166) for induction of systemic resistance (ISR)

Treatment[w]	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean[x]
Control					
TLN[y]	17.5	10.5	15.5	20.2	15.9 a
TLA[z]	247.5	61.2	103.2	118.7	132.7 a
89B-27					
TLN	8.8	8.2	9.5	14.5	10.3 b
TLA	87.7	43.3	57.3	60.2	62.1 b
90-166					
TLN	7.5	6.8	6.5	15.3	9.0 b
TLA	75.2	63.8	30.3	59.2	57.1 b
Psl-Psl					
TLN	4.5	3.8	4.2	7.3	5.0 c
TLA	19.8	12.0	19.8	51.7	25.8 b
Cor-Psl					
TLN	4.3	2.2	4.8	4.2	3.9 c
TLA	19.3	13.0	25.7	18.5	19.1 b

[w] All experiments were challenge-inoculated with *P. syringae* pv. *lachrymans*. Control is the noninduced control; *Psl-Psl* is a classic ISR control, with *P. syringae* pv. *lachrymans* used as the inducer; *Cor-Psl* is a classic ISR control, with *Colletotrichum orbiculare* used as the inducer.

[x] Means with different letters are significantly different at $P = 0.05$.

[y] TLN = total lesion number, mean of six replications. $LSD_{0.05} = 3.79$.

[z] TLA = total lesion area (square millimeters), mean of six replications. $LSD_{0.05} = 48.01$.

DISCUSSION

The results reported here indicate that ISR mediated by PGPR strains 89B-27 and 90-166 protected cucumber plants from angular leaf spot. Both lesion number and size were reduced by treatment with these PGPR compared to the noninduced disease control (Table 1; Figs. 1 and 2). In addition, populations of the pathogen in inoculated leaves were lower with PGPR treatment than in the noninduced control (Fig. 3). In the cucumber-anthracnose system, the classic ISR control protected plants significantly better than PGPR treatments; however, in the cucumber-angular

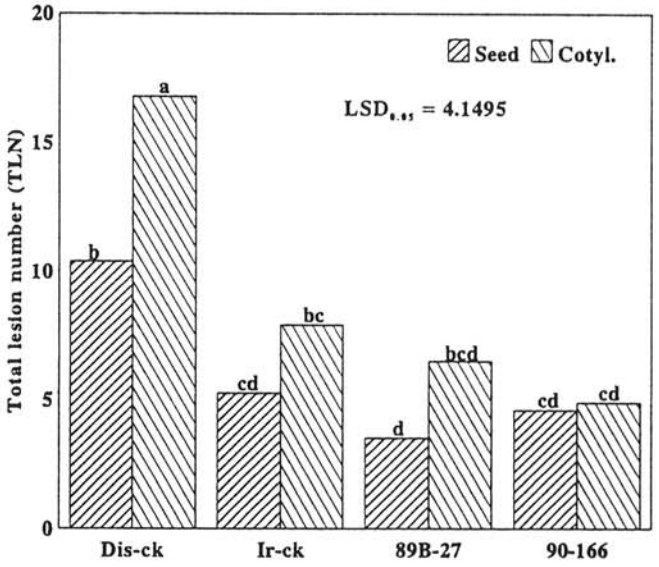

Fig. 1. Total lesion number (TLN) caused by *Pseudomonas syringae* pv. *lachrymans* on leaves challenge-inoculated by plant growth-promoting rhizobacteria (PGPR; *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166) seed and cotyledon treatments. Seed = seed treatment with PGPR; Cotyl. = cotyledon injection with PGPR; Dis-ck is a noninduced, pathogen-inoculated control; Ir-ck is a classic induced resistance control by preinoculation of *Colletotrichum orbiculare* on the first leaves.

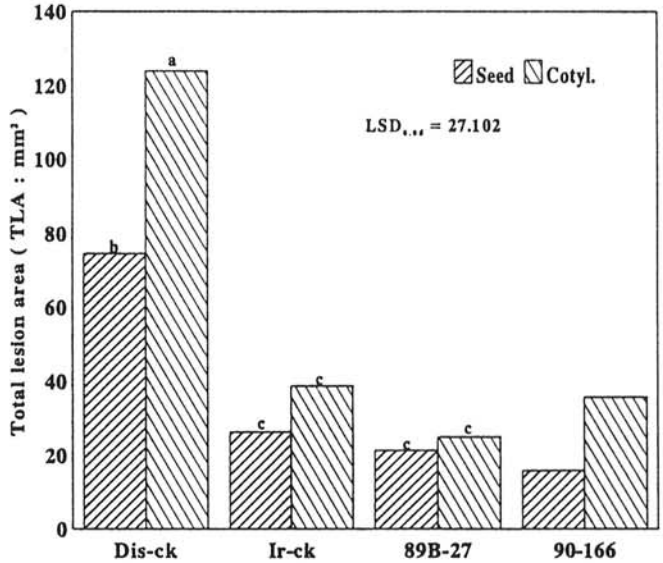

Fig. 2. Total lesion area (TLA) caused by *Pseudomonas syringae* pv. *lachrymans* on leaves challenge-inoculated by plant growth-promoting rhizobacteria (PGPR; *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166) seed and cotyledon treatments. Seed = seed treatment with PGPR; Cotyl. = cotyledon injection with PGPR; Dis-ck is a noninduced, pathogen-inoculated control; Ir-ck is a classic induced resistance control by preinoculation of *Colletotrichum orbiculare* on the first leaves.

leaf spot system used here, lesion size was not significantly different between the ISR control and PGPR treatments (Table 1).

There was no significant difference in ISR between seed treatment and cotyledon injection of PGPR (Figs. 1, 2, and 3). Traditionally, application methods of PGPR have been limited to seed or root treatments (1,18,26,27,32,33), and many studies have focused on correlations of plant root colonization and biological control activities of PGPR (12,30,31). Only a few investigations have been conducted on foliar applications of PGPR (3,19,20). Scientists in China reported that application of some PGPR strains on foliage could increase growth and protect plants from foliar diseases (19,20). However, these studies did not test whether induced resistance was an operable mechanism. Our research suggested that ISR also can be obtained by cotyledon injection of PGPR and that the resulting ISR is as effective as seed treatment. Neither PGPR strain was recovered from stems or leaves after cotyledon injection, suggesting that PGPR and the pathogen were separated spatially and the reduction of the disease was due to ISR. Foliar application of PGPR should be examined further as a means to deliver ISR in practical agriculture.

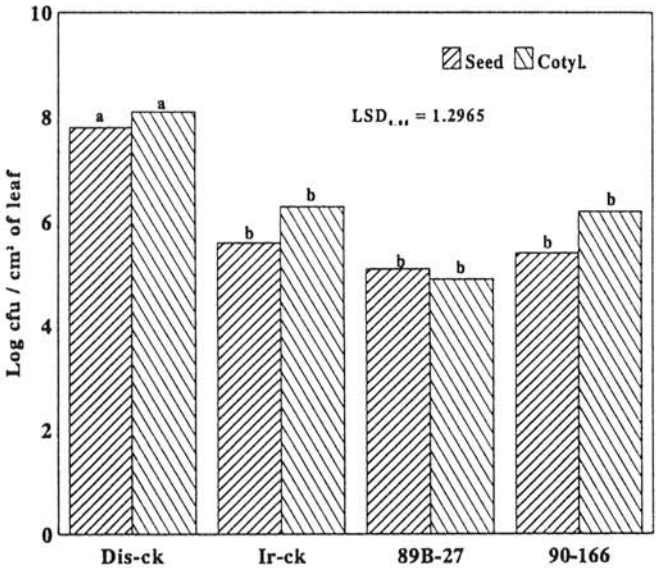

Fig. 3. Population densities of *Pseudomonas syringae* pv. *lachrymans* in leaves challenge-inoculated by plant growth-promoting rhizobacteria (PGPR; *Pseudomonas putida* 89B-27 and *Serratia marcescens* 90-166). Seed = seed treatment with PGPR; Cotyl. = cotyledon injection with PGPR; Dis-ck is a noninduced, pathogen-inoculated control; Ir-ck is a classic induced resistance control by preinoculation of *Colletotrichum orbiculare* on the first leaves. Data are from four experiments, each with three replications. Factorial analysis revealed no interaction between inoculation method and the four treatments. Different letters indicate a significant difference at $P = 0.05$ (data analyzed together).

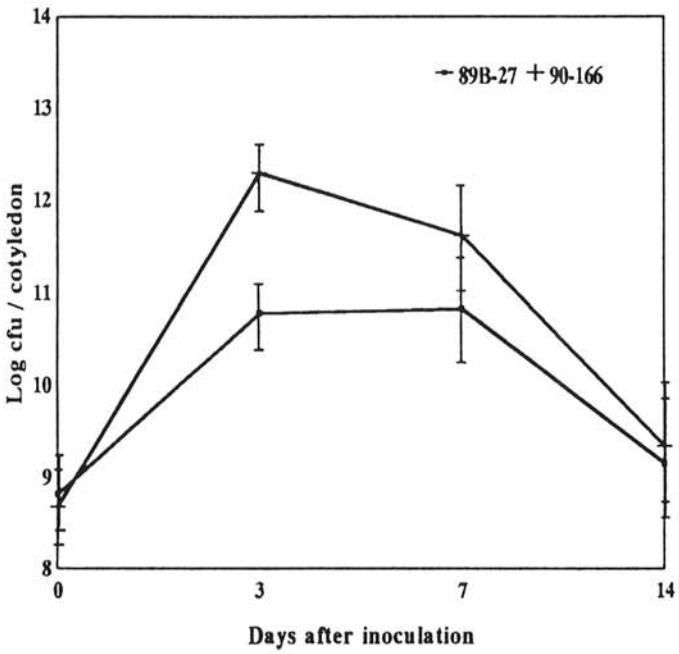

Fig. 4. Population densities of plant growth-promoting rhizobacteria rifampicin-resistant mutant strains 89B-27r3 (*Pseudomonas putida*) and 90-166r2 (*Serratia marcescens*) in cotyledons after cotyledon inoculation. Cucumber cultivar Straight 8 was used. Cotyledons were inoculated with the bacterial suspensions by injection 10 days after planting. Data are means of two experiments, each with three replications.

Reduction of TLN, TLA, and populations of the pathogen by PGPR-mediated ISR were highly correlated, suggesting that reduction of disease symptoms may be due to inhibition of infection by the pathogen or reduction of its development in the plant. Early work on classic ISR showed that lignification occurred after preinfection with inducers (8), indicating that ISR led to a physical barrier against fungi. PGPR also may induce a defense response in plants that leads to physical or biochemical barriers to the pathogen.

Although populations of strain 89B-27 and 90-166 increased rapidly in inoculated cotyledons within 1 week after inoculation (from approximately log 9 to 12 CFU per cotyledon), no necrotic lesions resulted within 14 days after inoculation. This lack of necrosis is fundamentally different from the classic ISR system, in which all reported inducing agents lead to the formation of local necrotic lesions (9,13,25). This key difference suggests that mechanisms involved in PGPR-mediated ISR may differ from those of classic ISR.

The results reported here show that PGPR-mediated ISR, like classic ISR, can protect cucumber against angular leaf spot of cucumber. Because classic ISR requires induction via preinoculation with pathogens, it is difficult to develop commercial products to be used in practical agriculture because of the obvious risk associated with introducing pathogens into fields. In contrast, PGPR represent a practical delivery system for ISR, and therefore, PGPR-ISR could be evaluated in an integrated pest management approach for controlling plant disease.

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