Induction of Systemic Resistance of Cucumber to *Colletotrichum orbiculare* by Select Strains of Plant Growth-Promoting Rhizobacteria

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ABSTRACT

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Ninety-four strains of plant growth-promoting rhizobacteria (PGPR) were screened for induction of systemic resistance using a model system of cucumber and anthracnose, caused by *Colletotrichum orbiculare*. Compared with a nonbacterized, challenged control, treatment of cucumber seeds with six PGPR strains resulted in a significant reduction in lesion size after challenge-inoculation with *C. orbiculare*. Four of the six PGPR strains that induced resistance in cucumber produced HCN in vitro. Antagonism in vitro toward *Pythium ultimum*, *Rhizoctonia solani*, and *C. orbiculare* on three media generally was absent with five PGPR strains and weak with one strain. Rifampicin-resistant mutants of the PGPR strains colonized roots at mean population densities of log 6.5 to 8.3 cfu g^{-1} of root at 7 days after planting and log 4.1 to 6.1 cfu g^{-1} at 21 days after planting. None of the strains was recovered from surface-disinfested petioles on the day of challenge with *C. orbiculare*. Roots from plants bacterized with PGPR strains showed less necrosis than the nonbacterized, challenged control. The results support the conclusion that some PGPR strains applied to seed can induce systemic resistance to *C. orbiculare*.

Additional keywords: Colletotrichum lagenarium.

Induced systemic resistance in plants has been demonstrated in over 25 crops, including cereals, cucurbits, legumes, solanaceous plants, trees, and small fruits, against a broad spectrum of pathogens (3,21-23,32,40). Cucumber has been used as a model for induced systemic resistance in several laboratories (3,10,21-23,28,36). Treatment of the first true leaf with necrosis-causing pathogens protects the cucumber plant against at least 13 pathogens representing fungi, bacteria, and viruses causing leaf (21,23) and root diseases (7). Resistance in systemically protected cucumber plants is often expressed by a significant reduction in both size and number of lesions caused by foliar pathogens, such as *Colletotrichum orbiculare* (Berk. & Mont.) Arx (synonym for *C. lagenarium* (Pass.) Ellis & Halst.) (23).

The term *rhizobacteria* was coined to describe rhizosphere bacteria that exhibit root colonization (35). Those rhizobacteria exerting a beneficial effect on plants are termed *PGPR* (plant growth-promoting rhizobacteria) (17). Investigations into mechanisms for the beneficial effects of most reported PGPR strains indicated that the PGPR increased growth indirectly by changing the microbial balance in the rhizosphere (18). Iron-chelating siderophores (14,15,34), antibiotics (6,43), and hydrogen cyanide (HCN) (1,37) are produced by some PGPR and have been implicated in reductions of plant pathogens and deleterious rhizobacteria with a corresponding improvement in plant growth. HCN also has been implicated in growth promotion by the discovery that certain deleterious rhizobacteria produce HCN, which may restrict plant growth, and that these deleterious rhizobacteria are inhibited by some PGPR strains (34).

The distinction between biological control and growth promotion is vague at best. The first reports of PGPR on potato noted that growth promotion was associated with a reduction of total fungal propagules on the rhizoplane (18). This suggested that select PGPR strains also could be used to reduce pathogen populations in the root zone. Some potato PGPR were subsequently shown to reduce populations of the bacterial pathogen, *Erwinia carotovora* (12). In a later study (20), PGPR strains that demonstrated growth promotion in field trials on canola (13) were examined for biological control activity against *Rhizoctonia solani* and *Pythium* in plant assays. Of the PGPR strains that demonstrated biological control activity, some exhibited antibiosis in vitro toward the pathogens but others did not, suggesting that the biocontrol PGPR strains consist of two groups: those that control disease by antagonism to the pathogen, and those that control the disease by mechanisms that do not involve production of toxic compounds, such as substrate or site competition or induced resistance. We hypothesize, therefore, that select PGPR strains may induce systemic resistance in the bacterized host to plant pathogens.

Anderson and Guerra (2) reported that bean roots colonized by a strain of *Pseudomonas putida*, which provided some protection from *Fusarium solani* f. sp. *phaseoli*, had 17-93% higher lignin content than nonbacterized seedlings. This finding demonstrates that PGPR strains may induce plants to increase host defense compounds. van Peer et al (42) recently reported evidence that a pseudomonad PGPR strain induced resistance in carnation. In this system, plants inoculated by pouring a PGPR suspension onto roots of cuttings in rock wool and stem-inoculated 1 wk later with *Fusarium oxysporum* f. sp. *dianthi* had a significantly lower incidence of Fusarium wilt. Antagonism and competition were ruled out as possible mechanisms due to the spacial separation of the PGPR strains and the pathogen.

The objective of the investigation reported here was to test the hypothesis that some PGPR strains could induce systemic resistance to a foliar pathogen when used as a seed treatment. Known PGPR strains were tested in a system consisting of cucumber and anthracnose disease, caused by *C. orbiculare*.

MATERIALS AND METHODS

Microbial cultures. *C. orbiculare* was obtained from Joseph Kuć, Department of Plant Pathology, the University of Kentucky. The pathogen was maintained for long-term storage at −80 C on green bean agar (9) slants covered with 50% glycerol. For experimental use, cultures were transferred to green bean agar plates that were incubated until sporulation was abundant (7-10 days at 28 C).

P. ultimum Trow and *R. solani* Kühn AG 4 were obtained from Gustafson, Inc., Dallas, TX. Both isolates induced seedling damping-off disease of cotton. The pathogens were maintained for long-term storage in sterile soil at 4 C and were grown on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI) at 28 C for experimental use.

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Ninety-four PGPR strains were provided by Esso Chemical Ag Biologicals, Saskatoon, Saskatchewan, Canada, and were identified by analysis of fatty acid methyl esters (4,8,31,33). The strains were previously shown to reduce disease incidence caused by *P. ultimum* or *R. solani* or to promote seedling emergence, plant dry weight, or yield of several crops (16,19,20,27,30,38,39). Bacteria were maintained for long-term storage at −80 C in tryptic soy broth (TSB) (Difco) with 20% glycerol. For use in the bioassays, cultures from −80 C storage were purified on tryptic soy agar (TSA) (Difco) by incubation for 24–48 h at 28 C. Single colonies were transferred to 20-50 ml of TSB in screw-cap centrifuge tubes, which then were incubated 24 h at 25 C with shaking at 150 rpm. Suspensions were centrifuged, and pelleted bacterial cells were resuspended in 2 ml of sterile 1% sodium alginate (high viscosity from Sigma Chemical Co., St. Louis, MO) for seed treatment of cucumber. For determination of root colonization by inducing PGPR strains, spontaneous rifampicin-resistant mutants were selected as previously described (19).

Cucumber-anthracnose assay. The cucumber-anthracnose system (26) was chosen as a model for testing PGPR strains as inducers of systemic resistance. Treatments consisted of bacterized seed and plants challenge-inoculated with *C. orbiculare*, nonbacterized seed and plants challenge-inoculated with *C. orbiculare* (*C. orbiculare* control), and nonbacterized seed and plants induced and challenged with *C. orbiculare* (standard induced resistance control). Seeds of a cucumber cultivar (*Cucumis sativus* L. 'Straight Eight') highly susceptible to anthracnose (25) were dipped either into the alginate suspension or in sterile alginate immediately before planting one seed per 10-cm-square plastic pot in Promix (Premier Peat, Rivière-du-Loup, Québec, Canada). Plants were maintained in a greenhouse until challenged with *C. orbiculare*. The second true leaf of each plant was challenge-inoculated 21 days after planting by applying 30 10-μl drops of a suspension containing log 4 conidia ml^{-1} of *C. orbiculare*. Plants were kept in the dark at 100% RH in a high humidity chamber for 30 h. Number of lesions and total lesion diameter (summed diameter of 30 lesions) on the challenged leaf were recorded 6 days later. For the standard induced resistance control, the first true leaf was inoculated with 30 5-μl drops of a log 6 conidial suspension ml^{-1} of *C. orbiculare*, and 7 days later the second true leaf was challenge-inoculated as previously described. In the initial experiment all strains were tested using two replicate plants per strain. Groups of 15–20 PGPR strains were tested for each pair of controls.

The consistency of disease suppression by the top-performing six PGPR strains from the primary screen was determined by repeating the experiments three times. Each experiment was designed as a completely randomized block consisting of six replications of eight treatments (the six PGPR strains, the *C. orbiculare* control, and the standard induced resistance control). Lesion number and total lesion diameter on plants treated with inducing PGPR were compared with the disease control.

Data were analyzed for significance using the general linear models with SAS software (SAS Institute, Cary, NC). Mean separation was by LSD = 0.05. Cumulative data from all three experiments also were analyzed for significance using the general linear models of SAS after determining homogeneity of variances.

HCN production and antagonism. Production of HCN was determined using a modification of the procedure of Millar and Higgins (29). Bacteria were grown on TSA supplemented with 4.4 g L^{-1} 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 water) in the lid of each petri dish. Dishes were sealed with Parafilm and incubated for 2–4 days. After incubation at 28 C for 48 h, HCN production was indicated by the presence of a colored zone around the bacteria. Reactions were scored as weak (yellow to light brown), moderate (brown), or strong (reddish brown) for each of the PGPR strains. The experiment was repeated once with similar results.

Antagonism in vitro of PGPR strains toward *C. orbiculare*, *P. ultimum*, and *R. solani* was tested by streaking the bacteria in a 4-cm line on each test medium. Mycelial plugs, 5 mm in diameter, of *P. ultimum* and *R. solani* were transferred to the most distal point from the bacteria on the plate. Mycelial plugs of *C. orbiculare* were transferred to the center of plates (approximately 3 cm from bacterial streaks) because of the slow

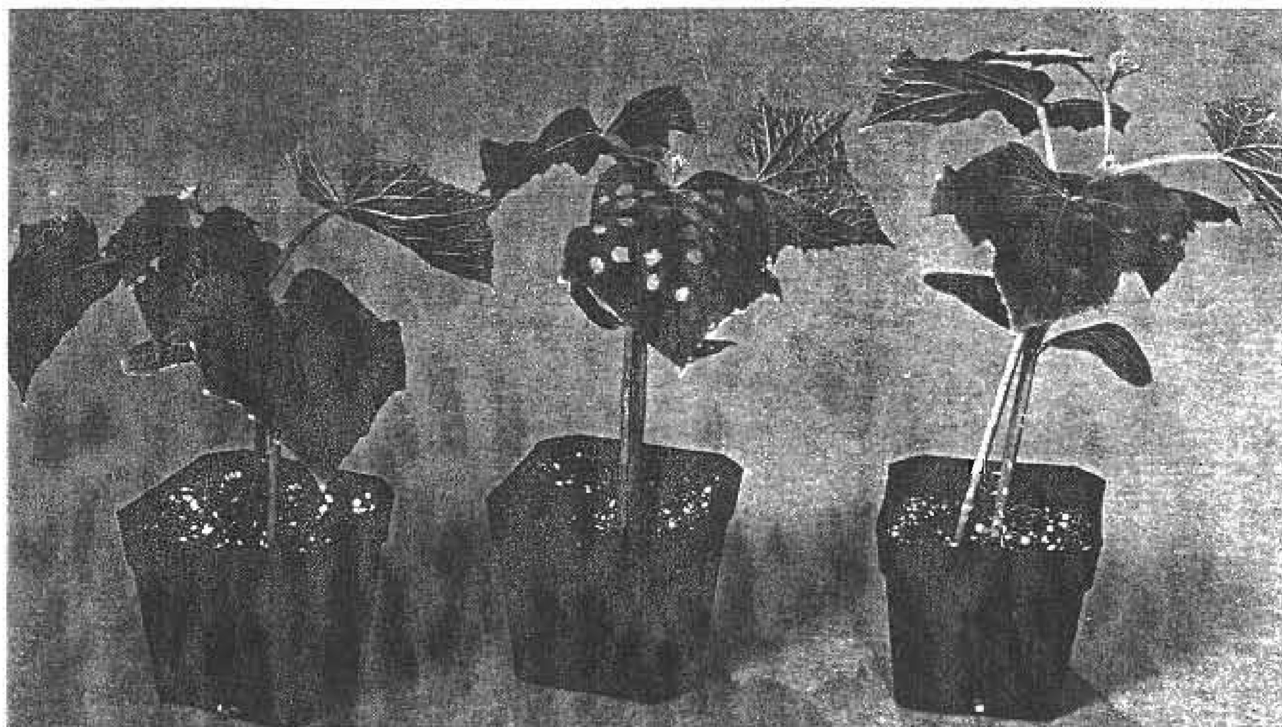

Fig. 1. Suppression of cucumber anthracnose after seed treatment with plant growth-promoting rhizobacteria (PGPR). The second true leaf of each plant was challenge-inoculated with 30 15-μl drops of a conidial suspension of *Colletotrichum orbiculare*. Treatments shown are as follows: left, standard induced resistance control (nonbacterized and induced with prior inoculation of first true leaf with *C. orbiculare*); middle, *C. orbiculare* control (nonbacterized and noninduced); and right, bacterized (seed treated with *Pseudomonas aureofaciens* PGPR strain 25-33 before planting). Growth promotion is evident with the PGPR treatment.

fungal growth rate. Three replicate plates were used for each medium, and the experiment was repeated once with similar results. Test media included TSA, PDA, and green bean agar. Plates were incubated at 28 C, and zones of inhibition were recorded after 2, 4, and 10 days for *P. ultimum, R. solani,* and *C. orbiculare,* respectively.

Plant colonization. Root colonization ability and possible systemic plant colonization of the six PGPR strains were determined using rifampicin-resistant mutants. Three cucumber seeds for each treatment were inoculated as described above, and plants were sampled 7, 14, and 21 days later. Whole root systems were shaken to remove loosely adhering Promix, then weighed and agitated in sterile 0.02 M phosphate buffer (PB), pH 7.0, for 20 min at 150 rpm. Serial dilutions were prepared in PB and plated onto TSA amended with 100 mg L^{-1} of rifampicin (rif-TSA) using a spiral plater (Spiral Systems, Inc., Bethesda, MD). After incubation for 24 h at 28 C, bacteria were enumerated with a laser colony counter (Spiral Systems) and bacterial enumeration software (Spiral Systems). Mean cfu g^{-1} fresh weight of root was calculated for each strain using log-transformed data. The experiment was repeated twice with similar results.

Possible systemic colonization by PGPR was investigated by sampling petioles of the second true leaf 21 days after planting. Petioles were surface-disinfested by shaking in 1.05% NaClO with Tween 20 (two drops, 100 ml^{-1}), followed by rinsing three times in 100 ml of sterile distilled water and grinding in 5.0 ml of sterile water with an autoclaved mortar and pestle. Serial dilutions were prepared and spiral-plated onto rif-TSA. Sterility controls, consisting of adding 1.0 ml of the final rinse water to 9.0 ml of TSB and incubating the TSB at 28 C for 48 h, were performed on each sample. The experiment was conducted three times with similar results.

Root health. An experiment was performed to determine if roots colonized by inducing PGPR exhibited root necrosis, since foliar necrosis has previously been consistently associated with induced systemic resistance by foliar pathogens. Treatments consisted of the six strains of inducing PGPR and a nonbacterized control, each replicated three times. PGPR were applied to cucumber seeds as described above. Roots were examined 27 days after planting. Roots were thoroughly washed, and 1-cm segments of main root with attached root hairs were cut from three regions per plant: upper root, midroot, and lower root. Three replicate plants were used per treatment. Root hairs of each segment were examined with a dissecting microscope (10×) for discoloration, and percent discolored root hairs per segment was calculated. Number of root hairs per segment ranged from 5 to 14 with a mean of 8. The experiment was repeated once with similar results.

RESULTS

Cucumber-anthracnose assay. In the preliminary screen of 94 PGPR strains for systemic effects on development of anthracnose lesions, treatment with six strains resulted in visible reductions in lesion development compared with the disease control (Fig. 1). These six strains represented the following taxa: *P. putida* (strain 34-13), *P. fluorescens* (strain G8-4), *P. aureofaciens* (strains 25-33, 28-9, and 36-5) and *Serratia plymuthica* (strain 2-67).

Results from three repeated trials (Table 1) indicated that, in general, seed treatment with the PGPR strains reduced the number and diameter of lesions caused by *C. orbiculare*. Significant reductions in mean lesion number were consistent with two of the strains in all trials, and in some of the trials with other strains. Four of the six PGPR strains significantly reduced lesion diameter in all trials (Table 1). Based on combined data for all three trials, lesion number was significantly reduced by five strains, and total

TABLE 1. Effect of seed treatment with selected plant growth-promoting rhizobacteria (PGPR) strains on cucumber anthracnose caused by *Colletotrichum orbiculare*

Treatment	Trial 1		Trial 2		Trial 3		Mean of 3 trials	
	Mean lesion number[a]	Mean TLD[b]	Mean lesion number	Mean TLD	Mean lesion number	Mean TLD	Lesion number	TLD
Pseudomonas fluorescens G8-4	19.5*[c]	74.2*	14.7*	42.7*	8.2*	20.0*	14.1*	45.6*
P. aureofaciens 25-33	23.8	139.9*	14.0*	40.3*	10.3*	26.0*	16.1*	68.8*
P. aureofaciens 28-9	25.0	107.3*	22.5	83.7*	...	...	23.9	95.4*
P. putida 34-13	22.0	88.8*	14.5*	46.2*	10.8*	27.3*	15.8*	54.1*
P. aureofaciens 36-5	13.0*	35.6*	14.2*	49.0*	10.7*	21.1*	12.6*	35.2*
Serratia plymuthica 2-67	27.0	146.8	16.5*	53.8*	14.8*	39.7*	19.9*	80.1*
C. orbiculare control	28.7	161.2	26.0	152.2	24.7*	110.3	26.4	141.6
Standard induced resistance control	13.7*	54.3*	4.3*	8.0*	2.5*	7.1*	6.8*	23.1*
$LSD_{0.05}$	8.5	73.9	5.2	38.1	5.9	24.8	3.8	26.5

[a] Mean of six replicates.
[b] Total lesion diameter.
[c] *, Significant ($P = 0.05$) difference from *C. orbiculare* control.

TABLE 2. HCN production and in vitro antagonism of plant growth-promoting rhizobacteria (PGPR) strains

PGPR strain	HCN production[b]	Zone of inhibition (mm)[a]								
		Tryptic soy agar			Potato-dextrose agar			Green bean agar		
		Pu[c]	Rs[d]	Co[e]	Pu	Rs	Co	Pu	Rs	Co
Pseudomonas fluorescens G8-4	Weak	0	5	0	0	0	0	0	0	0
P. aureofaciens 25-33	None	0	7	0	0	0	0	0	0	0
P. aureofaciens 28-9	Moderate	0	6	0	3	0	0	0	0	0
P. putida 34-13	Weak	3	7	0	0	0	0	0	0	0
P. aureofaciens 36-5	Strong	4	13	0	0	0	0	0	0	0
Serratia plymuthica 2-67	None	3	12	5	0	0	3	0	4	3

[a] Mean of three replications.
[b] Weak = yellow to light brown, moderate = brown, strong = reddish brown.
[c] *Pythium ultimum.*
[d] *Rhizoctonia solani.*
[e] *Colletotrichum orbiculare.*

lesion diameter was reduced by all six strains compared with the disease control.

HCN production and antagonism. Four of the PGPR strains produced some level of HCN in vitro (Table 2), while two strains showed no HCN production. Antagonism in vitro depended on the test medium and the challenged fungus (Table 2). Only strain 2-67 demonstrated antagonism toward all three challenged fungi on TSA, but the level of antagonism with this strain was weak, i.e., zones of inhibition of 1 to 5 mm except for antagonism toward *R. solani* on TSA. Frequency of antagonism among all strains was greatest on TSA, on which fungal growth was slowest.

Plant colonization. Rifampicin-resistant mutants of the six PGPR strains were recovered from cucumber roots at mean population densities ranging from log 6.54 to 8.28 cfu g^{-1} fresh weight of root 7 days after planting. Populations decreased 14 days after planting to log 6.05 to 6.68 cfu g^{-1} of root, and by 21 days after planting, populations ranged from log 4.09 to 6.13 cfu g^{-1}. No bacteria were detected on rif-TSA plates inoculated with suspensions of the petiole samples at 21 days after bacterization.

Root health. Examination of PGPR-colonized roots with a dissecting microscope revealed no evidence of root necrosis caused by PGPR (Table 3). In fact, treatment with all six inducing PGPR resulted in lower numbers of discolored root hairs than the nonbacterized control.

DISCUSSION

Seed treatment of cucumber with six PGPR strains consistently resulted in suppression of anthracnose caused by *C. orbiculare*. The PGPR strains colonized roots but were not detected in petioles of protected leaves, thereby suggesting that competition or antagonism were not operable mechanisms for the observed disease suppression. We conclude that protection in this system resulted from induction of systemic resistance by PGPR applied as seed treatments.

This conclusion agrees with that of van Peer et al (42) that biological control of Fusarium wilt of carnation by a pseudomonad PGPR strain was due to induced resistance because the pathogen was spatially separated (using stem inoculation) from the root-colonizing PGPR. In the carnation system, roots of established cuttings were treated with a suspension of *Pseudomonas* sp. strain WCS417r. Our results extend the concept by demonstrating that seed bacterization with PGPR strains may result in induced systemic resistance, thus potentially offering a practical way to immunize plants. In the carnation system (42), induction of resistance to *Fusarium oxysporum* f. sp. *dianthi* was lower with a susceptible cultivar than with a moderately resistant cultivar. In contrast, the PGPR strains used in our study consistently induced resistance of a highly susceptible cucumber cultivar.

The cucumber and anthracnose disease model system used in this study has several advantages for investigations into induced systemic resistance by root-associated microorganisms. Principal among these is that this model has been extensively studied in "classical" induced resistance systems (24) and, therefore, much is known about biochemical changes related to induced resistance. In addition, the use of a foliar pathogen unambiguously eliminates the possible involvement of antagonism and competition for infection sites and local nutrients as underlying mechanisms for biological control by root-colonizing bacteria. When challenge-inoculated at a rate of 30 10-μL drops of a log 4 conidia ml^{-1} suspension onto the second true leaf of cucumber, *C. orbiculare* forms discrete lesions that can be counted and measured, thereby producing quantifiable data for rating of treatments. The entire assay, from seed treatment with PGPR to rating of lesion development, requires 27 days, which is relatively rapid for foliar disease assays. Another advantage of the model system is that by including the standard induced resistance control (nonbacterized and immunized with *C. lagenarium*), the experiment contains a positive control. This is useful for indicating if an individual assay has been rendered nonconducive to expressing induced resistance because of adverse experimental conditions. Hence, the cucumber anthracnose model system should be useful in other studies designed to assess the potential contribution of induced resistance to biological control.

While bacterization with PGPR strains significantly reduced anthracnose development compared with the *C. orbiculare* control (Table 1), the level of protection by bacterization was less than that of the induced resistance control. This is not surprising considering that the PGPR strains must colonize roots and produce systemic effects in the plant in order to reduce the incidence of a foliar pathogen. Future investigations may lead to an increase in the level of protection afforded by PGPR that induce systemic resistance. This may occur through an increase in the population densities of PGPR on roots or by understanding the critical time for production of the trigger for induced resistance.

Previous work on induced resistance in cucumber indicated that development of necrosis was necessary for induction of resistance (24). The induced resistance control in this study caused necrotic leaf lesions after induction of the first true leaf with *C. orbiculare*. In contrast, PGPR seed treatments did not cause visible necrosis on stems, foliage, or roots, and treated roots had less discoloration compared with the nonbacterized control (Table 3). Plants treated with most of the PGPR strains had visibly enhanced leaf area and height compared with the disease control and the induced resistance controls (Fig. 1). Increases in several plant growth parameters have been reported in one other study where induced systemic resistance was indicated (40).

Défago and colleagues (5) have extensively investigated the mechanisms by which *P. fluorescens* PGPR strain CHAO provides biological control against *Thielaviopsis basicola* on tobacco. Using mutational analysis and complementation, this group demonstrated that production of HCN by strain CHAO accounted for about 60% of the biological control activity and was correlated with root hair proliferation by CHAO. Défago et al (5) suggested that, because CHAO also was found to colonize the root cortex, the strain may produce a stress effect in the plant leading to cyanide-resistant respiration and possible modification of tobacco metabolism resulting in enhanced host defense mechanisms. While four of the six PGPR strains reported here produced some HCN in vitro (Table 2), two strains that induced resistance had no HCN production. These results suggest that HCN production is not a required trait for PGPR-induced resistance. Further research, including analysis of mutants lacking HCN production, is needed to elucidate if HCN production is causally associated with induced systemic resistance activity.

Broad-spectrum fungal antagonism in vitro was not common among the inducing strains, which would support the conclusion that antifungal metabolites do not account for the observed biological control of *C. orbiculare*. This agrees with the conclusion of Kempe and Sequeira (11) that in vitro inhibition of *P. solanacearum* by various bacteria did not correlate with protection by tuber treatment with the same bacteria.

TABLE 3. Effect of plant growth-promoting rhizobacteria (PGPR) strains on cucumber root necrosis

Treatment	Number of root hairs showing discoloration[a]			
	Upper root[b]	Midroot[b]	Lower root[b]	Overall[c]
Pseudomonas fluorescens G8-4	4.0	0	10.0	4.0
P. aureofaciens 25-33	0	0	0	0
P. aureofaciens 28-9	4.8	8.3	0	5
P. putida 34-13	7.4	0	0	3
P. aureofaciens 36-5	4.0	4.8	0	3
Serratia plymuthica 2-67	4.3	5.3	0	3.3
Nonbacterized control	3.2	18.8	7.1	8.2

[a] Each value is the mean of three replications.
[b] For each root region, a 1-cm segment of the main root with attached root hairs was examined.
[c] Summary of all data from all three root regions.

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