

Induction of Systemic Resistance in Cucumber by Plant Growth-Promoting Rhizobacteria: Duration of Protection and Effect of Host Resistance on Protection and Root Colonization

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ABSTRACT

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Two plant growth-promoting rhizobacteria, *Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166, which previously induced systemic resistance (ISR) in cucumber 'Straight 8' against anthracnose caused by *Colletotrichum orbiculare*, were used to determine the duration of ISR in cucumber. ISR activity induced by strain 89B-27 occurred at the first-leaf stage, increased over time, and was maintained at least to the fifth-leaf stage. With strain 90-166, ISR was less stable, occurring at the second-, fourth-, and fifth-leaf stages. Cultivar specificity of ISR and colonization capacity of the two strains were studied in one resistant and three susceptible cucumber cultivars under greenhouse conditions. Strain

89B-27 induced systemic resistance in all three, whereas strain 90-166 induced systemic resistance in two of the three susceptible cultivars. Both strains failed to induce resistance in the resistant cultivar. Root colonization patterns of strains 89B-27 and 90-166 also differed. Populations of strain 89B-27 declined at a consistent rate over time, dropping from \log_{10} 8.2 CFU/g fresh weight of roots 1 week after planting to \log_{10} 3.2 CFU/g fresh weight of roots by 4 weeks after planting. In contrast, populations of strain 90-166 declined rapidly from \log_{10} 7.4 CFU/g fresh weight of roots 2 weeks after inoculation and then decreased more slowly to \log_{10} 3.2 CFU/g fresh weight of roots by 4 weeks after planting. There was no relationship between ISR activity and populations of the two strains on roots. ISR increased over time, while populations of the bacteria decreased.

Additional keywords: root-colonizing bacteria, systemic acquired resistance.

Induced protection in plants against various pathogens by biotic or abiotic inducers has been reported since the 1930s, when Chester (14) proposed the term "acquired physiological immunity." Since then, several terms have been used to describe the phenomenon of induced resistance, such as "systemic acquired resistance" (42), "translocated-resistance" (27), and "plant immunization" (33,47). Induced systemic resistance (ISR) is defined here as systemic protection of a plant by an inducing agent when applied to a single part of the plant (31). Classical inducers include pathogens (22, 24,32,34), heat-killed or attenuated pathogens (36), synthetic chemicals (37,44), metabolic products of hosts or infectious agents (25, 37), and incompatible pathogens (40).

Kuč et al. (35) reported that systemic protection of cucumber against *Colletotrichum lagenarium* was obtained by prior inoculation of cotyledons or the first leaf with the same pathogen. Later work showed that systemic resistance could be induced in a large number of crops, including cucurbit (34,43), legume (45,52), potato (16), tobacco (48), tomato (23), and fruit (27), against a broad spectrum of pathogens, including viruses (7,42), fungi (48), and bacteria (10).

Kloepper and Schroth (29) reported that certain root-colonizing bacteria could promote radish growth in both greenhouse and field trials and termed the bacteria "plant growth-promoting rhizobacteria" (PGPR). PGPR have been reported to control many soilborne pathogens (15,21,39). Colonization and population dynamics of PGPR in the rhizosphere and on roots are considered important aspects of biological control and have been studied in detail (6,9,28,

30,51). Mechanisms of biological control by PGPR generally involve competition (17) or production of bacterial metabolites such as siderophores, hydrogen cyanide, antibiotics, or extracellular enzymes that account for antagonism against the pathogens (29,46, 51).

Recently, specific PGPR strains were shown to act as inducing agents in different plant systems. Van Peer et al. (49) reported that a *Pseudomonas* sp. strain induced systemic resistance in carnation against Fusarium wilt. Wei et al. (50) indicated that seed inoculation of cucumber with select PGPR strains induced systemic resistance in cucumber leaves against anthracnose caused by *C. orbiculare* (Berk. & Mont.) Arx. Alström (1) found that systemic resistance was induced in bean plants against halo blight (*P. syringae* pv. *phaseolicola*) by seed treatment with *P. fluorescens* strain S97. Zhou and Paulitz (53) reported that root rot of European cucumber, caused by *Pythium aphanidermatum*, could be controlled by a *P. fluorescens* strain applied in a split-root system. Maurhofer et al. (38) showed that *P. fluorescens* strain CHA0 induced resistance in tobacco against tobacco necrosis virus. Collectively, these reports extend the known mechanisms for biological control of plant pathogens by PGPR.

Because PGPR may be applied as seed treatments, they have the potential to deliver the benefits of classic ISR, such as broad-spectrum pathogen control, in practical agriculture. To reach this potential, however, many questions must be answered first. For example, the effects of cucumber cultivars on PGPR-mediated ISR are unknown. The duration of PGPR-mediated ISR also is unknown. The objectives of this research were to determine the duration of PGPR-mediated ISR, the level of PGPR-mediated ISR in different cucumber cultivars, and the effects of cucumber cultivar on root colonization by PGPR.

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MATERIALS AND METHODS

Microbial cultures. Two PGPR strains, *P. putida* 89B-27 and *Serratia marcescens* 90-166, which previously demonstrated ISR activity in susceptible cucumber (*Cucumis sativus* L.) 'Straight 8,' were used (50). Cultures were maintained for long-term storage at -80°C in tryptic soy broth (TSB, Difco Laboratories, Detroit) with 20% glycerol. For experimental use, bacteria were incubated in TSB with shaking at 150 to 200 rpm at 28°C for 24 h. Cultures then were centrifuged at $5,000 \times g$ for 10 min, and the supernatant was discarded. Bacterial pellets were mixed with cucumber seeds (approximately \log_{10} 9 to 10 CFU per seed) before planting.

Strains 89B-27r3 and 90-166r2, two spontaneous rifampicin-resistant mutants of strains 89B-27 and 90-166, were selected on tryptic soy agar (TSA) with 100 μg of rifampicin per ml and maintained under the conditions described above. For seed inoculation, the mutants were incubated in TSB with 100 μg of rifampicin per ml with shaking at 150 to 200 rpm at 28°C for 24 h and were added to seed as described above.

Cultures of *C. orbiculare* (obtained from J. Kuć, Department of Plant Pathology, University of Kentucky, Lexington), were maintained for long-term storage at -80°C on green bean (GB) agar (20) plates covered with 50% glycerol. For experimental use, cultures were incubated on GB agar at room temperature for 5 to 7 days. Spores were washed with sterile distilled water and adjusted to \log_{10} 6 spores per ml for inoculation of first leaves in classic induced resistance control treatments and to \log_{10} 4 spores per ml for challenge inoculation on the other leaves.

Duration of PGPR-mediated ISR. The experimental design was a completely randomized design with three treatments and six replications (one plant per replication). Treatments included the two PGPR strains and a disease control, which was noninduced and challenge-inoculated only with the pathogen. Cucumber seeds were mixed with bacterial pellets, which resulted in \log_{10} 9 to 10 CFU per seed, prior to planting in 10-cm² plastic pots containing Pro-Mix soilless mix (Premier Peat Ltd., Rivière-du-Loup, Québec). Plants were grown in a greenhouse at 32/25 $^{\circ}\text{C}$ day/night. Cucumber true leaves 1, 2, 3, 4, or 5 on separate plants were challenge-inoculated with 30 10- μl drops of a spore suspension of *C. orbiculare* (\log_{10} 4 spores per ml) when the leaves were fully expanded. Only one leaf was sampled in each experiment to avoid confounding effects of induction by wounding or multiple pathogen inoculations. Challenged plants were incubated in humidity chambers in the dark at 25°C for 24 h before removal to the greenhouse. Diameters of lesions were measured 5 days after challenge-inoculation. The experiment was conducted three times. Data were pooled and tested for homogeneity of variance using Bartlett's test (19). Analysis of variance was performed using the general linear models in PC-SAS (SAS Institute, Cary, NC).

Effects of cultivar on PGPR-mediated ISR. Three cucumber cultivars susceptible to *C. orbiculare*, 'Straight 8' (ST), 'Amira' (AM), and 'Marketmore 80' (MM), and one resistant, 'County Fair 87' (CF) (Park Seed Co., Greenwood, SC), were used. Seeds were inoculated with the bacteria and planted in the greenhouse under the conditions described above. Two control treatments were included. The disease control consisted of noninduced plants inoculated only with the pathogen. The classically induced control consisted of nonbacterized plants induced by preinoculation of the first leaf with 10 10- μl drops of a spore suspension of *C. orbiculare* (\log_{10} 6 spores per ml). Three weeks after planting, fully developed second leaves were challenge-inoculated with 30 10- μl drops of a spore suspension of *C. orbiculare* (\log_{10} 4 spores per ml). Challenged plants were grown under the conditions described above. Mean total lesion diameters were analyzed using the procedures mentioned above. The experimental design was a 4×4 factorial within a randomized block design. Factors were four treatments and four cultivars. Each treatment had five repli-

cations, each consisting of a single plant, and the experiment was performed three times.

Effects of cultivar on colonization by rifampicin-resistant mutants 89B-27r3 and 90-166r2. The four cucumber cultivars described above were used to determine cultivar effects on root colonization by rifampicin-resistant mutants 89B-27r3 and 90-166r2. The experimental design was a 4×2 factorial, with four cultivars and two PGPR strains. Each treatment had five replications (one plant per replication). The bacteria were incubated and inoculated as described above. Bacterized seeds were planted in the same greenhouse. Reisolation of the bacteria was done from two seeds per replication at day 0. Entire root systems were sampled at 7 and 14 days after planting; about 1 g of roots was sampled at subsequent times. Seeds or roots were rinsed with tap water and washed three times with sterile distilled water. Washed roots were weighed, ground in 10 ml of sterile 0.85% NaCl solution, diluted, and spread on plates of TSA supplemented with 100 μg of rifampicin per ml. At day 28, the second leaves also were sampled to test whether the bacteria inoculated on seeds moved into the leaves. Entire leaves were removed, surface-disinfested with 1% sodium hypochlorite for 30 s, rinsed with sterile water three times, ground in 10 ml of sterile 0.85% NaCl solution, diluted, and incubated on plates of TSA with 100 μg of rifampicin per ml at 28°C in the dark. Rifampicin-resistant colonies grown in dilution plates were enumerated 2 days after incubation. Data were transformed to \log_{10} CFU/g fresh weight of roots and analyzed using the general linear models procedure in PC-SAS. A nonbacterized control of each cultivar was included to test the natural occurrence of rifampicin-resistant bacteria. The experiment was conducted twice.

RESULTS

Duration of PGPR-mediated ISR. The consistency of ISR over time varied with the PGPR strain used (Table 1). Strain 89B-27 significantly reduced total lesion diameter (TLD) compared to the noninduced control at the first-leaf stage. The protection of cucumber by strain 89B-27 generally was maintained from the first-

TABLE 1. Duration of systemic resistance mediated by plant growth-promoting rhizobacteria in cucumber cultivar Straight 8

Experiment	Treatment ^y	Mean total lesion diameter (mm)/leaf ^w				
		Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5
1	Dis-ck ^x	168.8	101.8	120.7	63.2	132.0
	90-166	145.5	84.7	114.2	38.1*	109.8
	89B-27	129.9*	40.1*	69.8*	20.7*	86.2*
	LSD _{0.05}	26.2	18.0	20.6	16.9	34.3
2	Dis-ck	122.2	104.3	57.3	68.5	115.8
	90-166	102.2*	69.7	57.1	46.9	34.4*
	89B-27	103.2*	30.0*	63.3	43.0	27.5*
	LSD _{0.05}	10.7	40.2	27.4	31.9	43.4
3	Dis-ck	59.4	128.7	142.5	67.3	122.3
	90-166	88.8	87.0*	127.2	20.7*	60.7*
	89B-27	48.5	67.3*	53.3*	20.6*	41.0*
	LSD _{0.05}	27.3	31.0	43.4	24.7	35.3
Mean ^y	Dis-ck	116.8	117.7	106.8	63.1	123.4
	90-166	112.2	80.4*	99.5	35.2*	63.3*
	89B-27	93.8*	45.8*	62.1*	27.6*	51.6*
	LSD _{0.05}	12.4	17.0	17.5	14.7	20.7
% protection ^z	90-166	—	28.0	—	44.2	48.7
	89B-27	19.7	59.0	41.8	56.3	58.2

^y Bacteria (*Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166) were inoculated on seeds prior to planting.

^w Mean of six replications. * = significantly less than the noninduced control ($P = 0.05$).

^x Dis-ck indicates noninduced control.

^y Mean of three experiments.

^z Calculated as mean percent change from disease control across three experiments.

to the fifth-leaf stage. The mean reduction of TLD after treatment with strain 89B-27 was 19.7% compared to the noninduced control at the first-leaf stage and increased at the second-, fourth-, and fifth-leaf stages. In general, protection by strain 90-166 was more variable than protection by strain 89B-27. Strain 90-166 reduced mean TLD in two of the three trials at the first- and third-leaf stages and in all three trials at the second-, fourth-, and fifth-leaf stages. The level of disease reduction with strain 90-166 treatment also increased from 28.0% at the second-leaf stage to 48.7% at the fifth-leaf stage.

Effects of cultivar on PGPR-mediated ISR. A significant decrease in the TLD was observed with strain 89B-27 in the three susceptible cultivars, ST, AM, and MM (Table 2). Strain 90-166 significantly decreased TLD in cultivars ST and AM in all experiments but was only effective in experiment 2 in cultivar MM (Table 2). Neither strain 89B-27 nor strain 90-166 had a significant effect on mean TLD in the resistant cultivar CF.

Analysis of data indicated that strain 89B-27 induced systemic resistance in the three susceptible cultivars, while strain 90-166 induced resistance in only two of the three cultivars. With strain 89B-27, there was no significant interaction among susceptible cultivars. Mean TLD decreased from 44 to 50% among the three susceptible cultivars when treated with strain 89B-27 compared to the noninduced control. Significant interactions were observed between strain 90-166 and the cultivars. In cultivar AM, mean TLD was reduced 80% by strain 90-166, which was significantly different from those of the noninduced control and strain 89B-27. However, in cultivar MM, strain 90-166 did not induce resistance. The levels of ISR mediated by strains 89B-27 and 90-166 were not as high as those of classic ISR by *C. orbiculare* in all three susceptible cultivars.

Effects of cultivar on colonization by rifampicin-resistant mutants 89B-27r3 and 90-166r2. No cultivar specificity in root colonization patterns by strain 89B-27r3 or 90-166r2 was observed. Colonization by strain 89B-27r3 was similar on all four cucumber cultivars. The mean population densities of strain 89B-27r3 on roots of the four cultivars were around \log_{10} 9.7 CFU/g fresh weight of roots at day 0 and declined steadily to around \log_{10} 8.2, 6.4, 4.4,

and 3.2 CFU/g fresh weight of roots at days 7, 14, 21, and 28, respectively (Fig. 1). Colonization densities of strain 90-166r2 declined rapidly in the first 2 weeks and then declined more gradually; mean populations of strain 90-166r2 were around \log_{10} 9.7, 7.4, 3.6, 3.4, and 3.2 CFU/g fresh weight of roots at days 0, 7, 14, 21, and 28, respectively (Fig. 2). Significant differences ($P = 0.05$) in population levels between the two strains occurred at days 7, 14, and 21; strain 89B-27r3 was more numerous. But at day 28, there was no significant difference between the two strains on any cultivars. Neither strain 89B-27r3 nor strain 90-166r2 was recovered from the leaves of any of the cultivars.

DISCUSSION

The results demonstrated that PGPR-mediated ISR resulted in significant protection of cucumber from anthracnose for the entire duration of the experiments. Significant reduction of TLD after treatment with strain 89B-27 occurred at the first-leaf stage, increased, and was maintained at least to the fifth-leaf stage more than 5 weeks after PGPR inoculation. ISR mediated by strain 90-166 was somewhat more variable among the three experiments but also was generally observed at all leaf stages tested (Table 1). These results indicate that PGPR-mediated ISR may lead to biological control of foliar pathogens at least through the middle of the growing season.

Previous work demonstrated that classic ISR was not cultivar specific but generally was more effective on resistant than susceptible cultivars (22,34). In contrast, our work showed that ISR mediated by some PGPR strains was cultivar specific. In the three susceptible cultivars used, strain 89B-27 significantly induced resistance in three experiments in all three cultivars, whereas strain 90-166 induced resistance in only two of the three cultivars in three experiments (Table 2). Strain 89B-27 showed no bacteria-cultivar interactions in susceptible cucumber cultivars. Reductions in TLD in the three susceptible cultivars by strain 89B-27-mediated ISR were around 44 to 50%, with no significant differences among the susceptible cultivars. With strain 90-166, the reduction in TLD in cultivar AM was 80% compared to the non-

TABLE 2. Induced systemic resistance mediated by two plant growth-promoting rhizobacteria strains against *Colletotrichum orbiculare* in four cucumber cultivars

Cultivar ^a	Treatment ^w	Mean total lesion diameter(mm)				% disease decreased ^y
		Exp. 1	Exp. 2	Exp. 3	Mean ^x	
ST	Dis-ck ^z	142.0	141.2	158.2	147.1 a	
	90-166	80.8	89.2	65.2	78.4 cd	46.7
	89B-27	79.4	77.6	63.2	73.4 cd	50.1
	Ir-ck	21.0	23.2	18.7	21.0 f	85.7
AM	Dis-ck	138.8	184.4	98.9	140.7 ab	
	90-166	37.2	20.9	20.2	26.3 f	81.3
	89B-27	55.8	94.8	63.4	71.7 cd	49.0
	Ir-ck	1.4	0.9	4.2	2.4 f	98.2
MM	Dis-ck	125.4	101.6	67.2	107.4 bc	
	90-166	132.0	50.4	159.8	107.5 bc	0.0
	89B-27	46.0	75.0	60.5	60.5 de	43.7
	Ir-ck	15.1	19.0	14.0	16.0 f	85.1
CF	Dis-ck	32.0	35.2	30.0	32.4 ef	
	90-166	29.0	26.0	31.6	28.9 ef	10.8
	89B-27	36.0	23.6	25.8	28.5 ef	12.0
	Ir-ck	21.3	44.6	32.9	32.9 ef	0.0
	LSD _{0.05}	37.6	32.4	39.1		

^a ST = 'Straight 8,' AM = 'Amira,' MM = 'Marketmore 80,' and CF = 'County Fair 87.'

^w Bacteria (*Pseudomonas putida* strain 89B-27 and *Serratia marcescens* strain 90-166) were inoculated on seeds prior to planting.

^x Means with different letters are significantly different at $P = 0.05$. Means are from six replicate plants.

^y Calculated as percent change from disease control across the three experiments.

^z Dis-ck indicates noninduced control; Ir-ck indicates no bacteria on seeds, preinoculated on the first leaves by *C. orbiculare*.

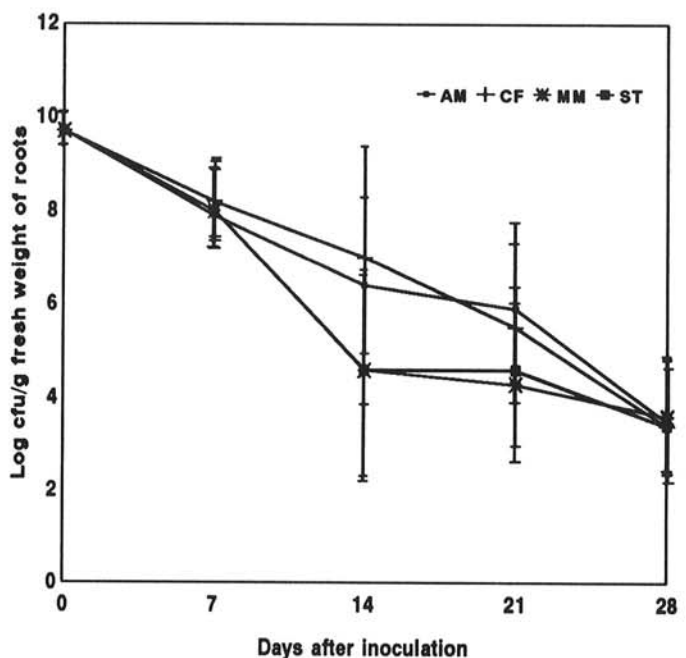


Fig. 1. Populations of plant growth-promoting rhizobacteria rifampicin-resistant mutant strain 89B-27r3 of *Pseudomonas putida* on roots of four cucumber cultivars grown from inoculated seed. AM = 'Amira'; CF = 'County Fair 87'; MM = 'Marketmore 80'; and ST = 'Straight 8.' Bars represent error based on standard deviations. Data are pooled results of two experiments.

induced control, which was significantly different from treatment with strain 89B-27 and the noninduced control. However, in cultivar MM, strain 90-166 did not show any ISR activity (Table 2). Interactions between cucumber cultivars and PGPR strain 90-166 occurred among susceptible cultivars.

In resistant cultivar CF, neither PGPR nor classic inducers showed any effect. The difference in our results from a previous report (34) of classic ISR on resistant cultivars may be explained by different relative levels of resistance. Kuć and Richmond (34) reported that 18 to 29 lesions per leaf developed in noninduced resistant cultivars used in their work. In contrast, in our study with resistant cultivar CF, disease was much more restricted, with an average of only 12 lesions per leaf on noninduced controls. This suggests that there is a lower limit beyond which ISR cannot add to the protection occurring due to heritable resistance. Alternative explanations could include: (i) PGPR did not or cannot induce resistance in these highly resistant cultivars; (ii) pathogenesis-related proteins such as chitinases and β -1,3-glucanases are already turned on in these cultivars; or (iii) mechanisms induced by PGPR are not additive or synergistic to those of heritable resistance. Further work is needed to clarify the interactions of host genetic resistance and ISR.

Plant species or cultivars have varied in their reaction to inoculation with beneficial or deleterious rhizobacteria (4,5,11,18,26). Chanway et al. (11,12,13) investigated the responses of plant genotypes to rhizobacteria in several plant systems, including annual and perennial plants. They found that genotypic specificity between spruce and rhizobacteria could determine the nature of the plant-growth response to inoculation. They also reported genotypic coadaptation in plant-growth promotion of forage species by *Bacillus polymyxa* and cultivar-specific growth promotion of spring wheat by *Bacillus* spp. (11). In addition, activities of harmful rhizobacteria have been reported to be cultivar specific (2,3). Aström and Gerhardson (3) reported that the activity of deleterious rhizobacteria in the wheat rhizosphere was cultivar specific. Early work provided evidence that a plant genotype-rhizosphere bacterial strain interaction may be based on genetic compatibility between plants and microbes (11,12). Our results on cultivar specificity of PGPR-mediated ISR agree with these reports.

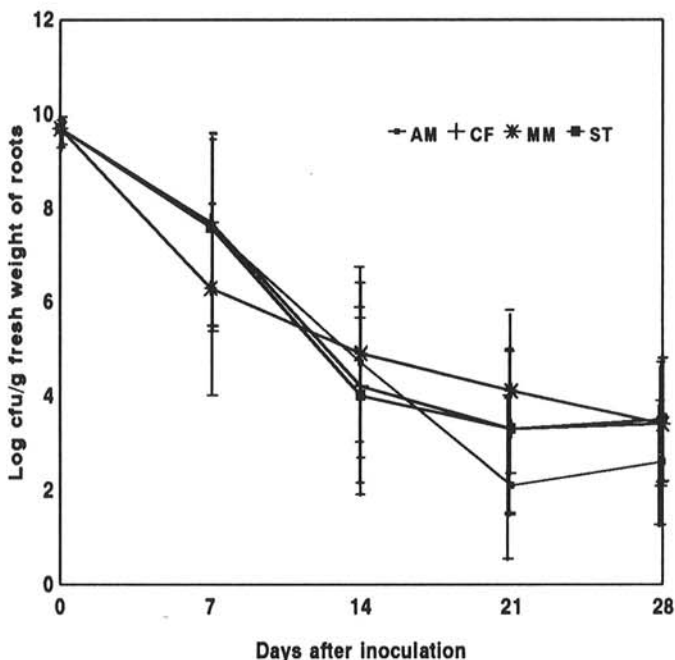


Fig. 2. Populations of plant growth-promoting rhizobacteria rifampicin-resistant mutant strain 90-166r2 of *Serratia marcescens* on roots of four cucumber cultivars grown from inoculated seed. AM = 'Amira'; CF = 'County Fair 87'; MM = 'Marketmore 80'; and ST = 'Straight 8.' Bars represent error based on standard deviations. Data are pooled results of two experiments.

In contrast, the colonization capacity of rifampicin-resistant mutants of PGPR strains studied on root systems of the four cucumber cultivars showed no cultivar specificity. Population dynamics of strains 89B-27r3 and 90-166r2 on roots of the four cultivars followed a similar pattern for each strain and showed no significant differences over time, regardless of the level of resistance (Figs. 1 and 2). There was no correlation between ISR activity and populations of the two PGPR strains. For instance, there was no significant difference in the populations of strain 90-166r2 between AM and MM; however, there was a significant difference in ISR activity between the two cultivars with strain 90-166 treatment. Another important point is that ISR mediated by strains 89B-27 and 90-166 increased over time although populations of the bacteria declined. Hence, the observed specificity in ISR may not be accounted for by cultivar-specific colonization.

Reddy and Rahe (41) reported that growth promotion and yield increase of onions by *B. subtilis* strain B-2 were not related to populations of the bacterium on roots. Although populations of B-2 declined rapidly within the first 2 weeks after inoculation and were maintained at very low levels later (an average \log_{10} 9.45 CFU per plant at 14 weeks after inoculation), shoot and root growth of onion seedlings were significantly increased. Contrasting results were reported by Bull et al. (8) who demonstrated a positive relationship between root colonization by *P. fluorescens* strain 2-79RN₁₀ and biological control of take-all, caused by *Gaeumannomyces graminis* var. *tritici*.

Our work demonstrated that ISR activities mediated by the two PGPR strains increased over time, suggesting that ISR activity and colonization capacity of PGPR strains probably are not correlated. Some earlier work that showed a positive relationship between PGPR populations and biological control activities might relate to production of antibiotics or related products by PGPR (8). Higher bacterial populations result in increased levels of antibiotics produced. In our research, ISR activity mediated by PGPR strains did not depend on high populations. Therefore, even though it may not be a good colonizer, a PGPR strain could be a good inducer and may be used as an active biological control agent.

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