Induced Systemic Resistance to Cucumber Diseases and Increased Plant Growth by Plant Growth-Promoting Rhizobacteria Under Field Conditions

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ABSTRACT

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Three field trials were conducted over a 2-year period to determine the capacity of plant growth-promoting rhizobacteria (PGPR) to induce systemic resistance against cucumber diseases, which was previously observed in the greenhouse. PGPR were applied as seed treatments alone or as seed treatments plus a soil drench at transplanting. Induced systemic resistance (ISR) activity, measured by significant reductions in the severity of angular leaf spot challenge-inoculated with *Pseudomonas syringae* pv. *lachrymans* on cucumber, occurred with three of three PGPR strains in 1992, with three of four strains in the first trial in 1993, and with four of four strains in the second trial in 1993. Most PGPR treatments also resulted in significant protection from naturally occurring anthracnose (*Colletotrichum orbiculare*), while ISR controls (plants previously inoculated with *C. orbiculare*) had a significantly greater incidence of anthracnose compared with nontreated controls. In all three trials, most PGPR strains (with the exception of one strain) significantly promoted early-season plant growth, as measured by length of the main runner and number of leaves per plant. Yield (cumulative fruit weight) was significantly increased by two of three PGPR strains in 1992, two of four strains in the first 1993 trial, and three of four strains in the second 1993 trial compared with nontreated controls. In contrast, the ISR control had no significant effect on promotion of early-season plant growth or yield compared with nontreated controls. These results demonstrated that PGPR-mediated ISR was operative under field conditions with consistent effects against challenge-inoculated angular leaf spot and naturally occurring anthracnose, along with significant effects on early-season plant growth promotion and yield enhancement.

Additional keywords: biological control, systemic acquired resistance.

Most bacterial biological control agents of plant pathogens function partially or completely through antagonism (2,6,22). Research over the past years has demonstrated that induced systemic resistance (ISR) can be an alternative mechanism to antagonism for achieving biological control of plant disease (25). There are several major differences in ISR compared with antagonism as a mechanism of biological control. First, the action of ISR is based on plant defense mechanisms that are activated by inducing agents (15), while antagonism relies on direct functions of the biological control agents such as production of antibiotics, siderophores, and hydrogen cyanide (HCN), as well as nutrient competition. Second, ISR, once expressed, activates multiple potential defense mechanisms, that include increases in activity of chitinases, β-1,3-glucanases, peroxidases, and other pathogenesis-related (PR-) proteins (19); accumulation of antimicrobial low-molecular-weight substances, e.g., phytoalexins (17,18); and formation of protective biopolymers, e.g., lignin, callose, and hydroxyproline-rich glycoproteins (11,12,13). Third, an important aspect of ISR is the wide spectrum of pathogens that can be controlled with a single inducing agent. In cucumber, treatment of the first leaf with a necrosis-forming organism protects the plant against at least 13 pathogens including fungi, bacteria, and viruses (7,16), while antagonism is generally not active against diverse pathogens. Fourth, ISR, by definition (15), protects the plant systemically following induction with an inducing agent to a single part of the plant, while other mechanisms of biological control are generally not systemic.

Induced systemic disease resistance has been studied mainly in laboratories and greenhouses. However, some reports indicate that ISR can protect crop plants under field conditions (3,4,8,24,26). In cucumber and watermelon, systemic protection following induction with *Colletotrichum orbiculare* (Berk. & Mont.) Arx was demonstrated against a challenge inoculation of the same pathogen in the field (3). In tobacco, extensive field tests over a 3-year period in Kentucky and Puerto Rico demonstrated that ISR protected plants against both metalaxyl-sensitive and metalaxyl-tolerant strains of *Peronospora tabacina* using stem-injection induction with sporangiosporal suspensions of the same pathogen (24,26). Widespread implementation of ISR has not been accomplished, partly because classical ISR employs pathogenic organisms as inducing agents.

In the past years, work in several laboratories has demonstrated that some plant growth-promoting rhizobacteria (PGPR) may act as inducing agents, leading to systemic protection against pathogens (1,20,21,27,28,29). PGPR may offer a practical way of delivering ISR to agriculture, but the feasibility of this approach has not been reported under field conditions. The objective of this study was to determine if PGPR-mediated ISR, which was previously observed in the greenhouse (20,21,28), also occurred under field conditions.

MATERIALS AND METHODS

Sources of PGPR strains and bacterization of plants. Four PGPR strains were used: *Pseudomonas putida* strain 89B-61,

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Serratia marcescens strain 90-166, *Flavomonas oryzihabitans* strain INR-5, and *Bacillus pumilus* strain INR-7. The strains were identified by analysis of fatty acid methyl esters using gas chromatography (23). Strain 89B-61 was selected by Agrium (Saskatoon, Canada) as a plant growth-promoting rhizobacterium; strain 90-166 was originally selected for biological control activity against *Rhizoctonia solani* on cotton; and INR-5 and INR-7 were originally isolated from internal parts of roots of cucumber. All strains were screened and selected for ISR activity in repeated assays using the cucumber anthracnose system described previously (28). Bacterial strains were maintained for long-term storage at –80°C in tryptic soy broth (TSB) with 20% glycerol. For preparing bacterial suspensions, cultures from –80°C were grown on tryptic soy agar (TSA) for 24 h at 28°C, and single colonies were transferred to TSB and incubated 24 h at 25°C with shaking at 150 rpm. Bacteria were pelleted with centrifugation for 5 min at 6,000 × *g* and resuspended in distilled water to give concentrations of 10^5 or 10^8 CFU/ml.

For seed treatment, seeds were placed in the bacterial suspension of 10^8 CFU/ml 30 min before planting. When soil drenches were used, 100 ml of the 10^5 CFU/ml bacterial suspension was poured into soil around each seed or plant.

Experimental design. A total of three field trials were conducted (one trial in 1992 and two trials in 1993) at the E. V. Smith Research Center, Horticulture Unit of the Alabama Agricultural Experiment Station near Shorter. The experimental designs were randomized complete blocks. All the plants were grown using cultural practices, including raised beds with black plastic mulch, methyl bromide-fumigated soil, drip irrigation, and fertilization as needed. The experiments started in early April and ended in mid-June in both years.

Trial 1 was conducted in 1992. There were five treatments with six replications per treatment and six plants per replication. The treatments included PGPR strains 89B-61, 90-166, and INR-5 and two controls: an ISR control (that was induced by previous inoculation of cotyledons with *C. orbiculare*) and a disease control (that was not induced and not treated with bacteria). The experiment used transplants, in which bacterized cucumber seeds were planted in 10-cm-square pots containing Promix soilless potting mix (Premier Peat, Riviére-du-Loup, Québec, Canada) in the greenhouse. The ISR control was induced in the greenhouse by inoculating cotyledons with *C. orbiculare* 2 weeks after planting. Cucumber plants were transplanted to the field 4 weeks after seeding and challenge-inoculated with *Pseudomonas syringae* pv. *lachrymans* 6 weeks after seeding.

In 1993, trials 2 and 3 were conducted. Both the trials were transplant experiments, in which cucumber plants were challenge-inoculated with *P. syringae* pv. *lachrymans* with the methods used in trial 1. Trial 2 was prepared using seed bacterization as in trial 1, while trial 3 received an additional PGPR application by adding 100 ml of bacterial suspension (10^5 CFU/ml) per plant at transplanting time. Randomized complete blocks were used with six treatments per experiment with six replications per treatment and 10 plants per replication in trials 2 and 3. Treatments included four PGPR strains, i.e., 89B-61, 90-166, INR-5, and INR-7, and two controls: an ISR control and a disease control.

Challenge inoculation. Challenge inoculum consisted of an isolate of *P. syringae* pv. *lachrymans* (causal agent of cucumber angular leaf spot), that was obtained from the Department of Horticulture, University of Wisconsin, Madison. The cultures from –80°C were grown on TSA for 24 h at 28°C, and single colonies were transferred to TSB and incubated 24 h at 25°C with shaking at 150 rpm. Bacteria were centrifuged and pellets were resuspended in distilled water, resulting in a concentration of 10^7 CFU/ml. Fully expanded new leaves were challenge-inoculated 2 weeks after transplanting by injecting 3.0 µl of inoculum suspension at each of 30 sites per leaf.

Data collected. With all three trials, ISR activity was monitored by recording lesion number and lesion diameter of angular leaf spot on the challenge-inoculated leaf 10 to 12 days after challenge inoculation. Naturally occurring cucumber anthracnose (caused by *C. orbiculare*) was also evident in the trials. In trials 2 and 3, the number and diameter of anthracnose lesions on the sixth leaf of main runners were measured at 60 days after seeding. In all three trials, data were also collected on the length of the main runner and the number of leaves on each plant as a measure of growth promotion at 50 days after seeding. Marketable cucumber fruits were picked and weighed twice weekly until the end of the growing season.

Statistical analysis. Data on disease severity, plant growth promotion, and yield were analyzed independently by year and trial. Analysis was performed with the general linear model (GLM) procedure, and treatment means were separated by the least significant difference (LSD) test at $P \leq 0.05$ with SAS software (SAS Institute, Cary, NC).

TABLE 1. Field trial 1 (1992) for testing induced systemic resistance and growth promotion with plant growth-promoting rhizobacteria

Treatments	Length of main runner (cm)[a]	Leaf number[a]	TLD[b] of angular leaf spot (mm)[a]	Cumulative fruit weight (kg)[a]
89B-61	96.0	64.9*[c]	110.3*	20.8*
90-166	101.5*	63.6*	107.2*	20.1*
INR-5	102.1*	59.8*	100.8*	18.4
ISR CK[d]	94.8	51.0	93.5*	14.9
Disease CK[e]	91.1	49.3	150.3	16.8
$LSD_{0.05}$	6.9	7.2	13.1	2.1

[a] Mean per plant from six replications with six plants per replication.
[b] TLD = total lesion diameter on challenge-inoculated leaf for angular leaf spot. Plants were inoculated 2 weeks after transplanting.
[c] * = Indicates significantly less disease incidence or greater yield than the disease control at $P \leq 0.05$.
[d] Induced systemic resistance control: prior induction on cotyledon with *Colletotrichum orbiculare*.
[e] Nonbacterized, noninduced control.

RESULTS

Results from trial 1 in 1992 (Table 1) showed that two of the three tested PGPR strains (90-166 and INR-5) significantly increased runner length, and all three strains significantly increased leaf number per plant compared with the disease control. All three PGPR strains and the ISR control demonstrated ISR activity by significantly reducing the total lesion diameter of angular leaf spot per leaf compared with the disease control. Two of the three PGPR treatments significantly increased total yield (fruit weight) compared with the disease control.

Results from trial 2 in 1993 (Table 2) showed that two of the three PGPR treatments used in trial 1 and the new strain INR-7 caused significant plant growth promotion with increased runner length and increased leaf number per plant compared with the disease control. Three PGPR strains and the ISR control demonstrated induced resistance activity with significantly reduced total lesion diameter of cucumber angular leaf spot per leaf compared with the disease control. The incidence of naturally occurring anthracnose was also reduced in three of the four PGPR treatments, while the disease incidence was greatest on the ISR control. Two PGPR treatments (strains 90-166 and INR-7) showed significant increase of total yield (fruit weight) compared with the disease control.

For trial 3 in 1993 (Table 3), all four PGPR strains showed significant plant growth promotion with increased runner length and increased leaf number per plant. All PGPR strains and the ISR control significantly reduced the total diameter of angular leaf spot lesions compared with the disease control. Again, naturally occurring anthracnose was greatest on the ISR control and

lowest on PGPR treatments. Three PGPR treatments (strains 89B-61, 90-166, and INR-7) caused significant total yield (fruit weight) increases compared with the disease control.

DISCUSSION

The results from field tests conducted over a 2-year period clearly demonstrated that PGPR-mediated ISR and plant growth promotion were operative under field conditions. Protection was observed against two pathogens, i.e., the causal agents of angular leaf spot and anthracnose. PGPR-mediated protection against the challenge-inoculated pathogen (*P. syringae* pv. *lachrymans*) was highly consistent, occurring at a statistically significant level ($P \leq 0.05$) with three of three PGPR strains in trial 1, three of four strains in trial 2, and four of four strains in trial 3. Disease protection by PGPR was generally associated with early-season plant growth promotion and yield enhancement.

In all three trials, the classical ISR control resulted in significant protection against the challenged pathogen (*P. syringae* pv. *lachrymans*) compared with the noninduced control (Tables 1 to 3). This confirmed previous work which demonstrated that one pathogen can induce systemic resistance against a heterologous pathogen (16). However, the classical ISR control did not significantly increase early-season plant growth or yield in any trial.

Naturally occurring anthracnose (caused by *C. orbiculare*) was severe in the fields during the growing seasons. Three of four PGPR strains in trial 2 and four of four strains in trial 3 resulted in significant protection against anthracnose (Tables 2 and 3). In contrast, classical ISR did not protect cucumber plants from late-season anthracnose. Actually, the incidence of anthracnose was significantly greater with classical ISR controls in trials 2 and 3 compared with the nontreated controls. There could be several explanations for this failure of classic ISR against *C. orbiculare* under field conditions. Besides the possibility that the duration of protection was less with pathogen induction than with PGPR induction, prior inoculation with *C. orbiculare* obviously provided a source of secondary inoculum on ISR controls. This could explain increased lesion number of the disease later in the season on the ISR controls. According to the theory of ISR, however, the disease still should not have developed to the highest level with both increased lesion number and lesion diameter on these ISR controls if the plants were actively expressing ISR. Also, it could be the possibility that the strain of *C. orbiculare* that had been previously inoculated on the ISR control was more virulent than the naturally occurring strain. Another explanation comes from the theory of "suppression of defense mechanisms". Some studies (5,10,14) suggest that virulent fungi may actively suppress the expression of plant defense reactions during successful infection. Daly (5) has pointed out that the action of host-selective toxins may be viewed as interfering with the normal resistance of the host. Similarly, the degradation of phytoalexins to nontoxic derivatives may also be regarded as a form of active suppression of plant resistance (14). Moreover, there have been indications that virulent fungi may produce "suppressors" to prevent expression of active resistance in their hosts. Perhaps the best characterized suppressors of host defense reactions are those produced by *Phytophthora infestans*. These are anionic and non-ionic glucans, of about 10 to 20 glucose units, that contain β-1-3 and β-1-6 linkages (9). All races of the tested fungus seem to be able to produce these compounds, that suppress the browning and terpenoid accumulation in compatible potato tubers (10). There may be a balance between "inducers" and "suppressors" to determine which will become predominant for expression of "induced resistance" or "induced susceptibility".

As plant pathologists, we are faced with the challenge of finding more effective, practical, and economical ways to protect plants from various diseases. Using PGPR as inducers has several advantages over classical ISR. PGPR are root-colonizing beneficial bacteria and the beneficial effects include biological control and plant growth promotion. In contrast, classical ISR by prior inoculation of a pathogen on plants may introduce a disease into fields, causing obvious risk to plants. Seed bacterization with PGPR strains is a practical way to deliver benefits of ISR, while classical ISR by hand-inoculation of a pathogen inducer to each plant is much more labor intensive.

The results reported here indicated that the application of PGPR-mediated ISR holds promise for practical disease management. In addition to using a single inducing bacterial strain, it may be possible to apply a mixture of inducing strains (bacteria-bacteria or bacteria-fungi) plus organic amendments. Future field trials should address several additional issues of PGPR-mediated ISR including the length of protection, the microbial ecology of the PGPR, and the spectrum of control against multiple pathogens or pests including fungi, bacteria, viruses, nematodes, and even insects. These studies will allow a full assessment of the potential applicability of PGPR-mediated ISR in integrated pest management strategies.

TABLE 2. Field trial 2 (1993) for testing induced systemic resistance and growth promotion with plant growth-promoting rhizobacteria

Treatments	Length of main runner (cm)[a]	Leaf number[a]	TLD[b] of angular leaf spot (mm)[a]	TLD[c] of anthracnose (mm)[a]	Cumulative fruit weight (kg)[a]
89B-61	92.7	63.2	134.4*[d]	34.3*	23.7
90-166	96.2*	64.2*	157.4	45.8	25.7*
INR-5	99.0*	65.0*	129.0*	36.3*	23.7
INR-7	95.9*	64.0*	125.3*	29.2*	23.9*
ISR CK[e]	84.9	44.7	139.6*	153.9#[f]	22.0
Disease CK[g]	89.6	55.7	161.9	61.9	20.0
$LSD_{0.05}$	4.7	7.7	19.1	23.7	3.8

[a] Mean per plant from six replications with 10 plants per replication.
[b] TLD = total lesion diameter on challenge-inoculated leaf for angular leaf spot.
[c] TLD = total lesion diameter from natural infection of anthracnose on the 6th leaf of main runner.
[d] * = Indicates significantly less disease incidence or greater yield than the disease control at $P \leq 0.05$.
[e] Induced systemic resistance control: prior induction on cotyledon with *Colletotrichum orbiculare*.
[f] # = Indicates significantly greater disease incidence than the disease control at $P \leq 0.05$.
[g] Nonbacterized, noninduced control.

TABLE 3. Field trial 3 (1993) for testing induced systemic resistance and growth promotion with plant growth-promoting rhizobacteria

Treatments	Length of main runner (cm)[a]	Leaf number[a]	TLD[b] of angular leaf spot (mm)[a]	TLD[c] of anthracnose (mm)[a]	Cumulative fruit weight (kg)[a]
89B-61	62.6*[d]	29.5*	56.0*	21.2*	37.4*
90-166	64.9*	30.1*	86.7*	30.4*	35.9*
INR-5	65.5*	31.5*	69.6*	22.4*	32.7
INR-7	65.6*	30.3*	48.7*	24.2*	37.1*
ISR CK[e]	49.6	20.5	45.6*	148.6#[f]	25.6
Disease CK[g]	55.9	25.0	121.2	57.5	27.3
$LSD_{0.05}$	5.6	4.4	10.0	25.1	8.2

[a] Mean per plant from six replications with 10 plants per replication.
[b] TLD = total lesion diameter on challenge-inoculated leaf for angular leaf spot.
[c] TLD = total lesion diameter from natural infection of anthracnose on the 6th leaf of main runner.
[d] * = Indicates significantly less disease incidence or greater yield than the disease control at $P \leq 0.05$.
[e] Induced systemic resistance control: prior induction on cotyledon with *Colletotrichum orbiculare*.
[f] Indicates significantly greater disease incidence than the disease control at $P \leq 0.05$.
[g] Nonbacterized, noninduced control.

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