Induction of Systemic Resistance in Cucumber Against Fusarium Wilt by Plant Growth-Promoting Rhizobacteria

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ABSTRACT

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Plant growth-promoting rhizobacteria (PGPR) strains 89B-27 (*Pseudomonas putida*) and 90-166 (*Serratia marcescens*) were tested for their ability to induce systemic resistance against Fusarium wilt, a vascular disease of cucumber, using a split-root assay. PGPR strains and *Fusarium oxysporum* f. sp. *cucumerinum* were inoculated on separate halves of roots of cucumber seedlings at the same time and then planted in separate pots. Both PGPR strains induced systemic resistance against *F. oxysporum* f. sp. *cucumerinum* as expressed by delayed disease symptom development and reduced number of dead plants after PGPR treatments compared to the nonbacterized, *F. oxysporum* f. sp. *cucumerinum*-inoculated controls 5 weeks after inoculation. *F. oxysporum* f. sp. *cucumerinum* was recovered from lower stems 2 weeks after root inoculation and from the first, second, and third petioles 5 weeks after inoculation in the nonbacterized control. In contrast, *F. oxysporum* f. sp. *cucumerinum* was isolated from stems of plants treated with PGPR only 4 weeks after inoculation and from the first petiole 5 weeks after inoculation, indicating that PGPR treatment reduced spread of the pathogen. Movement of PGPR in cucumber split-root systems was monitored with a bioluminescent derivative of 89B-27, strain L211, that was detected with a charge-coupled device camera. Strain L211 provided protection against *F. oxysporum* f. sp. *cucumerinum* at levels similar to the wild-type PGPR strain. L211 colonized cucumber roots up to 5 weeks after root inoculation and was not detected inside stems or petioles. The bacterium showed only limited movement within inoculated pots and did not move to the pots in which the pathogen was inoculated, demonstrating that the PGPR and pathogen remained spatially separated. We conclude that the two PGPR strains induced resistance systemically in cucumber against Fusarium wilt.

Additional keywords: cross-protection, immunization, *lux* marker, systemic aquired resistance.

Mechanisms of biological control of Fusarium wilt by beneficial microorganisms are complex. Most studies conducted previously have focused on using nonpathogenic fusaria or other antagonists (1,9,13–15,18) that exert biological control through mechanisms such as competition for nutrients or iron (4–6,16), competition for infection sites on roots (13), or production of antibiotics (8,10,19, 22,24).

Another mechanism for control of Fusarium wilt is induced resistance. Gessler and Kuć (7) in 1982 reported that systemic resistance in cucumber against *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cucumerinum* J.H. Owen could be induced by preinfection of first true leaves with *Colletotrichum orbiculare* or tobacco necrosis virus. The use of nonpathogenic fusaria to induce resistance (sometimes termed cross-protection) in plants against Fusarium wilt has been widely studied (1–3,18,23). Prior inoculation with a nonpathogenic *F. oxysporum* induced local and systemic resistance against Fusarium wilt in watermelon (2), cucumber (15), and tomato (23). Most of these studies, however, could not exclude mechanisms other than induced resistance because the pathogen and the biocontrol agents were not spatially separated.

Investigations of plant growth-promoting rhizobacteria (PGPR) as agents of induced systemic resistance (ISR) against various pathogens, including *F. oxysporum*, have been conducted recently (12,20,21,25). Van Peer et al. (20) reported that a *Pseudomonas* sp. strain suppressed Fusarium wilt in carnation. In this study, the pathogen was inoculated into stems after PGPR were applied to roots.

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To determine if ISR is a mechanism for biological control, it is necessary to use a system that spatially separates the PGPR and pathogens. Otherwise, one cannot exclude the possible role of antagonism or competition. The system also should involve introduction of the pathogen in a natural way, i.e., on roots to control soilborne pathogens if the inducing agent is eventually to be used in practical biological control.

Split-root systems, which allow spatial separations, have been used in some studies of ISR. The split-root system was used in studies of nonpathogenic *F. oxysporum* as an inducer of resistance against Fusarium wilt in tomato (9) and cucumber (13). Zhou and Paulitz (25) concluded that ISR was the mechanism for biological control of cucumber root rot, caused by *Pythium aphanidermatum*, by PGPR strains, based on studies in a split-root system.

The objectives of this research were to determine if PGPR strains 89B-27 (*Pseudomonas putida*) and 90-166 (*Serratia marcescens*), which previously demonstrated ISR activity against various cucumber foliar diseases (21), also induced systemic resistance against Fusarium wilt caused by *F. oxysporum* f. sp. *cucumerinum* and if the PGPR and the pathogen remained spatially separated throughout the bioassay using a bioluminescent marker system.

MATERIALS AND METHODS

Microbial cultures. Two wild-type PGPR strains, 89B-27 (*P. putida*) and 90-166 (*S. marcescens*), were used in the experiments. These strains previously induced resistance against *C. orbiculare* (21) and cucumber mosaic virus (12) after seed bacterization. For long-term storage, the strains were maintained at –80°C in tryptic soy broth (TSB) (Difco Laboratories, Detroit) with

20% glycerol. For experimental use, 24-h-old cultures in TSB were centrifuged at 5,000 × *g* for 10 min, the supernatant was discarded, and the pellets were resuspended in sterile water. Bacterial suspensions were adjusted to $\log_{10}$ 9 to 10 CFU/ml for use as root inoculants.

L211, a bioluminescent derivative of 89B-27, was obtained by biparental mating of a rifampicin mutant of strain 89B-27 and DH5α, a strain of *Escherichia coli* with pUCD623 in which Tn*4431* with *lux*CDABE was inserted (17). The transformed derivative, L211, emitted light that could be detected with a charge-coupled device (CCD) camera. Cultures were stored under the same conditions as described above. For experimental use, L211 was incubated in TSB with tetracycline at 7.5 µg/ml at 28°C for 24 h, and cultures were harvested as described above.

An isolate of *F. oxysporum* f. sp. *cucumerinum* was obtained from the Department of Horticulture at the University of Wisconsin. The isolate was stored at 4°C on potato-dextrose agar (PDA; Difco). For experimental use, the isolate was incubated on PDA at 25°C for 7 days. Microconidia were removed from the surface of the medium with sterile water, and the resulting suspension was filtered through eight layers of cheesecloth to remove mycelial fragments before adjusting the concentration to $\log_{10}$ 5.3 spores per ml with a hemacytometer.

Cucumber cultivar. *Cucumis sativus* L. 'Straight 8,' susceptible to Fusarium wilt, was used in this experiment. Seeds were planted in 10-cm^2 pots filled with soilless Pro-mix (Premier Peat Ltd, Rivière-du-Loup, Québec, Canada) and grown under greenhouse conditions at 32°C (day) and 25°C (night).

ISR by PGPR in the split-root system. Roots of 2-week-old cucumber seedlings were uprooted, washed with tap water, and carefully split into two halves with a dissecting knife. One half was dipped in 20 ml of the spore suspension of *F. oxysporum* f. sp. *cucumerinum*, and the other half was dipped in 10 ml of bacterial suspension. Care was taken to keep the halves separated during and after treatments. Each half of the treated root system was transplanted into separate 10-cm^2 plastic pots attached together on the outside with tape. The experiment was a completely randomized design with 4 treatments and 10 replications, each with 1 plant. Treatments included the two PGPR strains, a nonbacterized, pathogen-inoculated disease control, and a nontreated control. The transplants were placed in a high humidity chamber at 25°C for 5 to 7 days before being moved back to the greenhouse. For the disease control, one half of the split roots was inoculated with the pathogen, and the other half was treated with sterile water. For the healthy control, cucumber roots were split and transplanted as described above but without any treatment on the roots. Each pot was placed in a plastic bag to prevent release of the pathogen or PGPR from drain holes and possible contamination of other pots.

TABLE 1. Reduction of Fusarium wilt in cucumber, caused by *Fusarium oxysporum* f. sp. *cucumerinum* (Foc), by root treatment with plant growth-promoting rhizobacterial (PGPR) strains 89B-27 (*Pseudomonas putida*) and 90-166 (*Serratia marcescens*)[w]

Treatment[x]	No. of dead plants[y]				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean[z]
PGPR + Foc	9	7	5	6	6.75 a
89B-27 + Foc	9	3	1	2	3.75 b
90-166 + Foc	6	4	2	1	3.25 b
No PGPR, no Foc	0	0	0	0	0

[w] Susceptible cucumber 'Straight 8' was used.
[x] Ten plants per treatment.
[y] Number of dead plants was determined at the end of the experiments (4 to 6 wk after *F. oxysporum* f. sp. *cucumerinum* inoculation).
[z] Means with different letters are significantly different at $P = 0.05$. $LSD_{0.05} = 2.0795$.

The number of dead plants was recorded twice weekly after the first plant died (4 to 6 weeks after *F. oxysporum* f. sp. *cucumerinum* inoculation and PGPR treatments). The experiment was performed four times. Data were analyzed using the general linear models procedure in PC-SAS (SAS Institute, Cary, NC).

Separate experiments were designed to determine if the *lux*-derivative, L211, of strain 89B-27 retained ISR activity. The same split-root system was used, and all procedures were identical to those described above. Numbers of symptomatic leaves and dead plants were recorded twice weekly from 4 to 6 weeks after *F. oxysporum* f. sp. *cucumerinum* inoculation and PGPR treatments. Disease development on each plant was rated using the following scale: 5 = plant dead; 4 = 76 to 100% of leaves with symptoms; 3 = 51 to 75% of leaves with symptoms; 2 = 26 to 50% of leaves with symptoms; 1 = <25% of leaves with symptoms; and 0 = no symptoms. The disease index was calculated from the disease rating by the formula:

$$\text{Disease index} = \frac{\Sigma(\text{rating no.} \times \text{no. of plants in the rating})}{\text{Total no. of plants} \times \text{highest rating}} \times 100\%$$

Data were analyzed using the general linear models procedure in PC-SAS. The experiment was performed four times.

Effects of ISR on movement of *F. oxysporum* f. sp. *cucumerinum* inside plants. Development of *F. oxysporum* f. sp. *cucumerinum* inside plants was determined by isolation from the first through fourth internodes of stems and the first through fourth petioles following the growth of plants from all treatments 1 to 5 weeks after inoculation. Two plants were sampled from each treatment at each sample time. Segments (2 to 3 cm in length) of stems and petioles of plants were surface-disinfested with 1% sodium hypochlorite for 1 min and washed three times with sterile water. The segments of stem and petiole were split into two parts with a sterile dissecting knife. One half was placed onto PDA with rifampicin at 50 µl/ml and incubated at 25°C for 5 to 7 days for isolation of *F. oxysporum* f. sp. *cucumerinum*. The other half was used for isolation of L211.

Isolation of L211 from roots, stems, and petioles. To test whether bacteria inoculated on one side of the roots moved from the bacterized pot to the nonbacterized pot, isolation of L211 was attempted from external roots in both pots 1 to 5 weeks after inoculation. In addition, the first through fourth internodes of stems and the first through fourth petioles were sampled to test for bacterial movement within the plant. Segments of roots were washed three times with sterile water and incubated on TSA (tryptic soy agar) with tetracycline at 7.5 µl/ml. Segments of stems and petioles were treated as described above, except TSA with tetracycline at 7.5 µl/ml was used, and incubation was done at 28°C for 48 h.

RESULTS

ISR by PGPR in the split-root system. Analysis of the data from four experiments using the split-root system indicated that the number of dead plants was significantly lower after treatment with PGPR strains 89B-27 and 90-166 than with the nonbacterized disease control (Table 1). The mean number of dead plants for the four experiments was 6.8 for the nonbacterized disease control, 3.8 for strain 89B-27, and 3.2 for strain 90-166. Initial onset of disease was delayed, and final incidence was reduced after treatment with strains 89B-27 and 90-166 compared with the disease control (Fig. 1). The number of dead plants over time was generally less in all experiments using treatment with 89B-27 and 90-166 than in nonbacterized disease control. Forty days after inoculation with *F. oxysporum* f. sp. *cucumerinum* in experiment 3, for example, the number of dead plants was 5, 2, and 1 in the disease control, treatment with 90-166, and treatment with 89B-27, respectively (Fig. 2).

Both the bioluminescent derivative strain L211 and strain 90-166 significantly reduced Fusarium wilt in comparison to the nonbacterized control (Fig. 3). Average disease indices for two experiments were 0.74 for the nonbacterized control, 0.53 for treatment with strain 90-166, and 0.44 for treatment with strain L211.

Effects of ISR on movement of *F. oxysporum* f. sp. *cucumerinum* inside plants. *F. oxysporum* f. sp. *cucumerinum* was recovered from internal tissues of cucumber stems below the first internode in the nonbacterized control 2, 3, and 4 weeks after root inoculation (Table 2). At week 5, the pathogen was recovered from inside stems between the first and fourth internodes and from inside the first, second, and third petioles in the nonbacterized disease control. However, after treatment with L211, the pathogen was recovered only from internal stems below the first internode at 4 weeks after treatment and from inside the third petiole of one sample at week 5 (Table 2).

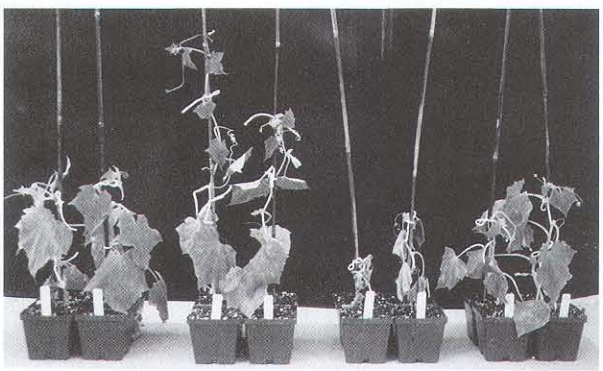

Fig. 1. Symptom of Fusarium wilt on cucumber 'Straight 8' in a split-root system. From left to right: treated with *Serratia marcescens* strain 90-166 and *Fusarium oxysporum* f. sp. *cucumerinum*; nontreated healthy control; nonbacterized disease control; and treated with *Pseudomonas putida* strain 89B-27 and *F. oxysporum* f. sp. *cucumerinum*.

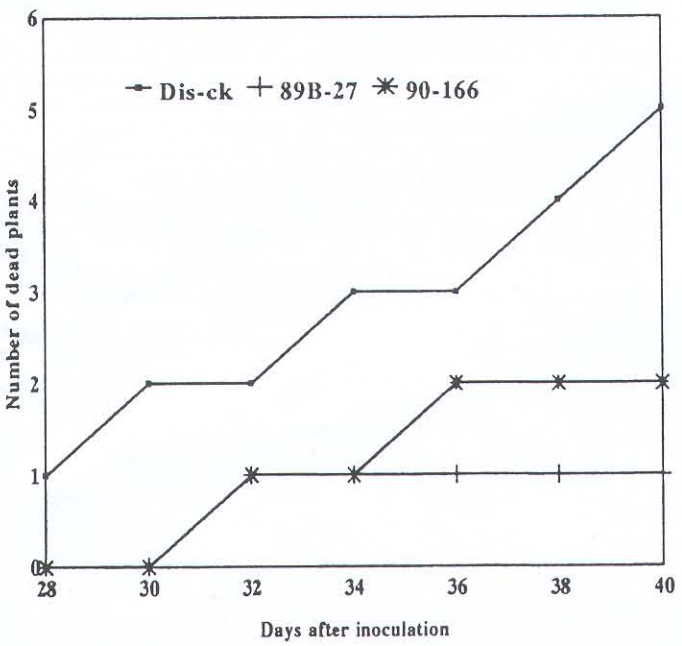

Fig. 2. Development of Fusarium wilt on cucumber by root inoculation with *Fusarium oxysporum* f. sp. *cucumerinum* in a split-root system. Cultivar Straight 8 was used. Dis-ck is a nonbacterized, pathogen-inoculated control; 89B-27 is *Pseudomonas putida* strain 89B-27 treatment; 90-166 is *Serratia marcescens* strain 90-166 treatment. No symptoms were observed on the nontreated healthy control. Data are from one of four experiments.

Isolation of L211 from roots, stems, and petioles. L211 was recovered only from bacterized parts of cucumber roots at 2, 3, 4, and 5 weeks after inoculation and was never recovered from nonbacterized parts of roots. Root pieces from bacterized parts were very bright when exposed for 10 min under the CCD camera, whereas roots from nonbacterized parts were not luminescent. L211 was not recovered from stems or petioles, with one exception when it was detected on a stem near the soil line.

DISCUSSION

PGPR strains 89B-27 and 90-166, which previously induced systemic resistance against foliar cucumber pathogens after seed treatment (12,21), significantly reduced Fusarium wilt of cucumber when applied as root treatments. This reduction in disease development appeared to be related to delayed movement of the pathogen within PGPR-treated plants compared to the nonbacterized disease control. Although the pathogen was recovered from the first internode 2 weeks after inoculation and from the first through fourth internodes after 5 weeks in the nonbacterized disease control, it was only recovered at week 4 from stems and at week 5 from the petioles of bacterized plants.

Lemanceau and Alabouvette (11) reported that some PGPR strains did not protect plants from pathogens effectively when they were applied alone but could improve disease control when applied with nonpathogenic *F. oxysporum*. Other previous work showed that PGPR strains suppressed Fusarium wilt in natural soil but not in sterile soil (4). These reports suggest that PGPR suppress Fusarium wilt indirectly by acting on nonpathogenic *Fusarium* spp. that have biocontrol potential. In contrast, Van Peer et al. (20) reported that Fusarium wilt of carnation was significantly suppressed by a *Pseudomonas* sp. strain alone. In their research, carnation roots were treated with PGPR, and the pathogen was introduced into plants by stem injection. In our research, both

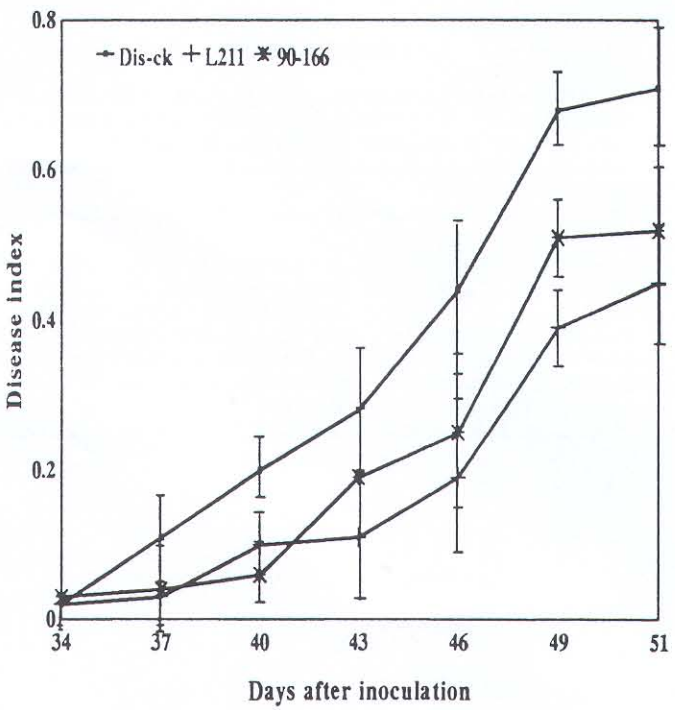

Fig. 3. Reduction in the disease index of Fusarium wilt of cucumber caused by *Fusarium oxysporum* f. sp. *cucumerinum* after treatment with plant growth-promoting rhizobacteria strains in a split-root system. Dis-ck is a nonbacterized, pathogen-inoculated control; L211 is a bioluminescent derivative of *Pseudomonas putida* strain 89B-27; and 90-166 is *Serratia marcescens* strain 90-166. Data are from two experiments. Disease index (DI) was calculated as DI = [Σ(rating no. × no. of plants in the rating)/(total no. of plants × highest rating)] × 100%

TABLE 2. The presence of *Fusarium oxysporum* f. sp. *cucumerinum* (Foc) in cucumber stems and petioles after root inoculation of the plants with L211, a bioluminescent derivative of the plant growth-promoting rhizobacterium *Pseudomonas putida* strain 89B-27[y]

Tissue	Treatment	Presence or absence of Foc after inoculation[z]			
		2 wk	3 wk	4 wk	5 wk
Stem	L211 + Foc	−−	−−	++	++
	Foc	++	++	++	++
Petiole	L211 + Foc	−−	−−	−−	−+
	Foc	−−	−−	−−	++

[y] Segments of stems or petiole tissue were incubated on potato-dextrose agar.
[z] At each sample time, two plants were tested per treatment. −+ indicates that *F. oxysporum* f. sp. *cucumerinum* was isolated in one of two samples.

PGPR and the pathogen were introduced by root inoculation. Results of our research showed that strains 89B-27 and 90-166 induced systemic resistance in cucumber against Fusarium wilt on their own.

Although the split-root system has been used to study induced resistance in plants against soilborne pathogens by nonpathogenic fusaria (9,13) and PGPR (25), it has not always been clear if spatial separation of PGPR and the pathogen was maintained. Using L211, a bioluminescent marker derivative of strain 89B-27, we confirmed spatial separation in our study. The split-root system is a good method to separate pathogens and PGPR strains spatially in studies on induced resistance of PGPR against soilborne pathogens.

Results of the root print experiment showed that strain L211 could colonize and live on roots for at least 5 weeks. L211 was limited to roots and did not move up to stems and petioles. Whether the ISR activity of the PGPR strains reported here is associated with transportable substances induced or produced by PGPR is unknown. Further studies should be designed to determine how specific induced plant responses relate to the observed reduction of *Fusarium* spread induced by PGPR.

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