Induced Systemic Resistance in Cucumber and Tomato Against Cucumber Mosaic Cucumovirus Using Plant Growth-Promoting Rhizobacteria (PGPR)

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ABSTRACT

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Plant growth-promoting rhizobacteria (PGPR) strains 89B-27 (*Pseudomonas fluorescens*) and 90-166 (*Serratia marcescens*), which previously demonstrated induced systemic resistance in cucumber against some fungal and bacterial diseases, were tested for their capacity to protect *Cucumis sativus* L. cv. Straight 8 from disease development of cucumber mosaic cucumovirus (CMV). Seed treatment with both PGPR strains significantly and consistently reduced mean numbers of symptomatic plants when CMV was inoculated onto cotyledons. Plants treated with PGPR did not develop initial symptoms 14 days after CMV inoculation and remained symptomless throughout the experimental period. In a comprehensive study with cucumber, no viral antigen could be detected by enzyme-linked immunosorbent assay (ELISA) in any asymptomatic PGPR-treated plants; whereas CMV was evident in every leaf of symptomatic plants. The same two PGPR strains were evaluated for effects on CMV symptom development in tomato in three experiments by measuring the disease severity at six observation dates. In all experiments, the area under the disease progress curve (AUDPC) was significantly lower with strain 89B-27 than in the nonbacterized control. The AUDPC with strain 90-166 was also significantly lower than with strain 89B-27. These results suggest that PGPR should be further evaluated for their potential to contribute toward management of viral plant diseases.

Additional keywords: bacterization, biological control, immunization, systemic acquired resistance

Historically, the most effective means to reduce damage to crops by viral diseases have been cultural practices and the introduction of natural resistance genes through breeding. Breeding for resistance included strategies against viruses and against vectors. Alternative strategies have been developed and implemented in recent years using transgenic plants containing viral coat protein genes and replicase-associated genes (5). Pathogen-mediated protection was derived from the concept of cross-protection (15), whereby plants infected with one viral strain were protected from subsequent infection by another related strain. Localized infection that results in protection from unrelated pathogens is known as acquired resistance or induced resistance (8,18). Since the 1970s, numerous reports have indicated that systemic resistance to a broad spectrum of pathogens could be induced in plants inoculated previously with pathogens such as fungi, bacteria, and viruses, incompatible pathogens, microbial metabolites, various plant-derived materials, and certain chemicals (4,6,7,22). Bergstrom et al. (2) showed that resistance in cucumber against cucumber mosaic cucumovirus (CMV) could be induced by previously inoculating plants with *Colletotrichum orbiculare, Pseudomonas syringae* pv. *lachrymans*, or tobacco necrosis virus (TNV).

Recent studies indicated that some plant growth-promoting rhizobacteria (PGPR) also act as inducers of systemic resistance in plants (1,20,21,23). In all of these studies, spatial separation of beneficial and pathogenic microorganisms was demonstrated, as PGPR strains could not be detected in stems or leaves (20,21), suggesting that the protection against leaf and stem diseases by PGPR strains was due to some form of induced systemic resistance. Maurhofer et al. (14) compared physiological changes in leaves due to systemic resistance induced by a classical method of leaf infection with a pathogen with resistance induced by PGPR application to the roots. Working with PGPR strains of *Pseudomonas* spp. and *Serratia* spp., Liu et al. reported that PGPR-mediated induced systemic resistance (ISR) resulted in protection against *C. orbiculare* (13), *Fusarium oxysporum* (11), and *P. s.* pv. *lachrymans* (12).

The objective of this research was to determine if PGPR strains that induced systemic resistance in cucumber to fungal and bacterial pathogens could also protect cucumber and tomato against infection by CMV. A preliminary account of a portion of this work has been published (9).

MATERIALS AND METHODS

Bacterial cultures. PGPR strains 89B-27 (*Pseudomonas fluorescens*) and 90-166 (*Serratia marcescens*) were used in all experiments. For long-term storage, cultures were maintained at –80°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) with the addition of glycerol to 20%. The PGPR were prepared for seed treatment by incubation in TSB at 28°C for 24 h with shaking at 175 rpm, followed by centrifugation at 6,000 × *g* for 10 min. The bacterial pellet was mixed with cucumber seeds at mean densities of 5×10^9 to 1×10^{10} CFU/seed.

CMV. The CMV Commelina strain (ATCC PV-30) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and used throughout all experiments. Inoculum consisted of CMV systemically infected symptomatic leaves of cucumber plants ground in 0.01 M sodium phosphate buffer (SPB), pH 7.0.

PGPR-mediated induced systemic resistance (ISR) in cucumber against CMV. A series of five initial experiments were conducted to test the hypothesis that PGPR could reduce CMV symptom development on cucumber. The susceptible cucumber (*Cucumis sativus* L.) cultivar Straight 8 was used in a completely randomized block design with four treatments and 10 replicates of single plants per treatment. Treatments included seed application with PGPR strains 89B-27 and 90-166, a nontreated CMV-inoculated disease control, and a control without any treatment (healthy control). Seeds were planted in 10 cm^2 plastic pots containing soilless Pro-Mix growing medium (Premier Peat Ltd., Rivière-du-Loup, Québec, Canada). Experiments were conducted under greenhouse conditions with 32/25°C day/night temperatures. Ten days after planting, cotyledons were lightly dusted with Carborundum and rub-inoculated with CMV inoculum. The healthy control plants were

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inoculated with sterile SPB instead of CMV. Inoculated cotyledons were immediately rinsed with water to remove sap extracts. Disease incidence was recorded as numbers of symptomatic plants. Mean numbers of symptomatic plants 14 days after CMV inoculation were determined. These values were analyzed for significant treatment effects using the general linear models procedure in PC-SAS (SAS Institute, Cary, NC). A sixth experiment was designed as before, with four treatments, 10 plants per treatment, to obtain additional information 4 weeks after CMV-challenge. From each plant, the main stem length (MSL) and total leaf area (TLA), measured by the AgVision system (Decagon Devices, Inc., Pullmann, WA), was recorded. In addition, three leaves, the second, third, and sixth youngest leaf of each plant, were tested individually for the presence of viral antigen by enzyme-linked immunosorbent assay (ELISA). In a virus distribution study, representative plants of each treatment, with and without CMV-symptoms, were selected to test all available leaves for viral antigen by ELISA.

ELISA. CMV coat protein antigen was detected using direct double antibody sandwich ELISA as described by Clark and Adams (3). Antiserum to the Commelina strain of CMV was obtained from ATCC (PVAS-30), and immunoglobulin G (IgG) was purified using the Bio-Rad Affigel system (Bio-Rad Laboratories, Hercules, CA). Conjugation of IgG to alkaline phosphatase (Type VIIT; Sigma Chemical Co., St. Louis, MO) was performed according to the manufacturer. Falcon flat-bottom plates were coated with anti-CMV IgG at 1.5 μg/ml and incubated at 4°C for at least 12 h. Antigen was expressed from leaf tissues by adding phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) at a ratio of 10:1 (ml of PBS-T:g of tissue), and triturated using a homogenizer (hand model Art. No. 400010; Bioreba AG, CH-4008 Basel, Switzerland). Antigen samples were incubated in the coated plates at 4°C for at least 12 h prior to addition of alkaline phosphatase-conjugated IgG diluted 1:850 in PBS-T. Following another 12-h incubation at 4°C, substrate (*p*-nitrophenylphosphate at 1 mg/ml in 10% diethanolamine, pH 9.8) was added and incubated for 1 h. Absorbance values at 405 nm were determined with a MR 700 Microplate reader (Dynatech Laboratories, Inc., Chantilly, VA).

Protection of tomato from CMV with PGPR seed treatment. A series of experiments was conducted to determine if PGPR strains 89B-27 and 90-166 induced systemic resistance against CMV in tomato (*Lycopersicon esculentum* Mill.). In contrast to the cucumber experiments, where only disease incidence was measured, severity was calculated for the tomato experiments. The susceptible cultivar Marion was used, and the experimental design was a complete randomized block with four treatments and 10 replicates of single plants per treatment. The four treatments were the same as described above. The bacterial strains were inoculated onto seeds prior to planting. CMV was challenge-inoculated onto the adaxial side of the first pair of leaves 3 weeks after planting using the same method as described above. Numbers of symptomatic plants were recorded 3 weeks after challenge-inoculation and continued twice a week for an additional 2 weeks. Disease was rated using the following scale: 10 = 100% of leaves showing mosaic symptoms; 8 = 50% of leaves showing mosaic symptoms; 6 = mosaic symptoms just starting; 4 = 50% of leaves appear puckered or curled; 2 = leaf puckering or curling just starting; 0 = no symptoms. Disease severity was calculated from the disease rating by the following formula: Disease severity = [Σ(rating no. × no. plants in rating) × 100%]/(total no. plants × highest rating).

The same experiment was conducted three times and always read over six observation dates, ending 5 weeks after challenge with CMV. For each experiment, disease progress was measured by the area under the disease progress curve (AUDPC) as described by Tooley and Grau (19), and data were analyzed using analysis of variances (ANOVA) in PC-SAS.

RESULTS

PGPR-mediated ISR in cucumber against CMV. Seed treatments with PGPR strains 89B-27 and 90-166 reduced the numbers of plants with mosaic symptoms in all five initial experiments (Table 1). Mosaic symptoms appeared on the youngest leaf about 7 days after inoculation with CMV; whereas disease development in PGPR-treated plants was delayed for up to 7 additional days. For the five experiments 2 weeks after CMV inoculation, mean numbers of symptomatic plants were significantly lower with PGPR treatments than with the disease control. Therefore, we conducted a sixth experiment to confirm whether the apparent resistance induced by treatment with PGPR correlated with reduced amounts of viral antigen (Table 2). The number of symptomatic plants did not change 14 days after challenge (DAC). PGPR-treated plants, which were symptomless 14 DAC, remained symptomless until the end of the study (28 DAC). In symptomless plants, no viral antigen was detected by DAS-ELISA; whereas leaf samples from plants showing CMV-symptoms always tested positive for CMV. Symptomatic plants had shorter main stem length and less total leaf area than nonsymptomatic and healthy control plants (Table 2). No CMV antigen was

Table 1. Plant growth-promoting rhizobacteria (PGPR)-mediated reductions in cucumber mosaic virus (CMV) symptom development in cotyledon-challenged cucumber plants

	No. of symptomatic plants[y]						
Treatment	**Trial**	**1**	**2**	**3**	**4**	**5**	**Mean[z]**
Disease control		7	6	8	9	10	8.0 a
PGPR 90-166		4	6	7	7	9	6.6 b
PGPR 89B-27		1	1	3	6	5	3.2 c
Healthy control		0	0	0	0	0	0.0 d

[y] Each treatment consists of 10 replicate plants. Data were recorded 14 days after CMV inoculation.
[z] Numbers with different letters indicate significant treatment effects, $LSD_{0.05} = 1.305$.

Table 2. Induction of systemic resistance against cucumber mosaic cucumovirus (CMV) in cucumber plants by plant growth-promoting rhizobacteria (PGPR) as a seed treatment 28 days after challenge (DAC)[w]

	No. of symptomatic plants[x]			Mean main stem length (cm)		Mean total leaf area (cm^2)		ELISA[y]	
Treatment	**7 DAC**	**14 DAC**	**28 DAC**	**S[z]**	**N**	**S**	**N**	**S**	**N**
Healthy control	0	0	0	...	94.9	...	839.6	...	0.01
Disease control	10	10	10	72.8	...	605.0	...	0.82	...
PGPR 89B-27	8	9	9	69.8	89.0	564.8	832.2	0.83	0.01
PGPR 90-166	5	6	6	70.0	86.5	565.3	801.1	0.80	0.01

[w] Data are from a single trial.
[x] Each treatment consists of 10 replicate plants.
[y] Enzyme-linked immunosorbent assay (ELISA) results are presented as absorbance values (405 nm) of means of three leaves of each plant.
[z] S = symptomatic plants, N = nonsymptomatic plants.

detected in any leaf from symptomless plants; whereas viral antigen was detected in all leaves of symptomatic plants (ELISA readings of 0.62 to 1.04).

Protection of tomato from CMV with PGPR seed treatment. CMV disease development was decreased at all six observation dates in the plants treated with both PGPR strains 90-166 and 89B-27 in all three experiments (Table 3). At the end of each experiment, 5 weeks after challenge-inoculation, disease severity was still reduced in the two PGPR treatments compared to the disease control. Mean disease severities from the three experiments were 66.7 for the nonbacterized disease control, and 42.0 and 21.3 for strain 89B-27 and 90-166 treatments, respectively, 35 days after CMV-inoculation. Disease progress, calculated by AUDPC, was significantly different ($P = 0.05$) for both PGPR treatments in each experiment (Table 3).

DISCUSSION

We have shown that PGPR strains, which were previously demonstrated to induce protection in cucumber against the fungal pathogens *C. orbiculare* (13,21) and *Fusarium oxysporum* (11), and the bacterial pathogen *P. s.* pv. *lachrymans* (12), also induced protection in plants against CMV under greenhouse conditions. Numbers of symptomatic cucumber plants following seed treatment with PGPR *Pseudomonas fluorescens* strain 89B-27 and *Serratia marcescens* strain 90-166 prior to CMV inoculation of cotyledons were lower than the nonbacterized disease control in six repeated experiments.

One major difficulty encountered when conducting research on viruses that induce systemic mosaic type of disease is choosing a suitable method of disease rating. Using symptoms, one has the option of measuring disease incidence or disease severity. For disease incidence, it is necessary to be able to differentiate clearly between symptomatic and asymptomatic plants. Alternatively, indirect disease measurements such as plant growth habits can be taken, as is done here with the investigations of PGPR-mediated ISR of cucumber against CMV (Table 2). Dealing with disease severity and quantification of viral symptoms is still an unsolved issue, since disease severity does not necessarily correlate to virus concentration (17). Therefore, quantitative ELISA readings are not particularly helpful. This situation is reflected with the studies conducted on tomato, where there were differences in disease severity between the PGPR-treated and control plants. There was no complete resistance, which makes a disease incidence measurement of no value in differentiating between treatments. In these tomato studies, the disease rating scale in association with AUDPC allowed detection of differences in disease development.

Our results extend previous studies with classical ISR against CMV on cucumber. Bergstrom et al. (2) reported ISR against CMV using the pathogens *C. orbiculare, Pseudomonas lachrymans*, and TNV as inducers. In their evaluation of CMV symptom development, Bergstrom et al. reported that faint chlorotic spots, the primary lesions of CMV, developed in the third, inoculated leaf approximately 2 to 3 days after inoculation. In contrast, in our study, systemic mosaic symptoms of CMV did not occur until 7 days after inoculation, when symptoms developed on the youngest leaves but did not occur on virus-inoculated leaves or cotyledons. The data of Bergstrom et al. (2) indicated a "tendency toward delay" for several days, and 100% infection was evident in both induced and noninduced treatments about 14 days after CMV inoculation. In contrast to those results, stable resistance was observed 14 days after inoculation in some plants in our studies. Mucharromah and Kuć (16) concluded that oxalate and phosphates induced resistance against CMV in cucumber by reducing the number of chlorotic lesions caused by CMV on the inoculated leaf. In contrast, we did not observe development of lesions from CMV on the inoculated leaves. Moreover, inoculation of cotyledons clearly showed that once plants were protected by PGPR treatment, mosaic symptoms did not develop. CMV infection was associated with altered plant growth habits, including a reduction in main stem length and total leaf area in non-PGPR-treated controls. PGPR-protected plants, however, showed the same growth pattern as plants from the healthy control. In addition, based on ELISA results, CMV appeared not to be distributed inside the inoculated but asymptomatic PGPR-treated plants. Whether the induced resistance is directed at virus replication or movement has not been determined. All leaves from diseased plants were symptomatic for CMV and accumulated relatively similar amounts of CMV antigen. In contrast, no viral antigen was detected in any PGPR-treated plants, which were apparently symptomless.

Both PGPR strains 89B-27 and 90-166 affected CMV-disease development in tomato. Disease severity in CMV-susceptible tomato cultivar Marion was significantly decreased by treatments with 89B-27 and 90-166 compared to the nonbacterized disease control. This is the first report of ISR against CMV in tomato and demonstrates that induced protection achieved by specific PGPR strains on one crop may be extended to different crops.

Protection against CMV by PGPR seed treatment appeared either to completely eliminate development of viral symptoms, as shown with cucumber cotyledon experiments, or to reduce disease severity, as shown in the tomato experiments. Although protection of both cucumber and tomato were statistically significant with application of both PGPR strains, the different levels of protection on different plant species may suggest that some level of specificity exists in the interactions between plant and bacteria. This specificity could result from differences in colonization patterns of PGPR strains on different plants or from different mechanisms of induction by treatment with different PGPR strains. Investigations of root colonization by these PGPR strains (10) indicated that there was no significant difference in colonization capacity of the two PGPR strains on cucumber. Therefore, it may be more likely that differences in ISR associated with the two PGPR strains result from different mechanisms of induction in cucumber and tomato. Further studies should be conducted to determine whether the results reported here extend to field conditions.

Table 3. Effect of plant growth-promoting rhizobacteria (PGPR) strains 89B-27 and 90-166 on disease severity of cucumber mosaic cucumovirus (CMV) on tomato cultivar Marion[w]

		Mean disease severity[y] (days after challenge with CMV)						
	Treatment[x]	18	22	26	29	32	35	AUDPC[z]
Trial I	Disease-C	58	64	70	70	74	74	114.0 a
	89B-27	30	38	44	44	44	50	70.5 b
	90-166	18	22	26	26	28	28	41.9 c
Trial II	Disease-C	62	62	64	64	68	68	109.7 a
	89B-27	40	42	44	46	46	54	75.9 b
	90-166	16	18	18	18	20	20	32.7 c
Trial III	Disease-C	30	50	54	54	58	58	87.2 a
	89B-27	14	22	32	32	36	36	47.9 b
	90-166	10	16	16	16	16	16	26.0 c

[w] CMV inoculation was conducted on the first pair of leaves. Data are from three experiments.
[x] Disease control was a nonbacterized, pathogen-inoculated control.
[y] Disease severity was calculated by the following formula: Disease severity = [Σ(rating no. × no. plants in rating) × 100%]/(total no. plants × highest rating).
[z] AUDPC = area under the disease progress curve. Values are means of 10 replicates per treatment over six observation dates. Values with different letters are significantly different ($P = 0.05$) within one experiment based on ANOVA in PC-SAS. LSDs for each experiment are: trial I = 14.44, trial II = 20.96, trial III = 11.48.

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