



Induction of resistance in tomato against cucumber mosaic cucumovirus by plant growth-promoting rhizobacteria

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Abstract. Studies were done to evaluate specific strains of plant growth promoting rhizobacteria (PGPR) for induced resistance against cucumber mosaic cucumovirus (CMV) in tomato. In greenhouse experiments where plants were challenged by mechanical inoculation of CMV, the percentage of symptomatic plants in the most effective PGPR treatments ranged from 32 to 58%, compared with 88 to 98% in the nonbacterized, challenged disease control treatment. Field experiments were conducted in 1996 and 1997 to evaluate 4 PGPR strain treatments based on superior performance in the greenhouse studies. In the 1996 field experiment, tomato plants treated with 3 PGPR strains exhibited a significantly lower incidence of CMV infection and significantly higher yields, compared with nonbacterized, CMV-challenged controls. In 1997, the overall percentage of plants infected with CMV in the control and PGPR treatments was higher than in 1996. CMV symptom development was significantly reduced on PGPR-treated plants in 1997 compared with the control, but the percentages of infected plants and tomato yields were not significantly different among treatments. These results suggest that PGPR-mediated induced resistance against CMV infection following mechanical inoculation onto tomato can be maintained under field conditions.

Key words: *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus subtilis*, induced resistance, induced systemic resistance, *Kluyvera cryocrescens*, plant defense

Introduction

Cucumber mosaic cucumovirus (CMV) is one of the five most important viruses affecting production of field-grown vegetables worldwide (Sherf and McNab, 1986; Tomlinson, 1987). CMV is difficult to control because of its extremely broad natural host range in excess of 800 plant species, and the ability to be transmitted in a nonpersistent manner by more than 60 species of aphids (Zitter, 1991; Palukaitis et al., 1992). In this mode of transmission, virus acquisition occurs during brief probes on infected plants by aphids

which are in turn able to transmit the virus quickly; however, aphids remain viruliferous for only a short time (Harris and Maramorosch, 1982). Weeds adjacent to vegetable plantings are often symptomless carriers of CMV and have been shown to be a source of infection for subsequent spread of CMV (Tomlinson, 1987). On tomato, symptoms of CMV infection include stunting of vegetative growth, distortion and mottling of new growth, and a characteristic shoestring-like leaf appearance (Zitter, 1991). A recent epidemic of CMV in Alabama resulted in a 25% yield loss in the north-central tomato-growing region of the state (Sikora et al., 1998). CMV epidemics have also been reported in tomato-growing regions of Italy (Kaper et al., 1990), Spain (Jorda et al., 1992) and China (Kearney et al., 1990). There are no sources of genetic resistance to CMV available in commercial fresh-market tomato cultivars (Sikora et al., 1998).

Plants have evolved complex and varied defense mechanisms for protection against herbivory and disease. These mechanisms may be constitutive (e.g. active throughout the plant's life) or induced following attack by herbivores or pathogens. Recent studies have suggested that inducible defenses in plants may have selective advantages over constitutive defenses (Agrawal, 1998 and references therein). While inducible defenses are often localized at the site of attack, plant defense mechanisms may be activated systemically throughout the plant following a localized infection or attack (Kessman et al., 1994). One of the first published reports of systemic resistance in plants was by Chester (1933), who used the term 'acquired physiological immunity'. Later, Ross (1961) reported that tobacco plants exhibited 'systemic acquired resistance' following local infection with tobacco mosaic virus. Other terms that have been used to describe systemic resistance in plants include 'translocated resistance' (Hubert and Helton, 1967), 'plant immunization' (Kuč, 1987), and 'induced systemic resistance' (Hammerschmidt et al., 1982). The term 'induced systemic resistance' (ISR) is used to denote induced systemic resistance by non-pathogenic biotic agents (Kloepper et al., 1992) and may differ mechanistically from resistance induced by other elicitors (reviewed in van Loon, 1998).

Kloepper and Schroth (1978) reported that certain root-colonizing bacteria could promote radish growth in greenhouse and field trials and therefore used the term 'plant growth-promoting rhizobacteria' (PGPR). Results of early studies with PGPR also demonstrated control of soilborne pathogens (Grusiddaiah et al., 1986; Ordentlich et al., 1987; Défago et al., 1990). PGPR act as antagonists against soil pathogens through competition (Elad and Chet, 1987), production of bacterial metabolites (e.g. siderophores, HCN, antibiotics), or production of extracellular enzymes that cause antagonism against pathogens (Kloepper and Schroth, 1978; Thomashow and Weller,

1988; Weller, 1988). In 1991, three laboratories independently demonstrated that certain PGPR strains protected plants through mechanisms associated with ISR against pathogens that cause foliar disease symptoms (Alstrom, 1991; van Peer et al., 1991; Wei et al., 1991). PGPR-induced resistance has subsequently been demonstrated against various fungal pathogens of cucumber (Liu et al., 1995a, b), and against bacterial wilt of cucurbits caused by *Erwinia tracheiphila* (Smith) (Zehnder et al., 1997 a, b). In the bacterial wilt system, reduced feeding by cucumber beetles on PGPR-treated plants was associated with a reduction in the concentration of cucurbitacin, a cucumber beetle feeding stimulant.

In greenhouse studies, Raupach et al. (1996) showed that two PGPR strains, which previously induced resistance in cucumber against fungal and bacterial diseases, also induced resistance in cucumber and tomato against CMV. Our study was done to screen additional PGPR strains for activity against CMV on greenhouse-grown tomato, and to determine if PGPR-mediated induced resistance could be extended to tomato grown in the field using commercial production practices.

Methods

Greenhouse experiments

PGPR seed treatment

Greenhouse experiments were done to evaluate 26 PGPR strains for induced resistance against CMV in tomato. PGPR strains were maintained at $-80\text{ }^{\circ}\text{C}$ in tryptic soy broth (TSB) with the addition of glycerol. For experimental use, strains were isolated onto tryptic soy agar (TSA) and were incubated at $28\text{ }^{\circ}\text{C}$ for 24 hours, then transferred to TSB and placed in a shaker at 150 rpm for 24 hours. The cultures were centrifuged at 6000 g for 5 minutes, and the supernatant discarded. Tomato seeds were mixed with the bacterial pellet, resulting in densities of approximately 5×10^9 cfu/seed. Seeds were planted into pots with soil-less planting mix. Control seeds were treated with 0.2M phosphate buffer.

PGPR soil drench

Tomato plants were transplanted into plastic pots containing planting mix two weeks after seeding. PGPR suspension treatments (100 ml containing approximately 5×10^8 cfu/ml) were poured into each pot immediately after transplanting. A water/buffer solution was applied to control plants.

CMV inoculation

A CMV isolate collected from tomato in North Alabama (J. Murphy, unpublished) was maintained in tobacco (*Nicotiana tabacum* cv. 'Burley 21') and used in all greenhouse experiments. The first two leaves (oldest two leaves) of each tomato plant were lightly dusted with carborundum and then rub-inoculated with CMV inoculum one week after transplanting. Inoculum consisted of CMV-infected tobacco leaf tissue ground in 50 mM KPO₄, pH 7.5, containing 10 mM sodium sulfite at a ratio of 1 g tissue:5 ml buffer.

Data

In each of the first two experiments, 13 PGPR strains were tested along with a disease control (CMV inoculation, no PGPR) and a healthy control (no CMV inoculation, no PGPR). At least 10 plants were included in each treatment. Plants were examined daily for CMV symptoms (leaf distortion, mosaic patterns, general stunting of the plant). The number of symptomatic leaves per plant and the number of plants with severe symptoms (e.g. >2/3 of symptomatic leaves) were recorded. Based on results of initial screening experiments, 16 of the most effective PGPR strains were evaluated in a third experiment, and the 8 strains exhibiting the highest level of protection were tested again in two additional trials (experiments 4 and 5). Based on these results, 4 strains were chosen for further evaluation in field experiments.

Field experiments

Experiment design and cultural practices

Field experiments were done in 1996 and 1997 to evaluate 4 PGPR strains, a disease control and a healthy control. The PGPR strains chosen for evaluation were *Bacillus pumilus* strain SE34, *Kluyvera cryocrescens* strain IN114, *Bacillus amyloliquefaciens* strain IN937a, and *Bacillus subtilis* strain IN937b. There were 6 replications per treatment arranged in a randomized block design, each consisting of 15 tomato plants (single row plots). Tomato plants were grown on raised beds, fumigated with methyl bromide/chloropicrin and covered with black plastic mulch (according to local tomato growing practices). Tomato beds were drip-irrigated.

Bacterization with PGPR and CMV inoculation of plants

In PGPR treatments, 'Mountain Pride' tomato seeds were mixed with the PGPR pellet (as described above) resulting in approximately 5×10^9 cfu/seed. Tomato seeds in the healthy and disease control treatments were dipped in distilled water before planting. Seeds were planted into plastic pots with soil-less planting mix as described above. In addition to the seed treatment, a PGPR soil drench (described above; 5×10^8 cfu/ml) was poured

onto each plant immediately after transplanting into larger pots (when plants were in the two leaf stage). A similar amount of water/buffer mixture was applied to the healthy and disease control treatment plants. All plants except those in the healthy control treatment were inoculated with CMV as described above one week before transplanting in the field.

Symptom rating

All plants in each treatment were examined weekly for virus symptoms using the following rating scale: 0, no symptoms; 2, leaf puckering or curling just beginning; 4, 50% of leaves on plant appear puckered or curled; 6, mosaic symptoms just beginning; 8, 50% of leaves showing mosaic symptoms; 10, 100% of leaves showing mosaic symptoms. Disease severity values were calculated using the formula (Yang et al., 1996):

Disease severity (Y) =

$$\frac{\sum (\text{disease grade} \times \text{number of plants in each grade}) \times 100}{(\text{total number of plants}) (\text{highest disease grade})}$$

Disease progression over time was measured using a formula for calculating the area under the disease progress curve (AUDPC):

$$\text{AUDPC} = \sum [(0.5) (Y_{i+1} + Y_i) (T_{i+1} + T_i)]$$

where Y = disease severity at time T, and i = the time of the assessment (in days numbered sequentially beginning with the initial assessment).

ELISA

Leaf samples were subjected to indirect enzyme-linked immunosorbent assay (ELISA). Leaves from the upper plant canopy were collected from each plant 32 days after transplanting in the field (90 leaf samples per treatment). Leaf samples were ground in 50 mM carbonate buffer (pH 9.6) and added to microtiter plates at a final dilution of 1:10 (g tissue:ml buffer). Plates were incubated overnight at 4 °C, then washed 3 times with PBS-T. Anti-CMV (primary antibody) was added to the plates at a concentration of 1 Fg/ml in PBS-T. The plates were again incubated overnight at 4 °C and washed 3 times with PBS-T. Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase was diluted to 1:7000 in PBS-T and added to the plates. The plates were incubated at 35 °C for 4 hours. Plates were triple-washed with PBS-T, and substrate (p-nitrophenylphosphate at 1mg/ml in 10% diethanolamine, pH 9.8) was added and reactions allowed to develop at room temperature.

Absorbance values were read at 410 nm on a Dynatech MR700 plate reader. Leaf samples were considered positive for the presence of CMV if the absorbance value exceeded a threshold value equal to the mean of the absorbance value of healthy control samples + (3) (standard deviation of the mean).

Plant height and yield measurement

Height of plants was measured 30 days after transplanting in the field. Marketable (non-damaged and mature) tomato fruit were weighed on 6 harvest dates during the season.

Results

Greenhouse experiments

The number of plants exhibiting CMV symptoms was reduced in several PGPR strain treatments, compared with the nonbacterized, challenged control. Based on a comparison of the numbers of symptomatic tomato plants in the 4th and 5th greenhouse experiments, 4 PGPR strains were chosen for further evaluation in the field (Table 1; data from experiments 1–3 not shown). The percentage of symptomatic plants in these PGPR treatments ranged from 32 to 58% percent, compared with 88 to 98% in the nonbacterized, challenged disease control treatment.

Field experiments

In the 1996 field experiment, AUDPC values, indicating disease symptom progression over time, were significantly lower in all PGPR treatments compared with the disease control (Table 2). Similarly, ELISA values in all PGPR treatments, and the percentage of infected plants (based on ELISA) in 3 PGPR treatments, were significantly lower than in the disease control. The percentage of infected plants in the disease control treatment was over 3-fold greater than in the IN937a and IN937b treatments. Plant height measurements taken 30 days after transplanting indicated that plant growth in the PGPR treatments was greater than in the disease control. The increased growth may have resulted from PGPR-induced resistance against CMV, PGPR-induced growth promotion, or both factors combined. Importantly, yields in the SE34, IN937a and IN937b treatments were significantly greater than in the disease control.

As in 1996, results of the 1997 field experiment indicated that AUDPC values and ELISA absorbance values were significantly lower in the PGPR treatments than in the disease control (Table 3). Overall, the percentage of plants infected with CMV was higher in 1997 than in 1996. In 1997, 62.2% of the nonchallenged, 'healthy' control plants tested positive for CMV by

Table 1. Results of preliminary greenhouse trials to select the most effective PGPR strains for induced resistance against cucumber mosaic cucumovirus (CMV) on tomato

PGPR strain or Treatment	Mean no. symptomatic plants \pm SEM ^a	
	4th experiment	5th experiment
BE55	3.5 \pm 1.3	6.0 \pm 1.4
IN266	4.5 \pm 2.1	7.0 \pm 0.8
SE34 ^b	4.0 \pm 1.4	5.8 \pm 1.7
IN937a ^b	4.2 \pm 1.2	5.0 \pm 2.1
IN937b ^b	3.2 \pm 1.0	4.8 \pm 1.7
TE5	4.0 \pm 1.6	6.5 \pm 1.3
IN114 ^b	3.5 \pm 1.3	5.8 \pm 1.7
89B-27	4.5 \pm 2.1	6.8 \pm 1.0
Nonbacterized, challenged control ^c	8.8 \pm 1.0	9.8 \pm 0.5
Nonbacterized, unchallenged control ^d	0	0

^a Means calculated based on 40 plants per treatment/experiment.

^b Selected for further evaluation in field trials.

^c Plants inoculated with CMV and not treated with PGPR.

^d Plants not inoculated with CMV and not treated with PGPR.

Table 2. Response of field tomato treated with select PGPR strains before challenge with cucumber mosaic cucumovirus (CMV), 1996

Treatment	AUDPC value	ELISA value	% Plants infected based on ELISA	Ave. plant height (cm)	Ave. yield (kg/plot)
SE34	12.23 c	0.18 c	30.0 b	41.3 a	14.0 a
IN114	21.3 b	0.30 b	58.8 a	36.9 b	10.3 b
IN937a	9.9 c	0.12 cd	21.1 b	41.2 a	14.8 a
IN937b	11.1 c	0.12 cd	17.7 b	41.4 a	14.2 a
Nonbacterized, challenged control	24.8 a	0.48 a	66.7 a	34.9 c	9.5 b
Nonbacterized, unchallenged control	0.8 d	0.05 d	4.4 c	41.1 a	14.1 a
LSD _{0.05}	2.80	0.09	13.4	1.22	2.4

Means within columns sharing the same letters are not significantly different ($p > 0.05$; least significant difference test).

Table 3. Response of field tomato treated with select PGPR strains before challenge with cucumber mosaic cucumovirus (CMV), 1997

Treatment	AUDPC value	ELISA value	% Plants infected based on ELISA	Ave. plant height (cm)	Ave. yield (kg/plot)
SE34	8.42 c	0.27 b	64.4 a	41.4 a	3.2 a
IN114	12.72 b	0.29 b	68.9 a	39.4 c	2.4 a
IN937a	9.07 bc	0.26 b	55.8 a	40.7 b	2.5 a
IN937b	10.71 bc	0.25 b	65.6 a	41.5 a	2.1 a
Noninduced, challenged control	18.25 a	0.37 a	83.3 a	38.3 d	2.0 a
Noninduced, unchallenged control	7.12 c	0.26 b	62.2 a	41.9 a	2.9 a
LSD 0.05	3.80	0.07	27.90	0.60	1.54

Means within columns sharing the same letters are not significantly different ($p > 0.05$; least significant difference test).

ELISA, compared with 4.4% in 1996. This suggests the occurrence of plant-to-plant spread of CMV by aphids within the field. Although the percentages of infected plants in the PGPR treatments were lower than in the disease control, differences were not statistically significant. Plant growth was significantly greater in the PGPR treatments compared with the disease control, but average tomato yields were not significantly different among treatments. Tomato yields overall were lower in 1997 than in 1996.

Discussion

These results provide evidence that PGPR-mediated induced resistance against CMV on tomato, previously reported from greenhouse experiments (Raupach et al., 1996) and confirmed here, can be obtained under field conditions. It is known that virus symptoms and concomitant negative effects on yields are most severe when plants are infected with virus in early growth stages (Matthews et al., 1991). In the 1996 field experiment, the incidence of CMV infection was lower on PGPR-treated plants (strains IN937a, IN937b and SE34) that were mechanically challenged with virus before transplanting in the field. In addition, tomato yields from PGPR-treated plants that were challenged with virus were not significantly different from yields on nonbacterized, unchallenged control plants. Although AUDPC and ELISA values in 1997 were significantly lower in PGPR treatments than in the nonbacterized, challenged control, it is not clear why the significant effects

of PGPR on the incidence of infected plants and on tomato yields, as seen in 1996, were not evident in the second-year experiment. In 1997, a much greater proportion of unchallenged control plants tested positive for CMV than in 1996. A possible explanation for the greater incidence of infection in 1997 is that the plants were subjected to higher levels of naturally transmitted CMV than in 1996. Aphid counts were not recorded in either year, but alate aphids were periodically observed on plants in treatment plots. It is possible that aphids migrating into the area acquired CMV by feeding on the mechanically inoculated tomato plants, or on CMV-infected weeds adjacent to the tomato plots. These aphids could have subsequently inoculated other tomato plants, effectively supplementing the levels of CMV in the challenged plants. Consequently, in 1997, PGPR-induced plant defense mechanisms may have been weakened in plants exposed to higher levels of CMV inoculum. Another explanation for reduced effectiveness of PGPR in 1997 could be that plants were naturally infected with a different strain of CMV, and that the PGPR strains tested were not as effective against the naturally occurring strain. Even though plants were provided with drip irrigation, drought conditions in 1997 may have accounted for reduced yields overall compared with 1996, and could have been a factor in reduced effectiveness of PGPR. Additional studies are needed to evaluate the stability and durability of PGPR for protection against CMV in the field under diverse cropping practices and environmental conditions and where natural infection by insect vectors occurs.

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