Microbe-Induced Resistance Against Pathogens and Herbivores: Evidence of Effectiveness in Agriculture

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Abstract

This chapter presents a summary of the results of experiments conducted in Alabama and Florida over a five year period to evaluate strains of plant growth-promoting rhizobacteria (PGPR) for induction of resistance against insect-transmitted diseases on field-grown cucumber and tomato. Experiments with cucumber demonstrated that treatment with PGPR significantly reduced the incidence of wilt symptoms caused by the bacterial pathogen Erwinia tracheiphila*, and also reduced numbers of the cucumber beetle vectors of the bacteria. Cotyledons from PGPR-treated plants contained significantly lower concentrations of cucurbitacin, a secondary plant metabolite and cucumber beetle feeding stimulant, than untreated plants. The PGPR-induced change in cucurbitacin metabolism may be associated with the production of other plant defense compounds during the induction of a systemic plant defense response. Subsequent studies with tomato were conducted to identify PGPR strains for induction of systemic resistance against cucumber mosaic cucumovirus (CMV} and whitefly-transmitted tomato mottle virus (ToMoV). Tomatoes treated with PGPR demonstrated a reduction in the development of disease symptoms, and often a reduction in the incidence of viral infection and increase in tomato yield. While preliminary, the results of these experiments in cucumber and tomato demonstrate that PGPR-mediated induced resistance represents a viable and environmentally-friendly approach to crop disease management, particularly for insect-transmitted diseases which are often difficult or impossible to control with pesticides.*

Introduction

Plant-associated microorganisms, including eubacteria, actinomycetes, and fungi are part of the natural ecosystem of healthy plants, occurring in the major habitats of the rhizosphere, leaf surfaces, and inside plant tissues. Some of these naturally occurring microorganisms develop symbiotic relationships with the plant in which the microbes live within plant tissues in a modified morphological state and contribute to plant growth and development, as in the case of mycorrhizal fungi and the nitrogen-fixing rhizobia on legumes. Much research over the past century has been devoted to using free-living plant-associated microorganisms to benefit plants. One such group of free-living microorganisms which has been extensively investigated are "rhizobacteria" which are the subset of rhizosphere bacteria known to aggressively colonize roots (Schroth and Hancock 1982). When used in relation to microbial inoculants, the term "root colonization" denotes an active process whereby bacteria survive inoculation into seeds or soil, multiply in the spermosphere in response to seed exudates rich in carbohydrates and amino acids (Kloepper et al. 1985), attach to the root surface (Suslow 1982), and colonize the developing root system in soils containing indigenous microorganisms. Therefore, rhizobacteria have been shown to be efficient microbial competitors that can displace native root-colonizing microorganisms (Kloepper and Schroth 1981) and persist throughout some or all of the crop season. Typically, introduced rhizobacteria colonize roots at the mid-stages of host-plant ontogeny at population densities of 10^3 - 10^6 colony-forming units (CFU)/ g root fresh weight (Bahme et al. 1988, Kloepper and Beauchamp 1992). Rhizobacteria are distributed in the rhizosphere in a lognormal pattern (Loper et al. 1984) and are sporadically dispersed along roots in microcolonies (Bahme and Schroth 1987).

The general effects of rhizobacteria on host-plants range from deleterious to neutral to beneficial (Glick 1995, Lazarovits and Nowak 1997). Rhizobacteria that exert beneficial effects on plant development are termed "plant growth-promoting rhizobacteria" (PGPR) (Kloepper and Schroth 1978) because their application is often associated with increased rates of plant growth. PGPR also provide benefits to plants by suppression of soil-borne pathogens (Schippers et al. 1987) and through induction of systemic resistance (see below). Under practical agricultural conditions, in non-sterile field soils, it is not possible to conclusively differentiate mechanistically between growth promotion and biological control, because some soil borne pathogens, such as Pythium spp., are present in nearly all soils. However, under controlled laboratory or growth room conditions, one can demonstrate direct plant growth promotion by some PGPR strains (Glick 1995). The ability of PGPR to elicit induced plant resistance varies among bacterial strains, and plants also vary in the expression of resistance upon induction by specific bacterial strains (van Loon et al. 1998).

Pioneering studies beginning in the 1950s by researchers in Russia, China and several western countries showed the potential of bacteria for plant disease management (reviewed in Backman et al. 1997). Building on this early

research, more recent studies have demonstrated the biocontrol activity of numerous PGPR strains against many soilborne pathogens, including *Aphanomyces* spp., *Pythium* spp., *Fusarium oxysporum, F. solani, Gaeumannomyces graminis* var. *tritici, Phytophthora* spp., *Sclerotium rolfsii,* and *Thielaviopsis basicola* (reviewed in Weller 1988, Schippers 1988). In 1985, Gustafson, Inc. (Plano, Texas) introduced the first commercial PGPR products in the United States using Broadbent's (Broadbent et al. 1977) *Bacillus subtilis* A-13 strain and related strains GB03 and GB07 (sold under the trade names Quantum@, Kodiak@, and Epic@, respectively). These products are registered for use on a number of different crops, including dicots and monocots, and are targeted for control of damage caused by fungal soil pathogens after seed-treatment fungicides have dissipated.

Mechanisms by which PGPR strains exhibit biological control against soil pathogens have been reported to include antibiosis through bacterial production of antifungal compounds (including antibiotics and hydrogen cyanide), competition for ferric iron, competition for infection sites, and production of lytic enzymes (Kloepper 1993). During the 1980s, work on mode-of-action of PGPR with biological control activity began to suggest that some PGPR strains may activate host defense systems, based on lack of direct antibiosis of the strains toward pathogens, or on correlation of biocontrol with plant growth promotion (Scheffer 1983, Voisard et al. 1989). In 1991, direct evidence supporting the conclusion that PGPR, which remain on plant roots, can induce resistance in plants to foliar or systemic pathogens was published independently for three pathosystems: cucumber and anthracnose (Wei et al. 1991), carnation and Fusarium wilt (van Peer et al. 1991), and bean and halo blight (Alström 1991).

"Systemic acquired resistance" (SAR) is a term first introduced by Ross (1961) to describe induction of resistance in tobacco by prior inoculation with tobacco mosaic virus. Since then, the term SAR has been commonly used in cases where induced resistance results from prior inoculation with necrotizing pathogens or application of chemical agents. Induction of SAR is characterized by an accumulation of salicylic acid (SA) and pathogenesis-related (PR) proteins (reviewed in van Loon et al. 1998). Some PR proteins (e.g., chitinases and glucanases) act directly on fungi by degrading cell walls, while the role of others has yet to be determined (Hoffland et al. 1995, van Loon et al. 1998, Hammerschmidt and Nicholson, this volume). SA has been implicated as a component of the SAR signaling pathway, but it does not appear to be the translocating, SAR-inducing signal (Vernooij et al. 1994, van Loon et al. 1998).

The term "induced systemic resistance" (ISR) is an alternative term sometimes used to denote induced resistance by non-pathogenic biotic agents, e.g., PGPR, in cases where SA signaling and accumulation of PR proteins do not occur (Pieterse et al. 1996, van Loon 1997, van Loon et al. 1998). PGPR-mediated ISR (PGPR-ISR) is similar to resistance induced by other agents (e.g., pathogens or chemical agents) in terms of disease suppression. However, ISR mediated by some PGPR is not associated with an accumulation of SA or PR

proteins, and so may involve a distinct signal transduction pathway (see van Loon et al. 1998 for discussion and references on this topic). van Wees et al. (1997) demonstrated that SA is not required for the triggering of ISR in *Arabidopsis* by certain rhizobacteria1 strains. Pieterse et al. (1998) subsequently demonstrated that rhizobacterially-mediated ISR follows a novel signaling pathway in *Arabidopsis,* in which jasmonic acid and ethylene are involved in the signal transduction pathway. Once activated, PGPR-ISR is maintained for prolonged periods against multiple pathogens; even if populations of the inducing bacteria decline over time (van Loon et al. 1998).

Plant diseases caused by insect-transmitted pathogens are among the most difficult challenges in pest management, particularly in high-value vegetable production where loss of yield can drastically reduce profits. Effective control of insect-borne disease with insecticides is difficult or often impossible because most plant disease vectors are highly mobile insects that may colonize fields before growers are aware of their presence. In addition, even low numbers of insects may result in high field incidence of disease, as occurs with cucumber beetles and bacterial wilt disease (Yao et al. 1996). Plant diseases caused by viruses that are transmitted by aphids in a non-persistent manner, e.g., cucumber mosaic cucumovirus, are not effectively controlled by insecticides because incoming viruliferous aphids can inoculate plants in seconds before they are affected by insecticide exposure (Matthews 1991).

The application of PGPR for crop protection is relatively new. Therefore, rather than develop a review chapter on this topic, we chose to summarize the results of some of our experiments with cucumber and tomato that demonstrate the potential of PGPR as a crop protection tool. These efforts were directed towards PGPR-mediated induced resistance as an alternative strategy for management of three insect-transmitted diseases that have proven difficult to control with conventional methods.

PGPR-ISR Against Cucumber Beetles and Bacterial Wilt Disease

Bacterial wilt of cucurbits is a systemic disease caused by the xylem-inhabiting bacterial pathogen *Erwinia tracheiphila* (Smith) Holland. Yield losses in cucumber and muskmelon, the most susceptible host crops, can be as high as 75% (Sherf and MacNab 1986). The bacterial wilt pathogen is transmitted by diabroticite beetles, including *Acalymma vittata* (F.), the striped cucumber beetle, and *Diabrotica undecimpunctata howardi* (Barber), the spotted cucumber beetle. Early studies (Rand and Enlows 1916) demonstrated that primary infection occurs through feeding wounds made by beetles and subsequent transfer of the pathogen from the insect mouthparts or feces. Blua et al. (1994) provided serological evidence that herbaceous weeds served as overwintering hosts for the pathogen, but this evidence has been disputed (De Mackiewicz et al 1998). More recently, Fleischer et al. (1999) demonstrated that cucumber beetles serve as overwintering reservoirs for *E. tracheiphila,* and that beetle aggregation on host-plants facilitates delivery of a sufficient dose for

infection to occur. Field studies in Alabama have demonstrated a positive, linear relationship between cucumber beetle density on cucumber plants and the incidence of bacterial wilt symptoms (Yao et al. 1996).

It is known that cucumber beetle feeding behavior is strongly influenced by cucurbitacins, a group of triterpenoid plant metabolites that occur in the plant family Cucurbitaceae (Chambliss and Jones 1966). Cucurbitacins act as a powerful feeding stimulant for cucumber beetles (Chambliss and Jones 1966, Metcalf 1986), and a strong, positive relationship has been established between cucurbitacin concentration and beetle feeding damage (Ferguson et al. 1983).

We first suspected that cucumber beetle feeding behavior was affected by PGPR treatment following cucumber field experiments in which PGPR afforded unexpected protection against bacterial wilt disease with large numbers of cucumber beetles present (Wei et al. 1995). We then initiated a series of experiments in cucumber to evaluate the effects of PGPR treatment on cucumber beetles and bacterial wilt disease.

Methods

Field Experiments. Field studies were conducted to assess the effects of PGPR treatment on populations of cucumber beetles, and to compare PGPR treatment with weekly applications of insecticide for control of cucumber beetles and bacterial wilt on cucumber (Zehnder et al. 1997a). For these experiments, PGPR strains were used that were shown previously to reduce disease incidence in cucumber caused by *E. tracheiphila* (Kloepper et al. 1993). Cucumber seeds were dipped in the pelleted bacterial cells or into distilled water (control) immediately before planting in plastic pots containing sterilized soilless planting mix. A dilute PGPR suspension (100 ml containing ~ $l0^8$ CFU/ml) was poured into each pot immediately after seeding. Seedlings ('Straight 8') were transplanted into the field at the 2nd leaf stage and grown in fumigated (methyl bromide + chloropicrin), raised beds with black plastic mulch and drip irrigation. Treatments in 1993 included the following PGPR: *Pseudomonas putida* strain 89B-61, *Serratia marcesens* strain 90-166, *Flavomonas oryzihabitans* strain INR-5, and *Bacillus pumilis* strain INR-7. Control treatments included an insecticide control (weekly sprays of esfenvalerate by backpack sprayer) and a untreated control. The 90- 166 and INR-7 strains were re-evaluated in 1994 along with the insecticide and untreated controls.

Greenhouse Experiments. Greenhouse experiments were conducted to determine if resistance against feeding by cucumber beetles was a factor in PGPR-induced protection against bacterial wilt that was previously observed in the field (see Zehnder et al 1997b for complete details). In free-choice experiments, screen cages designed in a 'cross'arrangement with 4 arms (Fig. 1) were used to confine cucumber beetles on PGPR-treated (seed treatment and

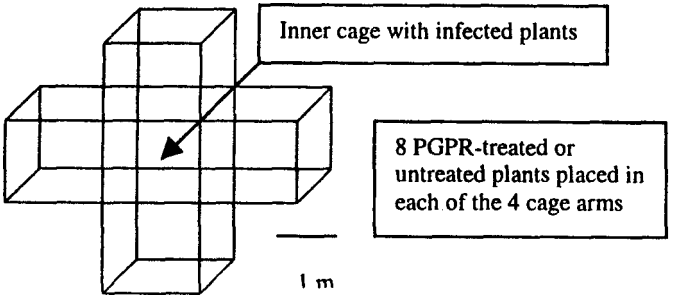

Figure 1: Diagram of screen cage used to confine cucumber beetles in free-choice experiments.

transplant drench with INR-7 strain) or untreated plants. PGPR-treated plants were placed in 2 arms/cage, and untreated plants in the other 2 arms/cage; 2 cages were used for each experiment (4 treatment replicates per experiment, 8 plants per replicate). Experiments were repeated twice. At the start of each experiment, 100 spotted cucumber beetles were confined on *E. tracheiphila*-infected cucumber plants in the center 'cage within a cage' for 48 h before doors were opened allowing beetles free access to all 4 cage arms. Data on beetle feeding damage and wilt incidence were recorded at 13 (experiment 1) or 17 (experiment 2) d after beetle release on uninfected plants. In separate no-choice experiments, beetle transmission of bacterial wilt was assessed in screen cages (1 .0 by 0.5 by 0.5 m) where beetles were allowed to feed only on PGPR-treated or non-treated plants. In these experiments, 25 spotted cucumber beetles were released in each cage and allowed to feed on 3 *E. tracheiphila*- infected cucumber plants placed in the center of each cage for 48 h before 5 healthy PGPR-treated or untreated plants were introduced (see Zehnder 1997a for complete details). No-choice experiments were conducted separately for each of 3 cucumber cultivars; 'Poinsett' bitter (BI), 'Poinsett' non-bitter (bi), and 'Straight 8' (with low levels of cucurbitacin C). Wilt incidence was assessed by determining the percentage of wilted leaves per plant in each cage 17-23 d after the introduction of test plants into the cages with beetles.

Cucurbitacin Analysis. Cucurbitacin 'C', the putative sole cucurbitacin in cucumber *(Cucumus sativus)* (Rice et al. 1981), was detected in samples of fresh or frozen cotyledon leaves from PGPR-treated (INR-7 and INR-5 strains) or untreated plants using HPLC analysis (see Zehnder 1997b for analytical methods).

Results

Field Experiments. In both years, average numbers of cucumber beetles (spotted and striped species combined) were significantly lower in the PGPR

Table 1. Results of cucumber field experiments with PGPR for control of cucumber beetles and bacterial wilt.

PGPR treatment	Mean no. beetles/plant		Mean % wilted vines	Mean fruit weight/plot (kg.)	
	1993	1994	1994	1993	1994
89B61	0.61cd	NT	NT	37.3a	NT
90-166	0.44 d	2.34 c	2.61 c	35.9 a	28.1 a
INR-5	0.56cd	NT	NT	32.7ab	NT
INR-7	0.73 bc	2.96 bc	3.35 bc	37.1 ab	26.5 ab
Insecticide Control[1]	0.89 b	NT	11.48 b	25.6 b	21.9 ab
Untreated	1.73 a	5.42 a	24.56 a	29.4 b	20.8 bc

NT, not tested. Means within columns sharing a letter in common are not significantly different ($P > 0.05$; LSD test). Beetle and wilted vine means derived from 6 replicates; 10 plants per replicate. Beetle data averaged over 6 sample dates; wilted vines recorded on 24 June, 1994. Plants sprayed weekly with esfenvalerate insecticide at the rate of 0.05 lb (AI)/acre.

treatments compared with the untreated control (Table 1). In 1994, when bacterial wilt symptoms were observed, the incidence of wilted vines was significantly lower in the PGPR treatments than in the untreated control. In both years, yields in the PGPR treatments were higher (significantly only for some PGPR strains) than in the untreated controls. It is interesting to note that some PGPR strains provided significantly greater protection against cucumber beetles and bacterial wilt than the weekly applications of esfenvalerate, the recommended insecticide treatment.

Greenhouse Experiments. In free-choice experiments, beetle-feeding damage on cotyledons was greatly reduced on PGPR-treated plants compared with untreated plants, and damage to stems was also less severe on PGPR-treated plants (Fig. 2A and 2B). Wilt symptoms on test plants in the 4 cage arms were observed between 7 and 12 d after beetle release, demonstrating that beetles acquired *E. tracheiphila* from inoculated plants in the 'center cage', and then successfully transmitted the pathogen to healthy test plants. The average number of wilted leaves per plant ranged from 1.13 to 2.59 on untreated control plants, but only from 0 to 0.28 on PGPR-treated plants (Fig. 2C). In the no-choice experiments, the average percentages of wilted leaves in the 3 cultivar experiments ranged from 52.8% to 85.3% on the untreated plants, but only from 7.6 to 13.1% on the PGPR-treated plants (Fig. 3). Thus, spread of *E. tracheiphila* on both bitter and nonbitter cucumber cultivars was significantly reduced by PGPR treatment, even when beetles were restricted to feeding only on PGPR-treated plants for a prolonged period.

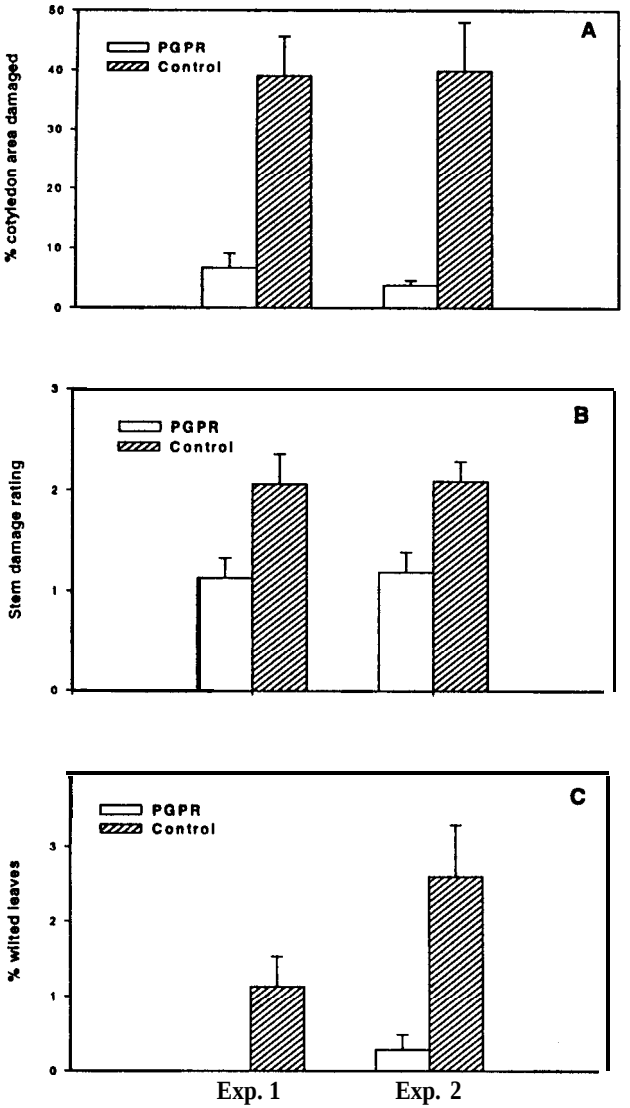

Figure 2: Cucumber beetle feeding damage and incidence of bacterial wilt symptoms on PGPR and untreated (control) cucumber plants. Infected cucumber beetles were released in greenhouse cages (see Fig. 1) and permitted to choose between treated and control cucumber plants (var. Straight 8, with low concentrations of cucurbitacin C). Experiments were repeated twice. (A) Mean percentage of cotyledon leaf area per plant with feeding damage. (B) Mean stem feeding damage rating per plant: 1 = < l/3 of stem from soil line to cotyledons damaged; 2 = 1/3 to 2/3 of stem damaged; 3 = > 2/3 stem with feeding damage. (C) Mean number of wilted leaves per plant.

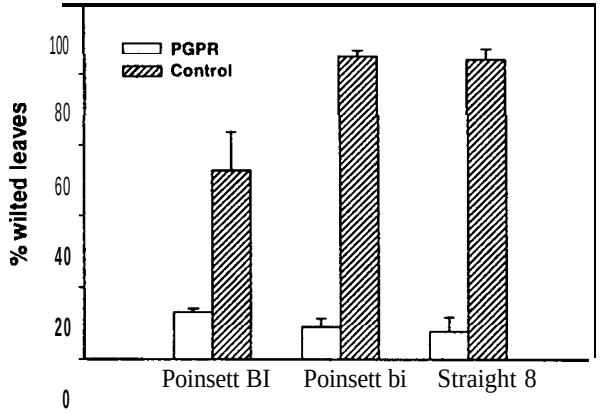

Figure 3: Comparison of mean percentage of wilted leaves per plant on 'Poinsett' bitter, non-bitter and 'Straight 8' (low concentrations of cucurbitacin C) cucumber plants in no-choice greenhouse cage experiments (see Fig. 1). Cucumber beetles infected with *E. tracheiphila* were released into cages with either PGPR-treated or untreated plants and allowed to feed for 17-23 days.

Table 2. PGPR-mediated reduction in cucurbitacin 'C' concentration.

	Mean cucurbitacin concentration (µg)	
PGPR treatment	Poinsett (bitter) cultivar	Straight 8 cultivar (non-bitter)
INR-7	117.3 b	27.1 c
INR-5	117.9 b	35.2 bc
Untreated	158.6 a	48.4 a

Means within columns sharing the same letter are not significantly different ($P > 0.05$; LSD test). Cucurbitacin 'C' values are µg cucurbitacin/g dry weight plant material. Means derived from 5 replicates per treatment. Data from Zehnder et al. 1997b.

Cucurbitacin Analysis. Cucurbitacin 'C' concentration was significantly lower in cotyledons of 'Poinsett' and 'Straight 8' cucumber treated with PGPR compared with untreated plants (Table 2). The greatest reduction occurred in 'Straight 8', with an average of 44% lower cucurbitacin in INR-7-treated plants compared with the untreated plants.

Discussion

The results of the field and greenhouse experiments demonstrated reductions in cucumber beetle feeding and spread of bacterial wilt were mediated by PGPR-induced resistance, and led us to hypothesize that the induction of resistance resulted in an unexpected physiological change in the

plant: reduced levels of the feeding stimulant cucurbitacin 'C'. Cucurbitacin levels were reduced as much as 44% on PGPR-treated plants, providing support for our hypothesis (Table 2). The metabolic pathway for cucurbitacin 'C' biosynthesis involves squalene synthetase, an enzyme that catalyzes conversion of squalene epoxide to cucurbitadienol, the simplest tetracyclic triterpene with a cucurbitane skeleton (Balliano et al. 1982). Squalene is also a precursor of sesquiterpene plant defense compounds (i.e., phytoalexins, Tjamos and Kuc 1982).

Although not yet confirmed, PGPR-induced effects on cucurbitacin production may be the result of a metabolic shift, in which an increase in the production of plant defense compounds may result in deficiencies in other compounds requiring the same chemical precursors or intermediates (Karban and Kuc, Stout and Bostock, Felton and Eichenseer, this volume). Squalene used to manufacture cucurbitadienol may be diverted to the production of phytoalexins during PGPR-mediated induction of resistance, resulting in reduced cucurbitacin production.

We hypothesize that PGPR treatment provides two levels of protection against bacterial wilt in cucumber. First, the reduction in feedant (cucurbitacin) synthesis in PGPR-treated plants makes these plants less palatable to cucumber beetles, which may result in a lower proportion of beetles that acquire and successfully transmit the pathogen. Second, PGPR may elicit the induction of other plant defense mechanisms (i.e., phytoalexin production and other compounds involved in ISR) against the pathogen after it has been introduced into the plant.

PGPR-ISR Against Cucumber Mosaic Virus

Worldwide, cucumber mosaic cucumovirus (CMV) is one of the five most important viruses affecting production of field-grown vegetables (Tomlinson 1987). CMV is often the most prevalent virus in surveys of plant virus infections and it has been implicated in disease epidemics of fruit, vegetable and greenhouse crops (reviewed in Palukaitis et al. 1992). 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 is difficult to control because of its extremely broad natural host range and the ability to be transmitted by more than 60 species of aphids (Zitter 1991). Bergstrom et al. (1982) demonstrated that previous inoculation of cucumber with *Colletotrichum orbiculare*, *Pseudomonas syringae* pv. *lachrymans*, or tobacco necrosis virus (TNV) could induce resistance against CMV. In greenhouse studies, Raupach et al, (1996) showed that two PGPR strains, which previously induced resistance in cucumber against certain fungal and bacterial diseases, also induced resistance in cucumber and tomato against CMV. The purpose of our study was to evaluate 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 were first conducted to evaluate the effects of 26 PGPR strains on the establishment of CMV infection in tomato. In these experiments, the PGPR strains (a plied to seed as pelleted bacterial cells in densities of approximately 5 x 10^9 cfu/seed) were tested along with a disease control (CMV mechanical inoculation, no PGPR) and a healthy control (no CMV inoculation, no PGPR). Plants were examined daily for CMV symptoms (leaf distortion, mosaic patterns, general stunting of the plant). Based on these results, 4 PGPR strains were chosen for further evaluation in field experiments: *Bacillus pumilis* strain SE34, *Kluyvera cryocrescens* strain IN114, *B. amyloliquefaciens* strain IN937a, and *B. subtilus* strain IN937b.

Field experiments were conducted in 1996 and 1997 to evaluate these PGPR strains, a disease control and a uninoculated ("healthy") control. 'Mountain Pride' tomato seeds were mixed with pelleted PGPR ceils resulting in approximately 5 x 10^9cfu/seed. Tomato seeds in the healthy and disease control treatments were dipped in distilled water before planting. Control seeds were treated with 0.2M phosphate buffer. Tomato plants were transplanted into pots containing planting mix two weeks after seeding. PGPR suspension treatments (100 ml containing approximately 5 x 10^8 cfu/ml) were poured into each pot immediately after transplanting. Water or a buffer solution was applied to control plants. CMV-KM inoculum, originally isolated from tomato in North Alabama, was used throughout these studies. CMV-KM inoculum consisted of systemically-infected tobacco leaves ground in 50mM potassium phosphate, pH 7.5, with 10mM sodium sulfite. Tomato leaves were lightly dusted with carborundum and inoculum was applied and rubbed onto the first two-tomato leaves/plant one week after transplanting into pots; transplants were set in the field 3 days after inoculation. Tomato plants were grown on raised beds, fumigated with methyl bromide and chloropicrin and covered with black plastic mulch. There were 6 replications per treatment arranged in a randomized block design, each consisting of 15 tomato plants (single row plots). All plants in each treatment were examined weekly for virus symptoms using a rating scale from 0-10, followed by a calculation of disease severity (Tian et al. 1985). Marketable (non-damaged and mature) tomato fruit were weighed on 6 harvest dates during the season.

Results

In the initial greenhouse experiments, the number of plants exhibiting CMV symptoms was reduced in several PGPR strain treatments compared to the uninduced, challenged control (data not shown). The percentage of symptomatic plants in the 4 PGPR strains selected for further evaluation in the field ranged from 32 to 58%, compared with 88 to 98% in the uninduced, challenged disease control treatment. We also observed a delay in the onset of symptoms in PGPR treatments compared with uninduced control plants.

Table 3. Effects of selected PGPR treatments on cucumber mosaic cucumovirus (CMV) infection and yield in field tomato.

	AUDPC[1] value		Mean yield (kg/plot)	
Treatment	1996	1997	1996	1997
SE34	12.2 c	8.4 c	14.0 a	3.2 a
IN114	21.3 b	12.7 b	10.3 b	2.4 a
IN937a	9.9 c	9.1 bc	14.8 a	2.5 a
IN937b	11.1 c	10.7 bc	14.2 a	2.1 a
Uninduced, challenged control	24.8 a	18.3 a	9.5 b	2.0 a
Uninduced, unchallenged control	0.8 d	7.1 c	14.1 a	2.9 a

Means within columns sharing the same letters are not significantly different ($P > 0.05$; LSD test). [1]AUDPC, area under the disease progress curve.

In the 1996 field experiment, AUDPC values, indicating disease symptom progression over time, were significantly lower in all PGPR treatments compared with the uninduced, challenged control (Table 3). Enzyme-linked immunosorbent assay (ELISA) indicated that the percentage of infected plants in the uninduced, challenged control treatment was over 3-fold greater than in the IN937a and IN937b treatments (data not shown). 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 were significantly lower in the PGPR treatments than in the uninduced, challenged control (Table 3). However, average tomato yields were not significantly different among treatments.

Discussion

These results suggest that specific PGPR strains can elicit induced resistance against CMV infection following mechanical inoculation onto tomato, and that protection can be maintained under field conditions. However, the level of PGPR-induced resistance observed was variable. In the 1996 field experiment, the incidence of CMV infection was significantly reduced and tomato yields were improved (relative to plants in the disease control) on PGPR-treated plants (strains IN937a, IN937b and SE34) mechanically challenged with virus before transplantation to the field. In 1997, AUDPC values were significantly lower in PGPR treatments than in the uninduced, challenged control, but the significant effects of PGPR on the incidence of infected plants and on tomato yields, as seen in 1996, were not evident. In 1997,62.2% of unchallenged control plants tested positive for CMV infection by ELISA, compared with 4.4% in 1996. A possible explanation for the greater incidence of infection in 1997 is that the plants were subjected to lower levels of naturally transmitted CMV in 1996.

Aphid counts were not recorded in either year. We have previously observed a high incidence of natural CMV infection on PGPR-treated plants in a field trial on a north Alabama tomato farm where the level of CMV inoculum was known to be extremely high. 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 CMV strain. We have not yet conducted experiments specifically to evaluate PGPR on tomato for induced resistance against CMV by natural aphid transmission, or to measure the effects of changing abiotic factors on PGPR-induced resistance. The level of protection resulting from treatment by a given PGPR strain may vary from one cropping season to the next depending on existing conditions.

PGPR-ISR Against Tomato Mottle Geminivirus

Tomato mottle, caused by the tomato mottle geminivirus (ToMoV) poses a major threat for both transplant and field production of tomato in west-central and south-west Florida (Abouzid et al. 1992, Polston et al. 1993). ToMoV is transmitted by adult sweet potato whiteflies, *Bemisia tabaci,* biotype B (also known as the silverleaf whitefly, *Bemisia argentifolii).* Symptoms of ToMoV in field-grown tomatoes include chlorotic mottling and upward curling of leaflets, and an overall reduction in plant height as well as the number and size of fruit (Polston et al. 1993). Similar to the CMV pathosystem, traditional management of ToMoV has been very difficult. Tomato cultivars resistant to ToMoV are not yet commercially available, and foliar-applied insecticides have not provided effective management, in part because of the development of insecticide-resistant whitefly biotypes. Prompted by our findings that treatment of tomato with PGPR resulted in reduced symptoms of CMV infection, trials were conducted to evaluate some of the same PGPR strains for induced resistance in tomato against ToMoV.

Methods

Field experiments were conducted during the 1997 fall tomato-growing season at the University of Florida Gulf Coast Research and Extension Center in Bradenton, Florida. Tomatoes were exposed to high levels of natural whitefly infestation and ToMoV infection throughout the production season. Spores of PGPR strains IN937b and SE34 were produced in culture and formulated as both a seed treatment and a powder by Gustafson Corp. (Plano, Texas). The PGPR powder was diluted with water according to the manufacturer's recommendations and incorporated into the planting mix before seeding. At 40 days after planting, each plant in each treatment plot was rated for disease severity using a scale of 0 to 5.0, and all samples were analyzed for ToMoV DNA by nucleic acid dot blot analysis (Polston et al. 1993). Leaf samples for

analysis of ToMoV DNA were also collected 80 days after planting. Tomatoes were harvested from all plots 80, 94 and 108 days after transplanting.

Results

The IN937b and SE34 PGPR treatments both resulted in reduced incidence of ToMoV and disease severity. Visual symptom ratings and the percentage of infected plants (based on dot blot analysis of leaves at 40 days after transplanting) indicated that the PGPR powder and powder + seed formulations were more effective than the PGPR seed formulations (Fig. 4 A, B). The severity of virus symptoms was significantly lower in all PGPR powder and powder + seed treatments than in the untreated control, but symptoms were not significantly different between the seed-only treatments and the control. There were no significant differences in symptom ratings between the PGPR powder-only and the PGPR seed + powder formulations. Contrast analysis of the percentage of plants testing positive for ToMoV DNA from leaf samples collected 40 days after planting generated similar results. By 80 days after planting, most plants in all treatments were infected with ToMoV, and differences in the percentages of infected plants among treatments were not significant (data not shown).

At the first harvest date (80 days after transplanting), tomato yields were higher in PGPR powder or seed + powder treatments than in the control or seed-only treatments; however, yield differences were statistically significant only between the IN937b powder treatment and the control (Fig. 4C). Analysis of tomato yield data from harvests at 94 and 108 days after transplanting did not indicate a significant effect of PGPR treatment on tomato yield. We suspect that PGPR-mediated resistance provided protection against ToMoV in the early stages of infection, but that continual whitefly infestation of plants (based on general observations, not direct insect counts) and inoculation of the virus eventually overcame the induced resistance response.

Discussion

The results with ToMoV in Florida demonstrate that specific PGPR strains can provide protection in the field against viruses in different groups with different insect vectors. The observed level of ToMoV disease symptom suppression resulting from PGPR treatment was encouraging given that the PGPR strains used in the Florida trial were selected based on screening for protection against CMV and not ToMoV. This illustrates the potential of PGPR to provide protection against multiple pathogens. Furthermore, vegetative cell treatments of PGPR were used in our previous experiments with CMV in tomato, and the PGPR spore seed and powder formulations used in the Florida trial had not previously been tested. The levels of whitefly infestation and ToMoV inoculum at the Bradenton field experiment site were greater than what typically occurs in commercial tomato fields (D. Schuster, personal

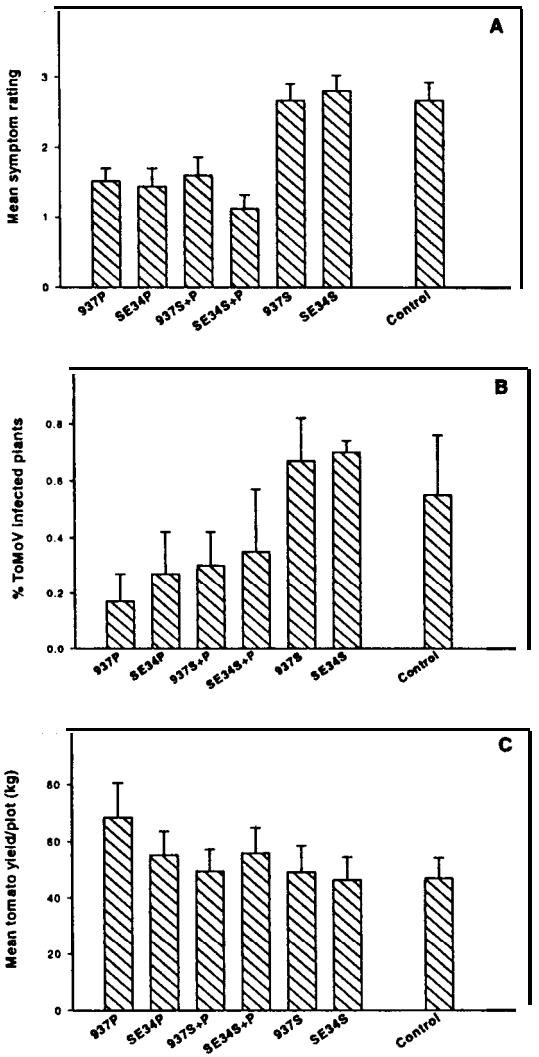

Figure 4: Evaluation of commercially prepared PGPR spore formulations IN937B and SE34 for induced resistance against tomato mottle geminivirus (ToMoV) in Florida field experiments in 1997. S, seed formulation; P, powder formulation; S+P, combination. (A) Mean virus symptom rating. (B) Mean percent of infected plants based on dot blot analysis. (C) Mean weight of marketable tomato fruit per plot on the first harvest date (Dec. 1, 1997).

observation). Thus, conditions in the Florida trial provided a severe test for the PGPR treatments.

Results of the Florida experiment also indicate that the PGPR powder formulations more effectively induced systemic resistance than the treatments in which PGPR were applied directly to the seed. We do not know whether the occurrence and relative speed of PGPR germination and root colonization is correlated with higher levels of PGPR-induced resistance in tomato. If this is the case, it is possible that germination and colonization occurred earlier in the powder formulation treatments resulting in an earlier ISR response. Nonetheless, formulations of PGPR powder provide a practical delivery system for ISR in crops because powder can easily be added to planting mix, or mixed with water and applied as a transplant drench application.

Synthesis

Increased public demand to reduce reliance on chemical crop protectants combined with more stringent government regulations over pesticide registration (e.g., Food Quality Protection Act passed by the U.S. Congress in 1996) has focused more attention on the development of biological agents for pest management in agriculture. PGPR have practical applicability as components of environmentally-sound integrated pest management programs because they can provide safe, persistent and broad-spectrum protection that can be easily delivered to the crop.

To our knowledge, our results with PGPR-ISR in cucumber provide the first evidence that PGPR-induced plants are protected against insect herbivory and disease. The finding that the reduction in cucumber beetle feeding was associated with reduced levels of the secondary plant compound cucurbitacin, a beetle feeding stimulant, is of particular interest because it suggests that plant metabolism is affected by PGPR-ISR. Similar observations have been made for plants in which defense responses have been induced by other agents, including pathogens or chemicals (reviewed in Sticher et al. 1997) or herbivory (reviewed in Karban and Baldwin 1997).

PGPR appear to elicit both disease and insect resistance in cucumber. In contrast, other elicitors of induced resistance have been associated with increased insect feeding. Tallamy (1985) demonstrated an increase in cucurbitacins following herbivory or mechanical injury to zucchini, *Cucurbita pepo*. Apriyanto and Potter (1990) also showed that pathogen-induced resistance against anthracnose *(Colletotrichum lagenarium)* in cucumber was associated with increased cucumber beetle feeding, although cucurbitacin levels were not affected. Thus, it appears that different metabolic pathways are involved in induced resistance in cucurbits, and that the specificity of induced resistance differs in response to induction by pathogens, herbivores or PGPR (see also Stout and Bostock, this volume).

In a recent survey of Alabama tomato growers, a majority of respondents indicated that their most serious pest management problem was control of aphid vectors of plant viruses (Bauske et al. 1998). Recent work at Auburn University has shown that PGPR strains, previously shown to induce

resistance against fungal and bacterial pathogens, also induce resistance against virus diseases in cucumber and tomato. Our results with tomato follow the work of Raupach et al. (1996) in which they reported a significant reduction in CMV symptoms on PGPR-treated tomato grown in the greenhouse compared with a untreated control. We have shown that PGPR-mediated induced resistance in tomato against two different viruses, CMV and ToMoV, can be maintained under field conditions; however, the level of virus disease protection may vary. We have not yet determined whether induced effects on aphid and whitefly feeding behavior and are involved with PGPR-mediated induced systemic resistance in tomato. It is conceivable that chemical signals elicited by PGPR-ISR in tomato may be translocated in the phloem and perceived by aphids or whiteflies during feeding, but this remains to be investigated.

Future Directions

Since 1985, with the first introduction of commercial PGPR products in the United States, the markets for PGPR have continued to increase. At present, 60-75% of the United States cotton crop is treated with *B. subtilis* bacteria targeted against soil borne pathogens (Backman et al. 1997). In China, 18 commercial PGPR strains or strain mixtures are sold, most of which are derived from the spore-forming genus *Bacillus* (Backman et al. 1997). *Bacillus* strains have been the most frequently exploited bacteria for commercial development because the resistant endospore produced by *Bacillus* spp. remains viable for long periods and is tolerant to extremes in temperature and pH, as well as to pesticides and fertilizers. However, the development of new formulation technologies has facilitated the use of biocontrol strains from other genera that have recently been introduced commercially (Backman et al. 1997).

In addition to improved formulation technology, the identification of effective combinations of bacteria for specific crop/pest systems is another research area that may lead to increased efficacy of PGPR products. Studies have shown that combinations of bacteria have been more effective than single strains for disease control and improved plant response (Pierson and Weller 1994, Sheng 1996, Raupach and Kloepper 1998). Another area of future development will undoubtedly include the genetic engineering of PGPR strains in which plant-beneficial genes are expressed, or in which such genes are expressed at an increased level. Genetically engineered PGPR may have an expanded spectrum of activity or level of control (Backman et al. 1997).

Another approach to achieve effective disease protection may be to use combinations of PGPR with other inducing agents that suppress diseases by complementary mechanisms (i.e., benzothiadiazole, Görlach et al. 1996, Tally et al., this volume). Additional studies in this area are needed to determine whether different inducers used simultaneously (e.g., PGPR and chemical inducers) enhance the induced defense response or act antagonistically (Karban and Kuc, Stout and Bostock, Felton and Eichenseer, this volume).

The enhancement of PGPR technology will be aided by a better understanding of the mechanisms involved with PGPR-ISR. Workers in the area of induced resistance are just beginning to compare and contrast the different mechanisms associated with plant resistance elicited by pathogens, chemicals, insects and non-pathogenic microbes (Stout and Bostock, this volume). In the case of PGPR-mediated resistance, it appears that different rhizobacterial strains induce resistance by different mechanisms (reviewed in van Loon et al. 1998). For example, bacterial production of salicylic acid is involved with ISR in some strains, but not in others (van Loon et al. 1998). van Loon et al. (1998) suggest that the mechanism by which a rhizobacterial strain induces resistance may vary depending on local conditions in the rhizosphere. Studies to elucidate the different mechanisms involved in the induction of systemic resisitance will enable us to develop and deploy PGPR products to their greatest advantage.

It remains to be determined if these strategies to optimize the effectiveness of PGPR as inducers of resistance will generate PGPR products that alone can consistently provide acceptable levels of disease protection. In the short term, at least, combinations of PGPR with other disease management tools (e.g., resistant or tolerant varieties, insecticides targeted against insect disease vectors, etc.) may prove to be the best strategy for the commercial use of PGPR. Furthermore, PGPR product development will be driven by economic considerations that may restrict its use to certain markets. PGPR represent a potentially valuable resource for IPM programs, particularly in high-value cropping systems like vegetables where regulations or lack of efficacy limit the availability of chemical crop protectants.

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