

RHIZOSPHERE POPULATION DYNAMICS AND INTERNAL COLONIZATION OF CUCUMBER BY
PLANT GROWTH-PROMOTING RHIZOBACTERIA WHICH INDUCE SYSTEMIC RESISTANCE TO
COLLETOTRICHUM ORBICULARE

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INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are root-colonizing bacteria which exert a beneficial effect on plant development. The reported beneficial effects of PGPR include plant growth promotion (Kloepper et al., 1991) and reductions in the incidence of soilborne diseases (Kloepper, 1991; Weller, 1988). Due to their effects on crops, PGPR hold promise for use in integrated strategies for implementing low-input sustainable agriculture. Implementation of large-scale field use of these bacterial inoculants will require decreasing the variability of field performance which appears to be innate with most inoculants consisting of single bacterial strains. One approach to reducing variability is to use mixed inoculants consisting of two or more bacterial strains with different mechanisms. This approach depends upon having identifiable differences in mechanisms. Most of the PGPR and bacterial biological control agents which have been reported to date were first selected by antibiosis *in vitro* toward fungal pathogens (Weller, 1988) and then tested for biological control activity in disease assays with the host plant. Hence, it is not surprising that most suggested mechanisms for growth promotion and biological control by rhizobacteria involve antibiotics, siderophores, HCN, or other compounds which can be broadly called antifungal compounds (Kloepper, 1991; Weller, 1988). Competition for infection sites or nutrients (other than iron) and parasitism are other mechanisms which have been reported to relate to biological control with a few rhizobacterial strains (Kloepper, 1991).

Induced systemic resistance (Kuo and Strobel; Scheffer et al., this volume), broadly defined as activation of latent defense mechanisms in plants prior to pathogenic attack, has been hypothesized in recent years to be an operable mechanism in several rhizobacterial systems. In the mid 1980s, Allelix Crop Technologies tested the biocontrol activity of PGPR strains which were originally selected for plant growth promotion activity without prescreening for antibiosis *in vitro* (Kloepper et al., 1991). PGPR strains which exhibited biological control activity were then tested for *in vitro* antibiosis to fungal pathogens, and about 40% lacked such activity, suggesting that mechanisms other than antagonism were operable in the assay. In another system, *Pseudomonas fluorescens* strain CHAO was found to provide

biological control of Thielaviopsis basicola on tobacco (Ahl et al., 1986). CHAO produces HCN which was shown to be associated with biocontrol by mutational analyses, and Voisard et al. (1989) suggested that HCN may induce resistance to Thielaviopsis.

Experimental evidence to support induction of resistance by rhizobacteria may come from two kinds of studies. One involves looking for elevated levels of compounds associated with plant defense mechanisms, after inoculation with PGPR. Anderson and Guerra (1985) reported that a strain of P. putida, which has biological control activity against Fusarium solani on bean, was associated with increased production of lignin in roots. Treatment of potato tissue-culture plants with a growth-promoting strain of Pseudomonas resulted in enhanced total plant lignification (Frommel et al., 1991) compared to non-inoculated controls. As lignin has been associated with increased host defense, these studies suggest that some PGPR may increase host defense. Albert and Anderson (1987) demonstrated that bean roots colonized by a P. putida PGPR strain had increased and altered production of peroxidases, which have been implicated in induced resistance. Pathogenesis-related (PR) proteins have also been implicated in host defense, and Hynes and Lazarovits (1989) reported that PGPR treatment of bean increased the level of a 60 KD PR protein in leaves.

The second kind of study which can support a role of induced resistance in biocontrol by rhizobacteria is a more direct pathological approach in which treatment with a rhizobacterial strain causes a reduction in disease caused by a subsequently inoculated pathogen which is spatially separated from the rhizosphere to avoid the possible involvement of competition or antagonism. The first reports that PGPR strains may reduce the incidence of disease caused by a spatially-separated pathogen were recently published. Van Peer et al. (1991) found that the fluorescent pseudomonad PGPR strain WCS417, when poured onto rockwool cubes containing rooted cuttings of carnation, decreased the incidence of wilt caused by stem inoculations with Fusarium oxysporum f. sp. dianthi. We previously reported (Wei et al., 1991) preliminary results indicating that select strains of PGPR could be applied to cucumber as seed treatments, resulting in a reduction in lesion area of anthracnose caused by Colletotrichum orbiculare. The conclusion was made in both of these studies that the observed biological control resulted from induced resistance. We now report recent results confirming the preliminary work and results from investigations into the population dynamics of PGPR which induce systemic resistance (hereafter referred to as "inducing PGPR").

MATERIALS AND METHODS

Sources of Microorganisms

C. orbiculare (Berk. and Mont.) Arx (previously known as C. lagenarium (Pass.) Sacc. and Roum.) was provided by Joseph Kuc, Department of Plant Pathology, the University of Kentucky. PGPR strains were obtained from the culture collection of Esso Chemical Ag Biologicals, Saskatoon, Saskatchewan, S7N 2X8, Canada and were previously shown to promote seedling emergence or yield of soybean (Glycine max) or canola (Brassica napus). The six inducing PGPR strains used in this study (G8-4, 25-33, 28-9, 34-13, 36-5, and 2-67) were selected from a collection of 94 PGRP strains based on preliminary work (Wei et al., 1991) indicating potential induction of systemic resistance and represented the following taxa: P. putida (34-13), P. fluorescens (G8-4), P. aureofaciens (25-33, 28-9, 36-5) and Serratia plymuthica (2-67).

Cucumber Induced Resistance Assay

The cucumber anthracnose system of Kuc et al. (1975) was selected as the

model for testing PGPR as inducers of systemic resistance. The first true leaf of cucumber seedlings was immunized by inoculating with 30 drops (5- μ l each) of a conidial suspension of *C. orbiculare* at \log_{10} 6 conidia/ml. Seven days later, the second true leaf was "challenged" by inoculating with 30 drops (10- μ l each) of a suspension containing \log_{10} 4 *C. orbiculare* conidia/ml. Six days after challenge, the number of lesions and the total lesion diameter on the challenged leaf were recorded.

The six inducing PGPR strains were grown in tryptic soy broth (TSB) (Difco) for 24 hr at 30 C. Cultures were centrifuged at 10,000 x g for 5 min, and pellets were resuspended in 2 ml of 1% sodium alginate. Cucumber seeds, cv. 'Straight Eight' were dipped into the alginate suspension immediately prior to planting in Promix (Premier Peat, Rivière-du-Loup, Québec, CANADA). Plants were maintained in a greenhouse and were fertilized weekly with 20:20:20 soluble plant food.

The experimental design was a completely randomized block with six replications. The treatments consisted of the six inducing PGPR strains, a control with no bacteria, induced and challenged with *C. orbiculare* (the "induced resistance control"), and a control with no bacteria, no induction, and challenged with *C. orbiculare* (the "disease control"). The induced resistance control was induced to confirm that each experiment was conducive to expressing induced resistance. The plant responses from treatments with the inducing PGPR were compared to the disease control. Data were analysed using the general linear models with SAS software (SAS Institute, Cary, North Carolina). The LSD at $P = 0.05$ was calculated to determine significant differences among treatment means.

Population Dynamics of Inducing PGPR

Experiments were designed to determine the population dynamics of inducing PGPR in the rhizosphere and inside roots during the time period from planting to challenge (21 days). Spontaneous mutants resistant to 100 mg/l rifampicin were selected for each inducing strain. Cucumber seeds were inoculated with the rifampicin-resistant inducing PGPR as described above, and the average population of each treatment was determined prior to planting by dilution plating of four seeds per treatments onto tryptic soy agar (TSA) amended with 100 mg/l rifampicin (rif-TSA) with a spiral plater (Spiral Systems, Inc., Bethesda, Maryland). Seeds were also sampled 24 hr after planting. To determine rhizosphere populations of inducing PGPR, whole root systems were sampled at 3, 7, 14, and 21 days after planting. Loosely adhering Promix was removed by gently shaking roots prior to weighing each root system. Roots were agitated for 15 min in phosphate buffer pH 7.0, and serial dilutions were prepared and spiral-plated onto rif-TSA. Samples were also plated onto TSA to determine rhizosphere populations of total bacteria.

Populations of inducing PGPR inside roots were determined by removing the tap root, weighing, and surface disinfecting by shaking in 1.05% NaClO with Tween 20 (2 drops/100 ml). Root sections were rinsed three times in 100 ml sterile distilled water and ground in 5.0 ml sterile water with an autoclaved mortar and pestle. Serial dilutions were prepared and spiral-plated onto rif-TSA and TSA. Sterility controls, consisting of adding 1.0 ml of the final rinse water to 9.0 ml TSB and incubating the TSB at 28 C for 48 hr, were performed on every sample. Previous tests demonstrated that this control detected contamination at a similar rate to controls consisting of placing surface-disinfected roots onto TSA plates.

Three replicate seeds or roots were used at each sample time for estimating populations. All inoculated plates were incubated 24 hr at 28 C, and bacteria were enumerated using a laser colony counter and Bacterial Enumeration software (Spiral Systems, Inc.). Mean cfu/g root or seed were deter-

Table 1. Results Showing the Effect of Inducing Plant Growth-promoting Rhizobacteria (PGPR) on Cucumber Anthracnose Caused by Colletotrichum orbiculare

Treatment	Mean Lesion Number/Leaf	Mean Total Lesion Diameter (mm)
PGPR Strain G8-4	14.7 ^a	42.7 ^a
PGPR Strain 25-33	14.0 ^a	40.3 ^a
PGPR Strain 28-9	22.5	83.7 ^a
PGPR Strain 34-13	14.5 ^a	46.2 ^a
PGPR Strain 36-5	14.2 ^a	49.0 ^a
PGPR Strain 2-67	16.5 ^a	53.8 ^a
Disease Control ^b	26.0	152.2
Induced Res. Control ^c	4.3 ^a	8.0 ^a
LSD ($P = 0.05$) =	5.2	38.1

^a Indicates significant reduction compared to the disease control. All values are the means of six replications.

^b The disease control consisted of plants without bacteria and challenged with C. orbiculare as described in the methods.

^c Induced resistance control consisted of plants induced by inoculating the first true leaf with a conidial suspension of C. orbiculare as described in the methods.

mined at each sample date using log-transformations of the data. The potential for inducing PGPR to colonize the challenged leaf (second true leaf) was determined. Seeds were treated with rifampicin-resistant mutants of all six inducing strains. Three-cm sections of petioles were removed from the second true leaf 21 days after planting. Sections were surface sterilized as described above, ground in sterile water in an autoclaved mortar and pestle, and spiral plated onto rif-TSA and TSA. Sterility controls were conducted as described above.

RESULTS

Cucumber Induced Resistance Assay

In multiple experiments, inoculation of cucumber seeds with the "inducing PGPR" resulted in significant reductions in mean number and diameter of lesions caused by C. orbiculare compared to the disease control. Typical results are shown in Table 1. In this experiment, treatment with five PGPR strains resulted in a significant reduction in number of lesions compared to the disease control, while significant reductions in total lesion diameter occurred with all six PGPR strains. In a previous investigations (Wei et al., 1991) this protection was found to be specific to the six PGPR strains used here, and 88 other PGPR strains lacked protection activity. In addition to providing protection from C. orbiculare, some PGPR strains resulted in increased plant growth. Cucumber plant height and leaf area were increased beginning at 7 days after planting with these strains (Fig. 1).

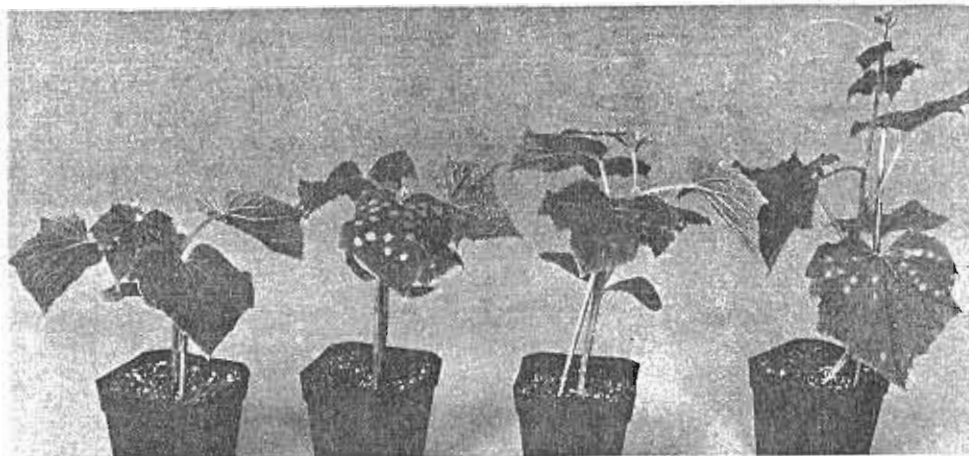


Fig. 1. Reduction in number and total diameter of lesions caused by *Colletotrichum orbiculare* and plant growth promotion following treatment of cucumber seed with plant growth-promoting rhizobacteria (PGPR). From left to right: Induced resistance control (induced with *C. orbiculare* on first true leaf 7 days before challenge), disease control (noninduced and no bacteria), two plants induced with PGPR strains. The second true leaf of all plants was challenged with *C. orbiculare*.

Population Dynamics of Inducing PGPR

Seed treatment with the alginate suspension of rifampicin-resistant PGPR resulted in mean populations of \log_{10} 8.0 to 8.5 cfu/seed, depending on the strain used. The rhizosphere population dynamics of the six inducing PGPR strains from planting to 21 days after planting (when challenge inoculation with *C. orbiculare* was made) are shown in Fig. 2. Rhizosphere colonization by all strains showed a similar pattern with an initial decrease of about 0.5 log units 1 day after planting, followed by an increase of 0.3 to 1.6 log units 3 days after planting and then a steady decrease to a final population density of \log_{10} 4.4 to 4.9 cfu/root system 21 days after planting.

The population dynamics of inducing PGPR inside cucumber roots varied markedly among strains (Fig. 3). Strain G8-4 was recovered from inside roots at all sample times, while strain 25-33 was not recovered at any sample time. The remaining four strains were not detected 3 days after planting but were recovered at one or more subsequent sampling dates.

DISCUSSION

Reductions in development of anthracnose lesions on leaves of cucumber 27 days after seed treatment with six PGPR strains demonstrates that some PGPR may induce systemic resistance. The inducing PGPR strains were not detected in petioles of protected leaves, thereby showing that competition or antagonism were not operable mechanisms for the observed control. The underlying mechanisms by which these strains induce resistance and how the plant defense compounds are affected are currently under investigation. While inducing PGPR significantly reduced lesion development compared to disease controls, this protection was less than that afforded by the induced

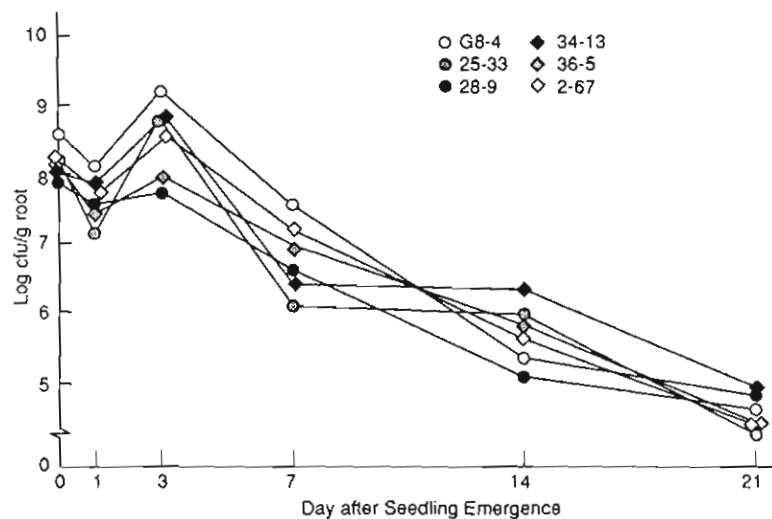


Fig. 2. Colonization of cucumber rhizospheres by plant growth-promoting Rhizobacteria (PGPR) which induce systemic resistance to Colletotrichum orbiculare

resistance controls (Table 1, Fig. 1). This is not surprising considering that PGPR must compete with other rhizosphere microorganisms and produce a trigger or signal for induced resistance which then must be translocated from roots to the second true leaf (challenged leaf).

Rifampicin-resistant mutants of the inducing PGPR strains colonized cucumber rhizospheres during the 21 days from planting to challenge at mean population densities of \log_{10} 8 to 4 cfu/g root (Fig. 2). The drop in population from 14 to 21 days after planting may not be important, if the signal or trigger for induced resistance already moved from the root to the stem by this period; however, further research is needed to determine the precise timing of signal initiation.

Although no PGPR strains were detected in petioles at the time of challenge with C. orbiculare, some strains were recovered from inside sur-

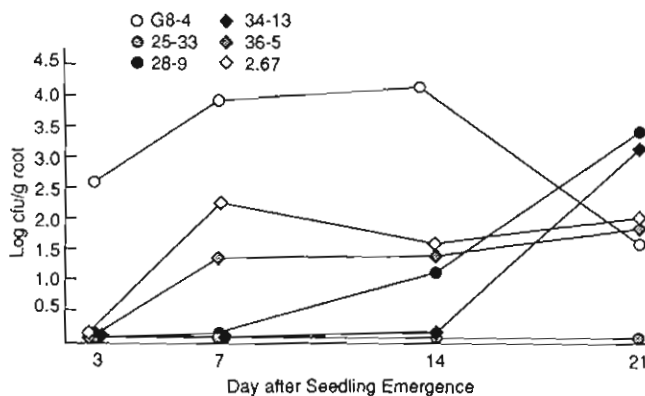


Fig. 3. Internal colonization of cucumber roots by plant growth-promoting Rhizobacteria (PGPR) strains which induce resistance to Colletotrichum orbiculare

face-disinfested roots (Fig. 3). The timing of internal root colonization varied among strains, ranging from colonization from 3 to 21 days after planting to no colonization. The relationship between internal plant colonization by inducing PGPR and protection in the cucumber assay requires further investigation.

These results confirm the report by van Peer et al. (1991) that PGPR may induce systemic resistance to a pathogen which is spatially separated from the root zone. Our study extends this finding to include seed treatment as a means for inducing resistance. The model system of cucumber and anthracnose disease, caused by C. orbiculare, allows for a quantifiable assessment of the potential for root-colonizing bacteria to induce systemic resistance against a foliar pathogen within 4 weeks of planting. This system should be useful in other studies designed to assess the potential contribution of induced resistance to biological control.

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