Virginia Journal of Science
Volume 41, Number 4B
Winter 1990

Evaluation of a *Pseudomonas fluorescens* Strain for Repression of Seedling Disease in Cotton

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ABSTRACT

A strain of *Pseudomonas fluorescens* (EG1053) antagonistic to *Pythium ultimum* (Trow) and *Rhizoctonia solani* (Kuehn), the major seedling pathogens of cotton (*Gossypium hirsutum* L.), was evaluated for disease repression in greenhouse trials. In both potting mix and soil, disease severity (presence of root lesions) and incidence (numbers of infected plants) declined with higher application rates of EG1053 in a peat-based carrier. Low application rates resulted in plants that were equivalent to noninoculated controls while higher rates produced plant stands equal or superior to fungicide treatments. The placement of EG1053 inoculum in relation to the proximity of cotton seeds affected both the degree of root colonization that occurred and the numbers of surviving plants. Applications of EG1053 provided larger plant stands and reduced seedling disease symptoms on surviving plants in both potting mix with amended pathogens and naturally infested cotton soils.

INTRODUCTION

The heterotrophic bacterium, *Pseudomonas fluorescens*, has been successfully used as seed and soil inoculants to biologically control several plant pathogens (Colyer and Mount, 1984; Ganesan and Gnanamanickam, 1987). Strains of *P. fluorescens* have been reported to control seedling disease of cotton (*Gossypium hirsutum* L.) and there is some evidence regarding possible mechanisms involved in the control process (Hagedorn *et al.*, 1989; Howell and Stipanovic, 1979). There are also reports of *P. fluorescens* isolates that demonstrate both plant growth promotion and yield increases under either gnotobiotic or field conditions (Kloepper and Schroth, 1981; Klopper *et al.*, 1980). The ability of *P. fluorescens* to reduce plant disease and promote plant growth has been attributed to several factors (or combinations) that include antibiosis, competition and displacement of the naturally occurring microflora, siderophore activity, and alterations in microniche factors such as pH and redox potential (Lambert *et al.*, 1987; Scher and Baker, 1982; Suslow and Schroth, 1982).

Cotton seedling disease impacts the quality of the plant stand early in the growing season, and is responsible for substantially reducing cotton yields (Bell, 1984). Annual yield loss estimates have ranged from 1% to 6% over a thirty year period and were 2.0% in 1987, for a loss of 291,042 bales of cotton (6.6×10^6 kg) in the U.S. (CDCR, 1987).

This report demonstrates that seedling disease repression in cotton is related to the quantity of *P. fluorescens* strain EG1053 applied. Repression was reflected in lowered disease severity on cotton roots and higher numbers of surviving plants. The results indicate that EG1053 grows rapidly on cotton roots and implicates root colonization as a mechanism in biological control.

MATERIALS AND METHODS

Bacterial Strain The *P. fluorescens* strain EG1053 was originally isolated from cotton field soil in Starkville, MS (Hagedorn *et al.*, 1989). The soil was a Marietta sandy loam (pH 8.1) classified as a fine-loamy siliceous, thermic, Fluvaquentic-Entrocrept. This isolate was in a collection of bacteria that were screened for ability to repress the seedling disease pathogens on cotton, and has been deposited in the Northern Regional Research Laboratory, ARS-USDA (Peoria, IL), under accession number NRRL-18336.

Fungal Isolates Strains of *P. ultimum* and *R. solani* were originally isolated from naturally infected cotton plants that demonstrated severe seedling disease symptoms. Cultures were maintained on potato dextrose agar (PDA), and tested for pathogenicity on cotton seedlings every six months.

Inoculum Preparation For root colonization assays, EG1053 was grown in trypticase soy broth (TSB) for 48 h at 30°C, followed by centrifugation and washing (once) in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% Bacto-Peptone (Difco), and then added to sterile (gamma-irradiated) granular peat (pH 6.8, provided by the Nitragin Co., Milwaukee, WI) to a level of 35% moisture (W/W). The peat was incubated at 30°C for three weeks prior to inoculation and EG1053 counts on King's Medium B (KB) were determined at seven day intervals. Fluorescent colonies were observed after 48 h incubation at 30°C. Additional peat formulations (non-sterile, pH 7.1) for the biocontrol tests that contained pre-determined numbers of colony forming units (CFU's)/g were provided by Ecogen Inc., Langhorne, PA. These formulations were used within 24 h and samples were plated on KB to determine numbers of EG1053 at planting. Biocontrol Assay. Plants were grown in either sterile potting mix that consisted of washed sand and commercial vermiculite (1:1 v/v) or cotton soils that were transported from Mississippi, South Carolina, Texas, and Virginia. Galvanized steel trays 60 cm by 30 cm were filled to a depth of 10 cm with potting mix or soil and untreated cotton seeds (cv. Stoneville 825) were planted in two rows (30 cm by 3.0 cm deep) per tray at a rate of 10 seeds per row. Four trays (replications) were employed for each test, and main treatments were application rates while subtreatments were the number of cells/g in the inoculum. One PDA plate each of *P. ultimum* (48 h at 25°C) and *R. solani* (72 h at 25°C) was blended in 500 ml sterile water and added to a volume of potting mix sufficient to fill two trays, and this provided counts between 10^3 and 10^4 propagules/g^{-1} of mix for each pathogen. The pathogens were not added to the soils since indigenous seedling disease pathogens were already present. Counts of fungal propagules were determined on PDA for the sterile potting mix and selective media for the soils: maize meal agar for *P. ultimum* and gallic acid agar for *R. solani* (Dhingra and Sinclair, 1985). The EG1053 peat inoculants were added to the trays at planting by weighing the correct amount of peat for each row and dispensing it, by hand, into the row as uniformly as possible. Plant growth tests included non-in-

oculated (with pathogens but no inoculant), peat alone (with pathogens but no EG1053) and fungicide controls (Terrachlor Super X (TSX), Olin Chemical Corp., and Ridomil, Ciba-Geigy). The granular fungicides were applied at a uniform rate of 11.2 kg/ha (0.8 g/row) as recommended for cotton by the manufacturers. Plants were grown in a glasshouse with 12 h light cycles of 25°C and 20°C at night, and harvested after 35 days. Roots of all surviving plants were rated based on the presence of *P. ultimum* root lesions and *R. solani* lesions and discoloration on the plant stem.

Inoculum Placement The effects of inoculum placement were evaluated by adding 0.25 g^{-1} of EG1053 inoculum (10^8 CFU's/g) at distances of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 cm^{-1} away from cotton seeds in potting mix plus the pathogens (4 replications). After 35 days, plants were harvested and roots were rated and cut into 100 mm segments to determine the populations of EG1053 as described above.

Statistical Analysis The experimental design was a randomized complete block with four replications. Application rates were main treatments while EG1053 cell numbers within rates were subtreatments. Analysis of variance (ANOVA) of treatment means was performed by the GLM procedure and mean separations were performed by Duncan's Multiple Range test when the overall F-test was significant at $P < 0.05$.

RESULTS AND DISCUSSION

Inoculum Production The sterile, granular peat supplied by the Nitragin Company was a suitable medium for cultivating *P. fluorescens* strain EG1053. Cell counts increased by approximately tenfold during the first two weeks of the incubation period and then doubled during the second two week period (data not shown). The initial inoculum added to the peat (10^6 to 10^9 cells/g) did not affect the rapidity of EG1053 growth in the material. Cell counts of the inoculants provided by Ecogen, Inc., ranged from 6.2×10^6 to 5.3×10^{10} CFU's/g.

The peat was an adequate carrier for EG1053 whether sterilized or not, although this material does contain large populations of indigenous microbes that have caused problems with the use of peat as a *Rhizobium* carrier (Turco *et al.*, 1986; Vincent, 1980). Sterilization of the peat tonnage used annually in agriculture is not practical but, without sterilization, resident microbes may overgrow the added inoculant strain. Peat usually contains indigenous *P. fluorescens* strains and this mandates selection of antibiotic resistance or some other distinguishing feature to permit a sensitive counting technique for an inoculant bacterium.

Plant Stands-Pottinq Mix Disease pressure was maintained at a high level as evidenced by the low percentage of surviving plants in the non-inoculated control (12.5% ave. stand) while the fungicide treatment provided plant stands that averaged 73.3% (Table 1). Increasing the inoculant application rate and the cell numbers within each rate was significantly correlated with larger plant stands (RSQR = 0.92). In the 5.6 kg/ha treatment, the inoculants provided plant stands that ranged from 26% (10^6 CFU's/g) to 47% (10^{10} CFU's/g), and these stands were statistically intermediate between the non-inoculated and fungicide controls. For the 8.4 kg/ha treatment, EG1053 provided plant stands ranging from 36% (10^6 CFU's/g) to 67% (10^{10} CFU's/g). These were all superior to the stands from the noninoculated control while one of the subtreatments (10^{10} CFU's/g) produced

TABLE 1. Percent plant stand as a result of application rate and cell numbers of EG1053 in peat inoculants.

Treatment	Inoculant Application Rate (kg/ha)				
	5.6	8.4	11.2	14.0	16.8
	Percent Stand				
Controls					
Non-Inoculated	11.0a*	12.5a	10.3a	13.8a	15.0a
Fungicides**	71.3d	70.0e	75.0c	77.5c	73.0d
Granular EG1053 (CFU's/g)					
10^6	26.3b	36.3b	43.8b	46.3b	48.8b
10^7	30.0b	42.5bc	53.8b	57.5b	60.0c
10^8	38.8bc	51.3cd	65.0c	73.8c	81.3de
10^9	43.7c	58.7de	67.5c	75.0c	85.0e
10^{10}	47.5c	67.5e	72.5c	78.8c	87.5e
LSD	9.0	12.0	10.0	11.0	11.0

*Entries followed by the same letter within each column are not significantly different at the 0.05 level (Duncan's Multiple Range Test) for Tables 1-4.

**The fungicides (Terrachlor Super-X and Ridomil) were applied in all tests at 11.2 kg/ha as recommended by the manufacturer (Tables 1-4).

plant stands equal to those from the fungicide treatment. The 11.2 kg/ha treatment provided plant stands that ranged from 43% (10^6 CFU's/g) to 72% (10^{10} CFU's/g). Three of the subtreatments (10^8, 10^9, and 10^{10} CFU's/g) provided stands equal to those from the fungicide. Similar results were obtained for the 14.0 kg/ha treatment as from the 11.2 kg/ha treatment. For the 16.8 kg/ha treatment, the inoculants provided plant stands that ranged from 48% (10^6 CFU's/g) to 87% (10^{10} CFU's/g) percent. One subtreatment (10^8 CFU's/g) was equal to the fungicide, and two subtreatments (10^9 and 10^{10} CFU's/g) provided stands superior to those from the fungicide (Table 1). The inoculant control (peat without EG1053) produced plant stands that were equivalent to the non-inoculated control (data not shown).

Plant Root Ratings-Potting Mix - Extensive disease was evidenced in the root ratings on the non-inoculated plants while ratings from the fungicide treatment were significantly higher (Table 2). The ratings were based on a visual evaluation to estimate the proportion of plants that displayed root and stem lesions (1.0 = 75-100% of plant roots and stems displaying lesions, 2.0 = 50- 75%, 3.0 = 25-50%, and 4.0 = 0-25%. For the 5.6 kg/ha treatment, the inoculants provided plants with root ratings that were intermediate between the non-inoculated and fungicide controls. At all other application rates, one subtreatment (10^6 CFU's/g) yielded root ratings that were significantly lower than those from the fungicide treatment while the other four subtreatments provided plants with root ratings that were equivalent to the fungicide treatment. Root ratings were significantly correlated with both increased application rates and higher numbers of EG1053 in the

TABLE 2. Plant root ratings as a result of application rate and cell numbers of EG1053 in in peat inoculants.

Treatment	Inoculant Application rate (kg/ha)				
	5.6	8.4	11.2	14.0	16.8
	Plant Root Ratings*				
Controls					
Non-Inoculated	1.0a	1.0a	1.1a	1.2a	1.0a
Fungicides	2.7c	2.6cd	2.5bc	2.8cd	2.9cd
Granular EG1053 (CFU's/g)					
10^6	1.4ab	1.7ab	1.9ab	2.0b	2.0b
10^7	1.7ab	1.9bc	2.0b	2.1bc	2.3bc
10^8	2.0bc	2.1bc	2.3b	2.6bc	2.8cd
10^9	2.3b	2.6cd	2.76bc	2.9cd	3.Ocd
10^{10}	2.2bc	3.0d	3.3c	3.4d	3.5d
LSD	0.8	0.7	0.8	0.7	0.7

*visual rating based on proportion of plant roots displaying disease symptoms: 1.0 = 75-100%, 2.0 = 50-75%, 3.0 = 25-50%, 4.0 = 0.25% (Tables 2-4).

inoculants (RSQR = 0.93). The inoculant control (peat without EG1053) provided plants with root ratings that were equal to the non-inoculated control (data not shown).

Plant Stands and Root Ratings-soil - To confirm the results that were observed with potting mix (Tables 1 & 2), three EG1053 application rates (10^8 CFU's/g in each) were employed for the soil tests (Table 3). Seedling disease pressure was high in the four soils as evidenced by the low average percent plant stands and root ratings in the non-inoculated controls. The fungicides provided plant stands and root ratings that were superior to the non-inoculated controls in each soil while the peat control produced plants that were equivalent to the non-inoculated control. For the granular EG1053 inoculants, the 5.6 kg/ha treatment produced plants that were equivalent to the non-inoculated control while the 16.8 kg/ha treatment provided plants that were equal to the fungicide and superior to the noninoculated controls for each soil. The 11.2 kg/ha treatment produced plants that were intermediate between the other two application rates (Table 3).

The application rate and cell concentration (in the inoculant) studies determined the minimal level of these two parameters required to provide plant protection from seedling disease that was equivalent to the fungicide treatment. At the same application rate recommended for the fungicides (11.2 kg/ha), EG1053 produced plants that were equivalent to those from the fungicide treatment at levels of 10^8 CFU's/g or greater (Tables 1-3). At lower application rates, the inoculants did not perform as well as the fungicides but higher cell concentrations did provide better levels of plant protection.

ABLE 3. Plant stands (%) and root ratings of cotton grown in four soils amended with EG1053.

Treatment	State							
	MS	SC	TX	VA	MS	SC	TX	VA
	% Plant Stand				Root Rating			
Controls								
Non-Inoculated	42.9a	32.3ab	47.6a	36.7a	1.7a	1.7a	2.0a	1.9a
Granular Peat	37.6a	28.4a	49.5ab	63.0b	2.0a	1.5a	2.1a	2.3ab
Fungicides	76.7c	66.1c	74.2c	61.4b	3.1b	2.6b	3.2b	2.7b
Granular EG1053 (kg/ha)								
5.6	39.4a	36.7ab	53.5ab	57.4b	2.2a	1.8a	2.3a	2.1ab
11.2	56.0b	47.8b	61.7b	64.9b	2.8ab	2.Oab	2.7ab	2.8b
16.8	81.3c	63.4c	76.9c	77.3c	3.3b	2.5b	3.4b	3.1b
LSD	13	15	12	11	0.8	0.7	0.8	0.6

Inoculum Placement - The placement of EG1053 inoculant appeared to be an important factor in successful root colonization and disease repression (Table 4). When the inoculant was placed further than 1.0 cm away form the seeds, plant stands were equivalent to the non-inoculated control. The proportion of 100 mm root segments colonized by EG1053 ranged from 95.6% (adjacent to the seeds) to 11.9% (3.0 cm from the seeds) and indicated that at least 80% of the root segments required EG1053 colonization before adequate disease repression occurred (plants equal to the fungicide treatment). These placement results may explain why the lower inoculant application rates failed to adequately repress seedling disease even though high numbers of cells were applied (Table 1). At 5.6 and 8.4 kg/ha rates, there is not sufficient peat to adequately contact every seed, and those seeds that do not receive any peat in close proximity will not be protected (Table 4).

SUMMARY

These studies demonstrate that *P. fluorescens* strain EG1053 can effectively protect cotton plants from seedling disease. The mechanisms of disease repression by EG1053 are unknown and no evidence of *in-vitro* antibiosis or direct fungal inhibition has been found (Hagedorn, 1988). Competition for root colonization sites has been associated with biocontrol agents (Loper *et al.*, 1985; Martin *et al.*, 1985) and, by comparison, EG1053 appears to be a rapid root colonizer. An antibiotic resistant mutant of EG1053 that would have permitted plate counts from the soils was not used because any such mutant could colonize and compete quite differently from the parental strain. Any differences cannot be acceptable when the potential field performance of a commercial strain is being evaluated. The experimental conditions in the potting mix were relatively non-competitive (no indigenous bacteria), but EG1053 had to compete against fungi that have been described as aggressive root invaders (Martin *et al.*, 1985). However, the non-sterile

TABLE 4. Effects of distance between seeds and EG1053 inoculant on root colonization, plant stands, and disease severity.

Treatment	% Root Segments Containing EG1053	% Plant Stand	Root Rating
Controls			
Non-inoculated	----	38.3a	1.2a
Fungicide	----	75.7c	3.1d
Distance EG1053 Inoculant Placed from Seeds (cm)*			
0.0	95.6	74.2c	3.3d
0.5	92.1	76.6c	3.2d
1.0	81.3	61.4bc	2.8cd
1.5	62.0	52.3ab	2.1bc
2.0	31.7	47.7a	1.4ab
2.5	16.4	39.2a	1.6ab
3.0	11.9	42.4a	1.3a
LSD	-	15	0.7

*The EG1053 inoculant contained 108 CFU's/g, and 0.25 g of the inoculant was placed varying distances from the cotton seeds.

soils contained large populations of indigenous bacteria that represented a realistic competitive microbial environment. The granular peat-based inoculants were applied to simulate in-furrow conditions, but resulted in only a small quantity of peat actually in contact with each seed. The extensive colonization of seedling roots by EG1053 may indicate the degree to which the strain was able to respond to the presence of young seedlings.

Future research will examine the ability of EG1053 to reduce the effects of seedling disease on cotton in field studies. To distinguish EG1053 from indigenous bacteria, the selection of spontaneous antibiotic-resistant mutants could be employed (Compeau *et al.*, 1988; Teintse *et al.*, 1980). Such mutants will be necessary if other fluorescent pseudomonads are present, but for non-fluorescent bacteria, mutants may not be required if the added bacteria do not overgrow the plating medium (Compeau *et al.*, 1988). Since EG1053 demonstrated cotton seedling disease repression, this strain lends itself to a molecular approach to determine possible mechanisms. Siderophores are one apparent choice (Scher and Baker, 1982) and the development of nonsiderophore mutants, as well as non-colonizing mutants, may demonstrate potential mechanisms involved in disease repression and offer research approaches to enhance repressive activities and the performance of EG1053 in the field.

ACKNOWLEDGMENT

This research was supported by Ecogen Inc., (Langhorne, PA), and the Biotechnology Institute of the Virginia Center for Innovative Technology.

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