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Biological Control of Fusarium Wilt on Cotton by Use of Endophytic Bacteria[1]

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One hundred seventy bacterial strains isolated from internal tissues of cotton, 49 strains with known biological control activity against *Rhizoctonia solani* in cotton, and 25 strains known to induce systemic resistance to *Collectotrichum orbiculare* in cucumber, were screened for biological control potential against vascular wilt of cotton caused by *Fusarium oxysporum* f. sp. *vasinfectum*. The strains were introduced as endophytes in the cotton cultivar Rowden. Cotton seedlings were bacterized 7 days after planting by piercing stems with a fine needle that had been brushed across actively growing colonies of each isolate. Ten days later, the plants were inoculated by stem injection with microconidia of the pathogen. Twelve days after pathogen inoculation, symptom expression was evaluated by rating disease severity with a 0 to 4 rating. Six strains reduced disease severity in two separate experiments. These strains were INR-6, JM-1128, JM-1137, CC-186, 89B-61, and JM-869, which were identified as *Aureobacterium saperdae, Bacillus pumilus, Phyllobacterium rubiacearum, Pseudomonas putida, P. putida,* and *Burkholderia solanacearum,* respectively. Colonization studies revealed that bacteria survived in cotton stems for up to 28 days. Five strains were tested for ability to move within the stem and two demonstrated limited movement, not exceeding 5.0 cm, 14 days after bacterization. Populations of log 3 and log 6 CFU of strain CC-471, introduced into plant stems, increased after 3 days to log 6.25 and to 6.63 CFU/g stem tissue, respectively. These results indicate that endophytic bacteria should be evaluated further for efficacy as biological control agents of vascular pathogens and that some endophytes may survive, multiply, and exhibit limited movement following introduction into cotton.

KEY WORDS: endophytic bacteria; biological control; *Fusarium oxysporum* f. sp. *vasinfectum;* bacterial colonization; cotton.

INTRODUCTION

Several early reports demonstrated that bacteria naturally inhabit healthy plant tissues. Endophytic bacteria have been found in fruits (Samish *et al.,* 1963), vegetables (Hollis, 1951; Samish and Dimant, 1959), stems (Fry and Milholland, 1990), and roots (Philipson and Blair, 1957). Other researchers (Mundt and Hinkle, 1976) found endophytic bacteria within seed and ovules of 25 of 27 plant species sampled, establishing their presence before germination. Since these reports, many researchers have found endophytes present in disease-free plants. Light microscopy of tetrazolium-reducing bacteria in roots of maize and other grasses (Patriquin and Döbereiner, 1978) revealed bacteria in cells of the cortex, in intercellular spaces between the cortex and endodermis, in xylem cells, and in and between pith cells. Other researchers (Jacobs *et al.,* 1985) located endophytes in parenchyma cells and xylem elements of sugarbeet roots and in the xylem of healthy alfalfa plants (Gagné *et al.,* 1989). It is apparent that some endophytic bacteria reside specifically in tissues colonized by vascular pathogens, such as *Fusarium oxysporum* Schlect. f. sp. *vasinfectum* (Atk.) Snyd. & Hans.

In addition to the fact that endophytes colonize plant tissues without inciting apparent disease (Sharrock *et al.,* 1991; Misaghi and Donndelinger, 1990; Dowler and Weaver, 1974), there are several other factors which make them potential agents of biological control. Bacterial endophytes have a natural and intimate association with plants. The internal tissues of plants provide a relatively uniform and protected environment when compared with the rhizosphere and phylloplane. In the rhizosphere and phylloplane, introduced bacterial populations must compete for nutrients with other microbes and endure fluctuations of temperature and moisture, as well as exposure to ultraviolet radiation on aboveground surfaces. In spite of these advantages, the potential of bacterial endophytes for biological control of plant pests has only recently been explored. The company Crop Genetics International (Columbia, Maryland) is currently developing a strain of the endophyte

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Clavibacter xyli subsp. *cynodontis* which has been modified by recombinant DNA techniques to produce the delta-endotoxin proteins of *Bacillus thuringiensis* subsp. *kurstaki* for control of the European corn borer in corn (Dimock *et al.*, 1989). Other researchers have proposed the use of spiroplasmas to control insect pests of plants (Hackett *et al.*, 1989). Poon *et al.* (1977) reported symptom inhibition of bacterial blight of rice hy an endophytic bacterium isolated from rice.

The objectives of this study were to determine whether bacterial endophytes could reduce severity of a model vascular disease and whether representative endophytic strains survive, move, and multiply in the host. The model system included a collection of 244 bacterial strains, the root pathogen *F. oxysporum* f. sp. *vasinfectum*, and the host cotton.

MATERIALS AND METHODS

Microorganisms

Two hundred forty-four bacterial strains were used in this study. One hundred seventy of these were isolated in a separate study (McInroy and Kloepper, 1990) from healthy field-grown cotton (cv. DES 119); 25 rhizobacterial strains were previously shown to induce systemic resistance in cucumber against *Colletotrichum orbiculare* (Wei *et al.*, 1991); and 49 were rhizobacterial strains with biological control activity against *Rhizoctonia solani* on cotton (Press, 1992). All strains were identified by gas chromatograph–fatty acid methyl ester (GC–FAME) analysis in combination with the Microbial Identification System (Microbial ID Inc., Newark, DE). The total bacterial collection contained strains in 32 genera (Table 1). Bacteria were maintained for long-term storage at −80°C in tryptic soy broth amended with 25% glycerol.

Eight isolates of *F. oxysporum* f. sp. *vasinfectum*, isolated from cotton seedlings with wilt symptoms, were tested for pathogenicity. The most pathogenic isolate was chosen for use in this study (data not shown). A single spore from a colony grown on potato dextrose agar was placed in 100 ml of Czapek-Dex broth (Difco, Detroit, MI) and shaken (150 rpm) for 2 days at 28°C. The liquid culture was poured through a double layer of autoclaved Chem Wipes (Kimberly-Clark, Roswell, GA) and the concentration of microconidia was determined using a hemacytometer (Bright-Line, American Optical Co.). The liquid culture was adjusted to a concentration of 10^6 spores/ml. Ten-milliliter aliquots of this suspension were centrifuged at 5000 rpm for 5 min, and the broth was poured off. The centrifuge tubes were placed in an ultracold freezer (−80°C) until use.

Introduction of Endophytes and Pathogen Inoculation

The cotton cultivar "Rowden," which is highly susceptible to Fusarium wilt (Glass, 1992), was used in all experiments. Prior to use, the bacterial isolates were cultured on tryptic soy agar (TSA) at 28°C for 2 days. A size 10/13 beading needle was brushed across the growing colonies, and the stem of the cotton seedling was pierced, but not traversed, with the needle approximately 0.5 cm above the soil line. All plants treated with bacteria were bacterized in this manner.

To prepare for pathogen inoculation, 10 ml of sterile H_2O was added to the concentrated *F. oxysporum* microconidia, returning the concentration to 10^6 spores/ml. This spore suspension was drawn into a 1-ml Tuberculin syringe, and the seedling stem was injected approximately 2.0 cm above the soil line. The stem was pierced, but not traversed, and the needle withdrawn so that a small droplet was left clinging to the stem over the needle wound.

Preliminary Screen

The entire collection of 244 strains was tested. Sixteen experiments with completely randomized designs (CRD) were done, each with 11–21 treatments (bacterial strains) and 12 replications (single plants) per treatment. Each experiment contained a *F. oxysporum*-inoculated, nonbacterized, control and a nonbacterized and noninoculated (healthy) control. Two cotton seeds were planted in a plastic pot (10 cm in diameter) in a soilless mix (Promix, Riviére-du-loup, Québec, Canada). Seven days after planting, the weaker of the two seedlings was removed, and the other seedling was bacterized with one of the endophytic strains. Ten days after bacterization, the plants were inoculated with *F. oxysporum*.

Twelve days after inoculation with *F. oxysporum*, symptom expression was evaluated. The bacterized plants were visually compared with non bacterized control plants infected with *F. oxysporum*. Disease severity was rated using a 0 to 4 rating scale (0, no disease; 1, 1–25% leaves with symptoms; 2, 26–50% leaves with symptoms; 3, 51–75% leaves with symptoms; and 4 ⩾ 76% leaves with symptoms). To avoid selecting isolates with a detrimental effect on plant growth, plants were also evaluated for growth promotion. One month after inoculation, all replications of each treatment were derandomized and grouped together on a bench beside the replications of both controls. Each treatment was rated as −, 0, or +, where − is stunted growth, 0 is no growth difference, and + is growth enhancement, compared to the healthy control. Treatments which were scored + typically had both increased height and leaf-surface area. Strains were selected for inclusion in the secondary screen based on lowered disease severity and/or increased plant growth compared to the diseased control.

Secondary Screen

Strains selected in the preliminary screen were tested again in four experiments; one with 9 strains, two with

10 strains, and one with 12 strains. In each, treatments (bacteria and the two controls described above) were arranged in a CRD with 12 replications. Two seeds were planted in Promix as before. Plants were thinned and bacterized 7 days after planting, inoculated with *F. oxysporum* at 17 days, and evaluated for disease at 29 days. Data from all experiments were analyzed using an analysis of variance with the Statistical Analysis System (SAS Institute, 1990) and the least significant differences were determined. All treatments in which disease ratings were significantly ($P \leq 0.10$) lower than the diseased control were chosen for advanced screening.

Advanced Screen

Ten strains from the secondary screen were selected for additional testing. The treatments were arranged in a randomized complete block (RCB) design with 12 replications. As before, two seeds were planted in each pot, thinned, and bacterized. The seedlings were inoculated with *F. oxysporum* 10 days after bacterization and evaluated for disease development 13 days after inoculation. This experiment was repeated with the 10 strains and 5 additional strains from the secondary screen. Data from all experiments were analyzed using an analysis of variance with (SAS Institute, 1990) and the least significant differences were determined.

Colonization Studies

Rifampicin-resistant mutants (rif^+) were spontaneously generated on TSA amended with 100 μg/ml rifampicin (rif–TSA) for INR-6, JM-1137, JM-1128, JM-979, JM-956, and CC-471. All mutants were transferred three times to rif–TSA and selected for growth rates similar to the wild-type strains.

To assess survival of reintroduced endophytes in cotton stems, an experiment with a RCB design and five replications of eight treatments was done. The treatments included the six rif^+ endophytic strains, a water-treated control, and an *Escherichia coli* strain as a negative control. Cotton plants were bacterized 1 week after germination with a needle as described above. A 2-cm-long sample of the stem (1 cm above and 1 cm below the injection point) was cut from the plant, weighed, rinsed for 5 min under running tap water, then soaked in 1% sodium hypochlorite for 4 min, washed three times in sterile water, and triturated in 1 ml of 0.02 *M* phosphate buffer (PB). Serial dilutions of the triturate were plated onto rif–TSA for the rif^+ strains, on eosin methylene blue agar (EMB) for the *E. coli* strain, and on rif–TSA and EMB for the water treatment. To assure adequate surface disinfestation, 0.1 ml of the third wash was transferred to TSB. The colonies were enumerated after 48 h and CFU/g tissue were determined. This process was repeated at 7, 14, and 21 days after bacterization.

To assess the movement of bacteria from the point of bacterization, two experiments were done, each with five replications and one of two strains: rif^+ INR-6 and rif^+ JM-1137. Bacterized plants, arranged in a CRD, were sampled at 0, 7, 14, 21, and 28 days after bacterization. Stem sections were surface disinfested, triturated in sterile PB, plated on rif–TSA, and colonies were enumerated. A 2-cm stem section (1 cm above and 1 cm below the bacterization wound) was plated at each date. Each week an additional 2-cm section was also sampled so that the section 3–5 cm above the wound was added after 7 days, 7–9 cm after 14 days, 11–13 cm after 21 days, and 15–17 cm after 28 days for a total of five distances from the point of bacterization. The colonies on rif–TSA were enumerated and CFU/g tissue determined. A similar experiment was done with three replications for strains rif^+ 89B-61, rif^+ CC-186, and rif^+ JM-1128. The plants were sampled at 7, 14, and 21 days after bacterization and at distances of −1–1 cm, 3–5 cm, and 7–9 cm from the point of bacterization.

An experiment was designed to assess endophytic bacterial multiplication inside cotton stems. Three treatments (a nonbacterized control and two bacterial concentrations) were arranged in a RCB design with four replications. Two concentrations of rif^+ CC-471 were made by serial dilution and determined by dilution plating. Plants were inoculated by placing the eye of a sewing needle into serial dilutions and piercing the cotton stem with the eye of the needle. The needle eye held a volume of 1.5 μl; therefore, the approximate number of bacteria delivered to each wound could be calculated. The number of bacteria delivered to each plant was 0, 10^3, or 10^6 CFU. Plants were sampled at 0, 3, 6, 9, 12, and 28 days after bacterization. Two-centimeter samples of stem tissue were removed, triturated in sterile PB, plated onto rif–TSA, and the colonies were enumerated. This experiment was repeated.

Data from all colonization experiments were transformed with the $\log_{10}$ (CFU + 1) transformation and analyzed (SAS Institute, 1990). Throughout these studies, the minimum detection limit ranged from $\log_{10}$ 1.7 CFU/g tissue to 2.5 CFU/g tissue. Replications which contained no detectable bacteria were included in the transformed data as 0 CFU/g tissue.

RESULTS

Biological Control Screens

Forty-one of the 244 strains tested in preliminary trials (Table 1) were chosen for further study based on their performance (Table 2). The disease severity of plants bacterized with these strains ranged from 0.75 to 3.67, while disease severity of control plants ranged from 2.08 to 3.67. Several of these strains were chosen because of apparent growth promotion. Of the 41 strains, 27% belonged to the genus *Burkholderia*, 24% to the genus

TABLE 1

Genera of 244 Endophytic Bacterial Strains Screened for Ability to Control Fusarium Wilt Disease Caused by *F. oxysporum* f. sp. *vasinfectum* on the Cotton Cultivar Rowden

Genus	%[a]	Genus	%	Genus	%
Acinetobacter	0.8	*Citrobacter*	0.4	*Micrococcus*	1.2
Aerococcus	0.4	*Clavibacter*	1.6	*Morganella*	0.8
Agrobacterium	0.4	*Corynebacterium*	0.4	*Phyllobacterium*	4.5
Arthrobacter	1.6	*Curtobacterium*	0.4	*Pseudomonas*	16.8
Aureobacterium	1.2	*Enterobacter*	3.3	*Rhodococcus*	0.4
Bacillus	18.1	*Erwinia*	4.5	*Salmonella*	4.1
Brevibacterium	0.4	*Flaoimonas*	0.4	*Serratia*	2.5
Brochothrix	0.4	*Flavobacterium*	0.4	*Staphylococcus*	0.8
Burkholderia	26.2	*Hydrogenophaga*	0.4	*Xanthomonas*	2.5
Cedecea	0.8	*Methylobacterium*	1.2	*Yersinia*	1.6
Chryseomonas	0.4	*Microbacterium*	0.4		

[a] Percentage of the 244 strains tested.

Pseudomonas, 12% to the genus *Bacillus*, and the remainder to 11 other genera.

Fifteen of 41 tested strains were selected in the secondary screen: 4 strains from Trial 1, 5 from Trial 2, and 6 from Trial 3 (Table 3). All 15 strains significantly ($P \leq 0.10$ or 0.05) reduced symptom expression when compared to the diseased control. No strains were advanced from Trial 4.

TABLE 2

Results of the Preliminary Screening of Bacterial Endophytes for Ability to Reduce Severity of Fusarium Wilt Disease Caused by *F. oxysporum* f. sp. *vasinfectum* in the Cotton Cultivar Rowden

Trial	Mean disease severity of control[a]	Number of bacterial endophytes in trial	Number of strains chosen for additional testing[b] (range of disease severity)
1	2.58	10	3 (1.50–2.00)
2	2.92	10	2 (2.00–2.08)
3	2.58	10	4 (0.91–2.58)
4	3.41	10	0
5	3.58	10	1 (2.50)
6	2.92	10	4 (2.33–2.75)
7	3.42	10	1 (3.08)
8	3.67	22	4 (3.00–3.67)
9	3.00	22	5 (1.17–2.50)
10	3.00	22	0
11	2.83	22	5 (1.17–2.33)
12	2.67	22	2 (1.17–1.83)
13	2.92	22	3 (1.50–2.67)
14	3.50	22	3 (1.33–2.17)
15	2.25	10	2 (1.25–1.67)
16	2.08	9	2 (0.75–1.25)

[a] Disease severity was rated using a 0 (no disease) to 4 (>76% leaves show characteristic symptoms) rating scale.

[b] Selected on the basis of lowered disease severity or increased plant growth compared to the control.

Twelve of the 15 strains tested in the advanced screen (Table 4) significantly reduced symptom expression ($P \leq 0.05$). Six strains reduced symptom expression compared to the diseased control in both Trials 1 and 2. Two of these strains, INR-6 (*Aureobacterium saperdae*) and 89B-61 (*Pseudomonas putida*), are known to induce systemic resistance in cucumber. Three of the strains, JM-1128 (*Bacillus pumilus*), JM-1137 (*Phyllobacterium rubiacearum*), and JM-869 (*Burkholderia solanacearum*), were originally isolated from within healthy cotton. Strain CC-186 (*Ps. putida*) is a rhizobacterial strain with biological control activity against *R. solani* Kühn on cotton.

Colonization Studies

All six model strains tested for ability to survive in cotton plants maintained detectable populations throughout the 21-day experiment (Fig. 1.) After an initial decrease in numbers by Day 7, the populations of JM-956, JM-1137, INR-6, JM-1128, and CC-471 leveled off and changed little from 7 to 21 days. The population of JM-979 decreased from log 3.36 CFU/g stem tissue at Day 14 to log 2.15 CFU/g stem tissue at Day 21. In direct contrast, the *E. coli* strain included as a negative control was recovered from plants only on the day of bacterization (log 6.51 CFU/g stem tissue) and was not detected in plants at any other time. No rif^+ bacteria were isolated from plants that had not been bacterized with rif^+ mutants.

In the experiments to determine the extent of movement of rif^+ INR-6 and rif^+ JM-1137, after 7 days neither strain was detected at a distance of 3–5 cm from the point of bacterization (Tables 5 and 6). By 14 days, both strains were detected at 3–5 cm. After 21 days no additional movement was detected. The other strains tested (rif^+ 89B-61, rif^+ CC-186, and rif^+ JM-1128) were detected at the point of inoculation throughout the study

TABLE 3

Results of the Secondary Screening of Bacterial Endophytes for Ability to Reduce Severity of Fusarium Wilt Disease Caused by *F. oxysporum* f. sp. *vasinfectum* in the Cotton Cultivar Rowden

Bacterial strain and mean disease severity[a]							
Trial 1		Trial 2		Trial 3		Trial 4	
CC-116	3.08	INR-5	3.00	JM-338	2.58	JM-451	3.08
CC-579	2.58	JM-832	2.67	JM-1129	2.00	JM-455	2.42
CC-187	2.58	INR-1	2.58	JM-118	1.92	JM-539	2.26
91B-164	2.42	JM-267	2.25	JM-117	1.92	JM-849	2.08
CC-177	2.33	CC-120	2.08*	89B-61	1.75*	JM-930	2.00
89B-76	2.25	CC-186	2.00**	JM-1128	1.75*	JM-944	2.00
91B-171	2.25	JM-872	2.00**	JM-979	1.67*	JM-931	2.00
JM-836	2.25	JM-1137	2.00**	JM-1122	1.50**	JM-949	1.91
91B-169	1.92*[b]	JM-869	1.92**	JM-339	1.25**	JM-856	1.75
JM-956	1.83**	—[c]	—	JM-1138	1.00**	JM-961	1.67
CC-471	1.75**	—	—	—	—	—	—
INR-6	1.58**	—	—	—	—	—	—
Diseased control	2.67	Diseased control	2.92	Diseased control	2.50	Diseased control	2.17
Healthy control	0.00	Healthy control	0.00	Healthy control	0.00	Healthy control	0.00

[a] Disease severity was rated using a 0 (no disease) to 4 (>76% leaves show characteristic symptoms) rating scale.
[b] Significantly different from the diseased eontrol. *$P \leq 0.10$ and **$P \leq 0.05$. All these strains were chosen for advanced testing.
[c] —, Not tested.

but were not detected in other stem sections distant from the point of hacterization (data not shown).

The results of the multiplication experiment indicated that rif^r CC-471 multiplied within stem tissue. When plants were bacterized with 10^3 CFU/plant, populations of log 0.96 CFU/g stem tissue were detected immediately after bacterization (Fig. 2). After 3 days, log 6.25 CFU/g stem tissue were detected. A smaller increase, from log 5.89 to 6.63 CFU/g stem tissue, was also observed in plants inoculated with 10^6 CFU/plant. The analysis of variances indicated significant differences ($P \leq 0.01$) between the two bacterial populations within stem tissues

TABLE 4

Results of the Advanced Screening of Bacterial Endophytes for Ability to Reduce Severity of Fusarium Wilt Disease Caused by *F. oxysporum* f. sp. *vasinfectum* in the Cotton Cultivar Rowden

Strain	Mean disease severity[a]		Identification[b]
	Trial 1	Trial 2	
INR-6	2.42*[c]	0.83*	*Aureobacterium saperdae*
JM-1128	2.42*	1.08*	*Bacillus pumilus*
JM-1137	2.33*	1.08*	*Phyllobacterium rubiacearum*
JM-1122	2.75	1.17*	*Phyllobacterium rubiacearum*
CC-471	—	1.75	*Pseudomonas corrugata*
CC-186	2.33*	1.33*	*Pseudomonas putida*
JM-339	3.00	1.33*	*Pseudomonas chlororaphis*
89B-61	2.50*	1.33*	*Pseudomonas putida*
CC-120	—	1.58	*Burkholderia cepacia*
JM-979	—	1.42*	*Burkholderia pickettii*
JM-956	—	1.50*	*Brochothrix thermosphacta*
JM-872	2.75	1.25	*Burkholderia pickettii*
91B-169	3.00	1.42*	*Pseudomonas putida*
JM-869	2.42*	1.42*	*Burkholderia solanacearum*
JM-1138	—	1.83	*Pseudomonas vesicularis*
Diseased control	3.42	2.00	
Healthy control	0.00*	0.00*	

[a] Disease severity was rated using a 0 (no disease) to 4 (>76% leaves show characteristic symptoms) rating scale.
[b] Identified by fatty acid analysis. See text for details.
[c] Significantly different from the diseased control *$P \leq 0.05$.

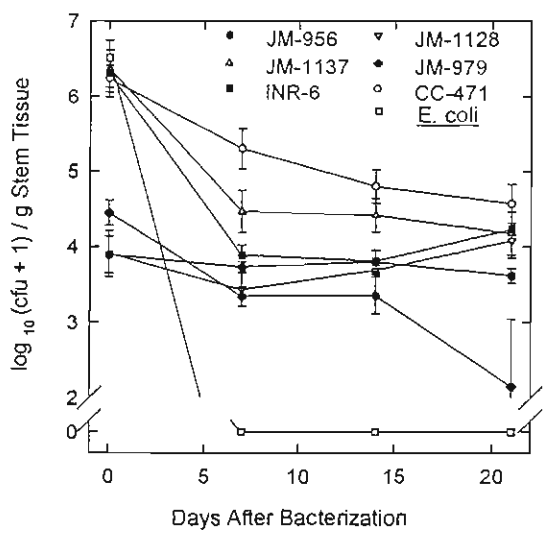

FIG. 1. Population dynamics of six rif^r endophytic strains and *E. coli* introduced into 7-day-old seedlings of cotton cultivar Rowden. Plants were bacterized by wounding stems with a needle brushed across colonies of actively growing bacteria.

TABLE 5

Spread of Strain rif^r INR-6 in the Stem of Rowden Cotton after Bacterization by Injection

Distance from bacterization point (cm)	Days after bacterization				
	0	7	14	21	28
−1–1	TNTC[a]	4.14 (±0.214)[b]	3.32 (±0.502)	2.56 (±1.471)	2.53 (±0.869)
3–5	NT[c]	0	1.74 (±1.012)	0.95 (±1.320)	0.30 (±0.670)
7–9	NT	NT	0	0	0
11–13	NT	NT	NT	0	0
15–17	NT	NT	NT	NT	0

[a] Too numerous to count.
[b] Mean log (1 + CFU/g stem tissue ± standard error).
[c] Not tested.

immediately after bacterization and between the two populations 3 days after bacterization ($P \leq 0.05$), but no statistically significant differences were detected after 3 days. Population densities remained relatively constant from 3 to 12 days. At 28 days population densities had dropped to log 2.62 and 3.05 CFU/g stem tissue for plants bacterized with 10^3 and 10^6 CFU/plant, respectively. Similar results were obtained when this experiment was repeated.

DISCUSSION

Kado (1992) defined bacterial endophytes as bacteria living in plant tissues without doing substantive harm or gaining benefit other than residency. As so defined, our strains are endophytic. The results of this study indicate that endophytic bacteria have biological control potential against Fusarium wilt of cotton. Six of the endophytic bacterial strains tested in this study significantly reduced symptom expression under greenhouse conditions. These strains included one endophyte and one rhizobacterium known to induce systemic resistance in cucumber (Wei *et al.*, 1991), three endophytic strains originally isolated from healthy field-grown cotton (McInroy and Kloepper, 1990), and one cotton rhizobacterium with biological control activity against *R. solani* (unpublished). In previously published studies, many microorganisms have been used to control Fusarium diseases, including bacteria (van Peer and Schippers, 1991; Scher *et al.*, 1984, Kempf and Wolf, 1989; Scher and Baker, 1980; Yuen *et al.*, 1985; Frommel *et al.*, 1991), fungi (Wymore and Baker, 1982; Schneider, 1984; Baker *et al.*, 1978), and combinations of bacteria and fungi (Park *et al.*, 1988; Lemanceau and Alabouvette, 1991). Generally, biological control agents of *Fusarium* spp. have been soil- rhizosphere- or root-colonizing microbes. This is the first report of the use of endophytic bacteria to control Fusarium wilt.

Results from the population studies of rif^r CC-471 suggest that introduced endophytic bacteria can reproduce in stem tissue and maintain at a certain carrying capacity. When introduced at a low population density (10^3), the population increased following introduction. For example, when populations were introduced at 10^3 CFU/plant, log 0.96 CFU/g stem tissue were detected

TABLE 6

Spread of Strain rif^r JM-1137 in the Stem of Rowden Cotton after Bacterization by Injection

Distance from bacterization point (cm)	Days after bacterization				
	0	7	14	21	28
−1–1	TNTC[a]	4.83 (±0.473)[b]	2.87 (±1.635)	2.99 (±0.993)	2.90 (±1.242)
3–5	NT[c]	0	2.38 (±1.443)	1.48 (±1.475)	1.28 (±0.808)
7–9	NT	NT	0	0	0
11–13	NT	NT	NT	0	0
15–17	NT	NT	NT	NT	0

[a] Too numerous to count.
[b] Mean log (1 + CFU/g stem tissue ± standard error).
[c] Not tested.

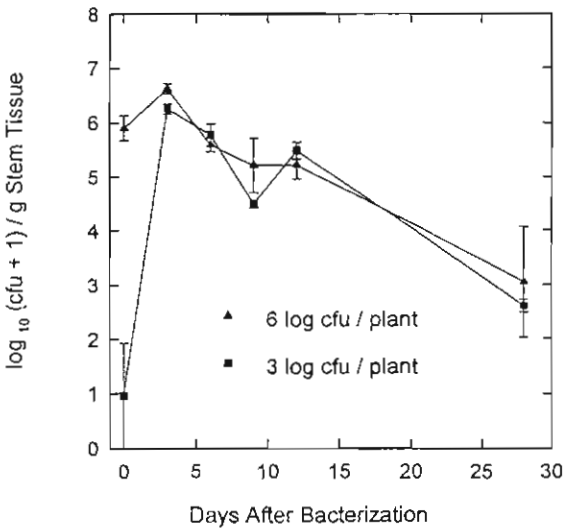

FIG. 2. Population dynamics of strain rif$^+$ CC-471 introduced into 7-day-old seedlings of cotton cultivar Rowden. Plants were bacterized by wounding stems with the eye of a sewing needle containing two different quantities of bacteria.

immediately after introduction (Fig. 2). Three days later, log 6.25 CFU/g stem tissue were detected. A smaller but similar increase was noted when populations were introduced at 10^6 CFU/plant.

Populations of introduced endophytes tended to decline slightly after 1 week and leveled off at log 3.5 to 5.5 CFU/g tissue (Fig. 1). A similar trend was reported by Gunson and Spencer-Phillips (1993). In their study, populations of rif$^+$ mutants, surviving endophytically in explants of arum lily, decreased after 5 days from the initial bacterization density of log 8–10 CFU to a stable population of log 5–7 CFU. Our figures are similar to those reported by Misaghi and Donndelinger (1990), who found that populations of endophytic *Erwinia* spp. introduced into cotton plants by seed treatment ranged from log 4.2 to 4.4 CFU/g fresh weight in flowers, bolls, and roots. These researchers did not attempt a quantitative assessment of bacterial populations in stems because trituration of woody stem tissues became increasingly difficult as plants matured and may result in the accumulation of phenolic compounds which prevent bacterial growth. McInroy and Kloepper (1990) also reported reductions of naturally occurring endophytic populations associated with increased age of tissue. They reported that total endophytic populations in cotton averaged log 3 CFU/g tissue at emergence, increased to log 5–6 throughout the growing season, and then decreased to log 3 at maturity. Further studies are required to determine if populations of endophytic bacteria actually decrease over time or if this apparent reduction is a result of extraction techniques.

Two strains, rif$^+$ INR-6 and rif$^+$ JM-1137, moved from the point of bacterization (Tables 5 and 6). Movement within stem tissue was slow, and very few (below detection limits) or no bacteria were established 7 cm from the point of bacterization. This is in agreement with some previous reports. Whitesides and Spotts (1991) also reported limited, but detectable, movement of up to 3 cm in stems of young pear trees. Gardner *et al.* (1982) noted limited movement of bacteria in citrus tree twigs. However, not all endophytic bacteria demonstrate limited movement. Dimock *et al.* (1989) reported that the endophyte, *C. xyli* subsp. *cynodontis*, colonized corn xylem systemically and could be detected throughout the plant, although population density was greatest at the basal stem.

The large standard errors are indicative of considerable variation among replications within some treatments of the colonization studies presented here (Tables 5 and 6, and Figs. 1 and 2). This is particularly true when populations were low because samples containing populations below the detection limit (approximately 2 CFU/g) were included in the data set as zero. Therefore, endophytic populations, particularly at lower levels, are probably underestimated.

There are several known mechanisms by which these endophytes could control Fusarium wilt. These mechanisms include production of antifungal compounds, siderophore production, nutrient competition, niche exclusion, and induction of systemic resistance (Cook and Baker, 1983). It is possible that several of these mechanisms play a role in the biological control demonstrated by these six endophytes. While the studies reported here cannot eliminate any of these mechanisms, our results suggest that induced systemic resistance may be an important factor in disease control. Although siderophore production is an important factor in some soils suppressive to Fusarium wilt (Kloepper *et al.*, 1980; Elad and Baker, 1985), it appears to affect chlamydospore germination and is unlikely to he an important factor in biological control with endophytes. The low dispersal of the bacterial strains tested also suggests that other mechanisms, such as production of antifungal compounds, nutrient competition, and niche exclusion, are less likely. It is possible that enhanced host defenses, via induced resistance, may be responsible for reduction of symptom severity. Cotton produces several phytoalexins which are important in resistance to disease and pests (Bell *et al.*, 1993), and van Peer and Schippers (1991) reported both induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by a rhizosphere colonizing *Pseudomonas* strain. One of the six strains which reduced wilt severity in both of the final screens, strain CC90-186, is a rhizosphere-colonizing *Pseudomonas* strain. In addition, two of the six stains, INR-6 and 89B-61, are known inducers of resistance to *Co. orbiculare* in cucumber (Wei *et al.*, 1991). Research is currently in progress to determine if systemic resistance is induced by the introduction of these endophytes.

Fusarium wilt disease of cotton is generally associated with the root-knot nematode *Meloidogyne incognita* (Kofoid & White) Chitwood (Jeffers and Roberts, 1993; Watkins, 1981). Current management strategies of Fusarium wilt disease have relied on control of *M. incognita* by use of genetic resistance and nematicides. However, resistant cultivars are not always available and the widespread use of nematicides has resulted in contamination of groundwater and deleterious effects on animal and human health (Heald, 1987; Johnson and Feldmesser, 1987). As a result of these problems, several of the most efficacious nematicides (aldicarb, ethylene dibromide, and dibromochloropropane) have been partially or totally removed from use by producers. The recent discovery that methyl bromide, a broad spectrum soil biocide, is destructive to the ozone layer of the earth (U.N. Environment Programme, 1992) underscores the need for alternative control methods. Endophytic bacterial biological control agents may provide an alternative control method for Fusarium wilt. Future research efforts will focus on a practical delivery system for beneficial endophytic bacteria.

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