Biocontrol Science and Technology (1995) 5, 407–416

Development of Delivery Systems for Introducing Endophytic Bacteria into Cotton

G. MUSSON, J. A. MCINROY AND J. W. KLOEPPER

Department of Plant Pathology, Alabama Agricultural Experiment Station, Biological Control Institute, Auburn University, Auburn, AL 36849–5409, USA

(*Received for publication 7 November 1994; revised manuscript accepted 20 April 1995*)

Experiments were designed to evaluate the effectiveness of several methods for delivering 15 endophytic bacteria into cotton stem and root tissues. The delivery methods included stab-inoculation of bacteria into stems, soaking seeds in bacterial suspensions, methyl cellulose seed coating, foliar spray, bacteria-impregnated granules applied in-furrow, vacuum infiltration and pruned-root dip. The success of delivery was gaged by recovery of the bacteria from internal plant tissues 2 weeks after the plants had been grown in a glasshouse potting mix. Following stab-inoculation into stems or radicles, 10 of the bacterial endophytes which previously exhibited biological control against fusarium wilt of cotton were successfully re-isolated from > 50% of the plants inoculated; however, this method was labor-intensive, involved wounding the plant and sometimes reduced plant growth. Four of the other methods established from six to eight of the 15 strains, and, with some strains, all methods effectively established endophytic bacteria, based on re-isolation of strains from internal tissues 2 weeks after inoculation. A method was developed which allowed more convenient isolation of endophytes from a large number of plants. The results suggest that introduction of beneficial endophytic strains into cotton plants could be accomplished by practical methods chosen specifically for each strain.

Keywords: *colonization, cotton, delivery system, endophytic bacteria*

INTRODUCTION

Of the habitats occupied by plant-associated bacteria, the zone of influence around roots, the rhizosphere, has been the primary source of potential biological disease control or plant growth-promoting strains. This is due to the high diversity and population densities of rhizosphere bacteria. However, other habitats of plant-associated bacteria, such as the phyllosphere, have also yielded potential biological control agents (Dimock *et al.*, 1989). Another habitat which is colonized by plant-associated microorganisms is the interior of root, stem and petiole tissues.

Endophytic bacteria have been defined as "bacteria that live within living plant tissues without doing substantive harm or gaining benefit other than securing residency" (Kado, 1992). Endophyte–plant relationships are diverse, with numerous bacterial species found within virtually every plant part in a multitude of plant species (Tervet & Hollis, 1948; Hollis, 1951; Philipson

Correspondence to J. W. Kloepper.

0958–3157/95/040407–10

& Blair, 1957; Pettit *et al.*, 1968; Cameron, 1970; Old & Nicolson, 1975; Mundt & Hinkle, 1976; McInroy, 1993). Because endophytic bacteria survive within cortical or vascular tissues (Patriquin & Döbereiner, 1978), their products, whether natural or formed as a result of genetic transformation, might move throughout the plant.

The first few weeks of cotton seedling development are a critical period in crop establishment. During this time, fungal pathogens such as *Rhizoctonia solani, Pythium ultimum, P. aphanidermatum, Fusarium oxysporum* f.sp. *vasinfectum* and *Thielaviopsis basicola* can cause damping-off and root rot diseases. Consequently, there is interest in finding bacterial strains with biological control or plant growth-promoting capabilities, such as *Bacillus subtilis* strain GB03, currently registered as a biological control agent for cotton (Backman *et al.*, 1994). *Clavibacter xyli* subsp. *cynodontis*, a xylem-colonizing endophyte of maize, was transformed with the genes encoding production of the insecticidal δ-endotoxin from *B. thuringiensis* subsp. *kurstaki* to act as a biological control agent against the European corn borer (Dimock *et al.*, 1989). If similar bacteria with biological control or plant growth-promoting activity can be found in internal plant tissues, as they can in the rhizosphere, these bacteria may have the unique capacity to elicit beneficial effects from within the plant. Also, as new beneficial bacterial strains are identified or genetically constructed, delivery of these strains at or near germination to specific plant tissues will be needed. Because some of these strains may function endophytically in nature, an understanding of methods specific for delivering endophytes will be necessary.

Numerous delivery systems have been reported for plant growth-promoting rhizobacteria (PGPR) (Kloepper & Schroth, 1981), including carboxymethyl cellulose (Kumar & Dube, 1992), alginate amended with skim milk and bentonite clay (van Elsa *et al.*, 1992) and in-furrow sprays (Zablotowicz *et al.*, 1992). However, little research has been devoted to delivery systems for bacterial endophytes. Gardner *et al.* (1985) inoculated rough lemon by applying bacterial inoculum as a soil drench around potted seedlings to study plant growth-promotion by rhizosphere bacteria. Antibiotic-resistant, xylem-resident bacteria were inoculated into alfalfa by several methods (Gagné *et al.*, 1987), including the addition of bacterial inoculum to the soil as a drench, wounding the roots with a knife then adding inoculum and inoculating stem stubble with a cotton swab. With cotton, Misaghi and Donndelinger (1990) recovered antibiotic-resistant strains of *Erwinia* spp. from stems, flowers, bolls and roots following vacuum infiltration of germinated cotton seeds. Seed treatment was used by van Peer *et al.* (1990) to inoculate tomato with rhizosphere and endophytic pseudomonads to determine their effects on plant growth. Although these methods introduced endophytes, they would be too labor-intensive for current commercial agricultural practices.

The objective of this study was to develop and compare systems for inoculation of rifampicin-resistant endophytic bacteria, originally isolated from cotton, back to cotton seeds or young plants. Additionally, a system for the surface-disinfestation of cotton tissues was developed to facilitate the study of endophytic bacteria.

MATERIALS AND METHODS

Bacterial Strains

Twenty-one strains of bacterial endophytes were used in this study (Table 1). Fifteen of these were previously found to reduce symptom expression in a fusarium wilt biological control assay on cotton (Chen *et al.*, 1995), and were originally isolated in 1990 and 1991 from within healthy cotton (*Gossypium hirsutum*) stems and roots (McInroy, 1993). The remaining seven endophytes were from the same original source, and were previously found by McInroy (1993) to colonize cotton stems or roots based on re-introduction of rifampicin-resistant mutants of each strain. The strains were identified by fatty acid analysis (Sasser, 1990). Mutants of the strains resistant to rifampicin (100 μg ml^{-1}) were selected from wild-type strains using the following procedure. Bacteria were grown for 24 h in tryptic soy broth (TSB) (Difco, Detroit, MI, USA) on a shaker at 25°C at approximately 200 rpm. Then, 0.1 ml was spread-plated on to tryptic soy agar (TSA),

TABLE 1. Bacterial strains used in attempts to establish endophytic populations in cotton seedlings

Strain	Strain identification[a]	Source[b]
89–B61	*Pseudomonas syringae*	Chen
JM–1128	*Bacillus pumilus*	Chen
JM–979	*Burkholderia solanacearum*	Chen
JM–1122	*Phyllobacterium rubiacearum*	Chen
JM–339	*P. chlororaphis*	Chen
JM–1138	*Brevundimonas vesicularis*	Chen
JM–1137	*P. rubiacearum*	Chen
CC90–120	*B. cepacia*	Chen
JM–872	*B. pickettii*	Chen
CC90–166	*P. chlororaphis*	Chen
JM–869	*B. pickettii*	Chen
91B–169	*P. chlororaphis*	Chen
JM–956	*Cellulomonas turbata*	Chen
CC90–471	*P. corrugata*	Chen
INR–6	*Aureobacterium saperdae*	Chen
JM–22	*Enterobacter asburiae*	McInroy
JM–93	*Clavibacter michiganensis* subsp. *insidiosus*	McInroy
JM–147R2	*Enterobacter cloacae*	McInroy
JM–147R3	*E. cloacae*	McInroy
JM–197	*E. asburiae*	McInroy
JM–198	*E. asburiae*	McInroy
JM–900	*E. asburiae*	McInroy

[a]Identified by fatty acid analysis (Sasser, 1990).
[b]Chen = endophytes previously found to reduce symptom development in a fusarium wilt bioassy on cotton (Chen *et al.*, 1995); McInroy = bacteria isolated from surface-disinfested, field-grown cotton and previously found to establish inside cotton following reintroduction (McInroy, 1993).

five plates/strain. After 8 h at 28°C, three drops of rifampicin (100 μg ml^{-1}) were placed on to the center of each plate. After further incubation for 3–5 days, resistant colonies were isolated from the zone of clearing and transferred to TSA supplemented with rifampicin at 100 μg ml^{-1} (TSArif100). Strain selection was based on colonies with similar morphology and growth to wild-type strains on TSA. Strains were subcultured on TSA to check for purity before storage at − 80°C in TSB with 20% (w/v) glycerol. Strains from − 80°C storage were cultured on TSA or TSArif100. For experiment 1, the strains were grown for 18 h in 10.0 ml TSBrif100 on a shaker at 22°C, centrifuged at 5000 × *g* for 10 min and resuspended in 50.0 ml of 0.02 M-phosphate buffer (PB), pH 7.0. For experiments 2 and 3, the strains were collected from the plates. The suspension concentrations were approximated either by dilution plating or by absorbance at 540 nm, compared with growth curves for *Bacillus* spp. or *Pseudomonas* spp. and adjusted to the required concentrations with 0.02 M-PB (experiment 2).

Cotton and Growing Conditions

Either non-acid-delinted, non-neutralized cotton seeds (experiment 1) or acid-delinted, neutralized seed (experiments 2 and 3) were used. Commercially acid-delinted cotton seeds of cultivars DP50, DES119 and Rowden were neutralized by placing 200 g of seed into a 600-ml beaker with 10 g of sodium bicarbonate; distilled water was added to bring the volume to 600 ml. The beakers were placed on a shaker for 7 min with occasional stirring. Seeds were air dried on paper towels and any damaged seeds were discarded. The seeds for all experiments were planted in the greenhouse in 10-cm diameter pots in Promix soil-less potting mix (Rivière-du-Loup, Québec, Canada).

Sampling and Surface-disinfestation of Plant Tissues

The sampling of plants in the first two experiments took place as follows; an alternative method (described later) was used in the third experiment. Approximately 2-week-old cotton plants from the greenhouse were rinsed under tap water to remove Promix prior to surface-disinfestation. Individual 2–3-cm stem and root segments were cut from the rest of the plant with a sterile scalpel. Additionally, 'whole plant samples' consisted of entire roots and stems with the leaves removed. The mean weight of three to five of the tissue pieces was recorded in each experiment. Individual tissue segments were placed into sterile test-tubes with 10 ml of aqueous H_2O_2 solution (20%, v/v) or $NaClO_3^-$ solution (1.05%, v/v) amended with 0.05% (v/v) Triton X-100 (Sigma, St Louis, MO, USA) (Misaghi & Donndelinger, 1990). Treatment for 4–5 min was sufficient to remove surface contamination from 90% of the tissue pieces (preliminary testing). Surface-disinfested pieces were aseptically transferred through three washes of 10 ml of sterile PB, for 1–3 min/wash.

To check for surface contamination, 0.1 ml of the third wash for each sample was transferred to 9.9 ml of TSB and incubated at room temperature on a shaker (approx. 200 rpm) or spread-plated on to TSA. After incubation at 28°C for 3 days, tubes and plates were examined, and samples with visible growth were not used in calculating the population densities of endophytes. This method was previously found to detect identical contamination percentages as those obtained by placing samples directly in TSB for a few minutes or printing directly on to TSA plates. Surface-disinfested pieces were triturated with 2–10 ml of PB, using autoclaved mortars and pestles. The resulting suspension was then spread-plated or spiral-plated (Spiral System, Inc., Cincinnati, OH, USA) on TSArif100 and TSA. TSA was used to check sterilant effects on indigenous populations, and to allow testing for possible rifampicin masking when suspected (McInroy *et al.*, 1992). Colonies were enumerated after 48 h at 28°C by hand-counting or by using a laser colony counter (Spiral System, Inc., Cincinnati, OH, USA).

Comparison of Delivery Methods

Experiment 1. An experiment was designed to compare seven delivery methods for inoculating seven bacterial strains. Rifampicin-resistant mutants of the seven strains listed last (source: McInroy) in Table 1 were used to inoculate cotton seeds and plants. Inoculation was performed as seeds or plants were transplanted from trays into individual 10-cm^2 pots 30 cm in depth. Six replicate seeds or plants were treated within each treatment. The controls consisted of sterile PB, as previously described, in place of bacterial inocula. All seeds were covered with 1 cm of Promix after treatment, and all pots were maintained in the greenhouse and watered twice daily. The treatments used were: seed treatment (seeds were submerged in the inoculum preparation for 0.5–1 h before planting); vacuum infiltration (seeds were submerged in the inoculum preparation and twice subjected to partial vacuum prior to planting); pruned-root dip (50% of the root mass was mechanically cut away, and the remaining root mass was submerged in the inoculum for 1 min prior to transplanting); stem injection (cotton was inoculated with 0.1 ml of the inoculum just above the root mass at the base of the stem, approx. 1 cm below the soil line); foliar spray (after transplanting, bacterial inoculum was sprayed on to the surface of all the leaves with a Sprā-tool misting apparatus) (Crown Industrial Products Inc., Hebron, IL, USA); soil drench (after the seeds were planted, the surrounding soil was drenched with approx. 20.0 ml of inoculum); and seed treatment and soil drench (a combination of seed treating and soil drenching, as previously described). For all inoculation methods, bacterial suspensions were adjusted to 10^7 colony-forming units (CFU) ml^{-1}. Sampling was carried out as described above, 14 days after planting.

A 7×8 factorial design with six replications arranged in a randomized, complete block design was used. Factors included the seven treatments and seven bacteria plus the control. The population data were analyzed for variance using the general linear models procedure of PC-SAS (Statistical Analysis System) software (SAS Institute Inc., Cary NC, USA, 1990). Populations below the minimum detectable limit, $\log_{10}$ 1.30 CFU g^{-1} of fresh weight, were treated as log 0 for calculating means.

Experiment 2. An experiment was designed to compare four delivery methods for introducing three strains of rifampicin-resistant endophytes: JM-1128, JM-339 and CC90–471. In the first method, 80–120 mg of a 48-h culture (TSArif100) of each strain were added to 1.0 ml of 2.0% (w/v) methyl cellulose (Sigma) in sterile Whirl Pac bags (Nasco, Twinsburg, OH, USA), with 15 cotton seeds/bag added. The bags were rolled vigorously on a counter, opened and poured into weighing boats to air dry under a laminar flow hood overnight. The second method was an in-furrow application of methyl cellulose/bacteria/vermiculite granules, made as described in the first method, except that in place of the 15 seeds, 3.5 g of sterile vermiculite were added to 10 ml of methyl cellulose. Bacteria-impregnated granules were crushed slightly after drying to separate aggregates. The third treatment was a 2-h seed soak (15 seeds each) in bacterial suspensions of approx. 10^6 CFU ml^{-1} of buffer containing 0.2% (v/v) Silwet (Union Carbide, Tarrytown, NY, USA). The fourth treatment was a foliar spray of approx. 10^5 CFU ml^{-1} (17.5 ml/plant) suspended in PB with 0.2% Silwet, 1 week after emergence. All plants in the experiment received no water on the day of the foliar treatment. The experimental design was a 4×4 factorial, where factors were the four delivery methods and three bacteria. Pots were arranged in a randomized, complete block design with 12 treatments (three strains and four methods) replicated four times. Each replicate consisted of two plants (subsamples).

Two weeks after planting, 'whole plant samples' were removed from the pots, surface-disinfested and triturated in 5 ml of buffer. Triturates were spiral-plated on TSA and TSArif100 with two plates/sample, incubated for 48 h at 28°C and the resulting colonies enumerated. Samples with no visible growth in the corresponding sterility checks were examined for growth, and the percentage of plants with colonization at the minimum detection limit was determined. The mean $\log_{10}$ CFU ml^{-1} was determined, and lack of detectable colonies (below detection limit) was treated as zero for calculating means.

The experiment was conducted once, and data were analyzed as a factorial using PC-SAS.

Experiment 3. An experiment was designed to compare five delivery methods for introducing rifampicin-resistant mutants of the 15 endophytic strains listed first (source: Chen) in Table 1. The first four of the five methods were identical to those used in the previous experiment, except that the foliar spray did not include Silwet. The fifth method was a stab of 7-day-old cotton stems with strains using a beading needle (size 10/13). The experiment was arranged into a completely randomized design with 75 treatments (five methods, 15 strains) with five seeds/pot planted.

After 2 weeks, 1.3-cm pieces were removed from the root just above the secondary root emergence zone and from the lower stem with a sterile scalpel. Twenty-four-well microtiter plates (Falcon, Lincoln Park, NJ, USA) were prepared by filling the six four-well columns as follows (Figure 1). Column 1 was filled with 2 ml of $NaClO_3^-$ solution amended with 0.05% Triton X-100. Columns 2 and 3 were filled with 2 ml of buffer with 0.05% Triton X-100. Columns 4 and 5 were filled with 2 ml and 1 ml of TSB respectively. Column 6 was filled with 1.5 ml of TSBrif100. Plant sections were placed into the first well (sterilant) in each row for 4.5 min. The tissues were then aseptically transferred through the buffer washes (wells 2 and 3) (1 min each) and placed into the surface-disinfestation check (well 4). The tissues were then transferred to well 5, and a flame-sterilized blunt metal tool was used to macerate the tissues within the well. Lastly, 0.5 ml of the triturate was transferred to well 6.

Three of the five plants in each treatment were sampled over a period of 4 days. In the first sampling, lower stem and upper root sections were assayed separately to determine the sites of colonization. In the other two samplings, both stem and root sections for single plants were processed together in one well. The plates were placed on a shaker for 3 days in the dark, and then examined for growth. Rows with wells which were positive for growth in the surface-disinfestation control well were not used in data analysis. Data were recorded as + or −, where + indicated that internal colonization was detected based on growth in well 6 (TSArif100). χ^2 analysis was performed on the data.

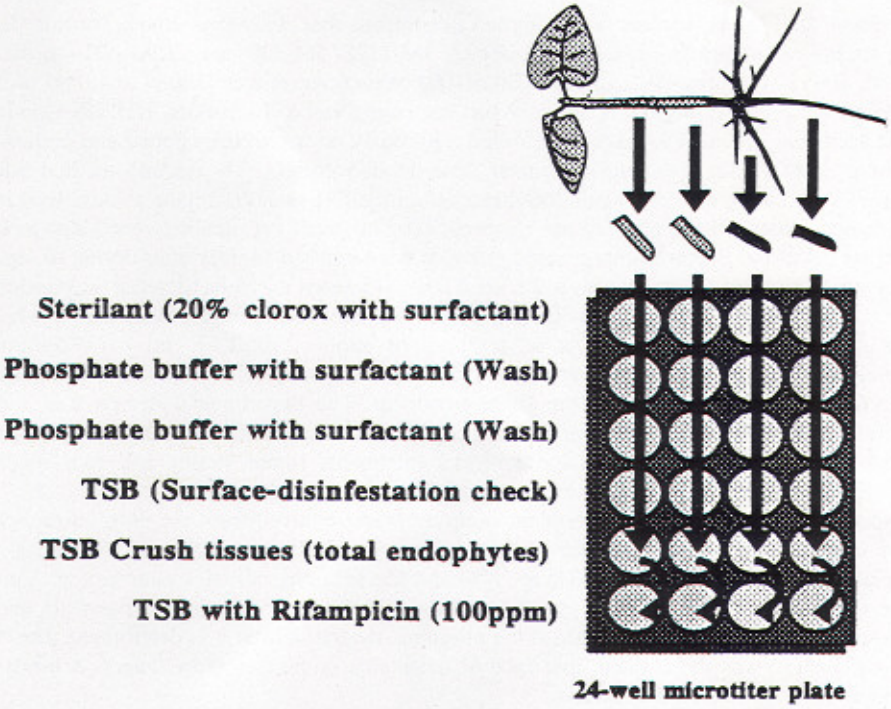

FIGURE 1. Method for sampling endophytic bacteria from cotton in experiment 3. All steps were conducted at room temperature. Tissue samples were placed in 20% clorox (household bleach) solution containing 0.05% Triton X-100 for 4.5 min, and were then rinsed twice in sterile buffer (1.0 min each time). Samples were then placed in TSB for 1 min, to check for external contamination, before being transferred to another TSB well. Samples were macerated in TSB before transferring 0.5 ml to the final well, which contained rifampicin- (100 μg ml^{-1}) amended TSB.

RESULTS

Comparison of Four Delivery Methods

Experiment 1. No single method led to establishment of all seven strains in cotton, based on re-isolation on TSArif100 (Table 2). The number of strains recovered from inoculated plants

TABLE 2. Comparison of methods for inoculating endophytic bacteria into cotton, experiment 1. Data are numbers of bacteria isolated from surface-disinfested stems ($\log_{10}$ CFU g^{-1} of fresh weight; means of six replicates)[a]

	Method[b]						
Strain	PD	SI	ST	VI	FS	SD	ST + SD
JM–22	ND	7.53	0.0	3.84	0.0	0.0	0.0
JM–93	0.0	0.0	0.0	0.0	0.0	0.0	0.0
JM–147R2	0.0	0.0	0.0	ND	5.34	0.0	0.0
JM–147R3	0.0	0.0	0.0	0.0	0.0	0.0	2.64
JM–197	6.74	2.99	0.0	0.0	0.0	0.0	0.0
JM–198	0.0	4.44	0.0	0.0	0.0	0.0	0.0
JM–900	2.03	3.59	0.0	0.0	0.0	0.0	0.0
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]0.0 = below detectable limit ($\log_{10}$ 1.30 CFU g^{-1}), ND = no data.
[b]PD = pruned root dip; SI = stem injection; ST = seed treatment; VI = vacuum infiltrated; FS = foliar spray; SD = soil drench.

TABLE 3. Internal colonization of cotton tissues by three rifampicin-resistant endophytic strains using four application methods, experiment 2

Bacterial strain	Recovery of introduced endophytes; mean $\log_{10}$ CFU g^{-1}[a] and (isolation frequency[b]) from different application methods[c]				
	Methyl cellulose seed treatment	Methyl cellulose/ vermiculite granules	Seed soak in Silwet	Foliar spray	Mean of all methods[d]
JM–1128	1.34 (5)	2.81 (8)	0.98 (4)	1.26 (3)	1.60a
JM–339	0% (0)	0.19 (1)	1.23 (3)	0.53 (2)	0.49b
CC90–471	2.34 (7)	1.87 (4)	2.43 (7)	3.68 (8)	2.58c
Mean[d]	1.23a	1.63a	1.55a	1.82a	

[a]Means of four replications, with two plants/replication. Values below the detection limit analyzed as zero.
[b]Number of plants from which the introduced endophyte was isolated, of eight plants sampled.
[c]Methyl cellulose seed treatment = seeds treated with bacteria suspended in 2% methyl cellulose; methyl cellulose/vermiculite granules = in-furrow application of granules prepared by suspending bacteria in methyl cellulose, which was then mixed with vermiculite; seed soak in Silwet = seeds were soaked for 2 h in a suspension of bacteria in 0.2% Silwet; foliar spray = 7 days after emergence, plants were sprayed to run-off with a suspension of bacteria in 0.2% Silwet.
[d]Means followed by different letters are significantly different at $P = 0.05$. $LSD_{0.05}$ for mean of bacterial strains = 0.78; $LSD_{0.05}$ for mean of methods = 0.91.

varied with the delivery system used, from no strains recovered with seed treatment or soil drench to four strains recovered from stem injection. Pruned-root dipping, however, was more effective than stem injection for strain JM-197.

Experiment 2. Each of the four methods used in experiment 2 led to establishment of endophytes in cotton plants (Table 3). Factorial analysis indicated that the methods of application for the whole experiment were not significantly different at $P = 0.05$. However, the differences in populations for each of the strains were significant at $P = 0.0001$, with the highest mean colonization by CC90–471. The method by strain interaction just failed to achieve significance at the 95% confidence interval ($P = 0.0557$). The highest mean populations ($\log_{10}$ 3.68 CFU g^{-1}) were recovered for strain CC90–471 following a foliar spray.

Experiment 3. Plants were colonized following delivery by each method (Table 4). Surface contamination of 9.7% of the plant samples was noted in the surface-disinfestation control wells (column 4) (Figure 1). Growth was observed in all fifth-column wells for the entire experiment (indigenous populations or rifampicin-resistant populations). Colonization, based on + or − growth, was observed in the ratio of 46% root, 18% stem and 36% both root and stem (data not shown). Of the 225 plants sampled (all three replications), 43% were colonized. Eight strains were recovered from at least 50% of replicate plants following introduction via methyl cellulose, seven from vermiculite, eight from seed soak, six from foliar spray and 10 from stab-inoculation. Two strains (CC90–166 and 91B-169) were generally recovered from plants following treatment with all five methods. χ^2 analysis indicated that the treatment methods were not significantly different, but that strains were significantly different in recovery across all methods ($P = 0.05$).

DISCUSSION

These experiments demonstrate that beneficial endophytes can be recovered from cotton plants, following introduction by numerous methods, without using labor-intensive inoculation treatments, such as stab-inoculation or vacuum infiltration. In the third experiment, each of the five

TABLE 4. Recovery of introduced endophytic bacteria from within 2-week-old cotton plants following introduction by five methods, experiment 3

Bacterial strain	No. of plants from which bacteria were recovered/ no. from which isolations were attempted[a] using different application methods[b]				
	Methyl cellulose seed treatment	Methyl cellulose/ vermiculite granules	Seed soak in Silwet	Foliar spray	Stab-inoculation
89B–61	2/3	2/3	3/3	C	2/3
JM–1128	3/3	C	2/3	3/3	3/3
JM–979	0/3	3/3	1/3	C	0/3
JM–1122	0/3	0/3	1/3	3/3	0/3
JM–339	2/3	2/3	3/3	1/2	3/3
JM–1138	C	0/3	0/3	0/3	0/3
JM–1137	1/2	0/3	0/3	0/3	0/3
CC90–120	1/2	2/3	1/3	1/3	3/3
JM–872	0/3	1/3	0/3	C	2/3
CC90–166	3/3	3/3	3/3	3/3	3/3
JM–869	0/3	1/3	2/3	3/3	3/3
91B–169	3/3	3/3	3/3	3/3	3/3
JM–956	0/3	1/3	1/3	C	0/3
CC90–471	1/2	1/3	3/3	C	3/3
INR–6	0/3	1/2	2/3	0/3	2/3
No. of strains detected at ≥ 50%	8	7	8	6	10

[a]Recovery on rifampicin-amended TSB from surface-disinfested stem samples which were not surface contaminated. C = two of the three samples were contaminated.

[b]See Table 3.

methods used for introduction resulted in six to eight of the 15 strains being recovered in at least 50% of the treated plants; stab-inoculation introduced 10 of the 15 strains. Each of the five methods tested in experiment 3 introduced some of the strains in all three samples. Results from experiments 1 and 2, which had higher minimum detection limits, were similar. In the second experiment, it was determined that the four methods of application tested were not significantly different from each other over the whole experiment. Hence, each method was equally effective for introducing all three strains.

The results from the three experiments also suggest that any one method will not be similarly effective for all strains, but that a method will have to be chosen specifically for the strain to be delivered. One strain, JM-339, attained the highest populations following a 2-h seed soak (experiment 2), while strain CC90–471 had maximum populations with the foliar spray (Table 3). Therefore, practical delivery of different strains to plants in a field may require different methods.

The specific bacterial traits that confer ability to colonize plants internally remain to be elucidated. It is interesting to note that strain CC90–147, which colonized the lower stem and upper root tissues in this study, was identified as *P. corrugata*, a taxon which is a vascular pathogen of tomato. This strain, selected in advanced screening for symptom reduction in a fusarium wilt assay (Chen *et al.*, 1995), was pathogenic to potato tissue culture plantlets tested in a gnotobiotic growth promotion assay (Musson, 1994). Another strain of *P. corrugata*, 2140, was reported to control take-all disease of wheat (Ryder & Rovira, 1993), and another was found to reduce post-harvest losses from silver scurf of potato caused by *Helminthosporium solani* (Chun & Shetty, 1994). The relationship of the biological control properties to the ability of the strain to colonize plant tissues has yet to be examined.

The mean populations of the three strains in experiment 2 were significantly different ($P = 0.0001$), indicating that some strains are better colonizers than others. Thus, strain-specific attributes appear to be important in determining whether a strain will colonize plants internally. For example, the mean colonization for CC90–471 was $\log_{10}$ 2.58 CFU g^{-1}, but that of strain JM-339 was only $\log_{10}$ 0.49 CFU g^{-1}. In experiment 3, strains 91B-169 and CC90–166 were found in all plants tested, following introduction by each of the five methods. These strains were easy to introduce, colonized lower stem and upper root tissues and were previously found to reduce symptom expression in a fusarium wilt biological control screen (Chen *et al.*, 1995). Hence, these strains would be good candidates for transformation with beneficial genes to target cotton diseases of the root and lower stem caused by *R. solani* or *Pythium* spp.

Several problems related to methods must be taken into account in studies on endophytes. Problems with minimum detection limits occurred due to the dilution of bacteria when the triturated plant tissue was suspended in PB for plating. Also, with young plants, stem tissue samples usually weighed less than 0.5 g, and older stem pieces could not be triturated because of the physical toughness of the tissue and the released phenolic compounds that could affect populations (Misaghi & Donndelinger, 1990). Therefore, in some experiments, populations of individual plants may have been below the detection limit. Another potential problem was that surface-disinfestation testing might not be sufficient to detect low populations that adhere to the external surfaces of the plant. Therefore, there is a need to examine further, and possibly improve, sterility-testing methods. Lastly, because sampling internal populations is more labor-intensive than sampling external populations, fewer replications can be processed in a single day, which may limit the accuracy of estimating mean internal populations. The microtiter plate method, utilized in experiment 3 and shown in Figure 1, resulted in a lowered detection limit and improved surface-disinfestation testing. Additionally, the test was rapid enough to allow sampling of 150 tissue pieces/day compared with 30–45 pieces/day with the methods used in experiments 1 and 2. Although the microtiter plate system minimized these three problems, it has not been used to quantify populations, but could be further adapted to allow quantification of bacteria through the most probable number technique.

ACKNOWLEDGEMENTS

The authors would like to thank C. Chen for assistance and advice with the experiments and Walter Mahaffee for conducting the χ^2 analysis. This research was supported by grant no. US-2026–92R from BARD, the United States–Israel Binational Agricultural Research and Development Fund.

REFERENCES

Backman, P.A., Brannen, P.M. & Mahaffee, W.F. (1994) Plant response and disease control following seed inoculation with *Bacillus subtilis*, in *Improving Plant Productivity with Rhizosphere Bacteria* (Ryder, M.H., Stephens, P.M. & Bowen, G.D., Eds) CSIRO, Australia, pp. 3–8.

Cameron, H.R. (1970) *Pseudomonas* content of cherry trees. *Phytopathology* **60**, 1343–1346.

Chen, C.Q., Bauske, E.M., Musson, G. & Kloepper, J.W. (1995) Biological control potential and population dynamics of endophytic bacteria in a cotton/fusarium wilt system. *Biological Control*, **5**, 83–91.

Chun, W.W.C. & Shetty, K.K. (1994) Control of silver scurf disease of potatoes caused by *Helminthosporium solani* Dur. & Mont. with *Pseudomonas corrugata*. *Phytopathology* **84**, 1090.

Dimock, M.B., Beach, R.M. & Carlson, P.S. (1989) Endophytic bacteria for the delivery of crop protection agents, in *Proceedings of a Conference on Biotechnology, Biological Pesticides and Novel Plant-pest Resistance for Insect Pest Management* (Roberts, D.W. & Granados, R.R., Eds) Boyce Thompson Institute for Plant Research, Ithica, NY, USA, pp. 88–92.

Gagné, S., Richard, H. Rousseau, H. & Antoun, H. (1987) Xylem-residing bacteria in alfalfa roots. *Canadian Journal of Microbiology* **33**, 996–1000.

Gardner, J.M., Chandler, J.L. & Feldman, A.W. (1985) Growth responses and vascular plugging of citrus inoculated with rhizobacteria and xylem-resident bacteria. *Plant and Soil* **86**, 333–345.

Hollis, J.P. (1951) Bacteria in healthy potato tissue. *Phytopathology* **41**, 350–366.

KADO, C.I. (1992) Plant pathogenic bacteria, in *The Prokaryotes* (BALOWS, A., TRÜPER, H.G., DWORKIN, M., HARDER, W. & SCHLEIFER, K.-H., Eds) Springer-Verlag, New York, NY, USA, pp. 660–662.

KLOEPPER, J.W. & SCHROTH, M.N. (1981) Development of a powder formulation of rhizobacteria for inoculation of potato seed pieces. *Phytopathology* **71**, 590–592.

KUMAR, B.S.D. & DUBE, H.C. (1992) Seed bacterization with a fluorescent *Pseudomonas* for enhanced plant growth and yield and disease control. *Soil Biology and Biochemistry* **26**, 539–542.

MCINROY, J.A. (1993) Endophytic bacteria of sweet corn and cotton. Masters thesis, Auburn University, Auburn, AL, USA.

MCINROY, J.A., WEI, G., MUSSON, G. & KLOEPPER, J.W. (1992) Evidence for possible rifampicin-resistance phenotype of marked bacteria *in planta*. *Phytopathology* **82**, 1177.

MISAGHI, I.J. & DONNDELINGER, C.R. (1990) Endophytic bacteria in symptom-free cotton plants. *Phytopathology* **80**, 808–811.

MUNDT, J.O. & HINKLE, N.F. (1976) Bacteria within ovules and seeds. *Applied and Environmental Microbiology* **32**, 694–698.

MUSSON, G. (1994) Ecology and effects of endophytic bacteria in plants. Masters thesis, Auburn University, Auburn, AL, USA.

OLD, K.M. & NICOLSON, T.H. (1975) Electron microscopical studies of the microflora of roots of sand dune grasses. *New Phytologist* **74**, 51–58.

PATRIQUIN, D.G. & DÖBEREINER, J. (1978) Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Canadian Journal of Microbiology* **24**, 734–742.

PETTIT, R.E., TABER, R.A. & FOSTER, B.G. (1968) Occurrence of *Bacillus subtilis* in peanut kernels. *Phytopathology* **58**, 254–255.

PHILIPSON, M.N. & BLAIR, I.D. (1957) Bacteria in clover root tissue. *Canadian Journal of Microbiology* **3**, 125–129.

RYDER, M.H. & ROVIRA, A.D. (1993) Biological control of take-all of glasshouse-grown wheat using strains of *Pseudomonas corrugata* isolated from wheat field soil. *Soil Biology and Biochemistry* **25**, 311–320.

SASSER, M. (1990) Identification of bacteria through fatty acid analysis, in *Methods in Phytobacteriology* (KLEMENT, Z., RUDOLPH, K. & SANDS, D., Eds) Akademiai Kiado, Budapest, pp. 199–204.

TERVET, I.W. & HOLLIS, J.P. (1948) Bacteria in the storage organs of healthy plants. *Phytopathology* **38**, 960–967.

VAN ELSA, J.D., TREVORS, J.T., JAIN, D., WOLTERS, A.C., HEIJNEN, L.S. & VAN OVERBEEK, L.S. (1992) Survival of, and root colonization by, alginate-encapsulated *Pseudomonas fluorescens* cells following introduction into soil. *Biology and Fertility of Soils* **14**, 14–22.

VAN PEER, R., PUNTE, H.L.M., DE WEGER, L.A. & SCHIPPERS, B. (1990) Characterization of root surface and endorhizosphere pseudomonads in relation to their colonization of roots. *Applied and Environmental Microbiology* **56**, 2462–2470.

ZABLOTOWICZ, R.M., PRESS, C.M., LYNG, N., BROWN, G.L. & KLOEPPER, J.W. (1992) Compatibility of plant growth promoting rhizobacterial strains with agrichemicals applied to seed. *Canadian Journal of Microbiology* **38**, 45–50.