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Control of *Rhizoctonia solani* and *Sclerotium rolfsii* in the greenhouse using endophytic *Bacillus* spp.

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Abstract

Isolates of different endophytic bacteria were recovered from surface-disinfected seeds obtained from commercial companies, plants in the field and tissue culture. The bacteria were isolated from seeds after stringent surface-disinfection. *Pseudomonas fluorescens* (isolate no. 14) from bean inhibited growth of all fungi tested and was fluorescent on King B medium. *Bacillus cereus* from *Sinapis* (isolate no. 65) inhibited growth of *Rhizoctonia solani, Pythium ultimum* and *Sclerotium rolfsii* and also exhibited chitinase activity. *Bacillus subtilis* from onion tissue culture (isolate no. 72) inhibited *R. solani* and *P. ultimum* growth. *B. cereus* from cauliflower (isolate no. 78) inhibited growth of *R. solani*. *B. pumilus* from sunflower (isolate no. 85) inhibited growth of *R. solani* and *S. rolfsii*. *B. cereus* (isolate no. 65) was introduced into cotton, and by using radioactive labelling we found that it was present for 16 days in the root-stem junction. It is most likely that these bacteria were still found 72 days after their introduction in the root and stem, at levels of $2.8 \cdot 10^5$ and $5 \cdot 10^4$ cfu g^{-1} fresh weight, respectively, when selective medium was used. There was no difference between control and treated plants in their height or in the fresh weight of roots, stems and leaves.

When cotton seedlings were inoculated with *B. cereus* (isolate no. 65), *B. subtilis* (isolate no. 72) or *B. pumilus* (isolate no. 85), disease incidence caused by *Rhizoctonia solani* was reduced in the greenhouse by 51%, 46% and 56%, respectively. In bean seedlings inoculated with *B. subtilis* (isolate no. 72), *B. cereus* (isolate no. 78) or *B. pumilus* (isolate no. 65), disease incidence caused by *Sclerotium rolfsii* was reduced by 72%, 79% and 26%, respectively, as compared to control. In both cotton and bean seedlings, these endophytes reduced the disease index more than 50%. These results indicate that endophytic bacteria can survive inside cotton plants and are efficient agents for biological control against plant pathogens under greenhouse conditions.

Introduction

A variety of endophytic bacteria have been isolated from interior parts of plant [Gagné *et al.*, 1989; Hollis, 1951; Misaghi and Donndelinger, 1990; Mundt and Hinkle, 1976; Philipson and Blair, 1957].

Seven different species of bacteria have been isolated from surface disinfected tubers [Hollis, 1951], roots [Philipson and Blair, 1957; Gardner *et al.*, 1982], seeds, ovules [Mundt and Hinkle, 1976] and stems [Whitesides and Spotts, 1991]. In alfalfa plants, *Pseudomonas* spp. and *Erwinia*-like bacteria have been shown to be normal residents of the xylem in numbers which are independent of plant age [Gagné *et al.*, 1987]. *Erwinia* spp. and *Bacillus* spp. have consistently been isolated from roots, young and old stems, and bolls and flowers of healthy cotton plants [Misaghi and Donndelinger, 1990]. In some citrus trees, species of *Pseudomonas* and *Enterobacter* have been found in the xylem sap [Gardner *et al.*, 1982].

Contamination with endophytic bacteria is a major problem in tissue culture [Cassells, 1991]. Endogenous bacteria have been found in many different plant tissues [Bastiaens, 1983]. An isolate of *Curtobacterium* spp. was found in aseptic *Dioscorea* shoot culture. These endophytic bacteria expressed β-glucuronidase (GUS)

and gave false positive results in a histochemical analysis generally used in transformation experiments with *Dioscorea* [Tör *et al.*, 1992]. Seedlings of silver and sugar maples which have been inoculated with a rifampicin-resistant strain of *Bacillus subtilis* were found to contain the bacteria 24 months later [Hall and Davis, 1990]. One mechanism by which endophytic bacteria are thought to penetrate the plants is through wounds on roots or stems [Hallaksela *et al.*, 1991; Whitesides and Spotts, 1991]. The precise role of endophytes in plants is not yet known. The presence of endophytic bacteria in tissue for extended periods of time suggests a possible symbiotic-like existence [Misaghi and Donndelinger, 1990; Whitesides and Spotts, 1991]. There are several examples of plant pathogens surviving on or in their host plants without producing symptoms [Leben, 1965]. Their ability to thrive within plant tissues provides them with numerous advantages, e.g. an environment with little competition, protection from environmental stresses, and a reliable food source.

The potential use of endophytes as biocontrol agents has been recently studied [Misaghi and Donndelinger, 1990]. Because of their systemic distribution throughout the plant via metabolic translocation it has been postulated that plants can be defended from pathogens by the manipulation of these naturally occurring microorganisms [Misaghi and Donndelinger, 1990]. Recently, Chen and co-workers [1995] have shown that after injecting cotton seedlings with endophytic bacteria, the severity of disease caused by Fusarium wilt is reduced. They also demonstrated that some endophytes can survive and multiply inside cotton plants. The present paper describes the isolation of several endophytic bacteria, their ability to inhibit the growth of fungi and their capacity to survive inside the plant over time.

Materials and methods

Isolation of endophytes

Endophytic bacteria were isolated from tissue culture of male sterile onion flowers (*Allium cepa* cv. 'Ben Shemen' kindly provided by Dr. H. D. Rabinowitch) and *Populus* cv. 'Heimburger', both of which developed contamination after several subcultures. When contamination appeared to originate from within the tissue, efforts were made to isolate the bacteria. The parameters for determining whether bacteria contamination was potentially endophytic were: contamination appearing only after a number of apparently clean transfers; contamination appearing initially form the exposed cut end of the tissue; and surface disinfection failing to produce aseptic cultures.

In addition, isolates were taken from seeds of *Raphanus raphanistrum*, cauliflower (*Brassica oleacea* L. cv. '202/A'), sunflower (*Helanthus annuas* L. cv. 'D-3'), cotton (*Gossypium herbaceum* L. cv. 'Akala'), bean (*Phaseolus vulgaris* L. cv. 'Cotender'), cucumber (*Cucumis sativus* L. cv. 'Delilah'), squash (*Cucurbita sativus* L. cv. 'sld'), muskmelon (cvs. 'E.D.' and 'Melia'), *Sinapis arvensis*, and Mimosa pudica. The seeds were obtained from commercial companies or collected from symptom-free plants. They were washed in soap and water, surface-disinfected with 20% (v/v) H_2O_2 for 3 min, transferred to 70% (v/v) ethanol for 90 s, soaked for 3 min in a solution of 10% (w/w) $NaOCl_2$ in 0.01% (v/v) Tween 20, and then transferred to 50% ethanol with 0.2% (w/v) $HgCl_2$ for 60 s. This treatment was followed by four thorough rinses with sterile distilled water. All chemicals were obtained from Sigma Chemical Co., St. Louis, USA.

To determine the effectiveness of surface-disinfection and to exclude the possibility of spore contamination and further germination in spite of the disinfection, seeds were incubated for 96 h at 28 °C on nutrient agar or trypic soy agar (NA and TSA, respectively) and plates were then incubated for an additional week. Only seeds not showing any bacterial growth were considered clean and were used in the experiments. Possible bacterial contamination was examined by incubation for additional week after seed removal. Seeds which showed no bacterial contamination were screened for endophytes. Seeds coats were removed aseptically. Embryos and cotyledons were cut into segments and incubated on TSA and NA for 48 h. Bacteria which appeared on the plates at this stage were considered to be endophytic.

Isolated endophytes were purified by subculturing individual colonies three times, and then stored in a glycerol solution at −70 °C [Whitesides and Spotts, 1991].

Bacterial characterization and identification

Isolation was carried out on either NA, TSA or synthetic medium [Monreal and Reese, 1969] containing colloidal chitin as sole carbon source [Rodriguez-Kabana *et al.*, 1983], to determine the presence

of chitinase activity. Some of the bacterial strains were identified by GC-FAME [Sasser, 1990] (analysis kindly performed by Dr. J. Kloepper at Auburn University, Auburn, Alabama, USA). The ability of the isolates to inhibit growth of fungal plant pathogens (*Rhizoctonia solani, Pythium ultimum, Sclerotium rolfsii* and *Fusarium oxysporum*) was determined on dual culture plates of PDA. The ability of bacteria to produce chitinase was determined on synthetic medium [Monreal and Reese, 1969] containing colloidal chitin as sole carbon source [Rodriguez-Kabana *et al.*, 1983].

The following five isolates were tentatively identified by GC-FAME and selected for further studies. *Pseudomonas fluorescens* (isolate no. 14) from bean, *Bacillus cereus* (isolate no. 65) from Sinapis, *Bacillus subtilis* (isolate no. 72) from onion tissue culture, *Bacillus cereus* (isolate no. 78) from cauliflower and *Bacillus pumilus* (isolate no. 85) from sunflower.

Inoculation of seedlings with endophytes

Cotton seeds were germinated for 3 days in vermiculite. The root tips were removed, and the seedlings placed in a Petri dish for 24 h, So that only the roots were in contact with an aqueous bacterial suspension containing $5 \cdot 10^8$ cfu mL^{-1} of the specified endophyte in water. Control treatments contained tap water only.

Detection of the endophyte Bacillus cereus

^{14}C-labelling of B. cereus. An overnight liquid NB culture of *B. cereus* (50 mL) was centrifuged (2500 *g*) for 10 min. The bacteria were resuspended in 20 mL of NB containing 50 μL of 0.2 mCi mL^{-1} ^{14}C-(U)-glucose and incubated for 1.5 h on a shaker at 200 rpm at 30 °C. After incubation, the cells were harvested by centrifugation, washed several times in sterile and finally washed in distilled water until no radioactivity could be detected in the supernatant [Bignell, 1989]. Seedlings were incubated with ^{14}C-labelled bacteria as described above. After 1, 12, 18 and 28 days, each plant with labelled bacteria was separated into leaves, stems and roots, and placed in scintillation vials. The tissues were frozen in liquid nitrogen and crumbled with a glass rod. Then 1 mL of 90% (v/v) methanol was added. The vials were placed in full sunlight for 8 h, then refrigerated for 48 h. The solution was counted in 20 mL of Insta-gel. (Packard Instruments, Downers Grove, IL).

Autoradiograms were made of intact plants containing ^{14}C-labelled bacteria. Each plant was placed on paper and covered with nylon, then laid on Kodak film paper in a light-tight metal exposure box. Seedlings autoradiographed 24 h after loading were exposed to the film for 3 days. Those autoradiographed 6 days of more after loading were exposed for one week.

Quantification of endophytic population. Cotton seeds cv. 'Akala' were treated with *B. cereus* as described above. Treated and control seedlings were planted in the greenhouse in polypropylene boxes (six seedlings per box) containing vermiculite no. 3. After 1, 8, 16, 32, 44 and 72 days, five plants were collected from treatment and control groups. The plants were surface-disinfected by washing them in soap and water, submerging in a 3% H_2O_2 solution containing 0.05% (w/v) $CuSO_4$ [Bloomfield, 1992] for 2 min, soaking for 90 s in 70% (v/v) ethanol, and then transferring to 2% (w/w) sodium hypochloride with 0.01% Tween 20 for 3 min. This treatment was followed by four thorough rinses with sterile distilled water.

Stem and root sections were separated for all except the 24-h samples. Plant sections were homogenized in 2 ml saline with a sterilized mortar and pestle. Aliquots (100 μL) of the various dilutions of the extracts were spread on a minimal medium containing colloidal chitin as a sole carbon source [Rodriguez-Kabana *et al.*, 1983], and colonies were counted. To confirm the identity of the bacteria, spore formation was examined by light microscopy and Gram-positive staining.

Greenhouse experiments

Incubated cotton or bean seedlings were planted in the greenhouse in polypropylene boxes (7×19×14 cm; eight seedlings per box) containing raw sandy loam soil which had been artificially infested with the fungal pathogens *R. solani* [Inbar and Chet, 1991; Sneh *et al.*, 1966] or *S. rolfsii* by mixing 50 mg of sclerotia with 1 kg of soil [Henis, 1984]. Control plants were grown in untreated soil. After 14 days of incubation, the percentage of infected seedlings with symptoms of damping-off disease was assessed. A visual index was used to describe the surviving plants, from 0 (no disease) to 5 (complete necrosis of the root and shoot junctions) [Sneh *et al.*, 1966].

All experiments were carried out under standard greenhouse conditions at 28–32 °C.

Table 1. Fresh weight (gram) of cotton plants loaded with *B. cereus* and control plants. The measurement at time zero was made on seedlings after 3 days in vermiculite. At times 0 and 1 day stem and root weights were measured together

	B. cereus			Control		
Time (days)	Leaf	Stem	Root	Leaf	Stem	Root
0	0.164 ± 0.133	0.045 ± 0.005		0.164 ± 0.133	0.045 ± 0.005	
1	0.17 ± 0.021	0.049 ± 0.006		0.15 ± 0.005	0.044 ± 0.007	
8	0.346 ± 0.023	0.097 ± 0.006	0.114 ± 0.010	0.291 ± 0.016	0.097 ± 0.005	0.092 ± 0.165
17	0.528 ± 0.071	0.188 ± 0.02	0.138 ± 0.027	0.59 ± 0.0743	0.192 ± 0.030	0.132 ± 0.011
24	0.785 ± 0.02	0.206 ± 0.045	0.138 ± 0.026	0.974 ± 0.071	0.295 ± 0.039	0.16 ± 0.012
33	1.148 ± 0.21	0.479 ± 0.061	0.258 ± 0.064	1.45 ± 0.163	0.520 ± 0.046	0.262 ± 0.018
44	1.88 ± 0.454	0.494 ± 0.102	0.228 ± 0.049	1.54 ± 0.26	0.578 ± 0.049	0.262 ± 0.042
72	12.7 ± 2.136	6.858 ± 1.178	1.802 ± 0.352	8.23 ± 1.347	4.544 ± 1.003	1.25 ± 0.168

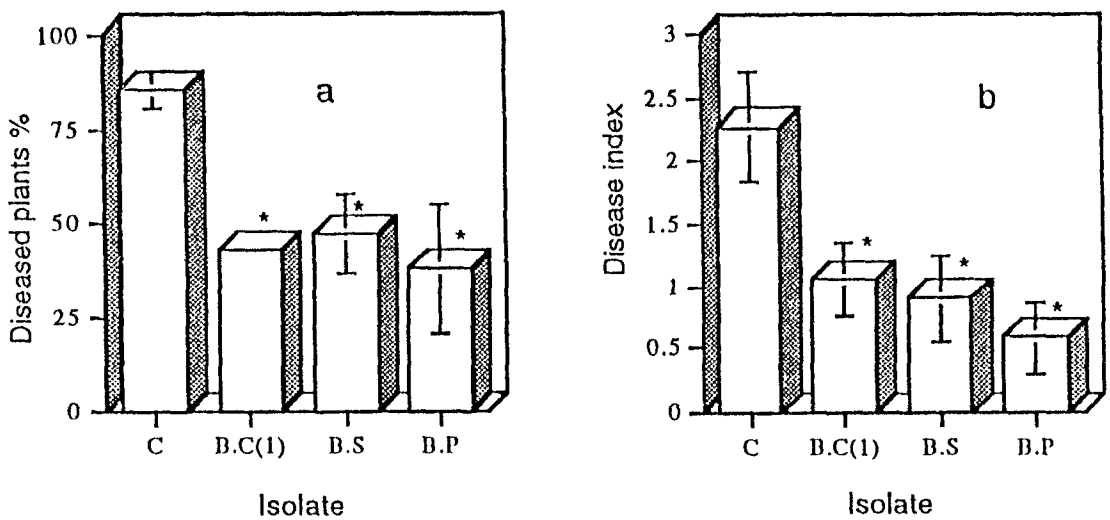

Fig. 1. The effect of application endophytic bacteria on: (a) damping-off disease of cotton caused by *R. solani*. Vertical bars topped with asterisks are significantly different from the control disease; (b) the disease index (0 = no disease, 5 = complete necrosis of the root-shoot junction). C = disease control; B.C(1) = *B. cereus* (isolate 65); B.S = *S. subtilis*; B.P = *B. pumilus*.

Statistical analysis

In each experiment, 4 pots of 8 plants per pot, the proportion of diseased plants per pot was subjected to the arcsine transformation before analysis. To compare degrees of disease in the different treatments, each plant was graded from 0 (healthy) to 5 (dead) and the mean grade calculated per pot [Sneh *et al.*, 1966]. The pot means were thereafter treated as continuous, approximately normal variables.

Treatment means were compared by analysis of variance. Thereafter, simultaneous comparisons of each treatment to the diseased control were performed by Dunnett test.

Results and discussion

Eighty-five bacteria were isolated following stringent disinfection to ensure that we were working with real endophytes. The following number of strains were isolated: seven each from cotton, bean and cucumber; 20 each from squash and melon; four from onion; three each from sinapis and sunflower; one from mimosa; two from poplar; five from raphanus and six from cauliflower. Endophytes were found in 16.7% of the seeds that were surface contaminant-free. Misaghi and Donndelinger [1990] reported the isolation of endophytic bacteria from two cultivars of cotton, and showed that the endophytes were present in seeds and

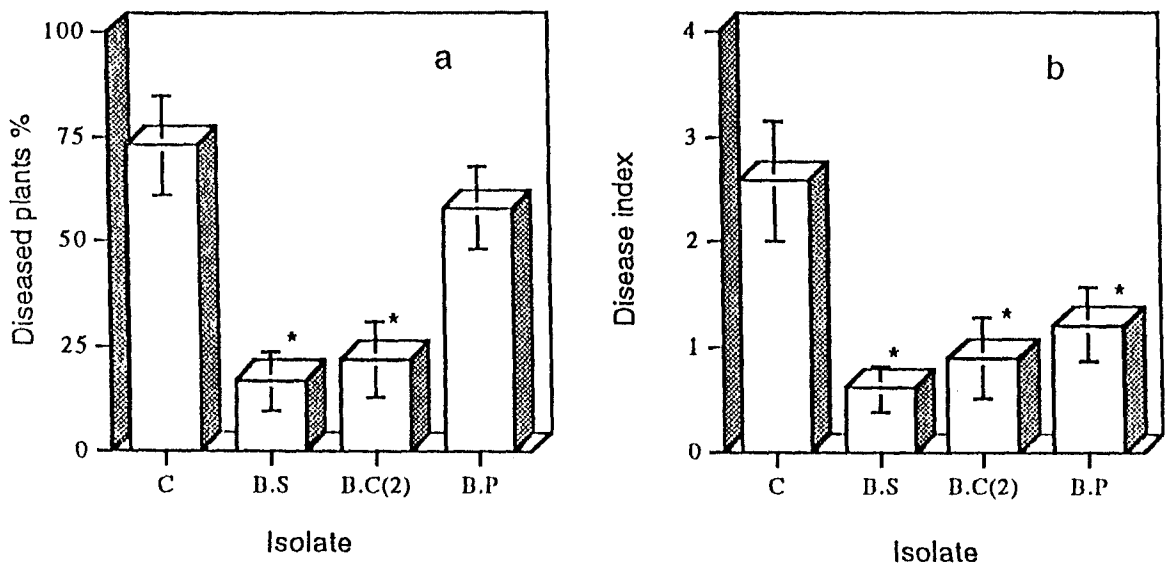

Fig. 2. The effect of endophytic bacterial application on: (a) damping-off disease of bean caused by *S. rolfsii*. Vertical bars topped with asterisks are significantly different from the control disease; (b) the disease index (0 = no disease, 5 = complete necrosis of the root shoot junction). C = disease control; B.S = *B. subtilis*; B.C(2) = *B. cereus* (isolate 78); B.P = *B. pumilus*.

Table 2. Height (cm) of cotton plants loaded with *B. cereus* and controls

Time (days)	*B. cereus*	Control
1	2.64 ± 0.29	2.7 ± 0.26
8	5.48 ± 0.29	4.4 ± 0.11
17	6.32 ± 0.64	5.72 ± 0.74
24	6.8 ± 1.21	7.76 ± 0.56
33	12.66 ± 0.75	13.18 ± 1.25
44	14.48 ± 1.33	14.54 ± 0.747
72	46.48 ± 3.93	39.44 ± 5.37

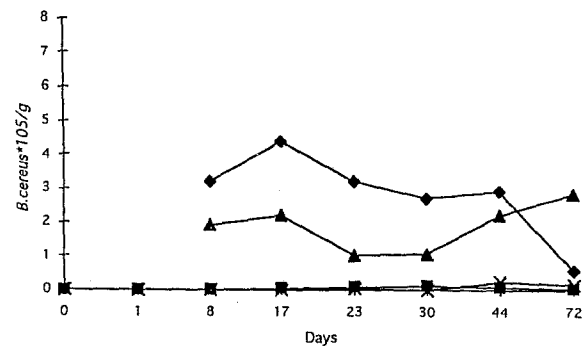

Fig. 3. *B. cereus* in cotton plants (cfu of *B. cereus* per gram fresh weight of plant part). —◆— Stem from cotton plant loaded with *B. cereus*; —■— Stem from control cotton plant; —▲— Root from cotton plant loaded with *B. cereus*; —×— Root from control cotton plant.

various tissues throughout the plant during all stages of its development. Mundt and Hinkle [1976] examined the presence of endophytic bacteria in different plant species. They found no endophytes in Mimosa or cucumber, whereas in squash and oats endophytes were found in 19 and 26% of the seeds, respectively. They succeeded in obtaining bacteria from 15% of herbaceous plants and from 16% of woody plants.

When the isolates were grown in dual culture, some showed no growth inhibition of the organisms tested. Some inhibited growth of several fungi while others inhibited growth of all fungi. For example, one of the isolates from bean, *Pseudomonas fluorescens* (no. 14) inhibited all fungi tested and was florescent on King B medium. *B. cereus* (no. 65) from *Sinapis* inhibited growth of *R. solani, P. ultimum* and *S. rolfsii*. It also exhibited chitinase activity. *B. subtilis* (no. 72) from onion tissue culture inhibited *R. solani* and *P. ultimum* growth. *B. cereus* (no. 78) from cauliflower inhibited growth of *R. solani*. *B. pumilus* (no. 85) from sunflower inhibited *R. solani* and *S. rolfsii*.

The second stage of selection was performed using cotton and bean seedlings as the plant material and *R. solani* and *S. rolfsii* as the pathogens, respectively. In cotton, the bacteria reduced disease incidence caused by *R. solani* by 51% (*B. cereus*, isolate no. 65), 46%

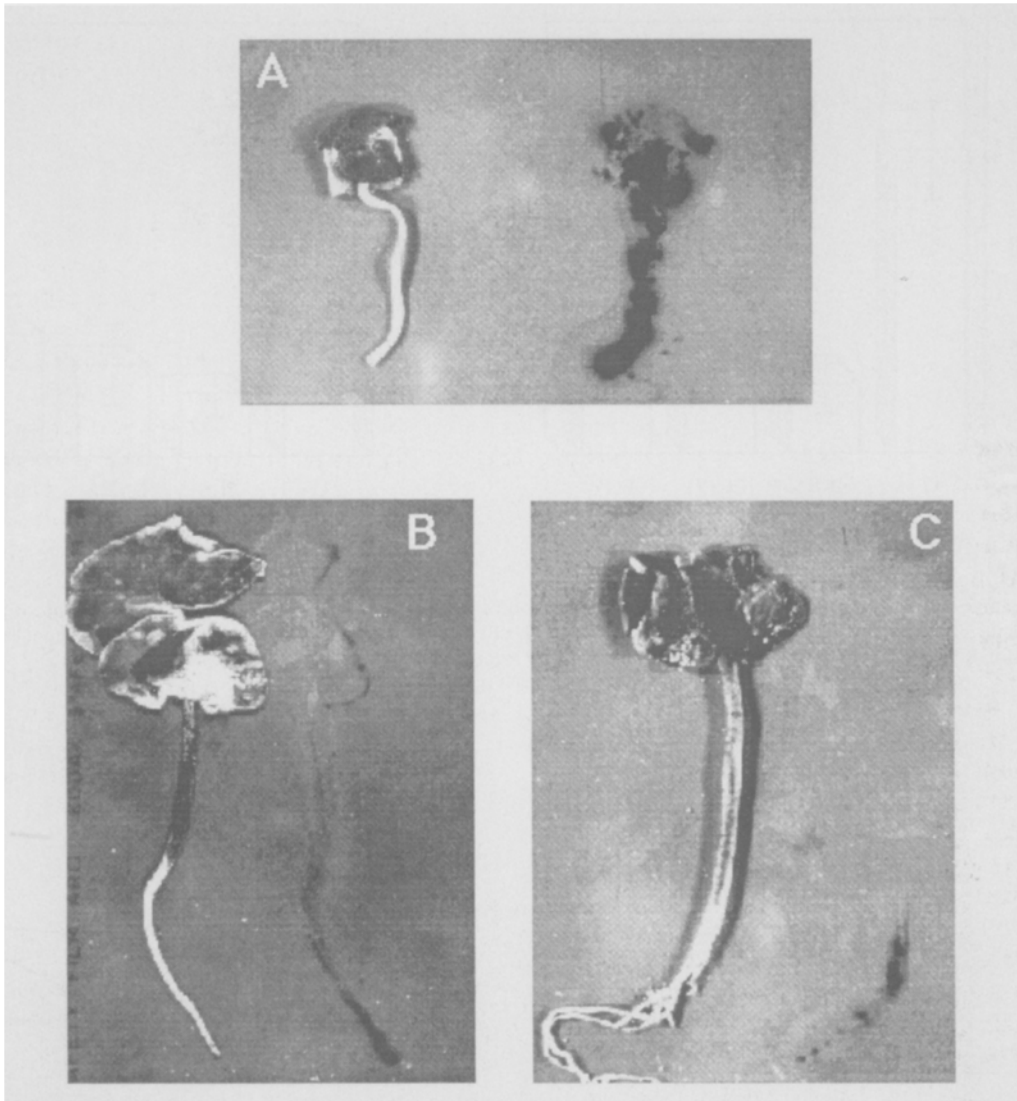

Fig. 4. Autoradiograms of cotton plants loaded with labelled *B. cereus*. In each photo the autoradiogram is on the right, the cotton plant is on the left. A – after 24 h; B – after 6 days; C – after 16 days.

(*B. subtilis*, isolate no. 72), and 56% (*B. pumilus*, isolate no. 85) when they were introduced into the plant during germination. Disease index, which expresses disease level, showed that seedlings inoculated with endophytic bacteria were less affected than the controls (1.05, 0.9, and 0.58 for the three *Bacillus* spp., respectively, as compared to 2.27 for the control) (Fig. 1). In bean seedlings, endophytes reduced disease incidence caused by *S. rolfsii* by 79% (*B. subtilis*, isolate no. 72), 72% (*B. cereus*, isolate no. 78) and 26% (*B. pumilus*, isolate no. 85) when they were 'loaded' into the plants during germination. As in cotton, the endophytes reduced the disease index over 50% (Fig. 2).

The use of rhizobacteria as biocontrol agents of soilborne plant pathogens has been clearly established [Chet *et al.*, 1991; Elad *et al.*, 1987]. Recently Chen *et al.* [1995] have shown that endophytic bacteria can reduce Fusarium wilt of cotton upon injection into the plant.

We succeeded in recovering the endophyte *B. cereus*, even after 72 days, from the roots and stems of cotton plants. Bacterial growth during that time was as follows: after an initial increase, there was a decrease in the number of bacteria in both stems and roots. However, from day 30 and on, the remaining bacteria began to multiply, causing a second increase in their numbers

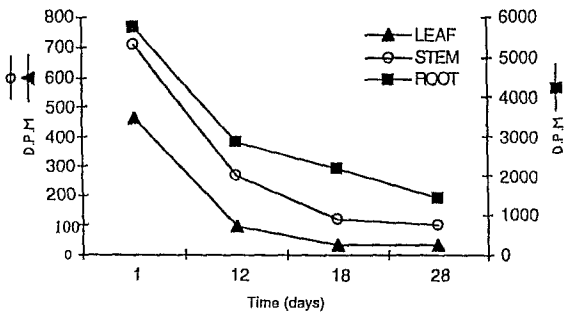

Fig. 5. Radioactive counting of cotton plants loaded with *B. cereus*.

within the roots (Fig. 3). After 24 h the bacteria had already spread to all plant parts, as seen by autoradiogram (Fig. 4A). After 6 days, most of the labelled bacteria were found in the roots and stems, with only a small amount in the leaves and upper part of the stems (Fig. 4B). After 16 days, it appeared mainly in the lower part of the stems and upper part of the roots (Fig. 4C). Most of the radioactivity was found in the stems and roots. The decrease in radioactive counts (Fig. 5) may have been due to the observed decrease in bacterial concentration (Fig. 3). When the number of the bacteria increased on day 30 the radioactivity had already been diluted. When we studied *B. cereus* on selective medium we obtained similar results. Following loading, the number increased to $6.4 \cdot 10^6$ in the roots and stems, then decreased to $3.19 \cdot 10^5$ and $1.93 \cdot 10^5$ in the stem and root, per gram fresh weight. The number of bacteria never decreased below. $5 \cdot 10^4$ bacteria g^{-1} fresh weight of stems and roots (Fig. 3). Misaghi and Donndelinger [1990] recovered an *Erwinia* sp. from stems, flowers, bolls and roots, 4 months after its introduction at a level of $2 \cdot 10^4$. *Clavibacter xylli* subsp. *cynodontis* was found to spread systematically throughout corn plants after its introduction. The bacteria was detected from different organs at a levels of $1 \cdot 10^7$ to $1 \cdot 10^9$ [Reeser and Kostaka, 1988]. In our study, introduction of *B. cereus* into cotton seedlings did not cause any visible damage, nor did it affect plant height or fresh weight (Tables 1, 2).

Using radioactive labelling, we showed that following its introduction, *B. cereus* spread throughout the plant. However, with time most of the label was found in the roots and stems, especially in the root-stem junction. Using selective media, we found the number of bacteria inside the root was higher than $1 \cdot 10^5$ g^{-1} fresh weight. These two types of evidence demonstrate the ability of the endophytic *B. cereus* from *Sinapis* to populate another plant species, namely cotton. All the bacterial isolates selected after stringent sterilization methods were tested for their ability to inhibit the growth of different bacteria and fungi. The non-host specificity of *Bacillus* spp. has been previously demonstrated and these bacteria have been found in various plant hosts [Hall and Davis, 1990; Hallaksella *et al.*, 1991]. Among the different *Bacillus* species found in the ovules and seeds of many different plant families, e.g. cereals (*Zea mays*), vegetables (*Cucumis sativus*) and woody plants (*Malus* sp.) [Mundt and Hinkle, 1976] were *B. cereus, B. pumilus*, and *B. subtilis, B. Brevis, B. pumilus,* and other *Bacillus* spp. have also been isolated from symptom-free cotton plants [Misaghi and Donndelinger, 1990] and from the xylem of rough lemon roots from young healthy and 'declining' trees [Gardner *et al.*, 1982]. The three *Bacillus* species isolated in our laboratory have also been found in *Picea abies* [Hallaksella *et al.*, 1991]. However, the results presented here demonstrate that endophytic bacteria isolated from various plants are capable of residing in other plants and have the ability to inhibit different fungal species. The results show that endophytic bacteria are capable of reducing post-emergence damping-off in various plants, when grown under controlled greenhouse conditions.

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