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 $\beta$-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 (*Helianthus annuus* L. cv. ‘D-3’), cotton (*Gossypium herbaceum* L. cv. ‘Akata’), 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*₂*O*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* in 0.01% (v/v) Tween 20, and then transferred to 50% ethanol with 0.2% (w/v) *HgCl₂* 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