Bacterial endophytes in cotton: location and interaction with other plant-associated bacteria

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Abstract: Investigations were conducted to determine if biological control agent Pseudomonas fluorescens 89B-61 could colonize cotton tissues systemically and if internal colonization by a known endophytic bacterium, Enterobacter asburiae JM22, was influenced by the presence of other plant-associated bacteria. Following seed treatment, Pseudomonas fluorescens 89B-61 colonized cotton roots both externally and internally at mean population densities of $8.7 \times 10^5$ CFU/g and $1.1 \times 10^5$ CFU/g, respectively. However, bacteria were not detected in cotyledons, leaves, or stems. After inoculation onto leaves, Pseudomonas fluorescens 89B-61 established a mean internal population density of $1.6 \times 10^5$ CFU/g leaf tissue. Following stem injection, Pseudomonas fluorescens 89B-61 did not colonize roots or leaves. Pseudomonas fluorescens 89B-61 was localized on the root surface concentrated in grooves between epidermal cells, below collapsed epidermal cells, and in intercellular spaces close to the root epidermis, as identified by immunogold labeling of the bacterial membrane. Combined application of E. asburiae JM22 with another endophyte, Paenibacillus macerans Tri2-10, resulted in significantly lower internal populations of E. asburiae JM22 compared with treatment with E. asburiae JM22 alone. However, when coinoculated with a rhizosphere colonist, Micrococcus agilis strain 2RD-11, the colonization density of E. asburiae JM22 was not negatively affected. The results suggest that the internal colonization of cotton by bacteria with biological control activity may be an important aspect in their capacity to protect host plants against plant pathogens. The extent of internal colonization was shown to be influenced by other bacterial colonists.

Key words: endophytic bacteria, location, interaction, cotton.

Introduction

Many studies have described the biocontrol potential of root-colonizing bacteria (Qiu et al. 1990; Sikora 1992; Kloepper 1993; Kloepper et al. 1991; Van Baren et al. 1993). Pseudomonas fluorescens 89B-61 is a root colonist which has been shown to reduce the incidence of Fusarium wilt of cotton (causal agent Fusarium oxysporum f.sp. vasinefectum) (Chen et al. 1995), cucumber anthracnose (causal agent Colletot-
trichum orbiculare), and bacterial angular leaf spot (causal agent Pseudomonas syringae pv. lachrymans) disease (Wei et al. 1996).

By contrast, Enterobacter asburiae JM22 was found to colonize the aerial parts of plants following seed treatment (Mahaffee et al. 1996; Quadt-Hallmann and Kloepper 1996). Immunological methods for the detection and localization of this model endophyte in plant tissue have already been developed (Quadt-Hallmann and Kloepper 1996). Therefore, E. asburiae JM22 was selected in studies to determine if the internal population densities of an introduced endophyte are affected by the presence of other plant-colonizing bacteria (rhizobacteria and endophytes). Interactions of bacteria with their host plant have been described by several authors (Elliott et al. 1984; De Freitas and Germida 1990; Mahaffee and Backman 1993). Bacterial population densities in the rhizosphere can affect the consistency of plant growth promotion and disease suppression by plant growth promoting rhizobacteria (PGPR) and biological control agents (Bull et al. 1991; Raaijmakers et al. 1995). These authors demonstrated a correlation between the population density of Pseudomonas strains and the level of suppression of take-all and Fusarium wilt, respectively.

The objective of this work was (i) determine if Pseudomonas fluorescens 89B-61 can colonize cotton (Gossypium hirsutum) systemically; (ii) localize the colonization sites of Pseudomonas fluorescens 89B-61 in cotton roots, and (iii) determine the effect of other plant-associated bacteria (rhizobacteria and endophytes) on the internal colonization of cotton by the endophyte E. asburiae JM22.

Material and methods

Bacterial inoculum

Three plant endophytes and one rhizobacterium were selected, namely E. asburiae JM22 and Paenibacillus macerans Tri2-10 isolated from cotton roots (McInroy and Kloepper 1995; Musson et al. 1995), Pseudomonas fluorescens 89B-61 isolated from canola (Brassica campestris) roots (Agrium, Saskatoon, Sask.), and Micrococcus agilis 2RD-11 isolated from the rhizosphere of cotton. Pseudomonas fluorescens 89B-61 has shown biological control against several pathogens (Chen et al. 1995; Wei et al. 1996), whereas E. asburiae JM22 has consistently lacked biocontrol activity (J.A. McInroy, personal communication). Micrococcus agilis 2RD-11 was selected because it appears to be exclusively a rhizosphere colonist and was never detected in the internal root tissues of cotton (W.F. Mahaffee and J.W. Kloepper, in preparation). All bacterial strains were stored in tryptic soy broth (TSB) (Difco, Detroit, Mich.) plus 20% glycerin at -80°C. Prior to use, bacterial strains were grown on tryptic soy agar (TSA) (Difco) for 24 h at 28°C.

Immunological methods

Pseudomonas fluorescens 89B-61-specific polyclonal antibodies were raised after the method of Quadt-Hallmann and Kloepper (1996) by Cocalico Biologicals (Reamstown, Pa.). Enzyme-linked immunosorbent assay (ELISA) and immunogold labeling were conducted after the method of Quadt-Hallmann and Kloepper (1996). Immunofluorescence microscopy procedures involved the use of semithin sections of cotton root tissues transferred to glass objective slides and incubated with blocking buffer (20 mM Tris(hydroxymethyl)-aminomethane hydrochloride (TBS) plus 2% bovine serum albumin, pH 7.4) for 30 min at room temperature. Sections were first coated for 30 min with a solution of primary antibodies and then with a solution of secondary antibodies (goat anti-rabbit antibodies) coupled with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, Mo.) for 30 min at room temperature. Samples were then kept in the dark to eliminate breakdown of the light-sensitive FITC. After two 5-min washings with sterile potassium phosphate buffer (PB) (pH 7.0) and one with double-distilled water, sections were sealed with mounting buffer (100 mL 0.1 M sodium phosphate buffer (pH 7.6) plus 50 mL double-distilled glycerine) and observed under a light microscope equipped with ultraviolet light and a FITC Texas-red filter.

Determining bacterial colonization in plants

Cotton plants (Gossypium hirsutum cv. DP 50) were grown in the greenhouse in commercially available ground clay (Moltan Company, Middleton, Tenn.), which was previously autoclaved. Plants were fertilized with 25 mL Peter’s fertilizer solution (20:20:20 N–P–K) (Scotts-Sierra, Maryvilles, Ohio) 1 week after planting. All the experiments were designed as randomized complete blocks. Root, stem, and leaf fresh weight were recorded, and the endophytic bacterial population density was estimated from surface-disinfested plant tissues and expressed as CFU per gram fresh weight tissue. Surface disinfestation of all plant samples was performed by shaking selected plant tissues in 1.05% NaOCl for 1 min, followed by three washes in sterile PB. As a sterility check, plant tissue was pressed onto TSA, and only plant samples that showed no bacterial growth after 24 h at 28°C were used in calculating population densities. To determine the level of internal bacterial colonization, plant tissue was triturated with sterile PB at a ratio of 1:5 w/v, and 100-μL aliquots of the tritrate were plated on TSA. After incubation at 28°C for 24 h, 10 random bacterial colonies that morphologically resembled Pseudomonas fluorescens 89B-61 per TSA plate were examined with ELISA against Pseudomonas fluorescens 89B-61 specific antibodies to confirm recovery. In addition, these suspected Pseudomonas fluorescens 89B-61 colonies and morphologically distinct colonies (of indigenous endophytic bacteria, 0–10 per plate) were identified by fatty acid methyl ester (FAME) analysis (Sasser 1990) after the method of McInroy and Kloepper (1995).

Three experiments, using different inoculation methods, were conducted to assess the internal colonization of cotton cultivar DP 50 by Pseudomonas fluorescens 89B-61. Plants for all experiments were grown as described above in autoclaved clay in the greenhouse. Experiment 1 was designed to test endophytic colonization following seed treatment. Seeds were soaked for 30 min in a bacterial suspension (100 mg bacteria and 2 mL of 2% methyl cellulose solution per 20 seeds) or in the same volume of methyl cellulose without bacteria (the control treatment) and then dried under a laminar flow hood. This inoculation system resulted in mean bacterial concentrations of 1.0 × 10⁷ CFU/seed as determined by dilution plating. The bacterized and control treatments were replicated eight times, with one plant per replication. The experiment was terminated 14 days after planting. Roots, stems, cotyledons, and leaves were sampled and processed as described above, and the internal population densities were quantified. Colonization sites in the root tissues of four seed-treated plants were analyzed immunocytologically using fluorescence and transmission electron microscopy. The root tissues of four nontreated plants served as a control.

Experiment 2 was designed to test endophytic colonization following application of bacteria to leaves. Leaves of 2-week-old cotton plants were inoculated with a suspension of Pseudomonas fluorescens 89B-61. A 1-ml suspension of Pseudomonas fluorescens 89B-61 in PB (1.0 × 10⁷ CFU/mL) was spread with a pipet over each of two leaves per plant and dispersed. Control plants were inoculated with PB. To avoid bacterial contamination of the clay growth medium, pots were covered with plastic wrap and secured around the stem. Plants were watered from the base of the pot. Each treatment consisted of five replications, each with one plant. Two weeks after inoculation roots and leaves were removed and processed as described.
Results

Population densities and colonization patterns of 89B-61 in cotton

The mean bacterial concentration for cotton plants after seed treatment with 89B-61 in experiment 1 was $1.1 \times 10^7$ CFU/g root tissue (Table 1) and ranged among replications from $3.9 \times 10^7$ to $3.3 \times 10^3$ CFU/g root tissue. Without surface disinfection, values of $8.7 \times 10^5$ CFU/g root tissue were reached (Table 1). Bacterial colonies of *Pseudomonas fluorescens* 89B-61 were not detected in stems, cotyledons, or leaves. All bacterial colonies recovered from the roots of seed-treated cotton plants reacted positively with the polyclonal antibodies raised against 89B-61 and were confirmed as *Pseudomonas fluorescens* by FAME analysis. Cotton plants inoculated with sterile PB were free of *Pseudomonas fluorescens* 89B-61 but had low populations of *Sphingomonas* spp., *Pantoea* spp., *Burkholderia* spp., *Bacillus* spp., or *Phyllobacterium* spp. as identified by FAME. These taxa were previously reported as indigenous endophytes of cotton (McInroy and Kloepper 1995). No cross reactions between these bacteria and the antibodies raised against *Pseudomonas fluorescens* 89B-61 were observed.

A schematic of the colonization sites of *Pseudomonas fluorescens* 89B-61 in cross sections of 2-week-old cotton roots after seed treatment ($1.0 \times 10^7$ CFU/seed) in comparison to those determined for *Enterobacter asburiae* JM22 in previous experiments (Quadt-Hallmann and Kloepper 1996).

<table>
<thead>
<tr>
<th>Plant tissue</th>
<th>ELISA*</th>
<th>Concentration of 89B-61 (CFU/g plant tissue)*</th>
<th>*The presence of 89B-61 was confirmed with ELISA (+, positive reaction; −, negative reaction) and also by gas chromatographical analysis of fatty acid methyl esters (not shown).</th>
<th>*Mean of eight replicates per treatment.</th>
<th>*No surface disinfestation.</th>
</tr>
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<tbody>
<tr>
<td>Roots</td>
<td>+</td>
<td>$8.7 \times 10^5$</td>
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<tr>
<td>Roots</td>
<td>+</td>
<td>$1.1 \times 10^5$</td>
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<tr>
<td>Stems</td>
<td>−</td>
<td>$&lt;5.0 \times 10^5$</td>
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<tr>
<td>Cotyledons</td>
<td>−</td>
<td>$&lt;5.0 \times 10^5$</td>
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<tr>
<td>Leaves</td>
<td>−</td>
<td>$&lt;5.0 \times 10^5$</td>
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*Experiment 3 was designed to test endophytic colonization following stem injection. Twenty microlitres of a suspension of *Pseudomonas fluorescens* 89B-61 ($1.0 \times 10^8$ CFU/mL) was injected into the base of cotton seedling stems 2 weeks after planting. This method was previously reported to differentiate among strains for endophytic colonization potential (Chen et al. 1995). Control plants were inoculated with sterile PB. Each treatment was replicated five times, with one plant per replication. Fourteen days after stem injection, the roots, upper stems, and leaves were harvested and the level of internal colonization by *Pseudomonas fluorescens* 89B-61 was determined as described above.*

**Fig. 1.** Schematic overview of the colonization sites of *Pseudomonas fluorescens* 89B-61 in cross sections of 2-week-old cotton roots after seed treatment ($1.0 \times 10^7$ CFU/seed) in comparison to those determined for *Enterobacter asburiae* JM22 in previous experiments (Quadt-Hallmann and Kloepper 1996). P/X, xylem and phloem.
Fig. 2. Transmission electron microscopy of a cotton plant seed treated with *Pseudomonas fluorescens* 89B-61 (1.0 × 10^7 CFU/seed), combined with immunogold labeling using primary antibodies against *Pseudomonas fluorescens* 89B-61 at a dilution of 1:500 and secondary antibodies coupled with colloidal gold at a dilution of 1:40. A single cell of *Pseudomonas fluorescens* 89B-61 shows an intensive gold label of the bacterial membrane, indicating a reaction of the antibodies with membrane proteins. B, bacterium. Scale bar, 0.5 μm.

Enterobacter. Stem injection with *Pseudomonas fluorescens* 89B-61 in experiment 3 did not lead to successful colonization of the cotton plants.

Interactions of *E. asburiae* JM22 with other plant-associated bacteria

When applied alone, *E. asburiae* JM22 reached a mean internal population density of 7.2 × 10^3 CFU/g fresh root tissue, which was significantly higher than that for *Paenibacillus macerans* Tri2-10 (4.3 × 10^4 CFU/g fresh root tissue) (Fig. 7). *Micrococcus agilis* 2RD-11 was not isolated from surface-disinfested root tissue, confirming it as a non-endophyte of cotton. When applied in combination with *E. asburiae* JM22, *M. agilis* 2RD-11 did not affect the internal colonization of *E. asburiae* JM22 (1.1 × 10^4 CFU/g root tissue) (Fig. 7). However, co-inoculations of *Paenibacillus macerans* Tri2-10 and *E. asburiae* JM22 lead to a statistically significant reduction of the internal population density of *E. asburiae* JM22 (5.9 × 10^4 CFU/g). Colonization by *Paenibacillus macerans* Tri2-10 was not statistically affected by *E. asburiae* JM22 (Fig. 7). Control plants were free of any of the applied strains (Fig. 7). *Enterobacter asburiae* JM22 also colonized cotton seedlings systemically. After seed treatment, mean internal population densities of *E. asburiae* JM22 within cotyledon tissues reached 5.2 × 10^3 CFU/g regardless of whether *E. asburiae* JM22 was applied singly or in combination with *M. agilis* 2RD-11 or *Paenibacillus macerans* Tri2-10. Neither *M. agilis* 2RD-11 nor *Paenibacillus macerans* Tri2-10 was recovered from stems, petioles, or leaves.

**Discussion**

Upon application via seed treatment, the two plant-associated bacterial strains used in this study exhibited different patterns of endophytic colonization. *Pseudomonas fluorescens* 89B-61 was restricted in colonization to the outer root cortex, whereas *E. asburiae* JM22 colonized throughout the cortex and inside the vascular stele in intercellular spaces close to the conducting elements (Quadt-Hallmann and Kloepper 1996). We propose that these differential patterns of internal colonization represent general patterns of endophytic bacterial colonization of plants; endophytes may be described as predominantly “cortical colonists” as typified by *Pseudomonas fluorescens* 89B-61 or as “systemic colonists” as typified by *E. asburiae* JM22. Evidence supporting the hypothesis that cortical colonists are distinct from systemic colonists for endophytic bacteria was found in another part of this study and in a previous study by Mahaffee et al. (1997). In this study, even when the cortical colonist *Pseudomonas fluorescens* 89B-61 was injected directly into stems of cotton, it did not colonize. Mahaffee et al. used immunofluorescent colony staining to determine colonization patterns of *E. asburiae* JM22 and *Pseudomonas fluorescens* 89B-27 in bean following application via seed treatment. Their results indicate that the systemic colonist, *E. asburiae* JM22, was detected in stems, petioles, and the apical meristem region, whereas *Pseudomonas fluorescens* 89B-27 was restricted to roots.

The efficacy of endophytes as biocontrol agents is not necessarily correlated with systemic colonization. Wei et al. (1996) reported that seed treatment of cucumber with *Pseudomonas fluorescens* 89B-61 resulted in systemic protection in the field against the leaf pathogen *C. orbiculate* via induced systemic resistance. Hence, to achieve biocontrol systemically using endophytic bacteria, it is not necessary to use a systemic colonist.

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Fig. 4–6. Transmission electron microscopy of a cotton plant seed treated with *Pseudomonas fluorescens* 89B-61 (\(1.0 \times 10^7\) CFU/seed), combined with immunogold labeling using primary antibodies against *P. fluorescens* strain 89B-61 at a dilution of 1:500 and secondary antibodies coupled with colloidal gold at a dilution of 1:40. Fig. 4. Electron-opaque apposition of an amorphous matrix on the cell walls of root epidermal cells (EP) is localized at the connection of two cells in the vicinity of a single cell of 89B-61. B, bacterium. Scale bar, 1 \(\mu m\). Fig. 5. Bacterial cells of *Pseudomonas fluorescens* 89B-61 are located on the root surface and inside intercellular spaces close to the root epidermis (E). Arrows denote bacteria. Scale bar, 10 \(\mu m\). Fig. 6. Single bacterial cell of *Pseudomonas fluorescens* 89B-61 occurs inside an epidermal cell (EP) which shows signs of necrosis. B, bacterium. Scale bar, 2 \(\mu m\).

Fig. 7. Population densities of *Enterobacter asburiae* JM22, *Micrococcus agilis* 2RD-11 (M.a.), and *Paenibacillus macerans* Tri2-10 (P.m.) in 2-week-old cotton roots after seed treatment when applied singly or in combination with JM22 (200 mg bacteria plus 2 mL of 2% methyl cellulose/20 seeds). Co, control.

Endophytic bacteria used in this study colonized plant tissues in lower concentrations than pathogenic bacteria, which can reach population densities as high as \(1.0 \times 10^7\) to \(1.0 \times 10^{10}\) CFU/g plant tissue in tomato and *Burkholderia solanacearum* (Grimault and Prior 1994) in tomato, eggplant, and pepper (Grimault and Prior 1994; Tsiantos and Stevens 1986). *Pseudomonas fluorescens* 89B-61, *E. asburiae* JM22, and *Paenibacillus macerans* Tri2-10 never exceeded concentrations of \(1.1 \times 10^4\) CFU/g root tissue.

Internal colonization by the systemic endophyte *E. asburiae* JM22 was influenced when co-inoculated with the endophyte *Paenibacillus macerans* Tri2-10 but not with the rhizobacterium *M. agilis* 2RD-11. When *Paenibacillus macerans* Tri2-10 was applied in combination with *E. asburiae* JM22, the population densities of both strains decreased compared to their single application. This suggests that these strains competed for the same resources within the plant, e.g., nutrients and space. In contrast, internal colonization of *E. asburiae* JM22 was not affected by rhizobacterium *M. agilis* 2RD-11, because these bacteria appear to exploit different ecological niches, namely internal root regions for *E. asburiae*.
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JM22 and the rhizosphere (outside the root) for M. agilis 2RD-11. The results reported here demonstrate that endophytic bacteria colonize internal plant tissues upon inoculation, even when they do not spread systemically in plants, and that different endophytic bacteria may have varying degrees of competitiveness.

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References


