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

A. Quadt-Hallmann, J. Hallmann, and J.W. Kloepper

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 8.7×10^5 CFU/g and 1.1×10^3 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 1.6×10^4 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.

Résumé : Des études ont été entreprises pour déterminer si le *Pseudomonas fluorescens* 89B-61, un agent de contrôle biologique, pourrait coloniser les tissus du coton de façon systémique et si la colonisation interne pour l'*Enterobacter asburiae* JM22, une bactérie endophyte connue, serait influencée par al présence d'autres bactéries associées aux plantes. Après le traitement de graines avec le *Pseudomonas fluorescens* 89B-61, les racines de coton ont été colonisées tant à l'extérieur qu'à l'intérieur à des densités de populations moyennes respectives de $8{,}7 \times 10^5$ UFC/g et de $1{,}1 \times 10^3$ UFC/g. Toutefois, les bactéries n'ont pas été détectées dans les cotylédons, les feuilles ou les tiges. Après inoculation sur les feuilles, le *Pseudomonas fluorescens* 89B-61 s'est développé à l'intérieur à une densité moyenne de $1{,}6 \times 10^4$ UFC/g de tissus foliaires. Injecté dans les tiges, cet agent de contrôle biologique n'a colonisé ni les feuilles ni les racines. Le *Pseudomonas fluorescens* 89B-61 a été localisé à la surface des racines, concentré dans des sillons entre les cellules épidermiques, sous des cellules épidermiques affaissées et dans des espaces intercellulaires près de l'épiderme racinaire, tel que détecté par immunomarquage à l'or colloïdal des membranes cellulaires bactériennes. L'application combinée de l'*E. asburiae* JM22 avec une autre espèce endophyte, le *Paenibacillus macerans* Tri2-10, s'est traduite par des populations internes significativement moindres de l'*E. asburiae* JM22 comparativement à l'inoculation de la souche JM22 seulement. Cependant, lorsque cette souche JM22 a été co-inoculée avec un colonisateur de rhizosphère, le *Micrococcus agilis* 2RD-11, la densité de colonisation de la souche JM22 n'a pas été affectée négativement. Les résultats suggèrent que la colonisation interne du coton par des bactéries agents de contrôle biologique peut être un aspect important dans leur capacité à protéger les plantes-hôtes contre les phytopathogènes. L'étendue de la colonisation interne a été influencée par d'autres bactéries colonisatrices.

Mots clés : bactéries endophytes, localisation, interaction, coton.
[Traduit par la rédaction]

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. *vasinfectum*) (Chen et al. 1995), cucumber anthracnose (causal agent *Colleto-*

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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 to (*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 triturate 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 1.0×10^7 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 (1.0×10^7 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

Table 1. Internal population density of 89B-61 in various plant parts of 2-week-old cotton following seed treatment (1.0×10^7 CFU/seed).

Plant tissue	ELISA[a]	Concentration of 89B-61 (CFU/g plant tissue)[b]
Roots[c]	+	8.7×10^5
Roots	+	1.1×10^3
Stems	−	$<5.0 \times 10$
Cotyledons	−	$<5.0 \times 10$
Leaves	−	$<5.0 \times 10$

[a]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).
[b]Mean of eight replicates per treatment.
[c]No surface disinfestation.

above to determine the internal population density of *Pseudomonas fluorescens* 89B-61.

Experiment 3 was designed to test endophytic colonization following stem injection. Twenty microlitres of a suspension of *Pseudomonas fluorescens* 89B-61 (1.0×10^8 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 injected 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.

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

Experiment 4 was designed to determine if the internal colonization by *E. asburiae* JM22 was affected by the presence of other endophytic or rhizosphere-colonizing bacteria. The experiment consisted of six treatments: sterile PB (control), *E. asburiae* JM22, *M. agilis* 2RD-11, *Paenibacillus macerans* Tri2-10, JM22 in combination with 2RD-11, and JM22 in combination with Tri2-10. Each treatment was replicated five times, with one plant per replication. The bacteria were applied as seed treatments to cotton cultivar DP 50 (as described above). In coinoculations, the concentration of each component was half its concentration as a single application. Fourteen days after planting, roots and cotyledons were harvested and the internal population density was estimated within these tissues. The bacterial strains were distinguished visually based on colony morphology. In addition, 10 colonies per sample of various morphologies were identified by FAME and colonies resembling the morphology of *E. asburiae* JM22 were verified by ELISA. After ELISA and gas chromatography, bacterial colonies resembling *E. asburiae* JM22 were counted to calculate mean endophytic population densities. Means were analyzed for significant differences using general linear models analysis in SAS-PC (SAS Institute, Gary, N.C.) and calculation of LSD at P 0.05. The experiment was repeated three times.

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 1.1×10^3 CFU/g root tissue (Table 1) and ranged among replications from 3.9×10 to 3.3×10^3 CFU/g root tissue. Without surface disinfestation, values of 8.7×10^5 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 and the endophyte *E. asburiae* JM22 is given in Fig. 1 (Quadt-Hallmann and Kloepper 1996). Polyclonal antibodies raised against 89B-61 formed an intensive gold label of the bacterial membrane, indicating a reaction of the antibodies with the membrane proteins (Fig. 2). *Pseudomonas fluorescens* 89B-61 was mainly located on the root surface and intercellular spaces close to the root epidermis (Fig. 3). No fluorescence was observed in intercellular spaces close to the conducting elements. Electron-opaque appositions of an amorphous matrix occurred on the epidermal walls in the vicinity of single cells of *Pseudomonas fluorescens* 89B-61 (Fig. 4). Cells in the vicinity of single cells of *Pseudomonas fluorescens* 89B-61 were also observed inside intercellular spaces close to the root surface (Fig. 5) and inside epidermal cells that showed signs of necrosis (Fig. 6).

Following leaf inoculation in experiment 2, bacterial cells of *Pseudomonas fluorescens* 89B-61 were detected within the leaves at a mean concentration of 1.6×10^4 CFU/g wet leaf tissue, ranging from 3.4×10^3 to 4.1×10^4 CFU/g. Systemic colonization of *Pseudomonas fluorescens* 89B-61 in noninoculated leaves or in the root system was not observed. Roots of three cotton plants inoculated with sterile PB and two plants inoculated with *Pseudomonas fluorescens* 89B-61 were colonized by low population densities (1.0×10^2 CFU/g) of bacteria from the genera *Erwinia*, *Actinobacillus*, and

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 (1.0×10^7 CFU/seed) in comparison to those determined for *Enterobacter asburiae* JM22 in previous experiments (Quadt-Hallmann and Kloepper 1996). P/X, xylem and phloem.

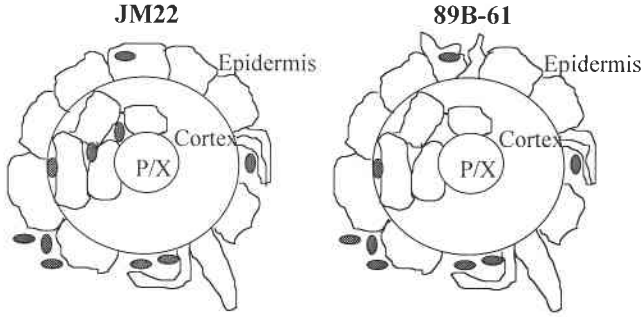
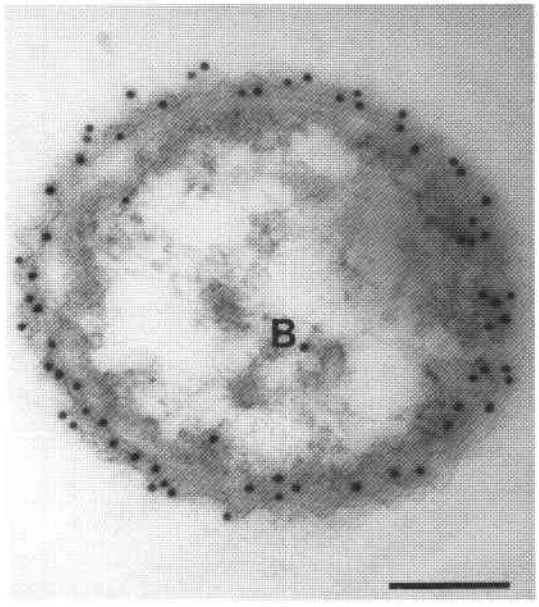


Fig. 2. Transmission electron microscopy of a cotton plant seed treated with *Pseudomonas fluorescens* 89B-61 (1.0×10^7 CFU/seed), combined with immunogold labeling using primary antibodies against *Pseudomonas fluorescens* 89B-61 at a dilution of 1:500 and secondary antibodies coupled with colloidal 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.

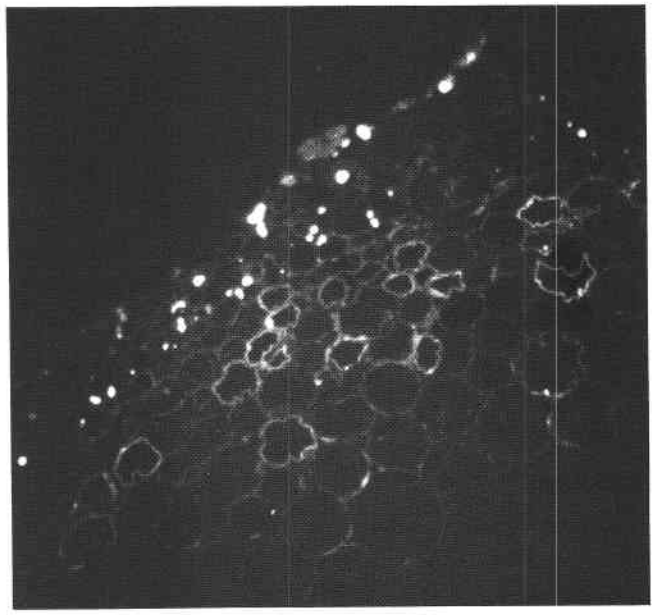


Enterobacter. Stem injection with *Pseudomonas fluorescens* 89B-61 in experiment 3 did not lead to successful colonization of the cotton plants.

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 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 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.

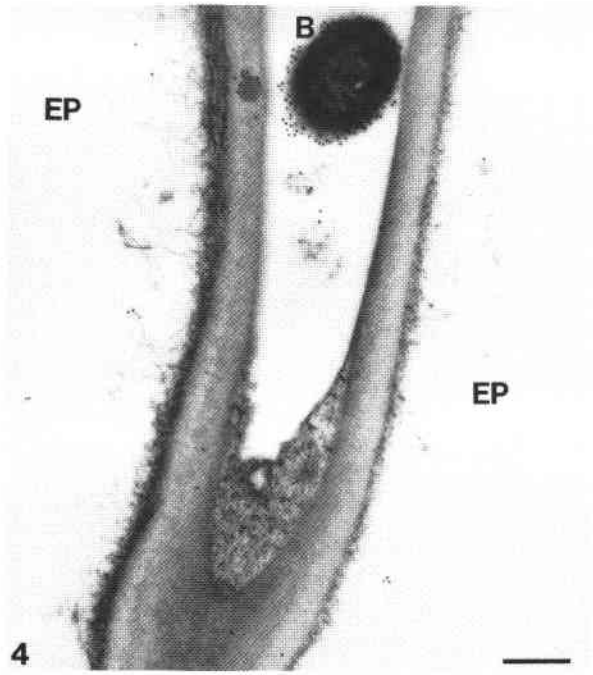
Fig. 3. Fluorescence microscopy using primary antibodies against 89B-61 at a dilution of 1:500 and secondary antibodies coupled with FITC at a dilution of 1:100. Bacterial cells of 89B-61 are located on the root surface as indicated by fluorescent areas.

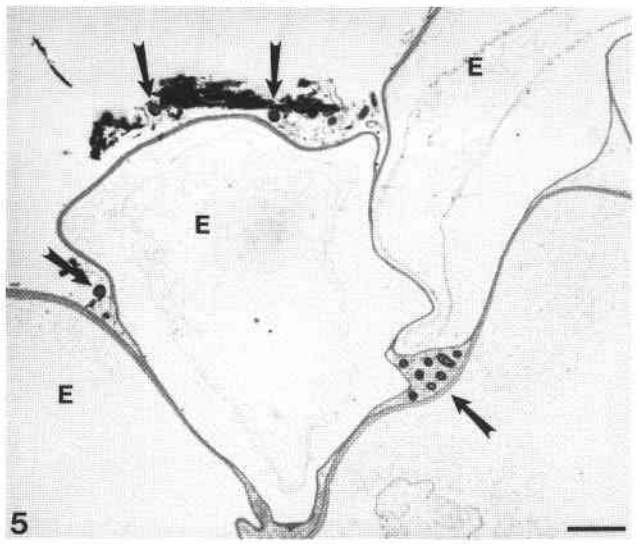


Discussion

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. orbiculare* via induced systemic resistance. Hence, to achieve biocontrol systemically using endophytic bacteria, it is not necessary to use a systemic colonist.





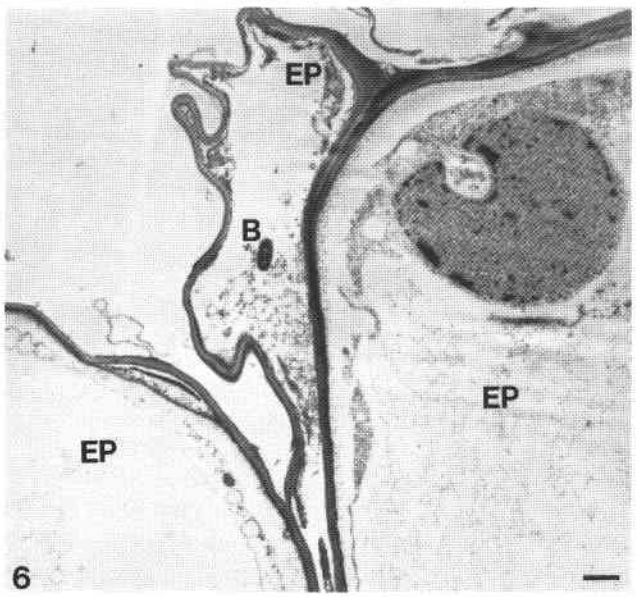
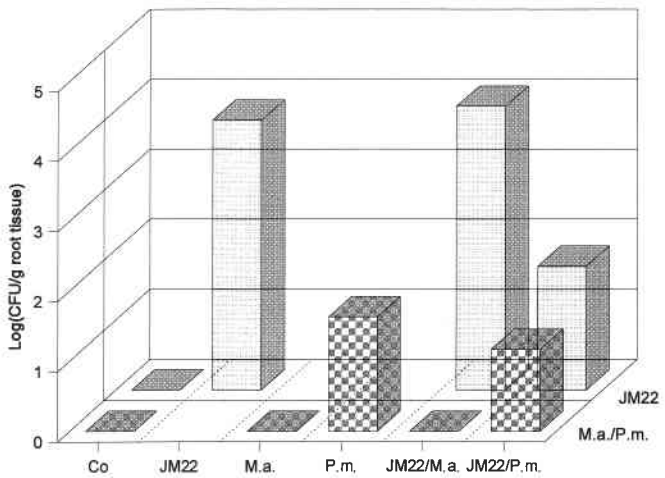


Fig. 4–6. Transmission electron microscopy of a cotton plant seed treated with *Pseudomonas fluorescens* 89B-61 (1.0×10^7 CFU/seed), combined with immunogold labeling using primary antibodies against *P. fluorescens* strain 89B-61 at a dilution of 1:500 and secondary antibodies coupled with colloidal 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 μm. 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 μm. 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 μm.

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 1.0×10^7 to 1.0×10^{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 1.1×10^4 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*

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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