Immunological detection and localization of the cotton endophyte *Enterobacter asburiae* JM22 in different plant species

A. Quadt-Hallmann and J.W. Kloepper

Abstract: Immunological methods were used to study the colonization of internal tissues of different plant species by the endophytic bacterium *Enterobacter asburiae* JM22. Polyclonal and monoclonal antibodies applied in enzyme-linked immunosorbent assay (ELISA), dot blot assay, tissue printing, or immunogold labeling were sensitive and specific enough to detect JM22 in plant tissues. Detection limits were 1.0×10^3 colony-forming units (CFUs)/mL for tissue printing, 1.0×10^4 CFUs/mL for ELISA and 1.0×10^5 CFUs/mL for dot blot assay. Polyclonal and monoclonal antibodies showed a positive immunological reaction with nearly all tested *Enterobacter* spp. In contrast with polyclonal antibodies, the monoclonal antibodies differentiated *Enterobacter* spp. and closely related genera like *Pantoea* or *Serratia*. Other bacterial genera, plant sap from nontreated field-grown crops, and soil solutions did not react with the antisera. When applied as a seed treatment, JM22 colonized roots, stems, and cotyledons of bean, cucumber, and cotton plants. Fourteen days after inoculation of cotton cotyledons or leaves, JM22 was detected inside the inoculated plant tissue and the bacteria moved to the roots. JM22 reached concentrations up to 1.0×10^5 CFUs/g in roots, 1.0×10^4 CFUs/g in stems, and 1.0×10^3 CFUs/g in cotyledons or leaves. Population densities of JM22 varied between the different plant species, being highest in bean and lowest in cotton. JM22 was detected with ELISA in different plant growth media. While sand, ground clay, and loamy sand showed high and comparable ELISA readings, the extinctions of sandy loam and Promix were significantly lower than the ones of the other three growth media, indicating a strong influence of soil mixes on immunological reactions. JM22 showed an intensive gold label in drop preparations of bacterial suspensions in phosphate buffer, plant sap, and ultrathin sections of plant tissue. After seed treatment, the bacteria were located on the root surface, concentrated in grooves between epidermal cells, below collapsed epidermal cells, within epidermal cells, and inside intercellular spaces in the root cortex close to conducting elements. Inoculation of leaves or cotyledons resulted in the occurrence of many gold labeled cells of JM22 on the petiole surfaces. *Enterobacter asburiae* colonizes different plant species and establishes endophytic populations in various tissues.

Key words: immunology, endophytic bacteria, colonization, localization, plant species.

Résumé : Des méthodes immunologiques ont été utilisées pour étudier la colonisation des tissus internes de différentes espèces de plantes par la bactérie *Enterobacter asburiae* JM22. Les anticorps monoclonaux et polyclonaux, utilisés en test immuno-enzymatique ELISA, en buvardages, sur empreintes tissulaires ou dans la détection avec les anticorps marqués à l'or, étaient sensibles et suffisamment spécifiques pour détecter la souche JM22 dans des tissus végétaux. Les limites de détection ont été de $1,0 \times 10^3$ unités formant colonie (UFC) pour les empreintes de tissus, de $1,0 \times 10^4$ UFC/mL pour les ELISA et de $1,0 \times 10^5$ UFC/mL pour les buvardages en points. Les anticorps monoclonaux et polyclonaux ont montré une réaction immunologique avec presque toutes les *Enterobacter* spp. testées. Contrairement aux anticorps polyclonaux, les anticorps monoclonaux ont différencié les *Enterobacter* spp. et des genres étroitement reliés, tels les *Pantoea* ou *Serratia*. D'autres genres bactériens, des sèves provenant de plantes non traitées cultivées au champ et des solutions de sols n'ont pas réagi avec les antisérums. Inoculée à des graines de semence, la souche JM22 a colonisé les racines, les tiges et les cotylédons dans les plants de haricot, de concombre et de cotonnier. Quatorze jours après l'inoculation des cotylédons ou des feuilles de cotonnier, la souche JM22 a été détectée à l'intérieur des tissus inoculés et les bactéries ont migré vers les racines. Les concentrations de la JM22 ont atteint jusqu'à $1,0 \times 10^5$ UFC/g dans les racines, $1,0 \times 10^4$ UCF/g dans les tiges et $1,0 \times 10^3$ UCF/g dans les cotylédons ou les feuilles. Les densités des populations de cette souche ont varié entre les différentes espèces de plantes, étant les plus élevées chez les haricots et les plus faibles chez les cotonniers. Les tests ELISA ont permis de détecter la JM22 dans différents milieux de croissance des plantes. Tandis que le sable, l'argile moulu et le sable limoneux ont présenté des lectures ELISA élevées et comparables, les extinctions dans le limon sablonneux et le Promix ont été significativement inférieures à celles des trois autres milieux de culture, ce qui indique que les mélanges de sols ont une forte influence sur les réactions immunologiques. La souche JM22 a présenté un marquage à l'or intensif dans les préparations

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en gouttes de suspensions bactériennes dans un tampon phosphate, dans des sèves végétales et dans des coupes ultra-minces de tissus végétaux. Après l'inoculation des graines, les bactéries furent localisées sur la surface des racines, concentrées dans les sillons entre les cellules épidermiques, sous les cellules épidermique effondrées, à l'intérieur des cellules épidermiques et à l'intérieur des espaces intercellulaires dans le cortex des racines, près des éléments conducteurs. L'inoculation des feuilles ou des cotylédons s'est traduite par l'occurrence de plusieurs cellules de JM22 marquées à l'or à la surface des pétioles. L'*E. asburiae* colonise différentes espèces de plantes et établit des populations endophytes dans différents tissus.

Mots clés : immunologie, bactéries endophytes, colonisation, localisation, espèces végétales.
[Traduit par la rédaction]

Introduction

Plant growth promotion and reduction of pathogen-caused disease symptoms by rhizobacteria are well documented (Rovira 1963; Brown et al. 1964; Merriman and Birkenhead 1977; Sakthivel et al. 1988; Weller 1988; Kloepper et al. 1991; Sikora 1992; Kloepper 1993). Several agricultural products containing rhizobacteria are marketed in China (Chen et al. 1995) and the United States (Backman et al. 1994).

Recent studies have shown that bacteria, which colonize external and internal plant tissues, demonstrate comparable beneficial effects on their hosts to those documented for rhizobacteria (Qiu et al. 1990; Sharrock et al. 1991; Van Baren et al. 1993). Preliminary studies indicate that some of these endophytes have biological control potential against pathogens (Chen et al. 1995; Pleban et al. 1995). To assess fully the practical utility of endophytic bacteria in integrated biological control strategies, specific marking systems are needed to detect and localize bacteria inside plant tissues and to allow differentiation of an introduced bacterial strain from the indigenous endophytic community. From the methods available for the differentiation of root-colonizing bacteria (Kloepper and Beauchamp 1992), we have chosen immunological detection systems for studies on endophytes because they do not depend on bacterial growth (Schmidt and Paul 1982), they circumvent problems associated with applying genetically modified microorganisms in agricultural ecosystems, and the original bacterial strain can be used without genetic alterations. Furthermore, immunological methods allow the simultaneous detection, specific identification, and precise localization of introduced bacteria.

The objectives of these studies were to (*i*) develop immunological marking systems for the endophytic bacterium *Enterobacter asburiae* strain JM22, (*ii*) test the feasibility of the application of the immunological methods for monitoring internal colonization of plant tissues, and (*iii*) determine the possibility of detecting bacteria in different plant growth media using specific antibodies. This work included investigations on population densities of JM22 in different plants and plant tissues after bacterial application via seed treatment or leaf inoculation.

Material and methods

Bacterial strain

Enterobacter asburiae JM22 was originally isolated from cotton (McInroy and Kloepper 1995*b*) and stored at –80°C.

Production of polyclonal and monoclonal antisera

Polyclonal antibodies were raised against living cells of JM22. Bacteria were cultivated for 24 h in tryptic soy broth (TSB) at room temperature, followed by three washes in sterile potassium phosphate buffer (0.02 M, pH 7.0) (PB). After resuspension of the pellet in 2 mL phosphate-buffered saline (pH 7.4, 0.85% NaCl) (PBS), the bacterial concentration was adjusted to 1.0×10^9 colony-forming units (CFUs)/mL. Immunization of New Zealand White rabbits was carried out by Cocalico Biologicals, Inc., Pa., following the injection scheme of Vruggink and Maas Gesteranus (1975).

The production of monoclonal antibodies was done with Balb/C mice at the Auburn University Hybridoma Facility, Ala. Bacterial cells were washed three times with sterile PB and then fixed with glutaraldehyde by the method of Allan and Kelman (1977). Glutaraldehyde-fixed cells of JM22 were chosen to improve bacterial detection in ultrathin plant tissue sections. The plant tissue was also fixed with glutaraldehyde. This way, comparable conditions are created for the detection of JM22. Four mice were injected subcutaneously or intraperitoneally with 200 µL of bacterial suspension (10^{10} CFUs/mL) per mouse, emulsified 1:1 with complete Freund's adjuvant. After 4 weeks, the second immunization was carried out with 250 µL of bacterial suspension (10^9 CFUs/mL) per mouse. Eight weeks after the first immunization, the mouse showing the highest antibody titre received a booster injection with 400 µL of bacterial suspension (5×10^9 CFUs/mL) and was used for hybridoma production. For the injections after 4 and 8 weeks, incomplete adjuvant was used. The fusion of myeloma and spleen cells, the propagation of cell clones, and the selection by screening with indirect ELISA followed the scheme of Jordan (1990). The procedure resulted in two different cell clones producing monoclonal antibodies against JM22.

Both Cocalico Biologicals and Auburn University Hybridoma Facility took care of the animals in accordance with approved guidelines of the NIH Guide for Care and Use of Laboratory Animals (1985, published by National Institute of Health, Bethesda, MD 20892, U.S.A.). The use of animals was reviewed and approved by the appropriate animal care review committee at the institutions where the experiments were carried out.

Enzyme-linked immunosorbent assay (ELISA)

Polyclonal antibodies

Antiserum was purified by ammonium sulphate precipitation and chromatography on a protein A – agarose column (Affi-Gel Protein A MAPS II Kit, Bio-Rad). The immunoglobulin G (IgG) concentration was adjusted using a spectrophotometer (Milton Roy) to an extinction of 1.4 at a wavelength of 280 nm, which is equivalent to approximately 1 mg protein/mL. The polyclonal IgG solution (1 mL) was used to couple the IgG with alkaline phosphatase (AP) (Avrameas 1969) for application in direct double antibody sandwich (DAS) – ELISA, tissue printing, and dot blot assay. DAS–ELISA followed the scheme of Casper and Meyer (1981).

The following buffers were used: coating buffer, 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$/L double distilled water, pH 9.6; washing buffer, PBS plus 0.05% Tween; sample and conjugate buffer, PBS plus 2% polyvinylpyrrolidone, 0.2% bovine serum albumin (BSA), and 0.05% Tween; substrate buffer, 10% diethanolamine adjusted to pH 9.8 with 0.1 M HCl.

The assay was performed in 96-well (flat bottom), polystyrene microtiter plates (Falcon 3912, MicroTest III™, Becton Dickinson, Calif.). The substrate *p*-nitrophenylphosphate (1 mg/mL) was used and hydrolyzed enzyme substrate was detected by measuring the absorbance at 405 nm in an ELISA reader (Dynatech MR 700) after 10-min and 1-h incubations at room temperature. The ELISA plates were then stored at 4°C for 16 h and read again. This additional incubation can increase the sensitivity of the assay without increasing nonspecific reactions (Giersiepen 1993). An absorbance higher than twice the negative control was considered positive.

Monoclonal antibodies

For immunological experiments with monoclonal antibodies, IgG from supernatants of growing cell clones were purified as described above and used in indirect ELISA. The buffer system was the same as for polyclonal antibodies. Microtiter plates were coated with bacterial suspensions or the analysis samples and incubated at 4°C for 16 h. Plates were then washed three times, after which wells were blocked with 1% BSA in conjugate buffer for 1 h and incubated with primary antibodies for 3 h at 37.5°C. After incubation, another three washes were conducted and secondary antibodies (goat anti-mouse) coupled with alkaline phosphatase were applied for 1 h at 37.5°C. After adding the substrate *p*-nitrophenylphosphate (1 mg/mL), color development was recorded with an ELISA reader as described above.

The sensitivity of ELISA with polyclonal and monoclonal antibodies was determined by comparing ELISA with dilution plating. Strain JM22 was serially diluted in sterile PB or in plant sap and 100-μL aliquots of each 10-fold dilution (10^{-1}–10^{-10}) were plated on tryptic soy agar (TSA). The plant sap was prepared by triturating plant tissue with sterile PB at a ratio of 1:1 w/v. The mixture of plant sap with JM22 was used to determine if contents of the plant sap interfered with the antigen–antibody reaction. For ELISA, 200-μL aliquots of each dilution were transferred into wells of ELISA plates. The cell concentration was determined for each dilution. These data were compared with corresponding ELISA extinctions and the detection limit for ELISA with polyclonal or monoclonal antibodies was reported in CFUs per millilitre.

Tissue printing

The tissue-printing technique, in which plant tissue is pressed on a nitrocellulose membrane, was used to detect bacterial colonization of roots, stems, cotyledons, and leaves. For leaves, the lower leaf side was rubbed with carborundum prior to pressing to remove the lower epidermal layer, allowing more distinct prints (Quadt et al. 1996). Tissue blots were blocked overnight at room temperature with PBS plus 2% BSA and 2% polyvinylpyrrolidone (pH 7.4). Blots were then washed three times with PBS plus 0.05% Tween (pH 7.4), incubated with primary polyclonal antibodies coupled with AP for 2 h, and washed again three times. Thereafter, the blots were immersed in a substrate solution consisting of 66 μL nitroblue tetrazolium solution (75 mg nitroblue tetrazolium/mL 70% dimethylformamide) and 25 μL 5-bromo-4-chloro-3-indolyl-phosphate solution (50 mg 5-bromo-4-chloro-3-indolyl-phosphate/mL 100% dimethylformamide) for color development. The enzyme–substrate reaction was stopped by washing the blot in 0.02 M Tris(hydroxymethyl)aminomethane hydrochloride (TBS) (pH 7.5).

Dot blot

The procedure for dot blots was the same as described for tissue prints. For the determination of detection limits for dot blots, a 10-fold dilution series (10^{-1}–10^{-10}) of approximately 100 mg bacteria in sterile PB as well as in plant sap was used. Plant sap was produced by triturating plant tissue with sterile PB at a ratio of 1:5 w/v. Aliquots of 100 μL were plated on tryptic soy agar (TSA) to count developing colonies and determine the bacterial concentration for each dilution, and 20-μL aliquots were used for the dot blot technique. Analysis of plant tissue samples was conducted by triturating tissue in five times (w/v) sterile PB and then placing 20 μL on nitrocellulose membranes.

Immunogold labeling

Plant material, cut in approximately 2 × 4 mm pieces, was fixed in Karnovsky's fixative (Karnovsky 1965) for 1–2 h and then washed five times for 15 min each with 0.2 M sodium cacodylate buffer (pH 7.35). Samples were then dehydrated with increasing ethanol – double-distilled water solutions: 20 min in 15, 30, and 50% ethanol, as well as two times for 20 min in 70, 90, and 96% ethanol. After the last dehydration step, samples were incubated 16 h in 1:3, 8 h in 1:1, and 16 h in 3:1 London resin (LR) white (medium grade) – ethanol solution, and finally for 3–5 days in 100% LR white (Electron Microscopic Sciences, Pa.). Polymerization was done in gelatin capsules at 50°C for 48 h. Ultrathin sections of 60–100 nm were cut with a diamond knife using a Sorvall Porter-Blum MT2-B Ultra Microtome (Du Pont Instruments).

The procedures for drop preparations of bacterial suspensions, triturated plant tissue, and immunogold labeling of ultrathin plant tissue sections followed the method described by Brown et al. (1993). Nickel grids with bacteria or ultrathin tissue sections were incubated on blocking buffer (20 mM TBS plus 2% BSA, pH 7.4) for 30 min at room temperature. The samples were then incubated on a solution of JM22-specific primary antibodies in TBS (20 mM, pH 7.4) for 16 h at 4°C, followed by treatment with a solution of secondary antibodies in TBS (20 mM, pH 7.4) for 30 min at room temperature. In these experiments, goat anti-rabbit and goat anti-mouse antibodies coupled with 10 nm colloidal gold particles (Electron Microscopic Sciences, Pa.) were used as secondary antibodies. After staining with a 2% solution of uranyl acetate, the sections were evaluated for bacteria labeled with gold with a Zeiss EM 10.

Specificity of polyclonal and monoclonal antisera

Antibody specificity was determined with ELISA by testing against the following: (*i*) seven bacterial strains commonly isolated from cotton such as *Serratia marcescens*, *Serratia plymuthica*, *Serratia proteamaculans*, *Burkholderia solanacearum*, *Agrobacterium radiobacter*, *Bacillus pumilis*, and *Bacillus megaterium* (McInroy and Kloepper 1995*b*); (*ii*) strains closely related to JM22 such as 2 *Pantoea agglomerans*, 37 *Enterobacter asburiae*, 40 *Enterobacter cancerogenus*, 5 *Enterobacter cloacae*, and 2 *Enterobacter intermedius*; (*iii*) five potentially pathogenic strains such as *Pseudomonas*, *Erwinia*, and *Xanthomonas*; (*iv*) plant sap of six noninoculated cotton and six noninoculated cucumber plants from different field plots; and (*v*) four soil samples diluted 1:10 with sterile PBS. Bacterial identification of the strains listed above was based on gas chromatographic analysis of fatty acid methyl esters (FAMEs) of total cellular fatty acids (Sasser 1990). Extraction and gas chromatography followed the methods of McInroy and Kloepper (1995*b*).

Bacterial colonization of plants

Cucumber (*Cucumis sativus* cv. SMR 58), bean (*Phaseolus vulgaris* cv. Kentucky Wonder), and cotton (*Gossypium hirsutum* cv. DP 50) were grown in heat-sterilized natural ground clay (Moltan Company, Middleton, Tenn.) under greenhouse conditions and fertilized once with 25 mL of Peter's fertilizer solution (20-20-20) (Scotts, Sierra, Marysvilles, Ohio) 1 week after planting.

Enterobacter asburiae JM22 was grown for each experiment on TSA for 24 h at 28°C. Seeds were treated with a mixture consisting of 100 mg bacteria and 2 mL of 2% methyl cellulose solution/20 seeds. The seeds were soaked in this suspension for 30 min, dried under a laminar flow hood, and planted. This procedure resulted in approximately 1.0×10^9 CFUs/seed for each plant species.

Surface disinfestation of all plant samples was carried out with 1.05% NaOCl for 1 min except for cucumber roots, which were

disinfested with 0.53% NaOCl for 1 min. After surface disinfestation, samples were immediately washed three times in sterile PB. Plant tissue was then pressed on TSA as a sterility check. If bacterial growth was observed on TSA, the disinfestation was considered as incomplete and the samples were discarded. In addition, complete surface disinfestation was checked for five selected plant samples by embedding, sectioning, and microscopic analysis of surface-disinfested tissue for the existence of bacteria on the tissue surface.

The experiments analyzing population densities and colonization patterns of JM22 in cucumber, bean, and cotton plants consisted of two treatments: seed treatment with JM22 and nontreated plants. For each plant species, the experiment was set up as a randomized complete block design with 15 replicates per treatment, divided into three subsamples of five plants each for analysis of (*i*) total bacterial population of rhizoplane and internal root tissue; (*ii*) endophytic colonization of roots, stems, cotyledons, and leaves after surface disinfestation; and (*iii*) distribution pattern of JM22 in roots and stems after surface disinfestation. The latter analysis was accomplished by separating root tissue into side roots and the main root and cutting stems into 2-cm pieces. After surface disinfestation, tissue was triturated in sterile PB at a ratio of 1:5 w/v and 100-μL aliquots were plated on TSA. TSA plates were incubated at 28°C for 24–48 h and total CFUs per gram of plant tissue was determined. To confirm colonization with JM22, 10 representative bacterial colonies from each TSA plate were checked with ELISA against JM22-specific antibodies. In addition, 200-μL aliquots of each trituration sample were analyzed directly with ELISA using the polyclonal antibodies against JM22. For testing the ability of the dot blot method to detect JM22 in plant tissue, aliquots of 20 μL of the trituration suspensions were placed on nitrocellulose membranes.

The influence of plant sap of different plant species on the growth of JM22 was also investigated. Four bean, four cucumber, and four cotton plants were triturated separately with sterile PB at a ratio of 1:1 w/v. Bacteria (200 mg) were suspended in 10 mL sterile PB and 0.5-mL aliquots of this suspension were added to 1 mL bean, cucumber, or cotton plant sap, as well as to four times 1 mL sterile PB. After 3 h at room temperature, samples were serially diluted (10^{-1}–10^{-10}) and 0.1-mL aliquots were plated on TSA. Plates were incubated at 28°C for 24 h and total CFUs per millilitre were determined for the different samples.

For the detection and localization of JM22 using tissue printing, five plants of each species (cucumber, bean, and cotton) were seed treated with JM22 and harvested 2 weeks after planting. After surface disinfestation, representative samples of roots, stems, cotyledons, and leaves were printed separately on nitrocellulose membranes. Comparable plant tissues from five nontreated plants of each species were used as negative controls. The tissue printing was carried out following the procedure described above. Colonization of plant tissue by JM22 was indicated by development of a purple color.

The experiments analyzing leaf application of JM22 consisted of two treatments: inoculation of cotyledons or leaves with bacterial suspension either with or without wounding. Therefore, 1 mL of JM22 suspension (1.0×10^9 CFUs/mL) with or without carborundum was pipetted on the leaf surface of two cotyledons or leaves per plant and dispersed. For cotyledons, the two treatments were replicated eight times and for leaves, four times. To prevent soil contamination from the leaves, the pot surfaces were covered with plastic wrap. Two weeks after inoculation, cotyledons and roots were harvested and surface disinfested. The tissues were then triturated with sterile PB at a ratio of 1:5 w/v and 100-μL aliquots were plated on TSA. After incubation at 28°C for 24–48 h, CFUs/per gram plant tissue were determined. Furthermore, 10 representative bacterial colonies per TSA plate were checked with ELISA to confirm the identity of JM22.

The ability of antibodies applied with immunogold labeling to detect JM22 was tested for bacteria in sterile PB, as well as in plant sap. Bacterial suspensions were prepared by suspending 10 mg of JM22 or *Pseudomonas fluorescens* 89B-61, both grown for 24 h on TSA, in 2 mL sterile PB. Plant sap from two cucumber, bean, and cotton plants seed treated with JM22 was prepared by trituration of the roots with PB at a ratio of 1:5 w/v. Bacterial suspensions and plant sap samples were used for drop preparations. Furthermore, we tried to detect the precise localization of JM22 inside the roots of four seed-treated plants of each species, as well as inside four JM22-inoculated cotton cotyledons. Root and cotyledon tissues for inoculated and noninoculated plants were embedded, sectioned, and analyzed by immunogold labeling as described above.

Detection of JM22 in plant growth media

Experiments were designed to analyze the feasibility of ELISA for the detection of bacteria in different growth media using antibodies raised against JM22. JM22 was chosen as a representative for other soil bacteria. Five different growth media were investigated: sand; natural ground clay; Pro-Mix soilless mix, a peat-based growth medium (75–85% peat) (Premier Peat Ltd., Rivière-du-Loup, Que.); sandy loam field soil; and loamy sand field soil. A suspension of JM22 in PB (5 ml; 1.0×10^9 CFUs/mL) were added to 10-g samples of the different growth media. After 1 h, growth media were mixed with 90 mL distilled water and thoroughly shaken for 5 min. Aliquots of 200 μL were checked with ELISA plates for detection of JM22. As a negative control, each growth medium was treated with sterile PB. Each treatment was replicated five times.

Results

Sensitivity of the marking systems

Optimal dilutions for polyclonal antibodies using ELISA were 1:1000 for IgG and 1:500 for IgG coupled with AP. Monoclonal IgG showed the highest ELISA extinctions for dilutions of 1:100.

The sensitivity of ELISA using polyclonal antibodies against JM22, determined by combining ELISA with dilution plating, showed a detection limit of 1.0×10^4 CFUs/mL for bacteria in PB if the plate was allowed to develop overnight at 4°C. For bacteria in plant sap the detection limit was 1.0×10^5 CFUs/mL. ELISA with monoclonal antibodies showed a higher detection limit of 1.0×10^6 CFUs/mL for bacteria in PB. The detection limits for the dot blot assays were 1.0×10^5 CFUs/mL for bacteria in PB and 1.0×10^6 CFUs/mL for bacteria in plant sap using polyclonal antibodies. Monoclonal antibodies recognized bacteria in dot blots only at 1.0×10^7 CFUs/mL. The most sensitive immunological method was the tissue printing. Color development on the nitrocellulose membranes, as a result of the specific antigen–antibody reaction, was consistently observed for plant tissues with minimum bacterial concentrations of 1.0×10^3 CFUs/mL, including cotyledons and stems of cucumber plants as well as roots of cotton plants.

Specificity of antisera

Both the polyclonal and monoclonal antibodies were able to differentiate *Enterobacter asburiae* JM22 from other bacterial genera, including *Pseudomonas*, *Erwinia*, *Xanthomonas*, *Burkholderia*, *Agrobacterium*, and *Bacillus*, with ELISA (Table 1). Polyclonal antibodies showed a positive immunological reaction for 75 *Enterobacter* strains and for the closely related genera *Pantoea* and *Serratia* (Table 1). In contrast, the monoclonal antibodies reacted positively with 81 of 84 *Enterobacter* strains tested but did not react with the more closely related *Pantoea* or *Serratia* strains, indicating a higher specificity than the polyclonal antibodies (Table 1). Neither

Table 1. Immunological specificity of polyclonal (PAb) and monoclonal (MAb) antibodies raised against JM22.

Antigens (bacteria species)	Total no. of tested strains	Immunological reaction: No. of strains positive with PAb	Immunological reaction: No. of strains positive with MAb
Enterobacter asburiae	37	35	37
Enterobacter cancerogenus	40	37	38
Enterobacter cloacae	5	2	4
Enterobacter intermedius	2	1	2
Pantoea agglomerans	2	2	0
*Serratia marcescens**	1	1	0
*Serratia plymuthica**	1	1	0
*Serratia proteamaculans**	1	1	0
Pseudomonas spp.	3	0	0
Erwinia uredovora	1	0	0
Xanthomonas maculans	1	0	0
*Burkholderia solanacearum**	1	0	0
*Agrobacterium radiobacter**	1	0	0
*Bacillus pumilis**	1	0	0
*Bacillus megaterium**	1	0	0

*Endophytic bacteria commonly isolated from cotton plants.

Table 2. Population density determined by dilution plating on TSA combined with ELISA and immunological detection of JM22 in tissues of 2-week-old plants after surface disinfestation.*

Plant species	Plant tissue	Dot blot PAb	Tissue printing PAb	Concentration of JM22 (CFUs/g plant tissue)
Bean	Root (no SD†)	+	+	10^7
Bean	Root	−	+	10^5
Bean	Cotyledons	−	−	10^3
Bean	Stem	−	+	10^4
Bean	Leaves	−	−	10^3
Cucumber	Root (no SD)	+	+	10^6
Cucumber	Root	−	+	10^4
Cucumber	Stem	−	+	10^3
Cucumber	Cotyledons	−	+	10^3
Cucumber	Leaves	−	−	<50
Cucumber	Flowers	−	−	<50
Cotton	Root (no SD)	+	+	10^6
Cotton	Root	−	+	10^3
Cotton	Stem	−	−	10^1
Cotton	Cotyledons	−	−	10^3
Cotton	Leaves	−	−	<50

Note: +, positive; −, negative.

* If not mentioned otherwise, plant tissue was surface disinfested in 1.05% NaOCl for 1 min

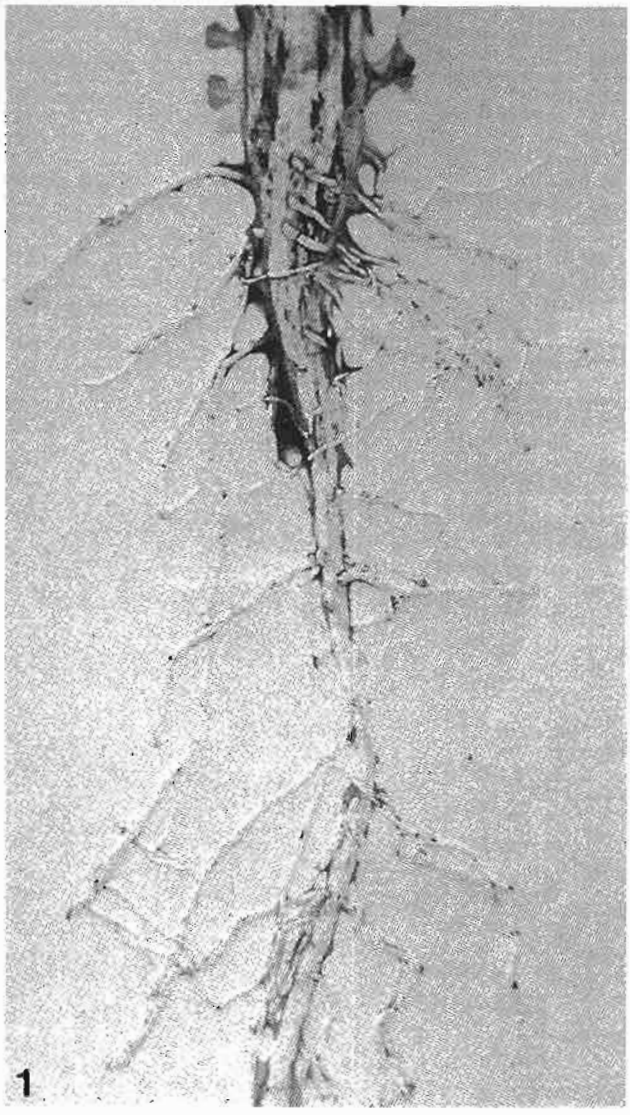
† Surface disinfestation.

type of antibody reacted with plant sap from field-grown cotton and cucumber plants, or with four different soil dilutions.

Bacterial colonization of plants

After embedding and sectioning surface-disinfested plant tissue, no bacteria were visible on the tissue surface or trapped under collapsed epidermal root cells. Strain JM22 was detectable by trituration of plant tissue and plating on TSA in roots, stems, and cotyledons of all plant species, as well as in bean leaves (Table 2). No bacteria were detected in cucumber flowers or leaves of cucumber and cotton plants. The highest bacterial concentration appeared in the root tissue, reaching

Fig. 1. Tissue print of a cotton root, which developed from a plant that was seed treated with JM22 (1.0×10^9 CFUs/seed). The high concentration of JM22 in upper root tissue is indicated by strong color development.



1.0×10^3 CFUs/g for cotton roots, 1.0×10^4 CFUs/g for cucumber roots, and 1.0×10^5 CFUs/g for bean roots. The population density of JM22 in stems and cotyledons of all plant species showed values of about 10^3 CFUs/g plant tissue.

Using tissue printing, we observed that the stem base and upper root showed the highest bacterial concentrations, indicated by strong color development. The high concentration of JM22 in upper root tissue is illustrated in Fig. 1 for the root of a cotton plant seed treated with JM22. High levels of variability in bacterial concentrations within and between plants were observed.

The lowest bacterial concentrations were detected in cotton plants. The inhibitory effect of cotton plant sap on bacterial growth was expressed by a significant reduction in population size on TSA following the bacterial treatment with sap of triturated cotton plants (Table 3). In contrast, the treatment of bacteria with cucumber sap resulted in a significant increase in population size on TSA (Table 3). Bean sap also tended to increase bacterial growth but not significantly (Table 3).

Table 3. Influence of plant sap* from different plant species on growth of JM22 on TSA† ($n = 4$).

Treatment	Log(CFUs/mL)
Phosphate buffer	9.01*a*
Bean sap	9.06*ab*
Cucumber sap	9.11*b*
Cotton sap	8.92*c*
LSD ($P < 0.05\%$)	0.06

Note: Means with the same letter are not significantly different at $P < 0.05\%$. LSD, least significant difference. LSD values are for comparison of means within the column above each value.

* Plant tissue diluted with sterile PB at a ratio of 1:1 w/v.

† Bacteria treated with plant sap for 3 h at room temperature and then plated on TSA.

Table 4. Endophytic colonization of cotton cotyledons and roots 2 weeks after inoculation of cotyledons with JM22 (with or without carborundum; $n = 8$).

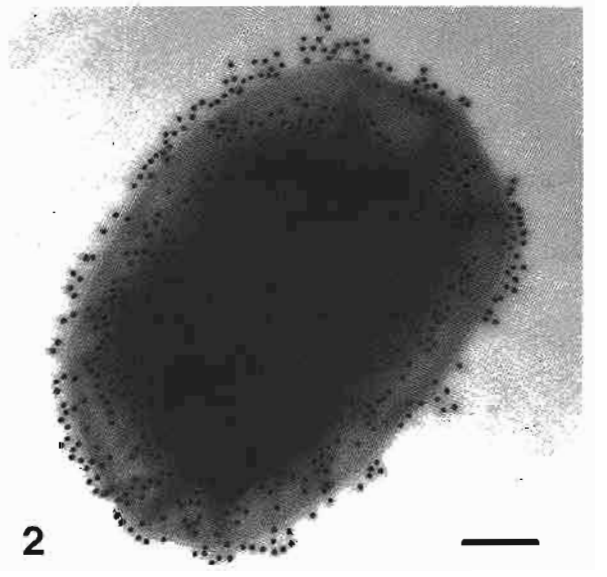
	Log(CFUs/g root)	Log(CFUs/g cotyledon)
Without carborundum	3.55*a*	2.85*b*
With carborundum	2.18*b*	5.72*a*
LSD ($P < 0.05\%$)	1.36	0.67

Note: Means with the same letter are not significantly different at $P < 0.05\%$. LSD, least significant difference. LSD values are for comparison of means within the column above each value.

Identification of JM22 directly in plant tissue using ELISA was only possible for bacterial concentrations higher than 10^5 CFUs/g plant tissue. Nevertheless, after plating and incubating plant sap on TSA, the developing colonies could always be identified with ELISA. Concentrations of JM22 lower than 10^6 CFUs/g plant tissue did not show a reaction with the dot-blot technique (Table 2). For cucumber cotyledons and stems as well as cotton roots, detection of JM22 with the tissue-printing technique was possible with bacterial concentrations of 10^3 CFUs/g plant tissue (Table 2).

After inoculation of the cotyledon surfaces, JM22 entered internal tissues of the cotyledons and colonized the roots (Table 4). Wounding of cotyledons with carborundum effected a significant increase in the endophytic population of JM22 compared with inoculation without carborundum (Table 4). In contrast, the population of JM22 in roots was significantly higher for plants inoculated without carborundum. This difference in JM22 density was only observed 2 weeks after inoculation. At 4 weeks after inoculation, no differences in bacterial concentrations of the roots were found for both inoculation methods with and without carborundum. Comparable to the population densities obtained after inoculation of cotyledons, leaf inoculation with JM22 resulted in bacterial populations of approximately 10^4 CFUs/g leaf or root tissue 2 weeks after inoculation, indicating bacterial penetration in leaves and movement from the leaves to the roots (Table 5). Wounding of

Fig. 2. Drop preparation and immunogold labeling of JM22 applying primary IgG at a dilution of 1:500 and secondary IgG at 1:40. Bacterial cells are uniformly labeled with colloidal gold. Scale bar = 0.3 μm.



leaves with carborundum during the inoculation also significantly increased populations of JM22 in the leaves.

Drop preparations combined with immunogold labeling of JM22 in sterile PB showed bacteria uniformly labeled with gold particles (Fig. 2). Bacteria from plant tissues showed a less intensive but still clearly visible gold label. *Pseudomonas fluorescens*, used as a negative control, was not labeled (not shown). Monoclonal antibodies showed a less intensive gold label of JM22 cells than polyclonal antibodies. In addition, monoclonal antibodies were bound only to membrane proteins, whereas polyclonal antibodies were bound to both membrane and flagellar proteins. The optimal dilution for polyclonal antibodies, monoclonal antibodies, and goat anti-rabbit or goat anti-mouse antibodies were 1:500, 1:100, and 1:40, respectively.

In ultrathin sections of JM22-inoculated cucumber, bean, cotton roots, and cotton cotyledons, unspecific reactions of the polyclonal and monoclonal antibodies with cell walls or cell organelles were very low. JM22 could be identified and precisely localized on root surfaces and inside root tissues of all plant species following a seed treatment. The bacteria colonized the root surface, and concentrated in grooves between epidermal cells and below collapsed root epidermal cells (Figs. 3 and 4). All bacterial cells showed an intensive gold label. In spite of the relatively low numbers of bacteria in plant tissue, single bacterial cells inside epidermal root cells of cotton plants could be detected. High numbers of JM22 cells were located in intercellular spaces of the root cortex close to the conducting elements (Figs. 5*a* and 5*b*). After inoculation of cotyledons, JM22 bacterial cells were detected on the surface of the inoculated plant tissue (not shown).

Detection of JM22 in plant growth media

Measuring the optical density at a wavelength of 405 nm for the different plant growth media after conducting ELISA, comparable extinctions were detected for sand, ground clay, and loamy sand with means ranging from 1.414 to 1.536 (Table 6). Sandy loam only reached ELISA readings with a mean of 0.750, which was significantly lower than the ones for sand, ground clay, and loamy sand, but significantly higher than the mean 0.378 for Pro-Mix. The mean extinctions of the negative controls were significantly lower than all other extinctions, confirming high specificity of the antibodies.

Table 5. Endophytic colonization of cotton leaves and roots 2 weeks after inoculation of leaves with JM22 (with or without carborundum; $n = 4$).

	Log(CFUs/g root)	Log(CFUs/g leaf)
Without carborundum	4.08*a*	3.68*b*
With carborundum	3.96*a*	4.73*a*
LSD ($P < 0.05\%$)	1.24	0.95

Note: Means with the same letter are not significantly different at $P < 0.05\%$. LSD, least significant difference. LSD values are for comparison of means within the column above each value.

Table 6. ELISA extinctions for JM22 in different growth media applying polyclonal antibodies.

Growth media	ELISA extinction (means of five replications)
Sand	1.460*a*
Ground clay	1.536*a*
Pro-Mix	0.378*c*
Loamy sand	1.414*a*
Sandy loam	0.750*b*
Negative control* × 2	0.020*d*
LSD ($P < 0.05\%$)	0.144

Note: ELISA plate was incubated for 16 h at 4°C; optical density was measured at 405 nm. Means with the same letter are not significantly different at $P < 0.05\%$. LSD, least significant difference. LSD values are for comparison of means within the column above each value.

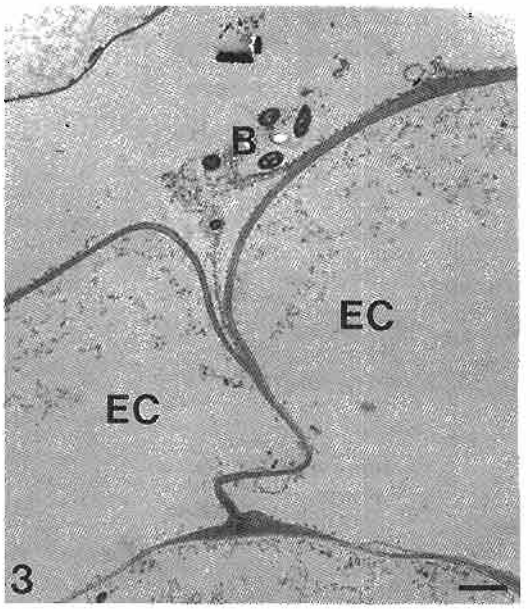
*Ground clay plus sterile phosphate buffer (pH 7.0) as a representative for the other growth media.

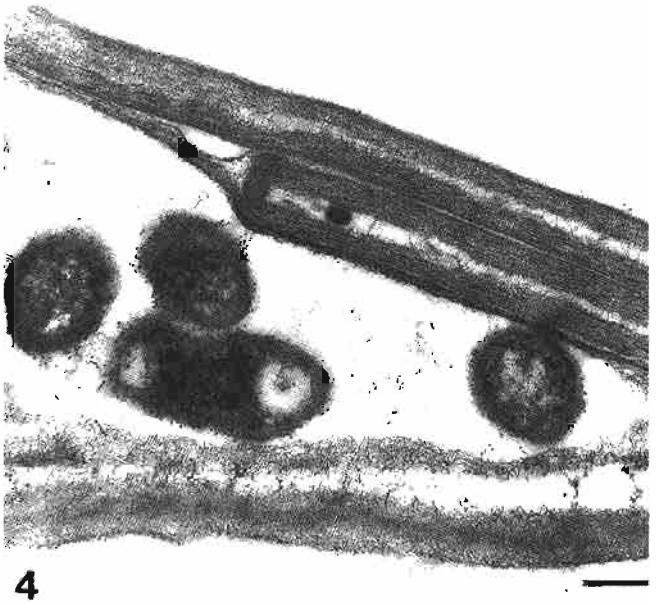
Discussion

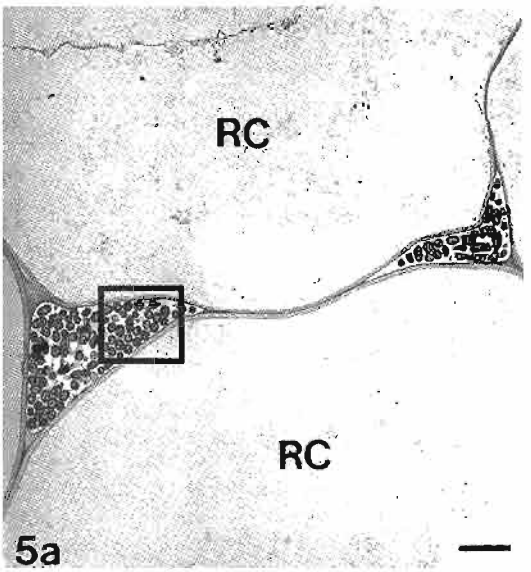
The results reported here demonstrate clearly that immunological methods can be used to detect introduced endophytic bacterial strains. These methods allowed the detection, identification, and precise localization of the model endophyte *Enterobacter asburiae* JM22 inside various tissues of different plant species. With seed treatment and leaf application it was possible to introduce JM22 into cotton plants. In addition, JM22 was detected in different plant growth media with ELISA using polyclonal antibodies.

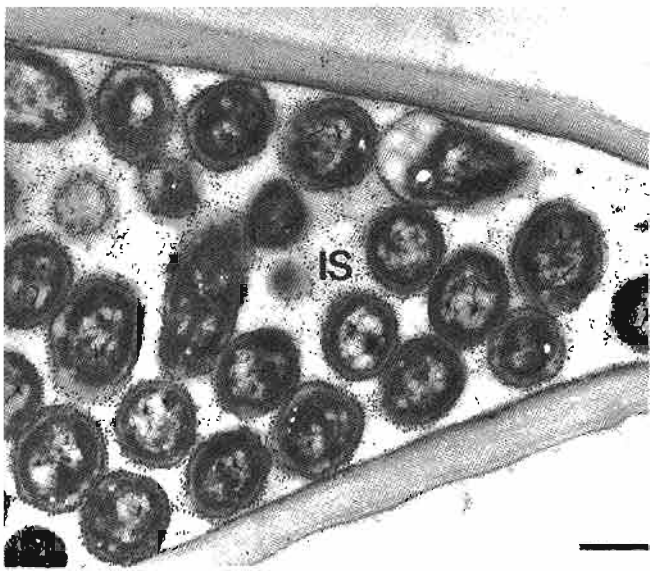
The detection limits determined here of 1.0×10^4 CFUs/mL for purified bacteria and 1.0×10^5 CFUs/mL for bacteria in plant sap with ELISA, indicating an influence of plant contents

Figs. 3–5. Transmission electron microscopy of ultrathin sections of the root from a cotton plant that was seed treated with JM22 (1.0×10^9 CFUs/seed). Identification of the bacteria by immunogold labeling applying primary IgG at a dilution of 1:500 and secondary IgG at 1:40. Fig. 3. JM22 is located on the root surface, concentrated in grooves between epidermal cells (EC). B, bacteria. Scale bar = 4 μm. Fig. 4. Gold-labeled cells of JM22 are located below collapsed epidermal root cells. Scale bar = 1 μm. Figs. 5*a* and 5*b*. High numbers of JM22 cells are located in intercellular spaces of the inner root cortex. (*a*) Root cortex cell (RC). Scale bar = 6 μm. (*b*) Higher magnification of the area in the black frame presented in *a*; gold label identifies bacteria as JM22. IS, intercellular space. Scale bar = 1 μm.









on the immunological detection, are comparable to the detection limits described in the literature for plant-associated bacteria (Schaad et al. 1990). Ruppel et al. (1992) reported a detection limit of 1.0×10^5 cells/mL for *Pantoea agglomerans*, a bacterium closely related to *Enterobacter asburiae*, in winter wheat or nutrient broth.

The monoclonal antibodies from our experiments showed higher specificity than the polyclonal antibodies, but they still cross-reacted with closely related bacterial genera. This is a common phenomenon, known to occur for antigens that differ by a single amino acid (Fazekas de St. Groth 1985). The immunization of mice with glutaraldehyde-fixed cells of JM22 resulted in only two cell clones producing monoclonal antibodies that seemed to be directed against epitopes common to all *Enterobacter* strains, as positive immunological reactions resulted with *Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter cloacae*, and *Enterobacter intermedius*. Monoclonal antibodies demonstrated a slightly higher detection limit than the polyclonal antibodies, which may also be a function of the reaction with a single epitope, resulting in fewer

bound antibodies, and lower levels of conjugate enzyme for substrate transformation.

Tissue printing and immunogold labeling were the most sensitive immunological procedures tested. For the tissue printing, this may be explained through the immediate transfer of bacteria to membranes reducing the exposure to plant cell components. In the case of immunogold labeling, the fixative used for embedding the plant tissue deactivates cellular contents such as hydrolytic enzymes, thereby immediately avoiding further degradation or blocking of bacterial epitopes. Immunological reactions of the bacteria are, therefore, preserved. Trituration of plant tissue, which is required for ELISA and dot blot assays, resulted in higher detection limits (lower sensitivity). This might be due to the release of specific vacuolar ingredients that may have a destructive effect on epitopes of polyclonal and monoclonal antibodies. The increase of the detection limit for bacteria in plant sap compared with bacteria in sterile PB using ELISA or the dot blot technique supports this conclusion.

The marking systems developed based on immunological methods confirmed the endophytic colonization of different plant species by JM22. In the past, the existence of endophytic bacteria has mainly been described by isolation of bacteria from surface-disinfested plant tissues (Misaghi and Donndelinger 1990; McInroy and Kloepper 1995*a*, 1995*b*), which is dependent on a complete surface disinfestation. Comparing several surface disinfestation procedures, Caetano-Anolles et al. (1990) observed that various treatments were effective in reducing bacterial contaminations on the root surface, but they never reached complete surface disinfestation. The results of the present electron microscopic investigations demonstrate reasons for the difficulties of a complete surface disinfestation: niches between numerous root hairs or below collapsed epidermal cells offer a protection for the bacteria against disinfectants.

Immunogold labeling allowed precise localization of strain JM22. It also overcame the difficulties of direct electron microscopic observation to distinguish microorganisms from cell organelles with any degree of certainty. The bacteria could be detected on the root surface, where they especially concentrated in the grooves between epidermal cells. Similar observations were made by Old and Nicolson (1978), who considered this as a response to greater secretion of nutrients at these junctures. Furthermore, cells of JM22 were located below collapsed epidermal cells, in epidermal cells, and in intercellular spaces of the root cortex close to conducting elements, confirming previous observations with JM22 in bean (Mahaffee et al. 1996) and other endophytes in different plant species (Patriquin and Döbereiner 1978; Gagne et al. 1989).

The results of our investigations concerning colonization of different plant tissues by JM22 after seed or leaf inoculation indicate that bacterial migration takes place inside the plants in two different directions: from the root to the shoot and vice versa. Similar results were obtained for the movement of *Pantoea agglomerans* in wheat (Ruppel et al. 1992). Nevertheless, the transport or movement of JM22 within plants needs further investigation. In the ultrathin root sections examined in this study, no cells of JM22 were visible in the phloem or xylem. In contrast, recent studies of Mahaffee et al. (1996) detected intracellular colonization of vascular tissues by JM22 within bean roots and suggested that endophytic bacteria may enter the vascular tissue by colonizing the undifferentiated root-tip tissue that eventually becomes vascular tissue. It remains uncertain whether single cells of JM22 move through the conducting elements of all plant species.

Leaf inoculation with carborundum significantly increased internal bacterial concentrations, indicating the importance of wounds for bacterial entrance. This may be due to increased plant metabolic activity in combination with nutrient leakage, as well as due to a simplified method of entry for the bacteria. Jacobs et al. (1984) and Mahaffee et al. (1996) also found increased bacterial populations in association with wounds, such as in the secondary root emergence zone where roots break through the cortex.

JM22 seems to develop a close association within the plant. The electron microscopic investigations of cucumber, bean, or cotton plants did not show any defense reactions in cells, although population densities up to 10^5 CFUs/g plant tissue were detected. Comparable interactions have been described for *Pantoea agglomerans* in wheat (Ruppel et al. 1992), *Azospirillum* in sorghum or wheat (Boddey and Döbereiner 1988), and *Azotobacter paspali* in *Paspalum notatum* (Döbereiner et al. 1972).

JM22 reached detectable concentrations inside different plants when applied as a seed treatment, although there was variation in population size between the plant species. Bean plants showed the highest population densities of JM22, followed by cucumber plants and then cotton plants. One explanation for such variation may be the influence of plant contents. Inhibitory effects of cellular contents on bacterial growth could be shown with our experiments, in particular for cotton plant sap. In contrast, treatment of bacteria with cucumber or bean sap promoted the growth of JM22 on TSA.

Inoculation of leaves is an alternative to seed application for introducing JM22 into plants. After the inoculation of cotton cotyledons or leaves, JM22 was detectable in the inoculated plant tissue and movement of JM22 to the roots was observed. Bacterial movement from the leaf through the midrib and petiole to the stem is already known for pathogenic bacteria such as *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker of kiwifruit (Serizawa and Ichikawa 1993). A combination of seed treatment and leaf application could be useful to reach higher population densities of beneficial plant-associated bacteria, which may improve their positive effects.

Immunological detection of bacteria in plant growth media may be influenced by the media type. Using antibodies raised against the endophyte JM22 as a representative for other soil bacteria, the lowest ELISA extinctions were obtained for Pro-Mix. Extinctions for sand, ground clay, sandy loam, and loamy sand were significantly higher. The reasons for this influence are probably based on different soil contents. Pro-Mix consists mostly of peat, which contains a high proportion of organic matter, and therefore, of many humic substances. Fulvic acids, humic acids, and humins are known for different adsorption mechanisms: (*i*) van der Waal's attractions, (*ii*) hydrophobic bonding, (*iii*) hydrogen bonding, (*iv*) charge transfer, (*v*) ion exchange, and (*vi*) ligand exchange (Choudhry 1984). In addition, humic substances are rich in stable free radicals, which most likely play important roles in reactions with other molecules (Choudhry 1984). One possibility is, therefore, that the soil contents adsorb the bacteria, so that they cannot react with antibodies. Another possibility is that the free radicals alter the

bacterial epitopes so that they are not longer recognizable by the antibodies. In addition, impurities of the sample solutions may affect the immunological detection, as it is already well documented for the isolation and detection of bacterial nucleic acids from soil (Van Elsas and Waalwjick 1991; Tsai and Olson 1992).

In conclusion, we demonstrated that with special attention to the soil type, immunological techniques can be used to detect bacteria in plant growth media. Furthermore, we confirmed that endophytic bacteria can enter and systemically colonize different plant species following introduction via seed treatment or leaf inoculation, and that colonization can be monitored using immunological methods. These techniques should have broad applicability for efforts to use endophytic bacteria as components in sustainable agriculture.

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References

Allan, E., and Kelman, A. 1977. Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. Phytopathology, **67**: 1305–1312.

Avrameas, S. 1969. Coupling of enzymes to protein with glutaraldehyde. Immunochemistry, **6**: 43–52.

Backman, P., Brannen, P.M., and Mahaffee, W.F. 1994. Plant response and disease control following seed inoculation with *Bacillus subtilis*. *In* Improving plant productivity with rhizosphere bacteria. Proceedings of the Third International Workshop on Plant Growth-Promoting Rhizobacteria, Adelaide, South Australia, 7–11 March, 1994. *Edited by* M.H. Ryder, P.M. Stephens, and G.D. Bowen. CSIRO, Australia.

Boddey, R.M., and Döbereiner, J. 1988. Nitrogen fixation associated with grasses and cereals: recent results and perspectives for future research. Plant Soil, **108**: 53–65.

Brown, I., Mansfield, J., Irlam, I., Conrads-Strauch, J., and Bonas, U. 1993. Ultrastructure of interactions between *Xanthomonas campestris* pv. *vesicatoria* and pepper, including immunocytochemical localization of extracellular polysaccharides and the AvrBs3 protein. Mol. Plant-Microbe Interact. **6**: 376–386.

Brown, M.E., Burlingham, S.K., and Jackson, R.M. 1964. Studies on *Acetobacter* species in soil. III. Effects of artificial inoculation on crop yields. Plant Soil. **20**: 194–214.

Caetano-Anolles, G., Favelukes, G., and Bauer, W.D. 1990. Optimization of surface sterilization for legume seed. Crop Sci. **30**: 708–712.

Casper, R., and Meyer, S. 1981. Die Anwendung des ELISA-Verfahrens zum Nachweis pflanzenpathogener Viren. Nachrichtenbl. Dtsch. Pflanzenschutzdienstes (Braunschweig), **23**: 49–54.

Chen, Y., Mei, R., Lu, S., Liu, L., and Kloepper, J.W. 1995. The use of yield increasing bacteria (YIB) as plant growth-promoting rhizobacteria in Chinese agriculture. *In* Management of soilborne diseases. *Edited by* R. Utkheade. M/S Kalyani Publishers, New Delhi, India. pp. 164–184.

Choudhry, G.G. 1984. Humic substances. Structural, photophysical, photochemical and free radical aspects and interactions with environmental chemicals. Gordon and Breach Science Publishers, Inc., London. pp. 64–78 and 102–104.

Döbereiner, J., Day, J.M., and Dart, P.J. 1972. Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum* – *Azotobacter paspali* association. J. Gen. Microbiol. **71**: 103–116.

Fazekas de St. Groth, S. 1985. Monoclonal antibody production; principles and practice. *In* Handbook of monoclonal antibodies: applications in biology and medicine. Noyes Publications, Park Ridge, N.J. pp. 1–10.

Gagne, S., Richard, C., and Antoun, H. 1989. Pathogenicity of root xylem bacteria of lucerne. Can. J. Plant Pathol. **11**: 22–27.

Giersiepen, R. 1993. Untersuchungen zur Charakterisierung des Kirschenblattrollvirus (CLRV) als Ursache einer Eschenvirose. Ph.D. thesis, University of Bonn, Bonn, Germany.

Jacobs, M.J., Bugbee, W.M., and Gabrielson, D.A. 1984. Enumeration, location, and characterization of endophytic bacteria within sugar beet roots. Can. J. Bot. **63**: 1262–1265.

Jordan, R.L. 1990. Strategy and techniques for the production of monoclonal antibodies. *In* Serological methods for detection and identification of viral and bacterial plant pathogens. A laboratory manual. *Edited by* R. Hampton, E. Ball, and S. De Boer. APS Press, St. Paul, Minn.

Karnovsky, M.J. 1965. A formaldehyde–glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell. Biol. **27**: 137–138.

Kloepper, J.W. 1993. Plant growth-promoting rhizobacteria as biological control agents. *In* Soil microbial technologies. *Edited by* B. Metting, Marcel Dekker, Inc., New York. pp. 255–274.

Kloepper, J.W., and Beauchamp, C.J. 1992. A review of issues related to measuring colonization of plant roots by bacteria. Can. J. Microbiol. **38**: 1219–1232.

Kloepper, J.W., Zablotowicz, R.M., Tipping, E.M., and Lifshitz, R. 1991. Plant growth promotion mediated by bacterial rhizosphere colonizers. *In* The rhizosphere and plant growth. *Edited by* D.L. Keister and P.B. Cregan. Kluwer Academic Publishers, Boston, Mass. pp. 315–326.

Mahaffee, W.F., van Vuurde, J.W.L., van der Wolf, J.M., Quadt-Hallmann, A., van den Brink, M., and Kloepper, J.W. 1996. Spatial and temporal colonization of *Phaseolus vulgaris* by the bacterial endophytes *Pseudomonas fluorescens* strain 89B-27 and *Enterobacter asburiae* strain JM22. Appl. Environ. Microbiol. In press.

McInroy, J.A., and Kloepper, J.W. 1995*a*. Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. Can. J. Microbiol. **41**: 895–901.

McInroy, J.A., and Kloepper, J.W. 1995*b*. Survey of indigenous bacterial endophytes from cotton and sweet corn. Plant Soil, **173**: 337–342.

Merriman, P.R., and Birkenhead, W.E. 1977. Effects of *Bacillus subtilis* and *Streptomyces griseus* on growth of vegetables. Aust. Plant Pathol. Newsl. **6**: 24.

Misaghi, I.J., and Donndelinger, C.R. 1990. Endophytic bacteria in symptom-free cotton plants. Phytopathology, **80**: 808–811.

Old, K.M., and Nicolson, T.H. 1978. The root cortex as part of a microbial continuum. *In* Microbial ecology. *Edited by* M.W. Loutit and J.A.R. Miles. Springer Verlag, Berlin. pp. 291–294.

Patriquin, D.G., and Döbereiner, J. 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Can. J. Microbiol. **24**: 734–742.

Pleban, S., Ingel, F., and Chet, I. 1995. Control of *Rhizoctonia solani* and *Sclerotium rolfsii* in the greenhouse using endophytic *Bacillus* spp. Eur. J. Plant Pathol. **101**: 665–672.

Qiu, X., Pei, Y., Wang, Y.N., and Zhang, F.X. 1990. Isolation of *Pseudomonas* from cotton plants and their effect on seedling diseases. Acta Phytophylacica Sin. **17**: 303–306.

Quadt, A., Löw, A., and Hamacher, J. 1996. Distribution of cherry leaf roll nepovirus (CLRV) in leaves of deciduous forest trees and herbaceous plants detected by tissue print immunopressblotting (TPI) of whole leaf blades. Z. Pflanzenkrankh. Pflanzenschutz. In press.

Rovira, A.D. 1963. Microbial inoculation of plants. I. Establishment of free-living nitrogen-fixing bacteria in the rhizosphere and their effects on maize, tomato, and wheat. Plant Soil, **19**: 304–314.

Ruppel, S., Hecht-Buchholz, C., Remus, R., Ortmann, U., and Schmelzer, R. 1992. Settlement of the diazotrophic, phytoeffective bacterial strain *Pantoea agglomerans* on and within winter wheat: an investigation using ELISA and transmission electron microscopy. Plant Soil, **145**: 261–273.

Sakthivel, N., Anuratha, C.S., Savithery, S., and Gnamanickam, S.S. 1988. Beneficial bacteria for plant disease management. Advances in research on plant pathogenic bacteria based on the proceedings of the National Symposium on Phytobacteriology held at the University of Madras, India, March 14–15, 1986. Today and Tomorrow's Printers and Publishers, New Delhi, India. pp. 213–220.

Sasser, M. 1990. Identification of bacteria through fatty acid analysis. *In* Methods in phytobacteriology. *Edited by* Z. Klement, K. Rudolph, and D. Sands. Akademiai Kiado, Budapest, Hungary. pp. 199–204.

Schaad, N.W., Süle, S., van Vuurde, J.W.L., Vruggink, H., Alvarez, A.M., Benedict, A.A., de Wael, L., and van Laere, O. 1990. Serology. *In* Methods in phytobacteriology. *Edited by* Z. Klement, K. Rudolph and D.C. Sands. Akademiai Kiado, Budapest, Hungary. pp. 155–186.

Schmidt, E.L., and Paul, E.A. 1982. Microscopic methods for soil organisms. *In* Methods of soil analysis. Part 2. Chemical and microbiological properties. *Edited by* A.L. Page, R.H. Miller, and D.R. Keeney. American Society of Agronomy, Inc., Madison, Wis. pp. 803–814.

Serizawa, S., and Ichikawa, T. 1993. Epidemiology of bacterial canker of kiwifruit. I. Infection and bacterial movement in tissue of new canes. Ann. Phytopathol. Soc. Jpn. **59**: 452–459.

Sharrock, K.R., Parkes, S.L., Jack, H.K., Rees-George, J., and Hawthorne, B.T. 1991. Involvement of bacterial endophytes in storage rots of buttercup squash (*Cucurbita maxima* D. hybrid 'Delica'). N.Z. J. Crop Hortic. Sci. **19**: 157–165.

Sikora, R. 1992. Management of the antagonistic potential in agricultural ecosystems for biological control of plant parasitic nematodes. Annu. Rev. Phytopathol. **30**: 245–270.

Tsai, Y.L., and Olson, B.H. 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58**: 754–757.

Van Baren, A.M., Andre, C., and Ishimaru, C.U. 1993. Biological control of the bacterial ring rot pathogen by endophytic bacteria isolated from potato. Phytopathology, **83**: 1406.

Van Elsas, J.D., and Waalwijk, C. 1991. Methods for the detection of specific bacteria and their genes in soil. Agric. Ecosyst. Environ. **34**: 97–105.

Vruggink, H., and Maas Gesteranus, H.P. 1975. Serological recognition of *Erwinia carotovora* var. *atroseptica*, the causal organism of potato blackleg. Potato Res. **18**: 546–555.

Weller, D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26**: 379–407.