Bacterial endophytes in cotton: mechanisms of entering the plant

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

Abstract: Investigations were conducted to determine how a systemic plant-colonizing bacterium *Enterobacter asburiae* JM22 enters cotton plant tissues. Passive uptake was excluded for JM22 by experimentation with glutaraldehyde-fixed (killed) bacterial cells applied to seeds and leaves; no bacteria were found internally or externally on roots or leaves. In contrast, application of live JM22 cells led to colonization of external and internal root and leaf tissues. Active penetration of JM22 in the absence of external wounding was demonstrated for cotton seedlings germinated on water agar and inoculated with the bacterial suspension. The mean internal bacterial population density for seedlings was 3.8×10^3 CFU/g surface-disinfected radicle tissue. Studies of in planta enzymatic activity demonstrated hydrolysis of wall-bound cellulose in the vicinity of JM22 bacterial cells. The same phenomenon was observed for a cortical root colonizing bacterium, *Pseudomonas fluorescens* 89B-61, a plant growth-promoting strain with biocontrol potential against various pathogens.

Key words: endophytic bacteria, cotton, cell wall hydrolysis.

Résumé : Les recherches menées par les auteurs ont visé à déterminer comment l'*Enterobacter asburiae* JM22, une bactérie qui colonise des végétaux de façon systémique, pénètre dans les tissus du coton. Une entrée passive de JM22 a été exclue, par suite d'une expérience comportant l'application à des graines ou à des feuilles de cellules JM22 fixées (tuées) à la glutaraldéhyde; aucune bactérie n'a été trouvée à l'intérieur ouo à l'extérieur des tissus racinaires ou foliaires. À l'opposé, l'application de cellules vivantes de JM22 s'eset traduite par une colonisation externe et interne des tissus racinaires et foliaires. Une pénétration active de JM22 est démontrée chez des plantules de coton indemnes de toute blessure croissant sur une gélose aqueuse inoculée avec une suspension bactérienne. La densité moyenne des populations bactériennes internes pour les plantules a été de $3,8 \times 10^3$ UFC/g de tissu racinaire désinfecté en surface. Des études sur l'activité enzymatique in planta ont révélé une hydrolyse de la cellulose des parois cellulaires dans le voisinage des cellules de JM22. Le même phénomène a été observé chez une bactérie colonisatrice du cortex racinaire, le *Pseudomonas fluorescens* 89B-61, une souche qui favorise la croissance des plantes avec un potentiel de contrôle biologique contre divers phytopathogènes.

Mots clés : bactéries endophytes, coton, hydrolyse des parois cellulaires.
[Traduit par la rédaction]

Introduction

The existence of endophytic bacteria inside different plant tissues is a well-documented phenomenon (Patriquin et al. 1983; Gardner et al. 1982; Gagné et al. 1987; Sharrock et al. 1991; Fisher et al. 1992; McInroy and Kloepper 1995*a*, 1995*b*). In general, endophytes have been defined as bacteria that are able to colonize living plant tissues without harming the plant or gaining benefit other than securing residency (Kado 1992). Several studies have shown that the interaction between plants and some endophytic bacteria was associated with beneficial effects such as plant growth promotion and biocontrol potential against plant pathogens (Lalande et al. 1989; Bashan et al. 1990; Xiao et al. 1990; Döbereiner 1992; Chen et al. 1995*b*; Hallmann et al. 1995; Pleban et al. 1995).

Although the precise location and spread of endophytic bacteria in plant tissues have been described by several authors (Old and Nicolson 1978; Patriquin and Döbereiner 1978; Jacobs et al. 1984; Gagné et al. 1989; Mahaffee et al. 1997; Quadt-Hallmann and Kloepper 1996), little is known about the mechanisms by which endophytes enter the plant. Huang (1986) summarized the avenues of entry for different plant pathogenic bacteria. Such pathways included stomata (Roos and Hattingh 1983), lenticels (Fox et al. 1971), wounds including broken trichomes, areas of emerging lateral roots (Jacobs et al. 1984; Mahaffee et al. 1997), and the germinating radicle (Gagné et al. 1987). Bacteria may also enter through undifferentiated meristematic root tissue (Hollis 1951; Mahaffee et al. 1997). The lack of penetration structures renders bacteria unable to exert mechanical or physical forces to penetrate intact epidermal cells (Goodman 1982). However, bacteria may enter intact plant tissue by invagination of the root hair cell wall, by penetration of the junction between root hair and adjacent epidermal cells, or by enzymatic processes

Received October 2, 1996. Revision received January 15, 1997. Accepted January 29, 1997.

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involving degradation of cell wall bound polysaccharides (Huang 1986). Alternatively, it may be proposed that bacteria enter the epidermis through passive plant uptake owing to transpiration. Further spread inside the plants may occur via intercellular spaces or conducting elements.

Endophytic colonization of cotton by *Enterobacter asburiae* JM22 and *Pseudomonas fluorescens* 89B-61 has recently been demonstrated after bacteria were applied to seeds or leaves (Musson et al. 1995; Quadt-Hallmann and Kloepper 1996; Mahaffee et al. 1997; Quadt-Hallmann et al. 1997). Strain JM22 colonized different plant species such as cucumber, bean, and cotton systemically, and moved from the roots to the stem, cotyledons, and finally leaves. Initially, bacteria were concentrated in grooves between epidermal cells on the root surface, within intercellular spaces, including spaces close to the conducting elements, and inside single root epidermal cells (Quadt-Hallmann and Kloepper 1996). Strain 89B-61 also demonstrated internal colonization of cotton root tissues, but bacteria were observed only in intercellular spaces close to the root surface with no evidence of further internal spread (Quadt-Hallmann et al. 1977). While the systemic colonist JM22 consistently lacked biocontrol activity (J.A. McInroy, personal communication), the plant growth promoting rhizobacterial (PGPR) strain and local cortical colonist 89B-61 exhibited biological control activity, including induced systemic resistance, against various pathogens in greenhouse experiments and field trials (Chen et al. 1995*a*; Wei et al. 1991, 1996). Interestingly, plants naturally infected with *Erwinia tracheiphila* showed significantly less wilting when induced with 89B-61 (Wei et al. 1996). Plant growth promotion in these experiments was expressed as a significant increase of runner length, number of leaves per plant, and total yield. In addition, protection against *Fusarium oxysporum* pv. *vasinfectum* was observed on cotton after treatment with 89B-61 (Chen et al. 1995*a*).

In an attempt to further elucidate the early events underlying plant root colonization by bacterial endophytes, the primary objective of this study was to determine how the model systemic colonist JM22 enters plant tissues, in relation to the production of cell wall hydrolyzing enzymes. A similar analysis was conducted with the local cortical colonist 89B-61, thereby facilitating a comparison of enzyme expression between an endophyte not exhibiting detectable effects on plants (JM22) and a PGPR strain with biocontrol activity (89B-61).

Material and methods

Bacterial strains

Enterobacter asburiae JM22 was originally isolated from cotton (McInroy and Kloepper 1995*b*) and *P. fluorescens* 89B-61 was isolated from canola plants by Agrium Biologicals (Saskatoon, Sask., Canada). Both strains were stored in tryptic soy broth (TSB; Difco, Detroit, Mich.) and 20% v/v glycerin at −80°C and were grown on tryptic soy agar (TSA; Difco) for 24 h at 28°C for each experiment. Two hundred milligrams (wet weight) of bacteria was mixed with 2 mL of 2% w/v methyl cellulose for seed application or 1 mL of sterile potassium phosphate buffer (pH 7.0) (PB) for leaf and seedling application.

Plant material

Surface-disinfected cotton seeds (*Gossypium hirsutum* cv. '*DP 50*') were used for all experiments. Cotton seeds were surface-disinfected by incubating in 5% NaOCl for 1 min, followed by three washes with sterile PB. A sterility check was then carried out by pressing seeds on TSA. Seeds showing bacterial growth were discarded. In addition, 20 surface-disinfected seeds were triturated with sterile PB, and 100-μL aliquots were incubated on TSA for 48 h at 28°C to check for naturally occurring bacteria within seeds. No growth was detected on any plates. Cotton plants were grown for 14 days in autoclaved natural ground clay (Moltan Company, Middleton, Tenn.) under greenhouse conditions and fertilized once with Peter's fertilizer solution (20:20:20 N−P−K) (Scotts-Sierra, Maryvilles, Ohio) 1 week after planting.

Immunological techniques

Poly- and mono-clonal antibodies were raised against JM22 in previous experiments (Quadt-Hallmann and Kloepper 1996). Enzyme-linked immunosorbent assay (ELISA), tissue printing, and immunogold labeling combined with transmission electron microscopy followed the procedures described by Quadt-Hallmann and Kloepper (1996).

Three experiments were conducted to determine how JM22 or 89B-61 enter cotton plant tissue. In experiment 1, possible passive uptake of JM22 was tested using live and glutaraldehyde-fixed cells of JM22 for seed and leaf application. Fixation with glutaraldehyde killed bacteria but did not affect their antigenic properties. To prepare glutaraldehyde-killed inoculum, JM22 cells grown for 24 h at room temperature in TSB were washed three times by centrifugation at 12 000 × *g* for 20 min and resuspended in sterile PB. Two millilitres of the bacterial suspension (1×10^7 CFU/mL) was then dialyzed first against 2% w/v glutaraldehyde (Sigma, St. Louis, Mo.) in PB for 3 h at room temperature in a Spectra/Por membrane tubing (MW cutoff 3500; Spectrum Medical Industries, Inc., Tex.) and then against several changes of PB for 20 h at 4°C. The suspension was centrifuged again, the bacterial pellet was resuspended in PB, and the solution was adjusted spectrophotometrically at 640 nm to a concentration of approximately 10^7 CFU/mL. Complete inactivation of the bacteria was confirmed by plating 100 μL of the suspension on TSA.

Surface-disinfected cotton seeds were then coated with a mixture of 200 mg living or inactivated bacteria in 2 mL of 2% methyl cellulose per 20 seeds, dried under a laminar flow hood, and planted. Two weeks after planting, roots, stems, cotyledons, and leaves of five plants from each treatment were surface disinfected as described for seeds, with the exception that the NaOCl concentration was 1.05%. Thereafter, they were checked with ELISA for presence of JM22. Comparable plant tissues of five other plants from both treatments were analyzed with tissue printing, again using JM22-specific antibodies. In addition, roots of four plants seed treated with glutaraldehyde-fixed cells of JM22 and of four plants seed inoculated with living cells of JM22 were prepared for microscopic observation.

Leaf inoculation was also conducted with JM22 living and glutaraldehyde-fixed cells by pipetting 1.0 mL of each bacterial suspension (1×10^7 CFU/mL) onto the surface of two leaves per plant. Each treatment was replicated four times. After 2 weeks, one inoculated leaf from each plant was embedded for transmission electron microscopy. Ultrathin leaf sections were evaluated for bacterial presence after immunogold labeling with JM22-specific antiserum with a Zeiss 10 transmission electron microscope.

In experiment 2, the potential of JM22 to enter radicles without wounds was tested. Cotton seeds were germinated on water agar (2%) for 48 h at room temperature. Two treatments were conducted: seedlings were dipped in a bacterial suspension with 1×10^7 CFU/mL or in sterile PB. The first treatment was replicated 20 times, because difficulties with surface disinfection were expected owing to the high bacterial concentration on the surface of the seedlings. The control treatment with sterile PB was replicated 10 times. After1 h, root tissues were surface-disinfected, pressed onto TSA as a sterility

check, and triturated after dilution with sterile PB at a ratio of 1:5, and 100-μL aliquots were plated onto TSA. The plates were incubated for 24 h at 28°C. Root samples that did not pass the sterility check were discarded. Up to 10 morphologically representative colonies from each of the other samples were checked with ELISA using JM22-specific antibodies. In addition, these colonies were identified based on fatty acid methyl esters of total cellular fatty acids (Sasser 1990). Extraction and gas chromatography followed the description of McInroy and Kloepper (1995*b*). Colonies resembling the ones identified as JM22 were counted to determine CFU per gram of root tissue.

In experiment 3, the production of cell wall degrading enzymes in planta was analyzed. Surface-disinfected cotton seeds were germinated for 2 days on water agar and dipped in suspensions of JM22 or 89B-61, both with a concentration of 1×10^7 CFU/mL. Root tissues were embedded for microscopic observation following the procedure described by Benhamou and Bélanger (1995). Sampling was carried out 2, 6, and 24 h after bacterial treatment. Ultrathin root sections were analyzed after cytochemical labeling with an exoglucanase (β-1,4-D-glucan cellobiohydrolase, EC 3.2.1.91), purified from a cellulase (from the fungus *Trichoderma harzianum*), and complexed to colloidal gold at pH 9.0 (Benhamou et al. 1987). For direct labeling of cellulosic β-1,4-glucans, ultrathin sections were first incubated on a drop of phosphate buffered saline (pH 6.0) (PBS) containing 0.02% w/v of polyethylene glycol (PEG) for 5 min at room temperature. Thereafter, sections were transferred to a drop of the gold-complexed probe for 30 min at room temperature in a moist chamber. After washing with PBS and rinsing with distilled water, grids were contrasted with uranyl acetate and lead citrate. A JEOL 1200 EX transmission electron microscope (Tokyo, Japan) was used to investigate the extent of cellulose degradation in root sections.

Results

Passive uptake of JM22 (experiment 1)

After seed treatment, neither live nor glutaraldehyde-fixed JM22 was detected by ELISA in roots, stems, cotyledons, or leaves of cotton plants. With tissue printing, JM22 was consistently observed in root, stem, and cotyledon tissue of cotton plants treated with living bacteria. The presence of bacteria was indicated by development of a lilac color in the areas containing antigen. No bacterial cells were detected in comparable tissues of plants seed-treated with glutaraldehyde-fixed cells and no bacteria were observed in ultrathin root sections of these plant tissues. Bacteria were also absent on the root surface. Seed application with living JM22 cells resulted in colonization of the root as described by Quadt-Hallmann and Kloepper (1996). Many bacteria were located on the root surface, concentrated in the grooves between epidermal cells, below collapsed epidermal cells, and in intercellular spaces close to the root surface and conducting elements. Single bacteria were detected inside epidermal cells, which appeared to be viable.

Following leaf application, ultrathin sections of leaves inoculated with glutaraldehyde-fixed bacterial cells of JM22 did not show any colonization with bacteria. In contrast, after inoculation with living cells of JM22, bacteria were observed on the leaf surface, concentrated in grooves between epidermal cells (Fig. 1). All bacterial cells showed an intensive gold label (Fig. 2). In the vicinity of several bacteria, the epidermal cell wall showed signs of deformation (Fig. 2). Sometimes, single bacteria were localized adjacent to stomata (Fig. 3). In ultrathin sections, no bacteria were detected inside the leaf tissue.

Penetration of JM22 without wounds (experiment 2)

A complete surface disinfection of the radicle tissue was obtained for only eight of the 20 JM22-treated cotton seedlings. From these 8 root tissue samples, bacterial colonies with a single morphology developed on TSA and reacted positively in ELISA against JM22-specific antibodies. These colonies were identified as *Enterobacter asburiae* based on their fatty acid methyl esters. The bacterial population density of JM22 inside radicle tissues reached a mean of 3.8×10^3 CFU/g, ranging from 2.5×10^2 to 2.2×10^4 CFU/g. Control seedlings dipped in sterile PB were free of bacteria.

Production of cell wall degrading enzymes (experiment 3)

Ultrathin root sections of cotton seedlings dipped in suspensions of JM22 or 89B-61 showed many bacterial cells on the root surface. Bacteria also accumulated in intercellular spaces close to the root surface. Some bacterial cells were observed in intercellular spaces and inside cortical parenchyma cells close to the conducting elements as early as 6 h after inoculation (Fig. 4). The treatment of ultrathin tissue sections with exoglucanase coupled to colloidal gold lead to regions of the cell wall without any gold labeling in the vicinity of bacterial cells (Figs. 5–7), indicating hydrolysis of cellulose in these areas. This was observed for both JM22 and 89B-61. However, the hydrolysis seemed to be stronger in the vicinity of bacterial cells of 89B-61.

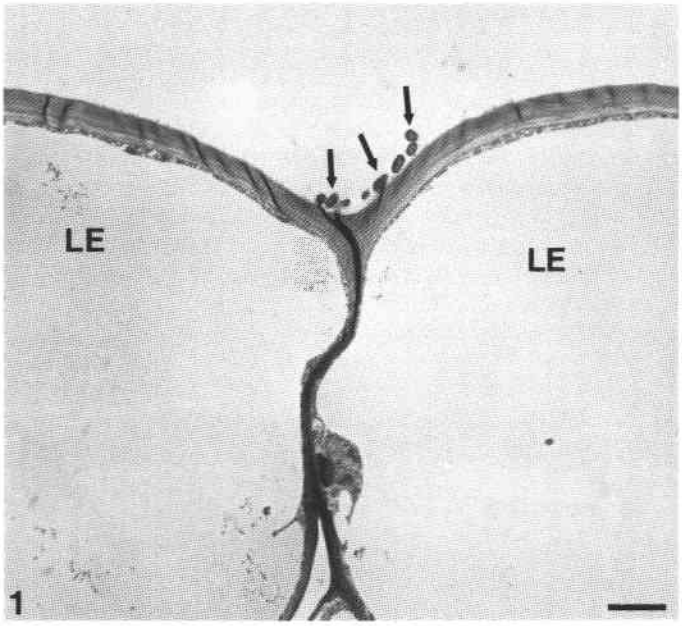
Discussion

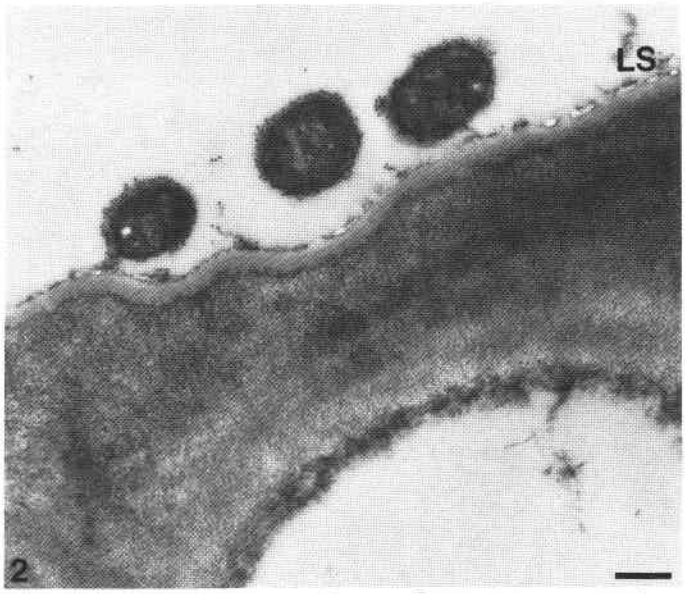
The systemic bacterial endophyte *E. asburiae* JM22 expressed the capacity to penetrate cotton root tissues. According to our results, passive uptake of JM22 is unlikely, since seed treatment and leaf inoculation with glutaraldehyde-killed bacteria did not result in internal colonization. There were also no glutaraldehyde-fixed bacteria observed on root or leaf surfaces, indicating that living bacteria are essential in the process of cell–cell recognition preceding bacterial entry.

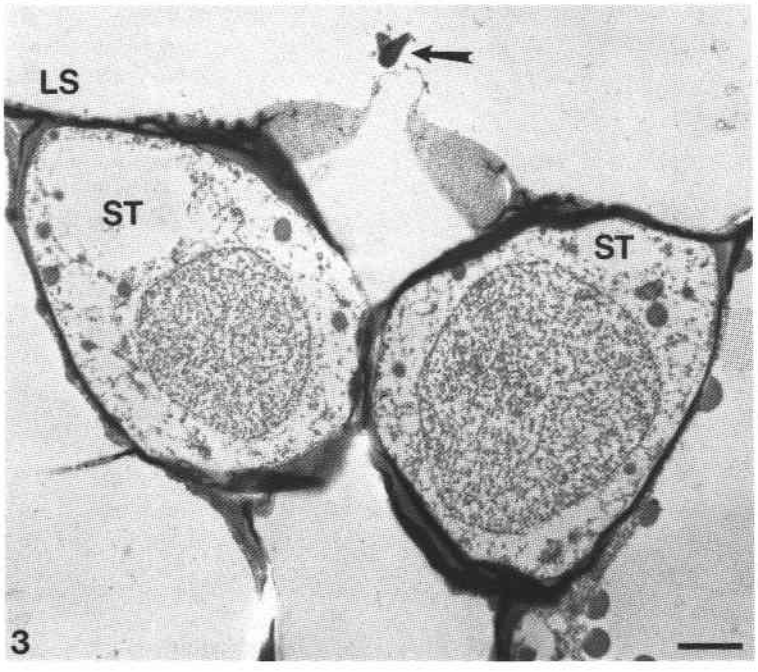
The capacity of JM22 to penetrate plant tissue in the absence of wounds was shown for 2-day-old cotton seedlings germinated on water agar and dipped in the bacterial suspension. This method avoided wounding due to abrasion by soil particles, pathogen attack, or lateral root formation. Inoculation of cotton leaves with a suspension of JM22 plus carborundum to cause wounds significantly increased bacterial populations within leaves compared with inoculation without carborundum (Quadt-Hallmann and Kloepper 1996). Furthermore, Schmidt (1978), Jacobs et al. (1984), Agarwhal and Shende (1987), and Mahaffee et al. (1997) detected larger external and internal bacterial populations in the vicinity of wounds produced by emerging lateral roots indicating the role of these sites for bacterial entry. The 2-day-old cotton seedlings analyzed in our experiments had not yet reached the age of lateral root emergence, thereby excluding this as a possible avenue of entry for JM22.

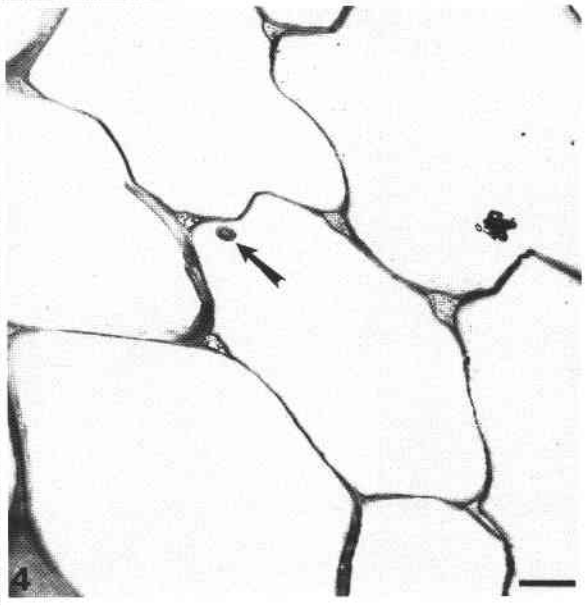
Indirect evidence for the production of cellulase by JM22 and 89B-61 was detected at the ultrastructural level. Although hydrolysis of cellulose seemed to be stronger in the vicinity of 89B-61 cells, both bacterial strains induced local cellulose degradation. Polysaccharide-degrading enzymes are well known to contribute to the invasiveness of many plant-pathogenic bacteria (Rombouts and Pilnik 1972; Mount 1978; Collmer et al. 1982; Scala and Zoina 1983; Solheim

Figs. 1–3. Cotton plant leaf-inoculated with suspension of *E. asburiae* JM22 (1×10^7 CFU/mL). Cross sections of an inoculated leaf. Transmission electron microscopy combined with immunogold labeling applying primary antibodies against JM22 at a dilution of 1:500 and secondary antibodies coupled with colloidal gold at a dilution of 1:40. Fig. 1. Bacterial cells of JM22 (arrows) are located on the leaf surface. LE, leaf epidermal cell. Scale bar = 6 μm. Fig. 2. Intensive gold label of the bacterial membrane identifies the bacteria on the leaf surface as JM22. Note deformation of the epidermal cell wall beneath bacterial cells. LS, leaf surface. Scale bar = 1 μm. Fig. 3. Single cell of JM22 (arrow) in association with a stomate (ST). LS, leaf surface. Scale bar = 4 μm. **Fig. 4.** Cotton seedling germinated on water agar and dipped in a suspension of *E. asburiae* JM22 (1×10^7 CFU/mL). Cross section of the radicle. Transmission electron microscopy combined with immunogold labeling applying primary antibodies against JM22 at a dilution of 1:500 and secondary antibodies coupled with colloidal gold at a dilution of 1:40. Single cell of JM22 (arrow) located inside an intact root cortical cell. Scale bar = 6 μm.



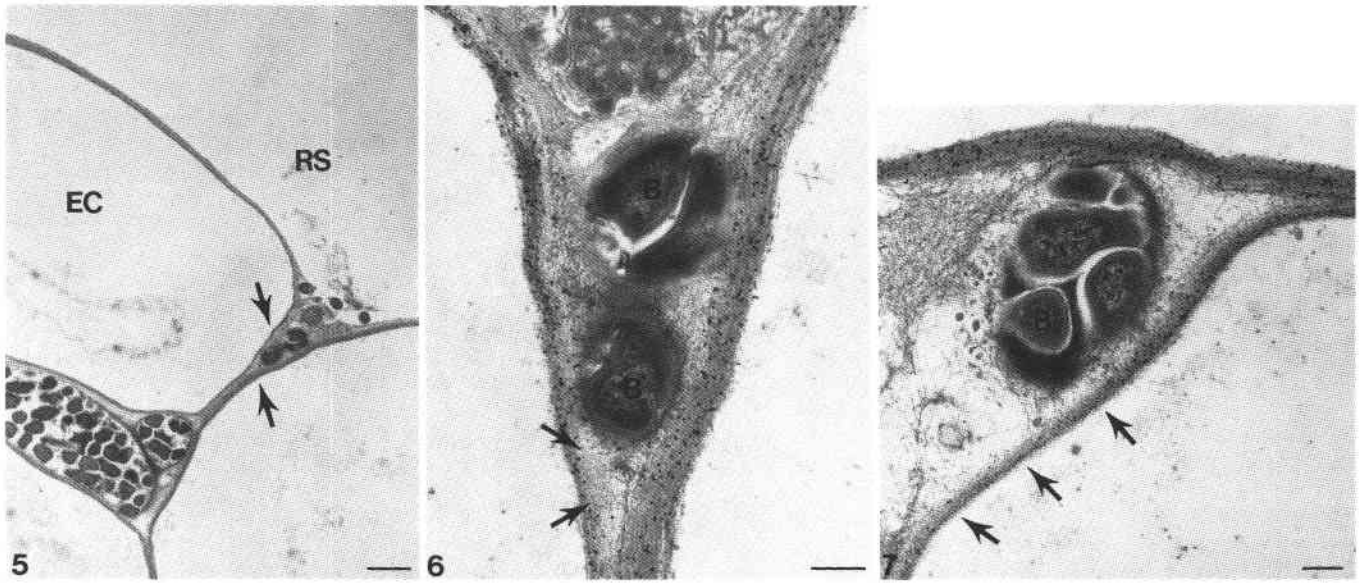






and Fjellheim 1984; Dean and Timberlake 1989; Lanham et al. 1991). Production of cell wall degrading enzymes was also detected in planta for another *P. fluorescens* strain with biocontrol potential (Benhamou et al. 1996). However, Bell et al. (1995) demonstrated that the frequency of hydrolytic enzymes was higher for soil bacteria than for xylem-inhabiting bacteria, and they concluded that it was unlikely for endophytic bacteria to gain plant entry via production of hydrolytic enzymes. However, there may be a difference between hydrolytic enzyme production in vitro and in planta. Studies

Figs. 5–7. Cotton seedling germinated on water agar and dipped in a suspension of *E. asburiae* JM22 (1×10^7 CFU/mL). Cross section of the radicle after 6 h. Transmission electron microscopy combined with cytochemical labeling of cellulose applying an exoglucanase (purified from a cellulase produced by the fungus *Trichoderma harzianum*) coupled to colloidal gold. Fig. 5. Bacterial cells of *P. fluorescens* 89B-61 located in the grooves between two root epidermal cells (EC) and in intercellular spaces close to the root surface (RS). Scale bar = 5 μm. Fig. 6. Higher magnification of the area indicated with arrows in Fig. 5. Deposition of gold particles over the host cell walls specifically detecting cellulose. Cell wall bound cellulose hydrolysis (arrows) adjacent to bacterial cell. B, bacteria. Scale bar = 1 μm. Fig. 7. Host cell wall intensively labeled. Absence of the deposition of gold particles indicates areas of cellulose hydrolysis (arrows). B, Bacteria. Scale bar = 1 μm.



by van Peer et al. (1990) showed distinct differences concerning lipopolysaccharide patterns, cell envelope protein patterns, and other biochemical characteristics between *Pseudomonas* species recovered from within tomato plants and those isolated from the root surface.

Besides the hydrolysis of cellulose, pectin may also be degraded owing to bacterial activity. Pectate lyase digests can inhibit a hypersensitive reaction in tobacco (Baker et al. 1990). Neither JM22 nor 89B-61 induced marked cellular alterations upon internal colonization. Nevertheless, localized hydrolysis of cellulose was detected. Although endophytic bacteria may have some attributes of pathogens and several endophytes can cause hypersensitive reactions in the plant, they can be beneficial for plants by inducing systemic resistance against pathogens.

The results presented in this study provide additional evidence regarding the mechanisms by which local and systemic bacterial endophytes enter plant tissues, thus further elucidating the early events of bacteria–plant interactions, which may be associated with their beneficial effects on plant growth or plant health.

Acknowledgements

This work was partially funded by the German Research Association (Deutsche Forschungsgemeinschaft, Project No. Qu 84/2-1) and by the USDA Binational Agricultural Research and Development (US-2026-91R).

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