Interaction of rhizobacteria with leafy spurge (*Euphorbia esula* L.) callus tissue cells

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Abstract

The interaction of two rhizobacterial isolates,*Pseudomonas fluorescens* isolate LS102 and *Flavobacterium balustinum* isolate LS105 with leafy spurge cells at the cellular level was studied using scanning and electron microscopy. Leafy spurge callus tissue inoculated with either isolate showed considerable changes compared to non-inoculated tissue. The attachment of rhizobacteria to cell surfaces was associated with the elaboration of fibrillar material which may anchor bacteria to surfaces and contribute to mediation of the phytotoxic effect caused by rhizobacteria. At the ultracellular level, inoculated callus tissue showed numerous cell alterations including vesiculation and convolution of the plasmalemma, cell wall degradation and disorganization of the cytoplasm, similar to those detected in the whole plant. It is concluded that callus tissue may provide an excellent working model to investigate the mode and/or mechanism of action of potential biocontrol agents on their host plants.

Introduction

Leafy spurge is a noxious weed that infests millions of hectares in the Northern Plains of the United States and the Prairie Provinces of Canada (Best et al., 1980). The relative ineffectiveness of chemical control and its high cost (Beck et al., 1993) has led to investigations of biological control as an alternative method to manage the weed. Such a new approach is based on initiating disease epidemics by pathogens of the host within the target weed population. Manipulation of microorganisms as biocontrol agents requires an investigation on their interaction with the host plant at the cellular level.

Systems involving tissue cultures and bacteria have been successfully used to study various bacteria-plant interactions such as in the crown gall disease complex in grapes caused by *Agrobacterium* (Pu and Goodman, 1993) and in the *Bradyrhizobium*- soybean symbiosis (Child and Kurz, 1978). The use of tissue cultures for the study of host-pathogen interactions has the advantage of allowing deliberate manipulations of the environment in which the pathogen interacts with the host

and of uniformly exposing cultured plant cells to pathogens without wounding.

Tissue cultures of leafy spurge have been shown to be suitable for investigation of the activity of allelochemicals, which were found to be representative of results obtained in whole plant experiments (Hogan and Manners, 1990, 1991). We previously developed and reported a method for screening rhizobacteria for deleterious effects on leafy spurge tissue culture (Souissi and Kremer, 1994), but did not describe the host-bacteria interaction at the cellular level. Rhizobacteria screened for bioactivity using callus tissue were also detrimental toward leafy spurge seedlings thereby verifiying the effectiveness of the callus model in detecting deleterious activity toward intact host plants. The objective of this study is to examine the interaction of leafy spurge cells with *Pseudomonas fluorescens* isolate LS102 and *Flavobacterium balustinum* isolate LS105, two deleterious rhizobacterial isolates previously isolated from roots of leafy spurge seedlings (Souissi and Kremer, 1994). Effects of these rhizobacteria were investigated over

time using scanning (SEM) and transmission (TEM) electron microscopy to determine their impact on leafy spurge at the cellular level.

Materials and methods

Bacterial cultures

Ps.fluorescens isolate LS102 and *Fl.balustinum* isolate LS105 previously isolated from roots of leafy spurge seedlings (Souissi and Kremer, 1994) and tested for their deleterious effects on leafy spurge growth were used during this study. Bacteria were grown on King's B agar medium (Schaad, 1980) for two days and a loop of the fresh cultures was used to inoculate 50-ml flasks containing King's broth. Flasks were shaken overnight at 27°C on a rotary shaker at 140 rpm. Cultures were harvested by centrifugation at 7,000 xg for 20 min at 4°C. Cell pellets were resuspended in phosphate-buffered saline (PBS; 10 mM K_2PO_4-KH_2PO_4, 0.14 M NaCl; pH 7.2) and used to inoculate callus tissue of leafy spurge.

Plant material

Leafy spurge callus tissue originated from stem tissue cultured at the USDA-ARS Bioscience Research Laboratory in Fargo, ND. Callus was grown in the dark at 27°C and maintained on Gamborg's B5 agar medium (Gamborg et al., 1968) supplemented with 1 mg l^{-1} 2,4-D (2,4-dichlorophenoxy acetic acid). Callus was subcultured to fresh medium once every three weeks.

One week after subculturing to fresh medium, callus pieces were inoculated with 1 ml of 10^8 CFU bacterial suspension on the top side where active callus growth was occurring. Tissue samples were taken at 24 and 48 h after inoculation and prepared for SEM and TEM as described below.

Microscopic studies

Samples were fixed in 2.5% glutaraldehyde (100 mM phosphate buffer, pH 7) for 4 h at room temperature, washed in the same buffer for 1 h then postfixed in 1% osmium tetroxide for 4 h. For SEM, samples were dehydrated through a graded ethanol series (20, 40, 60, 80, 95 and 100%), critical-point dried in liquid CO_2 and examined in a JEOL JSM-35 scanning electron microscope operating at 20 kv. Tissue samples taken for TEM were fixed as above, dehydrated in acetone and

embedded in Epon. Sections were made with an ultra-microtome equipped with a diamond knife. Thick sections, 2.5 to 3 μm, were stained with toluidine blue and examined by light microscopy to select areas for sectioning. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-100B transmission electron microscope operating at 100 kv.

Results

Visual observations

Two days after inoculation, callus inoculated with *Ps.fluorescens* isolate LS102 was covered by bacteria. Bacterial colonies were observed on the surface of the B5 agar medium surrounding the inoculated callus tissue (data not shown). Callus inoculated with *Fl.balustinum* isolate LS105 was not covered by bacterial colonies, but turned light brown, whereas the color of callus tissues inoculated with LS102 remained unchanged. LS105 reduced callus growth but to a lesser extent than LS102. Previous studies (Souissi and Kremer, 1994) showed that populations of both rhizobacteria isolates inoculated to leafy spurge tissue culture reached > 10^9 CFU g-1 of tissue after 6 days and reduced growth of callus by over 45% compared to the control tissue.

Microscopic studies of inoculated callus tissue

Scanning electron microscopy of uninoculated leafy spurge callus tissues revealed that most of the plant cells were elongated with smooth surfaces (Figure 1A), occasionally puckered by small shallow pits (inset). Occasionally, spherical cells were seen and small particulates of various shapes were found on the surface of some cells.

Forty-eight hours after inoculation, numerous bacteria attached to cell surfaces of inoculated callus tissue (Figures 1B and C). Cells of isolates LS102 and LS105 were typically rod-shaped and single-celled. Bacterial cells were not evenly distributed on the surface of inoculated tissue, some areas had more bacterial cells than others (Figure 1B). There was no specific pattern to the distribution of bacteria on host cells although most occurred individually rather than as aggregates or microcolonies. Both isolates showed a similar pattern of attachment to cell surfaces. Some bacteria attached laterally while others were attached in a polar orientation. Very few fibrils were seen in callus tissue inoc-
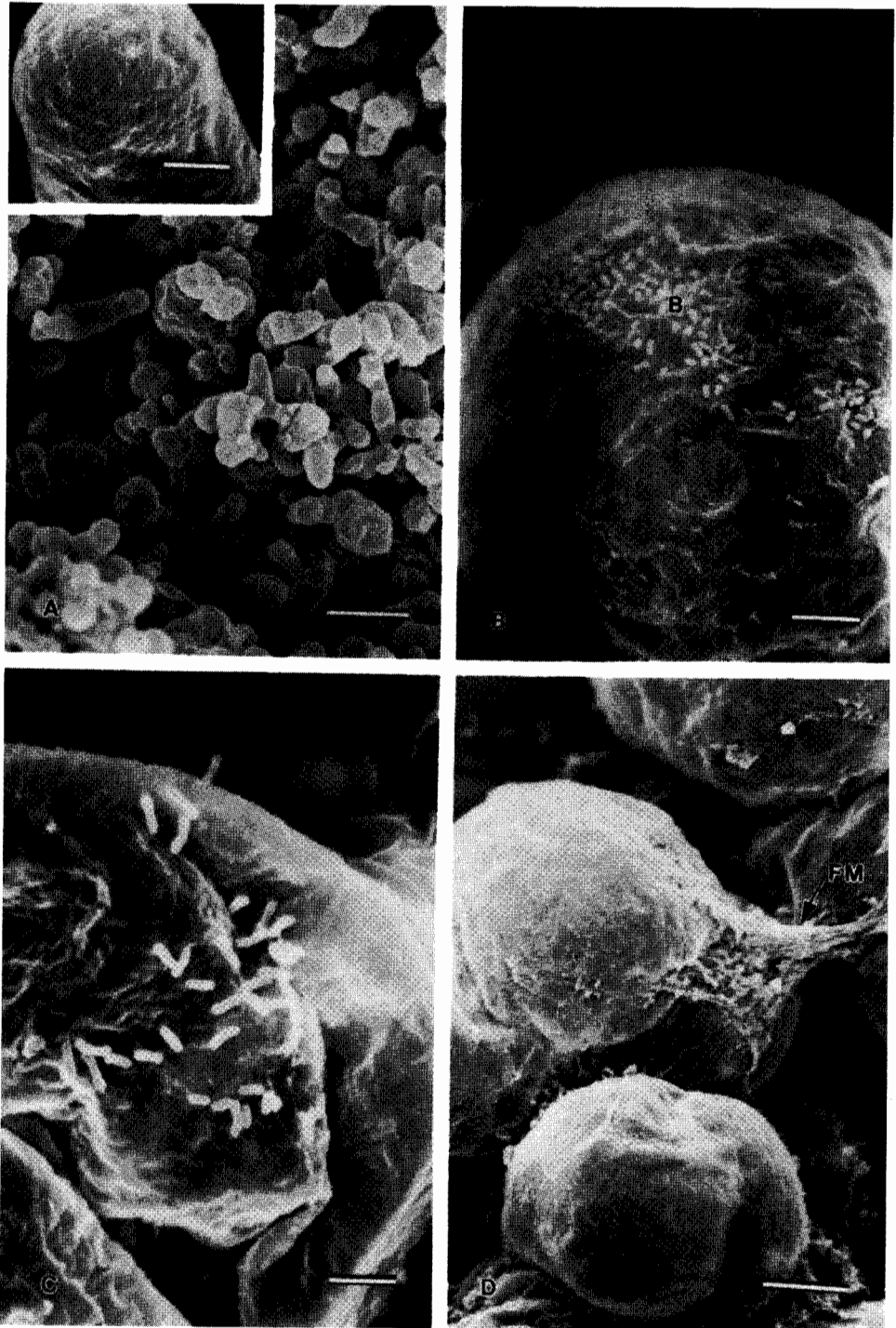
Figure 1. SEM of leafy spurge callus tissue inoculated with *Ps.fluorescens* isolate LS102 and *Fl.balustinum* isolate LS105 at 48 h. (*A*) Non-inoculated callus tissue where most of the cells were elongated (inset; bar=10 μm) and had smooth surfaces. Bar=100 μm. (*B*) Individual bacterial cells (*B*) of isolate LS102 attached to the surface of leafy spurge cells in a random orientation. Bar=5 μm. (*C*) Bacterial cells of isolate LS105 attached to leafy spurge cells. Bar=10 μm. (*D*) Development of thick fibrillar material (FM) in tissue inoculated with isolate LS102. Bar=10 μm.
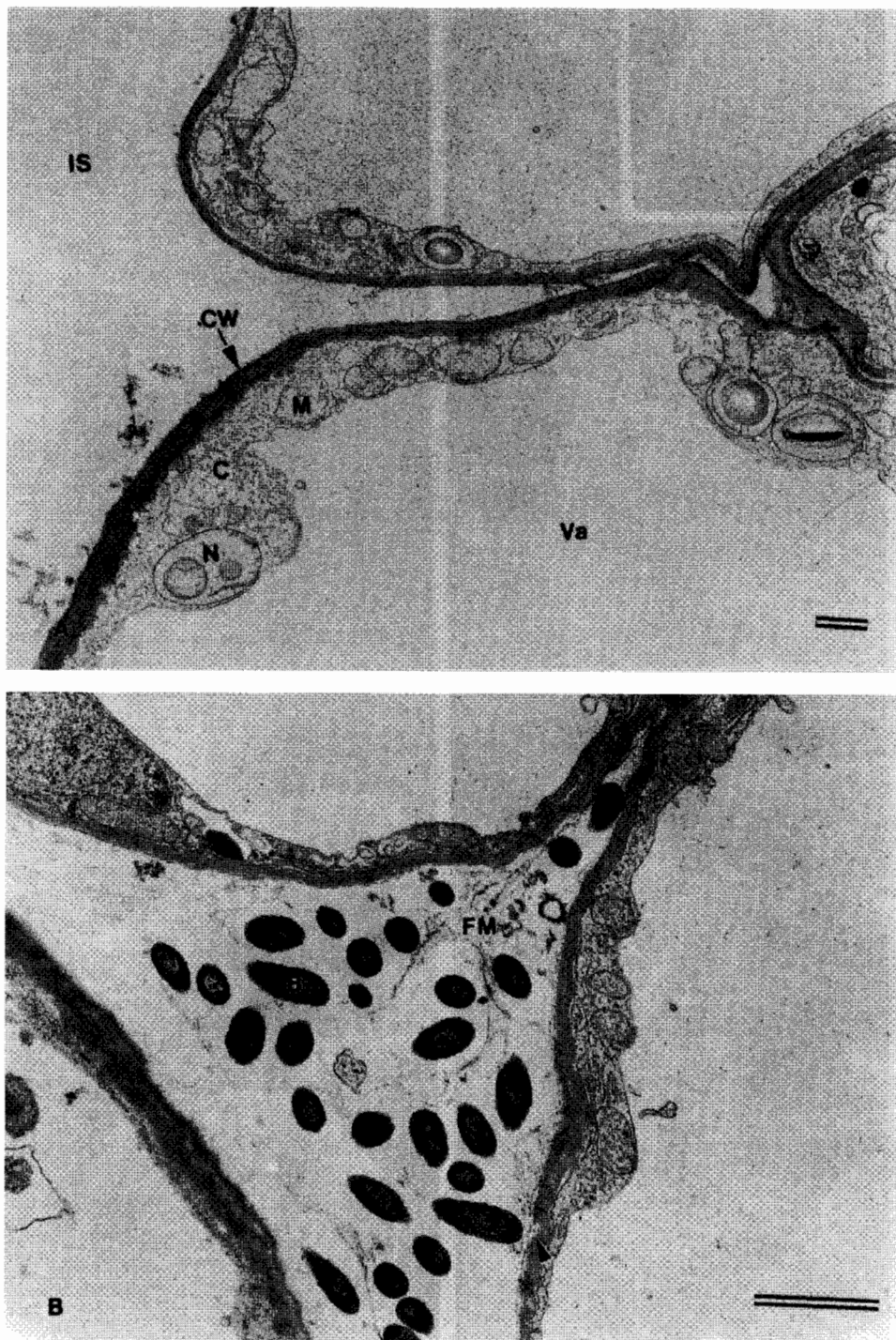
Figure 2. Ultrastructural observations from TEM of leafy spurge callus tissue inoculated with isolate LS102 at 24 h. (*A*) Non-inoculated callus tissue after spraying with buffer. All organelles were present and were normal. (*B*) bacterial cells surrounded by a net-like matrix of fibrillar material are present in the intercellular spaces. Adjacent cells appear normal apart from the alteration of some wall areas (arrowhead). Bar, 1 μm. B, bacteria; C, cytoplasm; CW, cell wall; FM, fibrillar material; IS, intercellular space; M, mitochondria; N, nucleus; Va, vacuole.
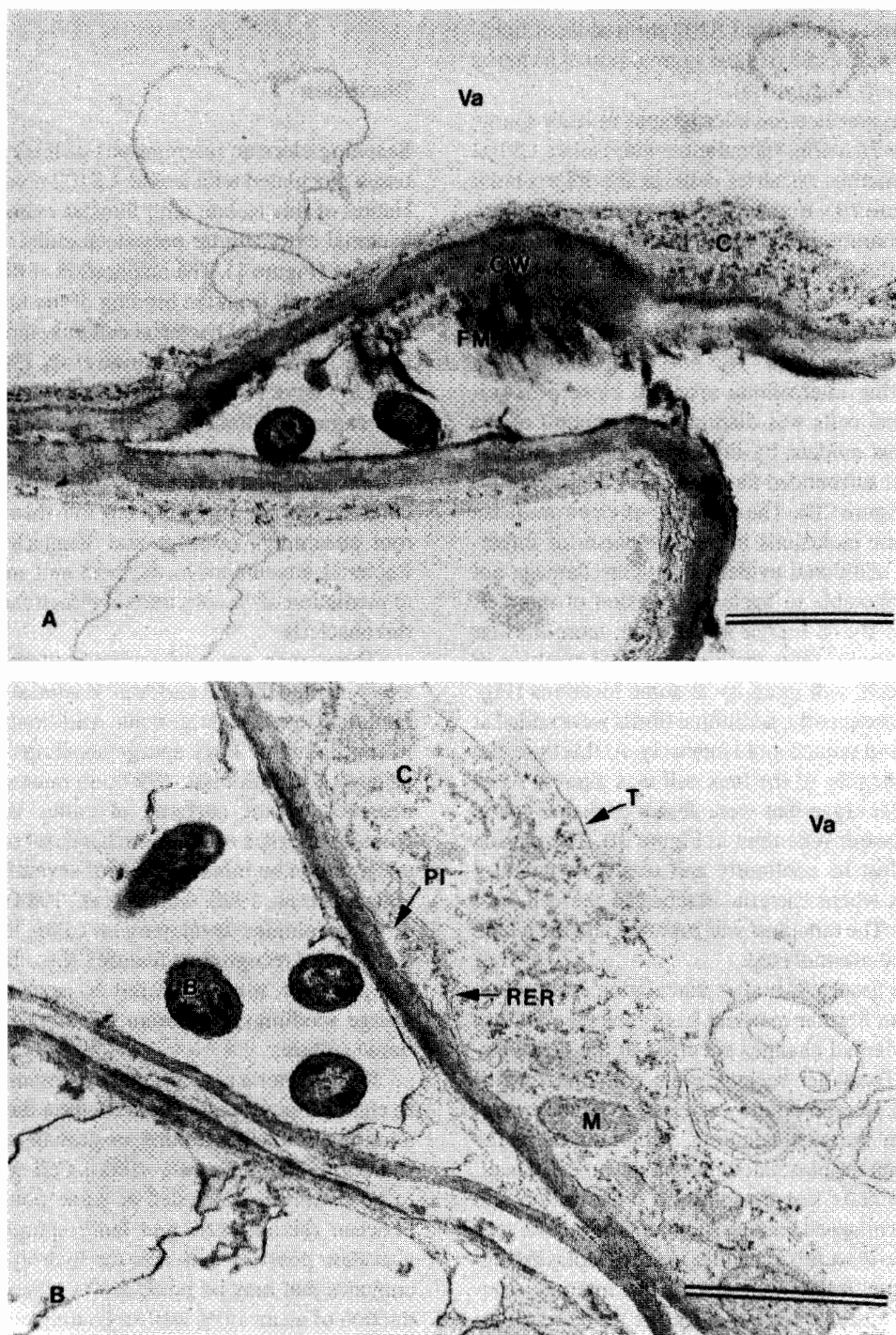
Figure 3. Ultrastructural interactions of isolate LS102 with leafy spurge cells 48 h after inoculation. (*A*) note the apparent erosion of the cell wall in proximity of bacteria. (*B*) Loss of structural integrity of cells adjacent to bacteria. Bar=1 μm. B, bacteria; C, cytoplasm; CW, cell wall; FM, fibrillar material; M, mitochondria; Pl, plasmalemma; RER, rough endoplasmic reticulum; T, tonoplast; Va, vacuole.

ulated with *Fl.balustinum* isolate LS105. In contrast, the surface of many cells of callus tissue inoculated with *Ps.fluorescens* isolate LS102 showed thick fibrillar material that resulted in the aggregation of bacterial and callus cells (Figure 1D).

Transmission electron micrographs of leafy spurge callus tissue 24 h after inoculation with isolate LS102 showed numerous bacterial cells in the intercellular spaces (Figure 2B). Both host and bacterial cells exhibited normal structural features. In non-inoculated tissue, all cellular organelles were present and were normal (Figure 2A). The major difference between inoculated and non-inoculated tissue occurred in the host cell wall structures. The host cell wall of some cells bordering intercellular space in close proximity to bacterial cells was disrupted. Disruption of the cell wall was evident by the appearance of fibrillar material that surrounded the bacteria in the intercellular space (Figure 2B). The presence of electron-dense or osmophilic inclusions in the cytoplasm of infected tissue is additional evidence of tissue damage not directly attributable to bacterial invasion of intercellular spaces. By 48 h after inoculation, more fibrillar materials extruded from the host cell wall resulting in loss of the cell wall integrity at some locations (Figure 3A). Consequently, additional fibrils were added at the surface and stained more intensely. At this time, the structural integrity of the host cell was altered. Most of the cellular organelles were absent or disintegrated i. e., endoplasmic reticulum in Figure 3B. The plasma membrane lost its continuity and was convoluted at some points where bacteria attached to the cell wall (Figure 3B). The tonoplast was ruptured and the whole cell appeared plasmolyzed.

In callus tissue, 48 h after inoculation with isolate LS105, loose fibrillar material from the cell wall was observed. Marked changes occurred in the host plasmalemma. The latter became more convoluted (Figure 4A). Membrane-bound vesicles, apparently originating from the plasmalemma, accumulated in the space between the plasmalemma and the host cell wall. Most of the cells lost cytoplasmic organization and completely collapsed leaving fragments of membranes and other pre-existing organelles in the cytoplasm (Figure 4B). Some of the mitochondria lost internal structure, others appeared ruptured. Unlike tissue inoculated with isolate LS102, the tonoplast did not rupture, however, the shape was irregular and severely convoluted (Figure 4B). Vacuoles were unusually filled with granular material. This material also accumulated in

the cytoplasm and became associated with membrane (Figures 4A and B).

Discussion

Scanning electron micrographs of leafy spurge callu tissue inoculated with isolate LS102 revealed the asso ciation of this isolate with fibrillar material, probabl bacterial extracellular polysaccharides (EPS), on ce surfaces (Figure 1). The elaboration of fibrils by bacte ria may result from the binding of the lipopolysaccha ride component of bacterial cell envelopes to recepto sites on the plant host (Matthyse et al., 1982). Microfib rils may serve to anchor bacteria to plant cell surface and entrap additional bacteria. A study of root colo nization of wheat and barley by *Pseudomonas fluo rescens* indicated the possible involvement of a majo outer membrane protein from the rhizobacterium i root attachment (DeMot and Vanderleyden, 1991 Bacterial attachment to the host cell may contribut to mediation of the phytotoxic effect(s) caused by th rhizobacteria.

There was no preferential pattern of bacteria attachment to the cell surfaces. Bacterial cells attache randomly to leafy spurge cells. Additionally and unlik in root tissue of leafy spurge seedlings infected wit isolate LS102 (Souissi, 1994), no microcolonies wer observed on the surfaces of callus tissue. Micro colonies are sites of optimum bacterial replication an are common on infected roots of several plant specie (Begonia et al., 1990; Gulash et al., 1984). The absenc of microcolonies developing on callus tissue suggest that proper recognition features (i.e., ligand-bindin receptors) or stimuli offered by root cells of leaf spurge seedlings are absent or poorly developed i tissue culture.

Rhizobacteria may affect their host in a way simila to classical phytopathogenic bacteria through the pro duction of enzymes, toxins, or growth regulating sub stances (Goodman et al., 1986). Cell walls appeare to be dissolved or eroded at some points of contac between rhizobacteria and leafy spurge cells. Suc alteration possibly indicates the hydrolytic activity o enzymes that may be produced by rhizobacteria. Pro duction of an array of wall-degrading enzymes durin pathogenesis by pathogenic bacteria has been previ ously established (Collmer and Keen, 1986; Goodma et al., 1986). The active enzymes cleave polymers i the primary cell wall and middle lamella, facilitatin pathogen penetration and colonization of the host tis
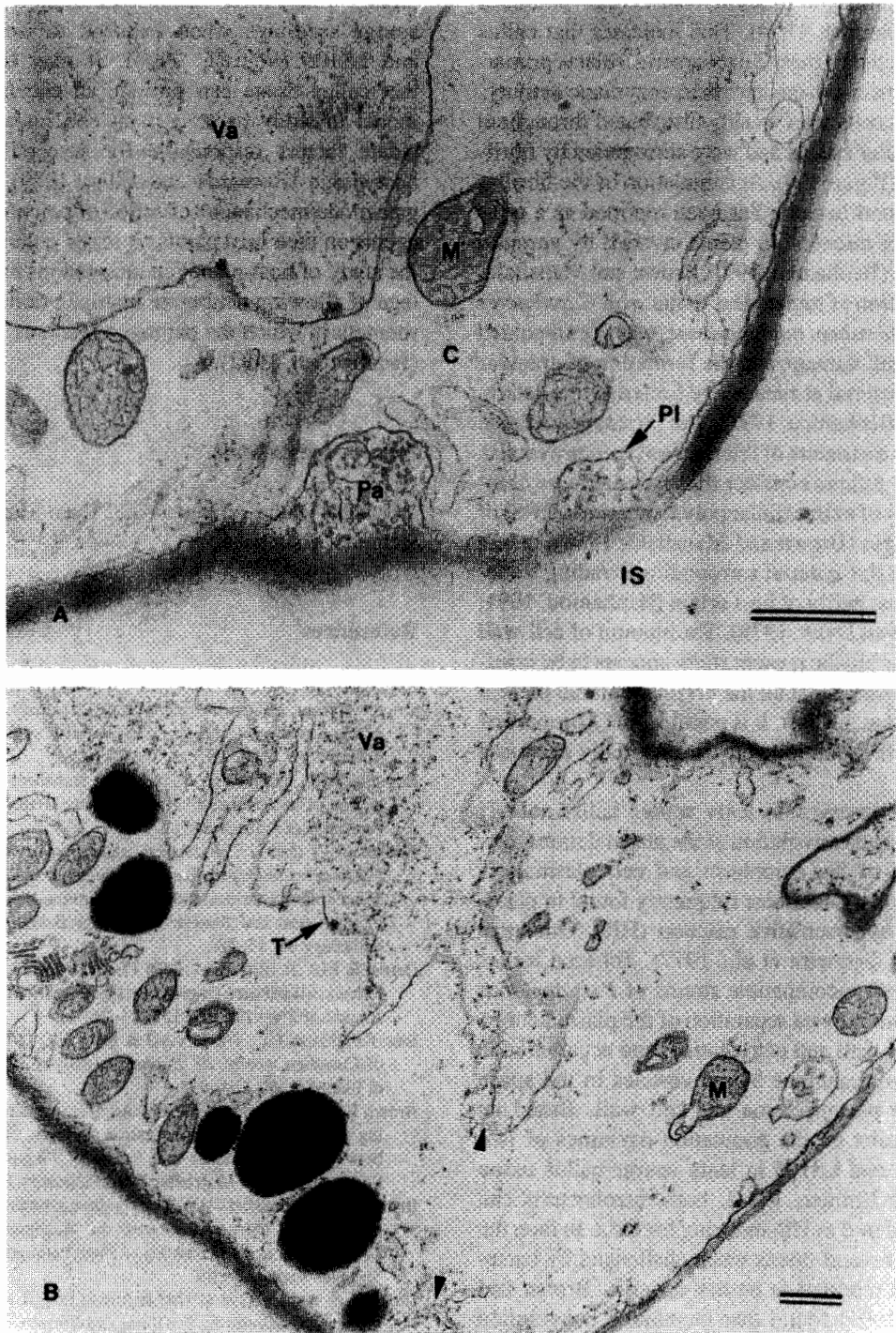
Figure 4. TEM of leafy spurge callus tissue inoculated with *Fl.balustinum* isolate LS105 at 48 h. (*A*) Convolution of the plasmalemm
and accumulation of vesicles in the space between the plasmalemma and the cell wall (papilla-like structure). (*B*) Alteration of host cellula
organelles such as mitochondria. Note the association of membranes with granular material (arrowheads). Bar=0.5 μm. C, cytoplasm; IS
intercellular space; m, mitochondria; Pa, papilla-like structure; Pl, plasmalemma; T, tonoplast; Va, vacuole.

sue. We previously showed that both isolates LS102 and LS105 produced proteases but neither produced pectinases (Souissi, 1994). This indicates that callus tissue is affected by these rhizobacteria isolates primarily through mechanismsother than enzymatic activity.

Bacterial cells were evenly distributed throughout the intercellular spaces and were surrounded by fibrillar material (Figure 2A). Accumulation of the fibrillar material around bacteria has been reported as a typical feature of infection in plants invaded by vascular wilt bacteria (Benhamou, 1991; Brown and Mansfield, 1988). Infection of tomato leaf tissue with *Clavibacter michiganense* subsp. *michiganense*, was accompanied by marked cell damage and the formation of granular or fibrillar material at sites where bacteria were actively growing (Benhamou, 1991). The literature available on the origin and nature of this material is controversial. While some authors consider this material to be composed mainly of extracellular polysaccharides (EPS) of bacterial origin (Brown and Mansfield, 1988), others reported that the material surrounding invading bacteria was at least partly of host origin (Benhamou, 1991; Huang and Van Dyke, 1978). The amount of cell wall disruption during the present study appears to be insufficient to conclude that all the fibrillar material present originated from the host. It is possible that a proportion of this material is attributed to the bacterially-produced EPS.

Changes observed in leafy spurge cells, notably vesiculation and convolution of the plasmalemma, disorganization of the cytoplasm and cell plasmolysis (Figures 3 and 4), are most frequently found in plants undergoing hypersensitive reaction (HR) (Goodman et al., 1986; Sequeira et al., 1977). Tobacco leaves infiltrated with incompatible strains of *Pseudomonas solanacearum* showed separation of the plasmalemma from the cell wall and convolution, and accumulation of numerous membrane bound vesicles in the space between the plasmalemma and cell wall. Based on previous results on the population dynamics of isolates LS102 and LS105 in leafy spurge callus tissue (Souissi and Kremer, 1994), both rhizobacteria can not be considered as HR-inducing bacteria. In fact, the response of several plants when challenged by bacteria has been reported to be non-specific. Brown and Mansfield (1991) found that membrane convolution was initiated in all bean cultivar/bacterium combinations used, and did not appear to be a determinant of subsequent plant responses, such as HR.

In the present study, the effects of selected deleterious rhizobacteria on leafy spurge callus tissue

at the cellular level have been demonstrated. These effects were similar to those detected in whole leafy spurge seedlings when exposed to isolates LS102 and LS105 (Souissi, 1994). It may be concluded that callus tissue can provide an excellent working model to study bacteria-plant cell interactions, elucidate factors responsible for susceptibility of the host tissue (Bernards and Ellis, 1989) and investigate mode/mechanism of action of potential biocontrol agents on their host plants. A tissue culture system for the study of host-pathogen interactions has the advantage of allowing deliberate manipulations of the environment in which the pathogen interacts with the host (Jacobi et al., 1982).

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