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Scanning and transmission electron microscopy of root colonization of morningglory (*Ipomoea* spp.) seedlings by rhizobacteria

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Abstract
Hydroponically-grown ivyleaf morningglory (*Ipomoea hederacea*) seedlings inoculated with deleterious rhizobacteria (DRB) were studied to observe colonization of roots using scanning and transmission electron microscopy. The DRB, *Bradyrhizobium japonicum* isolate GD3, previously isolated as a DRB producing high concentrations of indole-3-acetic acid (IAA), and *Pseudomonas putida* isolate GD4, were compared with a plant growth promoting rhizobacterium (PGPR), *Bacillus megaterium* isolate GP4. Scanning electron microscopy revealed that the colonization of isolates GP4 and GD4 were consistently distributed on the surface of roots; however, isolate GD3 was deeply localized into surface furrows of roots. Transmission electron microscopy showed considerable alterations of root cells including vesiculation, partial cell wall degradation, and cytoplasm disorganization. The average population density of isolate GD4 on the root surface was about 10 and 100 times greater than GP4 and GD3, respectively. Root elongation of seedlings inoculated with isolates GD3 and GD4 after 7 d of growth was significantly inhibited by ca. 26% and 90%, respectively, compared to the control. This study showed that inhibition of morningglory root growth by isolate GD3 might be related to production of high concentrations of IAA although other phytotoxins likely contributed to inhibiting root elongation of morningglory inoculated with isolate GD4. Rhizobacteria able to suppress morningglory growth may be effective as biological control agents to supplement herbicide weed management in crops where morningglory is difficult to control.

Keywords: Deleterious rhizobacteria, *Pseudomonas*, *Bradyrhizobium*, scanning electron microscopy, transmission electron microscopy, colonization

1. Introduction

The introduction of glyphosate-resistant soybean has changed traditional herbicide management from that based largely on soil-applied residual herbicides for specific weed species to one of almost complete reliance on postemergence applications of herbicides for nonselective control of weeds. Glyphosate [N-(phosphonomethyl) glycine; Roundup] is a foliar-applied, broad spectrum, non-selective herbicide that controls a wide array of grass and broadleaf weeds. A single postemergence application is usually sufficient to control weeds in glyphosate-resistant crops planted in narrow rows (Ateh and Harvey, 1999; Culpepper et al., 2000; Wait et al., 1999).

However, previous studies have shown that several annual weeds, such as ivyleaf morningglory (*Ipomoea hederacea*), velvetleaf (*Abutilon theophrasti*), hemp sesbania (*Sesbania exaltata*), and acanthaceas (*Dicliptera chinensis*) are more difficult to control with glyphosate than many other common weeds (Krausz et al., 1996; Jordan et al., 1997; Yuan et al., 2002). Frequently, annual weed species tolerant to glyphosate lead to increased application rates of glyphosate or additional applications of other herbicides for residual weed control in glyphosate-resistant crops (Johnson et al., 2002).

An alternative to herbicides is biological weed control using deleterious rhizobacteria (DRB). Kremer et al. (1990) reported a wide variety of DRB which inhibited *in vitro* seedling growth of velvetleaf (*Abutilon theophrasti*), morningglory (*Ipomoea* spp.), cocklebur (*Xanthium canadense*), pigweed (*Amaranthus* spp.), common

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lambsquarters (*Chenopodium album*), smartweed (*Polygonum* spp.), and jimsonweed (*Datura stramonium*). The effects of these DRB on host seedlings varied depending on root-colonizing ability, specific phytotoxin production and resistance to antibiotics produced by other rhizosphere microorganisms. Metabolites that have been implicated in deleterious activity include hydrogen cyanide (Alström and Bruns, 1989; Bakker and Schippers, 1987), phytohormones including indole-3-acetic acid (IAA) (Loper and Schroth, 1986; Schippers et al., 1987), and other unidentified phytotoxins (Bolton and Elliott, 1989; Fredrickson and Elliott, 1985a,b).

Growth suppressive activity based on production of high amounts of IAA has been described for several DRB including a transgenic rhizosphere pseudomonad (Dubeikovsky et al., 1993), *Enterobacter taylorae* (Sarwar and Kremer, 1995), and *Pseudomonas putida* (Barazani and Friedman, 1999; Xie et al., 1996). Liu et al. (1982) reported that a gene (*iaaP*) necessary for IAA synthesis by *Agrobacterium* spp. induced crown gall tumor formation or "hairy roots" in host plants. Gaudin et al. (1994) showed that the IAA biosynthesis pathway in bacteria (i.e., *Agrobacterium*, *Pseudomonas*, *Bradyrhizobium*), in which tryptophan is converted to IAA, was much simpler than pathways in plants. The host plants may provide a favorable environment for bacteria to proliferate and produce excessive amounts of IAA thus weakening the plant and promoting root colonization.

Colonization of root surfaces of weed seedlings by DRB has been described in a limited number of studies using electron microscopy that provides topographical and morphological images of the colonization features of rhizobacteria. Souissi et al. (1997) reported that colonization and attachment of the rhizobacteria, *P. fluorescens* isolate LS102 and *Flavobacterium balustinum* isolate LS105, to the root surfaces of leafy spurge (*Euphorbia esula*) were associated with microfibrils likely anchoring and entrapping bacterial cells. Transmission electron microscopy showed cell walls of the host plant apparently degraded at some points of contact between rhizobacteria and leafy spurge cells. Li et al. (2002), using scanning electron microscopy, demonstrated that selected DRB strains, *P. fluorescens*, *P. putida*, and *Stenotrophomonas maltophilia*, densely colonized the roots of green foxtail (*Setaria viridis*) and significantly reduced *in vitro* seedling growth by 50%.

Ivyleaf morningglory or entireleaf morningglory is a noxious weed and one of the most competitive weed species in soybean production (Dowler, 1992). Morningglory species are not greatly susceptible to glyphosate and may become increasingly tolerant as widespread use of glyphosate-resistant crops continues (Shaner, 2000). Glyphosate-resistant soybeans (Roundup Ready) harbor a diverse community of bacteria in the rhizosphere, which may include DRB, with weed-suppressive activity. If DRB that suppress morningglory growth exist in the soybean rhizosphere, these might be exploited for biocontrol and supplement weed control with glyphosate.

The rhizobacteria communities in agroecosystems are very diverse and include species of *Pseudomonas*, *Enterobacter*, *Bacillus*, *Bradyrhizobium* and many other genera (Li and Kremer, 2000; Sarwar and Kremer, 1995). For this study, we selected rhizobacteria classified as *Pseudomonas* and *Bradyrhizobium*, both with different effects on seedling growth of morningglory, to better understand the diverse root colonization patterns that influence the rhizobacteria-plant interaction. Although *Bradyrhizobium* species are well known for biological nitrogen fixation in association with soybean, they can have other effects on growth of legumes and non-legumes (Antoun et al., 1998; Werner, 1992). The objective of this study was to investigate the effects of selected rhizobacteria from different soil ecosystems on the root growth, colonization, and root cellular structure of ivyleaf morningglory seedlings.

2. Materials and Methods

Rhizobacteria cultures

Pseudomonas putida isolate GD4 was previously shown to effectively suppress seedling growth of several weeds, whereas *Bacillus megaterium* GP4 promoted weed seedling growth (Li and Kremer, 2000; Li et al., 2002). *Bradyrhizobium japonicum* isolate GD3, originating from Roundup Ready soybean roots, suppressed the seedling growth of ivyleaf morningglory (Kim and Kremer, in preparation). Isolate GD3 was isolated by using an IAA screening method based on an *in situ* membrane assay (Bric et al., 1991) for detecting IAA-producing bacteria. This color indicator assay revealed that isolate GD3 qualitatively produced high levels of IAA. Each bacterial isolate was cultured on King's B agar medium and incubated at 27°C for 48 h prior to use in assays (King et al., 1954).

IAA assays

IAA production by the bacterial cultures in half-strength King's B broth was measured colorimetrically using Salkowski's reagent (Gordon and Weber, 1951). For the IAA assays, a 24-h broth culture of each isolate was diluted in sterile water to an O.D. of 0.5 at 500 nm. The suspension (1 ml) was added to 14 ml of half strength King's broth in a 30 ml tube. Controls were prepared by substituting sterile water for the bacterial suspension. Tubes were capped, vortexed, statically incubated in the dark at 27°C for 72 h, and centrifuged for 10 min at 6,000 rpm. Salkowski's color-developing reagent was prepared by mixing 0.5 M $FeCl_3$ (2 ml) with 35% perchloric acid (98 ml) (Gordon and Weber, 1951). IAA present in the culture broth supernatants (3 ml) was reacted with the reagent (2

ml) to yield a pink-colored product after 30-min incubation, which was quantitatively measured on a spectrophotometer at 530 nm.

Seedling growth and inocula preparation

Ivyleaf morningglory seeds were surface sterilized by immersing in 70% ethanol for 2 min, rinsing in sterile water, immersing in 1.25% sodium hypochlorite for 4 min, rinsing 4–6 times with sterile water and blotting on autoclaved filter paper. The surface-sterilized seeds were germinated in Petri dishes containing 1.5% agar. Petri dishes were wrapped with parafilm and incubated at 27°C overnight. Bacteria were grown on tryptic soy agar for 24 h to provide inocula for pre-germinated seeds in the hydroponic system. For inocula preparation, bacterial cultures were suspended in peptone broth (0.1%) and spectrophotometrically adjusted to 10^8 cells ml^{-1} at 500 nm.

The hydroponic system

The effects of DRB isolates on ivyleaf morningglory were monitored in a hydroponic system by measuring root elongation of seedlings growing in nutrient solution. The pouches (Northrup-King) are made of plastic bags (16 × 18 cm) with germination paper wick inserts and contain 20 ml of nutrient solution (Hoagland and Arnon, 1938). Potassium nitrate (0.5 mM) was added to the nutrient solution and the pH was adjusted to 6.7. Three pre-germinated morningglory seeds were aseptically placed on the paper towel wick and inoculated with 2 ml of bacterial inoculum two times at an interval of 2 days. The pouches were placed at ambient temperature (19–24°C) under a 12 h light and 12 h dark period supplemented with fluorescent lamps. Five replicate pouches were prepared per treatment.

Electron microscopic studies

Seedlings of 7-d old morningglory were randomly selected from growth pouches of each treatment for electron microscopic examination. Tissue samples from inoculated and non-inoculated seedling roots of ivyleaf morningglory were fixed in 2% glutaraldehyde (made up in 0.1 M cacodylate buffer) in the refrigerator (8°C) for 1.5 hr. Samples were washed two times in the same buffer for 10 min, postfixed in 1% OsO_4 for 4 hrs, and dehydrated as follows: 30%, 50%, 70%, 85%, and 95% ethanol for 15 min; 100% ethanol, two times for 15 min each. For scanning electron microscopy, the Critical Point Drying (CPD) method, sputter coating, and an Amray 1600 scanning electron microscope operating at 20 kv were used. For transmission electron microscopy, samples were fixed as above, dehydrated in acetone, and embedded in Epon 812 resin. Thin sections were made by ultramicrotome equipped with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM-100B transmission electron microscope. Root vascular systems and rhizobacteria colonization patterns were observed by TEM and SEM, respectively.

Variables measured and statistical analysis

Root elongation was measured periodically during a 6-d period after the hydroponic system was set up. Fresh plant top weight was measured after 7 d of growth. Root systems not used for SEM or TEM were suspended in phosphate-buffered saline (PBS; 0.01 M K_2HPO_4, 0.14 M NaCl, pH 7.2) and agitated vigorously on a vortex shaker for 5 min. The populations of each bacterial isolate on roots were determined by serially diluting root suspensions in PBS, spread-plating onto duplicate plates of King's B agar, and incubating for 72 hr at 27°C. The study was set up in a completely randomized block design. Three DRB and a control were tested for root colonization and seedling fresh weight. The data were subjected to analysis of variance and, where the F-test was significant, treatment means were separated using Fisher's protected LSD (α=0.05).

3. Results and Discussion

Rhizobacteria properties

A comparison of IAA production revealed wide differences among the rhizobacterial isolates used in the study (Table 1). Isolate GD3, originating from a soybean rhizosphere, was an extremely more prolific IAA producer than isolate GP4, presumably a PGPR. The variability in IAA production by rhizobacteria has been documented previously (Sarwar and Kremer, 1995).

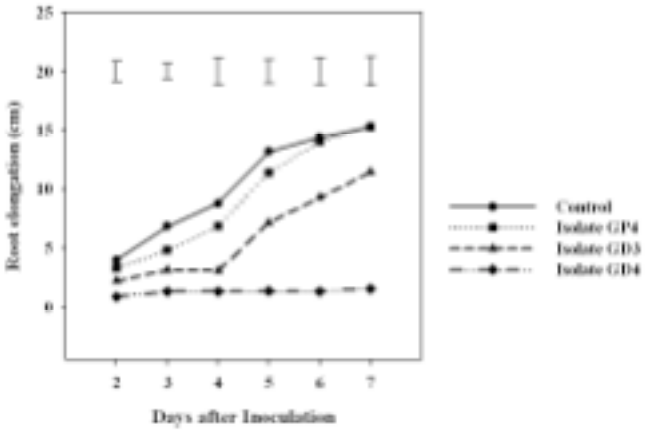

Figure 1. Root elongation of morningglory seedlings inoculated with *B. megaterium* isolate GP4, *B. japonicum* isolate GD3, and *P. putida* isolate GD4 in the hydroponic system. Treatments different from each other at P<0.05 (5 to 7 days after inoculation) are denoted with different letters.

Table 1. IAA production, root colonization, and shoot weight of ivyleaf morningglory affected by selected rhizobacteria.

Treatment	IAA[1] (mM)	Root colonization[2,3] Log_{10} (cfu)	Plant shoot fresh weight[2] (mg/plant)
Control	0	0	280 a
Bacillus megaterium GP4	0.28 c[4]	5.1 ab	260 a
Bradyrhizobium japonicum GD3	64.0 a	4.8 b	225 a
Pseudomonas putida GD4	6.9 b	6.2 a	60 b

[1]Indoleacetic acid production in broth culture determined by colorimetric assay (Gordon and Weber, 1951). [2]Data collected at 7 d after seedling emergence in the greenhouse study. [3]Colonization expressed as colony-forming units (c.f.u.) per g root fresh weight. [4]Means within a column followed by the same letter are not significantly different at the 5% level based on mean separation by least significant difference (LSD) test.

Growth responses of ivyleaf morningglory to the three rhizobacterial isolates varied in seedling root growth and fresh top weight (Fig. 1 and Table 1). Seven days after inoculation, root elongation was significantly inhibited by isolates GD3 and GD4, which reduced growth by ca. 26% and 90%, compared to the control, respectively (Fig. 1). Isolate GD4 not only severely inhibited root growth but also significantly reduced fresh top weight of morningglory determined 7 d after inoculation (Table 1).

All rhizobacteria colonized the roots of morningglory seedlings within 7 d after inoculation (Table 1). Isolate GD3 colonized morningglory at the lowest population level probably because it normally colonizes the soybean rhizosphere. However, isolates GD4 and GP4, originating from other host weeds, colonized morningglory roots at high population levels. These high numbers may result from bacterial growth stimulation by specific root exudates (Brimecombe et al., 2001). Additionally, rhizobacteria may be specifically attracted to roots through chemotaxis (Begonia and Kremer, 1994), contributing to establishment of high cell densities on the root surface. Therefore, these aggressively colonizing rhizobacteria are likely to severely damage weed seedlings due to phytotoxins produced by the high numbers of rhizobacteria on the root surface (Kremer and Kennedy, 1996). Deleterious activity was noted on root surfaces inoculated with isolate GD4 where abnormally dark brown and wrinkled lesions were observed (data not presented), typical symptoms of phytotoxic damage (Goodman et al., 1986).

Scanning electron microscopic observations

Primary root sections of ivyleaf morningglory examined by SEM revealed that cells of isolates GP4 and GD4 were consistently distributed on the surface of roots (Figs. 2B and D); however, isolate GD3 was partially localized into surface furrows of roots (Fig. 2C). Surface furrows appeared to be located at epidermal cell junctions. Root seedlings free of inoculant bacteria typically revealed a smooth, undamaged epidermal root surface (Fig. 2A). Visual examination of roots inoculated with isolates GD3 and GD4 appeared to be more distorted than with isolate GP4 (data not shown).

Root surfaces from isolate GD4-inoculated seedlings were colonized with many clusters of cells associated with fibrillar material (arrows), which contributed to the formation of "microcolonies" (Fig 2D). Microcolony formation by DRB on root surfaces frequently occurs with effective colonization (Begonia et al., 1990). Fibrillar materials are likely extracellular polymeric substances (EPS) composed of proteins and nucleic acids as well as polysaccharides (Wingender et al., 1999). When rhizobacteria are entrapped in such matrices, production of high IAA concentrations is possible, shown previously for rhizobacteria colonizing maize roots (Benizri et al., 1998).

Ultrastructural observations with TEM

Light microscopic examination of root sections revealed bacteria within endodermal cells, however, no bacteria were found in cortex tissues. Thin sections of the endodermis examined by TEM had considerably altered root cells showing degradation of plasma membranes (or plasmalemmae) and partial cell wall and cytoplasmic disruptions (Fig. 3B). Extracellular materials (arrowhead) released by bacteria were observed, similar to polysaccharides reported in a previous study (Souissi et al., 1997). The interaction between the extracellular substances of bacteria and cell wall components of the host cell appeared to damage the cell wall. Cell damage was not observed in control tissues (Fig. 3A) in which cell walls and plasmalemmae were distinct and continuous. In cells infected by isolate GP4, bacteria were surrounded by host cell organelles or fused with cell organelles (Fig. 3C). The cell wall was not distorted.

4. Conclusion

A hydroponic system was used for SEM and TEM where pre-germinated ivyleaf morning glory seedlings were inoculated with rhizobacteria. Root elongation of seedlings inoculated by *B. japonicum* isolate GD3, and *P. putida* isolate GD4 was significantly inhibited (Fig. 1). Colonization of the root surface by bacteria is a necessary step to subsequent interactions between bacteria and host

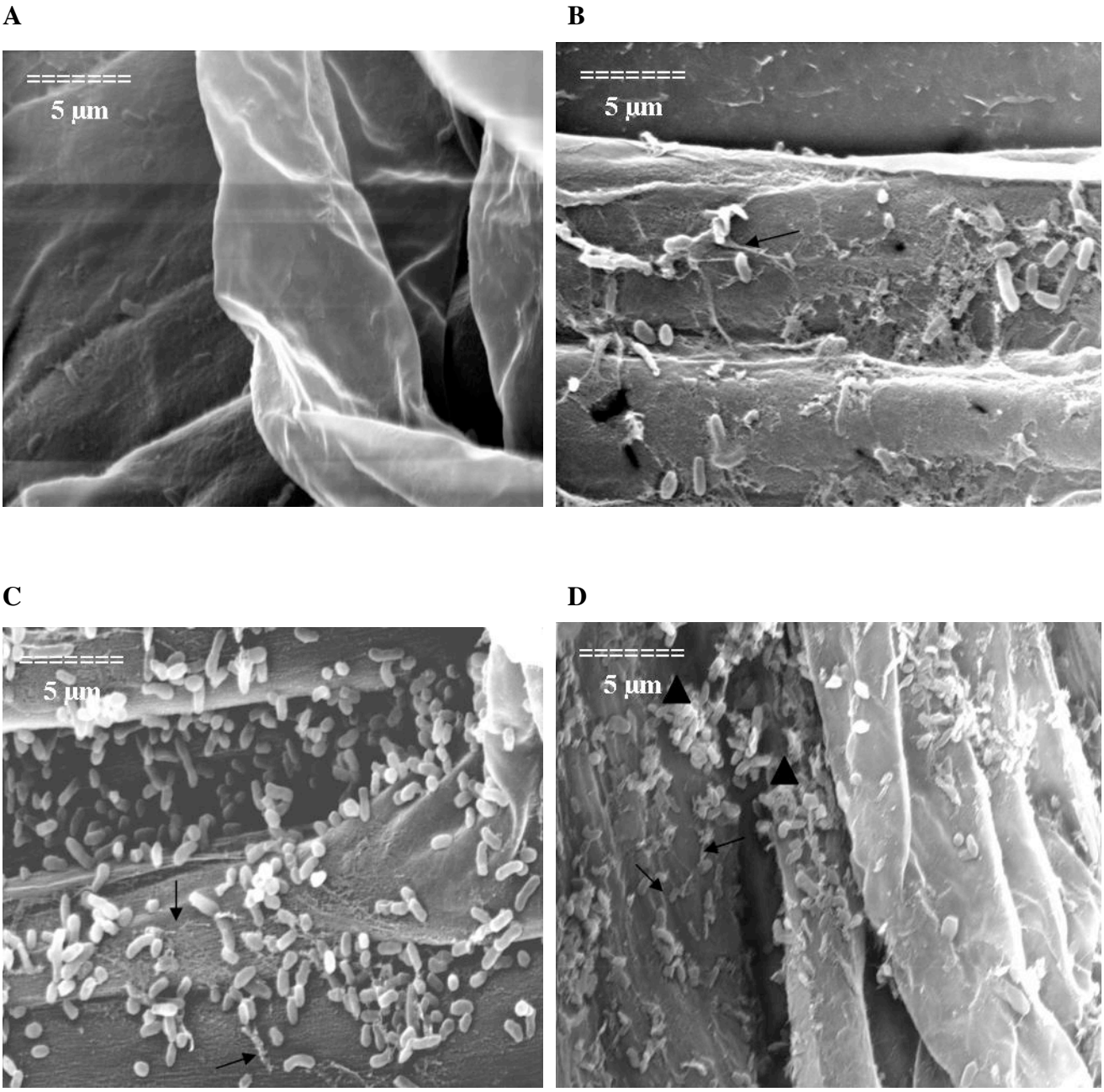

Figure 2. SEM of root surface of morningglory seedlings seven days after inoculation. (A) Root surface of non-inoculated seedlings free of bacteria; (B) *B. megaterium* isolate GP4 (B); (C) *B. japonicum* isolate GD3, and (D) *P. putida* isolate GD4. Examples of distinct fibrillar material released by bacteria are denoted by arrows in B, C, and D; formation of microcolonies or clustered cells are denoted by arrowheads in C and D. Bar=10 μm.

cells. Bacterial attachment to plant surfaces begins with attraction by seedling root exudates including amino acids, sugars, organic acids, and phenolic fractions (Begonia and Kremer, 1994). The ability of rhizobacteria to migrate chemotactically to substances released by seedling roots of ivyleaf morningglory may lead to higher bacterial colonization of roots. In studying the mechanisms of *P. putida* and *B. japonicum* attachments to the root surface,

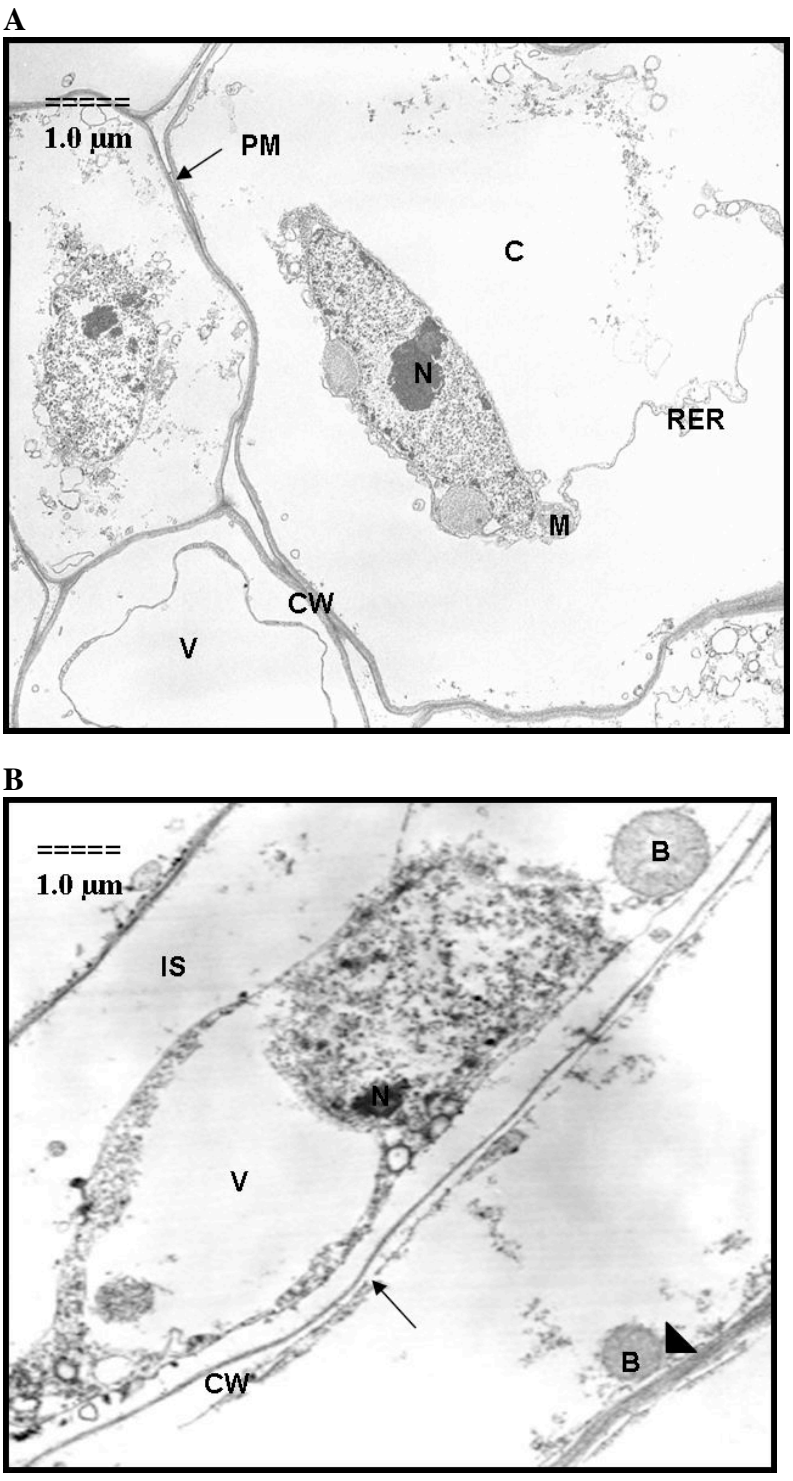

Figure 3. TEM of morningglory seedling root tissue seven days after inoculation. Non-inoculated root tissue (A); root tissue inoculated with isolate GD4 (B) in which partial plasma membrane and cell wall dissolution (arrow) and the association between bacterial cells and root cell wall components (arrowhead) are evident; root tissue inoculated with isolate GP4 (C) in which bacterial cells are surrounded by host cell organelles. B, bacterium; C, cytoplasm; IS, intercellular space; M, mitochondrion; N, nucleolus; RER, rough endoplasmic reticulum; PM, plasma membrane; V, vacuole. Bar=1.0 μm.

Begonia and Kremer (1994) suggested that chemotaxis and IAA produced by bacteria might be responsible for weed growth suppression. Such mechanisms would enhance the capability to colonize and adversely affect host tissue as observed by SEM and TEM. Rhizobacteria may affect plant hosts via mechanisms similar to phytopathogenic bacteria through production of enzymes, phytotoxins, or phytohormones (Loper and Schroth, 1986; Schippers et al., 1987).

As shown in our study, cell walls of morningglory roots appeared to be dissolved or eroded perhaps due to hydrolytic action of enzymes produced by the inoculant rhizobacteria.

C

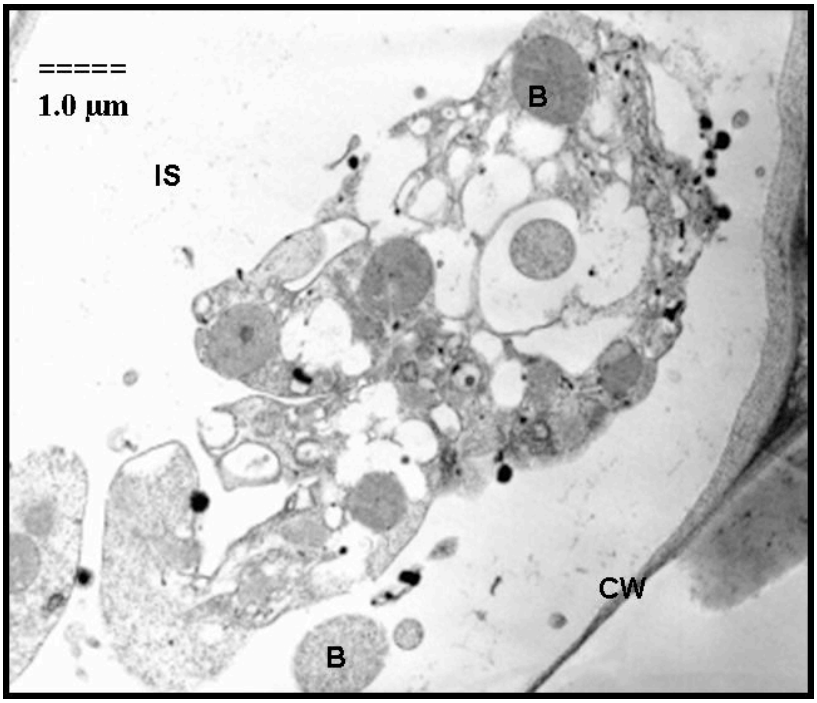

Figure 3. See legend on previous page.

A previous study reported that a *Pseudomonas fluorescens* isolate deleteriously affected leafy spurge growth through production of proteases (Souissi et al., 1997). Rhizobacteria in the present study may be potential biocontrol agents for managing morningglory, which is difficult to control with herbicides in many cropping systems, and may be very tolerant to glyphosate applied at recommended rates to glyphosate-resistant crops. Furthermore, *B. japonicum* isolate GD3 may readily establish high populations in soybean rhizospheres, colonize developing roots of morningglory seedlings adjacent to soybean, and subsequently suppress morningglory growth, thereby supplementing herbicide control of this weed.

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