

Ultrastructural localization and identification of *Azospirillum brasilense* Cd on and within wheat root by immuno-gold labeling

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Abstract

Azospirillum brasilense Cd localization in wheat roots was studied by light microscopy, by scanning, and by transmission electron microscopy. *A. brasilense* Cd cells were specifically identified immunocytochemically around and within root tissues. *A. brasilense* Cd cells found both outside and inside inoculated roots were intensively labeled with colloidal gold. In non-axenic cultures other bacterial strains or plant tissue were not labeled, thereby providing a non-interfering background. The roots of axenic grown wheat plants were colonized both externally and internally by *A. brasilense* Cd after inoculation, whereas non-axenic cultures were colonized by other bacterial strains as well. *A. brasilense* Cd cells were located on the root surface along the following zones: the root tip, the elongation, and the root-hair zone. However, bacteria were located within the cortex only in the latter two zones. In a number of observations, an electron dense material mediated the binding of bacterial cells to outer surfaces of epidermal cells, or between adjacent bacterial cells. *A. brasilense* Cd were found in root cortical intercellular spaces, but were not detected in either the endodermal layer or in the vascular system. This study proposes that in addition to root surface colonization, *A. brasilense* Cd forms intercellular associations within wheat roots.

Introduction

The genus *Azospirillum* has the ability to form a potentially beneficial interaction with a variety of higher plants (Patriquin *et al.*, 1983). This interaction can form two different associations: an external one and an internal one. In an external association, the bacteria form an aggregate mode of root colonization, and are sometimes embedded in the mucigel layer on the root surface. (Berg *et al.*, 1979; Jain and Patriquin, 1984; Patriquin, 1981; Patriquin *et al.*, 1983; Schank *et al.*, 1979; Umali-Garcia *et al.*, 1980). Live roots, dead roots or even synthetic particles can be colonized (Bashan, 1986b; Bashan *et al.*, 1986). In an internal association,

bacteria penetrate the root interior spaces (Döbereiner and Day, 1976; Okon and Hardy, 1983; Patriquin, 1981; Patriquin and Döbereiner, 1978). In these reports on the mode of colonization in both external and internal root tissues, identification of the bacteria has not been substantiated. Thus, it remained doubtful whether the bacteria detected were the inoculated *Azospirillum* or the many other rhizosphere bacteria capable of colonizing roots (Bashan, 1986a; Reinhold and Hurek, 1988; Reinhold *et al.*, 1986; Suslow, 1982).

The use of an immunofluorescent technique (Schank *et al.*, 1979) gave a partial qualitative solution for identification of *Azospirillum* in roots, but the method was limited by stellar autofluorescence

and non-specific binding of the conjugate. On the other hand, ultrastructural studies may reveal the exact interaction between bacteria and plants, but special care should be taken to specifically identify the observed bacteria. Thus, ultrastructural study should be combined with an identification method (Foster, 1986).

The usefulness of the immunogold cytochemistry combined with transmission electron microscopy for highly specific precise ultrastructural identification of cell substances and their localization is well established for animal tissue (de Mey *et al.*, 1981; Roth, 1983; Roth *et al.*, 1978). However, utilization of this technique in plant-bacteria interaction is rare (Van Laere *et al.*, 1985), scarce in soil microbiology (Jones *et al.*, 1987), and to the best of our knowledge, it was performed only on *Azospirillum* cultures *in vitro* (Levanony and Bashan, 1989).

The aims of the present study were to visibly detect, locate, identify and follow the distribution

of *A. brasilense* Cd on and within roots at the ultrastructural level by using the specific colloidal-gold immunocytochemistry method. Preliminary report of these findings were presented elsewhere (Bashan and Levanony, 1988a).

Material and methods

Organisms, growth conditions and bacterial inoculation

Wheat seeds (*Triticum aestivum* cv. 'Deganit') were disinfected in 1% NaOCl for 5 min, rinsed in sterile tap water under a Laminar Flow, and grown in sterile glass test tubes, as described in Fig. 1a. The plants grew in a controlled growth chamber (Model EF7H, Conviron, Controlled Environments Co. Canada) at $23 \pm 2^\circ\text{C}$ and 12h light ($130 \mu\text{E}_{\text{in}} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$). After 48–72 h the water in the test tubes was replaced by double washed

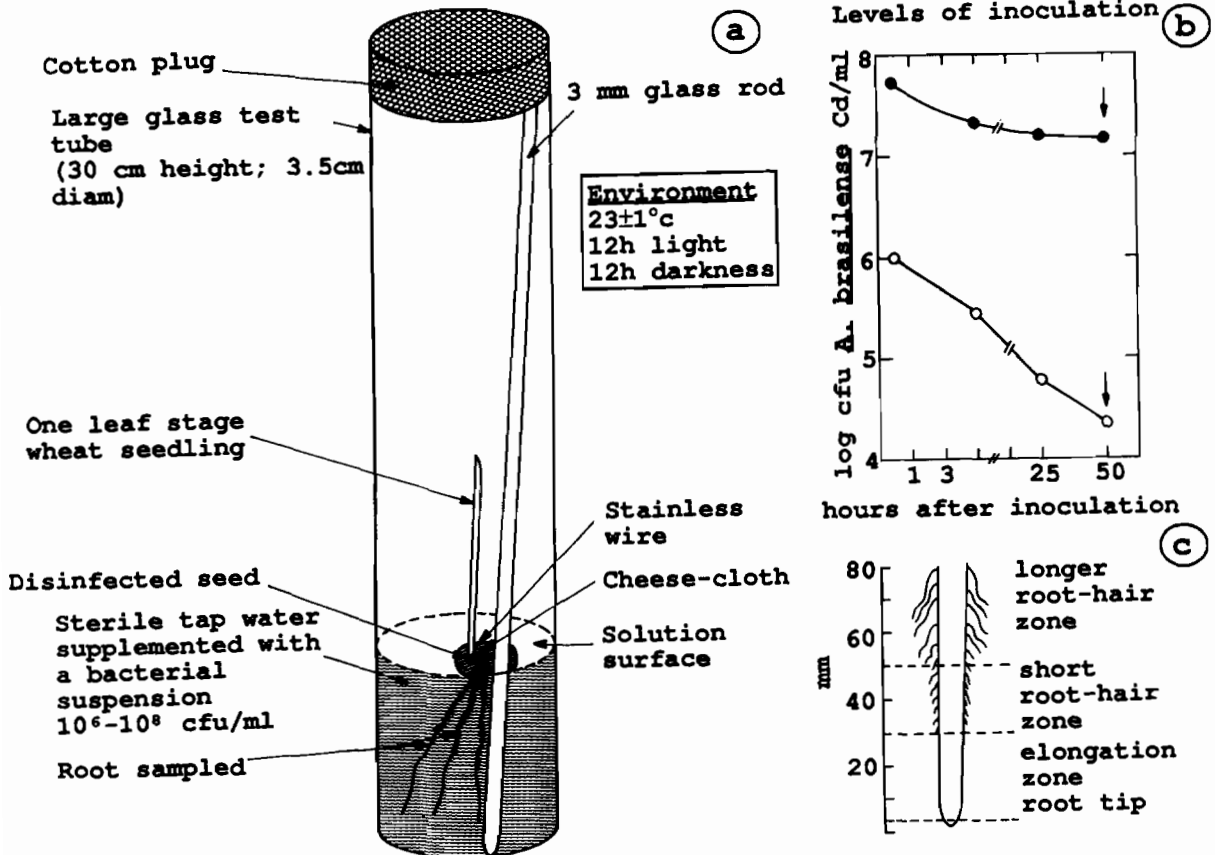


Fig. 1. Schematic representation of an axenic and non-axenic (a) hydroponic system used for plant growth. (b) The level of bacterial inoculation during the course of the experiment. Initial bacterial number of 10^8 cfu mL⁻¹ (●) and 10^6 cfu mL⁻¹ (○). Arrows indicate sampling time, and (c) location of zones used for ultrastructural observations.

Azospirillum brasilense Cd (ATCC 29710) suspensions containing usually 10^6 (the optimal inoculation level of this bacterial strain (Bashan, 1986d)) or in few cases 10^8 colony forming units mL^{-1} . Bacteria were cultured from a single cell colony as previously described (Bashan and Levanony, 1985) and a decrease in the level of bacteria in the inoculation suspension was monitored during the course of the experiment (Fig. 1b). Control plants were grown in water only. Non-axenic hydroponic experiments were conducted at the same time by using wheat seeds without any prior disinfection treatment. The seedlings developed from these seeds in tap water (water population of 150–200 bacterial cells/mL) had total root population of 10^4 – 10^7 bacterial cells/g fresh wt of roots. The exact species of these naturally occurring bacterial contaminants were not defined since they varied from one seed batch to the other. In general, these field-harvest untreated seeds contained mainly populations of fluorescent pseudomonads, *Bacillus* sp. and *Erwinia* sp. A previous immunological survey of the natural microflora of wheat seeds had showed that none of these seed-contaminant bacterial species had any cross reaction with the specific antibodies raised against *A. brasilense* Cd at their normal concentrations detected on wheat roots (Levanony and Bashan, 1989; Levanony *et al.*, 1987). No significant difference was found between the numbers of *A. brasilense* Cd in and on wheat roots grown in axenic and non-axenic cultures (10^4 – 5×10^5 cfu g^{-1} fresh wt root, surface population and 10^6 – 4×10^7 cfu g^{-1} fresh wt root, internal population). Forty eight h after inoculation, roots of both types of cultures were removed under a stereoscopic microscope (model SMZ-1B, Nikon, Japan). Each root was divided into three zones as described in Fig. 1c: (i) the root-tip and elongation zone, 10–30 mm long; (ii) the short root hair zone, 20–30 mm long; and (iii) the proximal zone of longer root-hairs, 15–35 mm long (Zone sizes were dependent on morphology of each root). These root segments were immediately fixed and prepared as described below for light and electron microscopy. The orientation of the segments was retained throughout all preparation procedures. In addition, mixed bacterial cultures were obtained by stir mixing of equal numbers (10^8 cfu mL^{-1}) of *A. brasilense* Cd with one (each time) of the naturally occurring bacteria isolated from wheat roots.

Light microscopy

In order to investigate the wheat plantlet's root anatomy, paraffin sections were obtained. Roots of plantlets were divided into three zones as described above and fixed in FAA (ethyl alcohol 95% — 50 mL, glacial acetic acid — 5 mL, formaldehyde 37–40% — 10 mL, distilled water — 35 mL) for 14 h at room temperature under vacuum to enhance fixative penetration. The roots were then dehydrated in a gradual series of ethanol (70% — 24 h, $2 \times 100\%$ — 1 h, 100% — 14 h). Xylene was gradually introduced (first with absolute ethanol:xylene 3:1, 2:1 and 1:1 — 1 h in each solution and then with xylene — 1 h). Afterwards, the root zones were embedded in paraffin and oriented in the blocks in their original order. Seven μm sections were first stained with safranin and then with fast green, and mounted on a glass slide with permount (NJ Fisher).

Scanning electron microscopy (SEM)

Inoculated and control root segments were prepared for scanning electron microscopy as described previously (Bashan *et al.*, 1986), using only an inoculum concentration of 10^6 cfu mL^{-1} .

Transmission electron microscopy (TEM)

The different root zones were cut into pieces of 10 mm and fixed in 5% glutaraldehyde (Polysciences, Warrington, Pa.) in 0.1 M cacodylate buffer pH 7.2. Roots in fixative were vacuum infiltrated in a desiccator and placed on a miniature rotary shaker (Penetron, Sunkay Laboratories, Tokyo) for 90 min. at room temperature. The roots were rinsed in the same buffer, postfixed with 1% osmium tetroxide (Polysciences) in the same buffer for 1 h at room temperature, and then rinsed and left overnight in the buffer at $4 \pm 1^\circ\text{C}$. Roots were block-stained (2% uranyl acetate (Polysciences) in distilled water) on the shaker for 10–35 min. in the dark. After several washing in distilled water, the roots were dehydrated in two changes each of an ethanol series (50%, 70%, 90% and 100%), for 10 min, and embedded in Spurr's Resin mixture (Spurr low viscosity embedding kit, Polysciences, Spurr, 1969), in flat embedding blocks. The va-

rious root portions were embedded to maintain their original orientation in the blocks.

Thick sections ($1\ \mu\text{m}$) were stained with 1% Toluidine-blue (Mallinckrodt, USA) and examined in a light microscope (Zeiss, Oberkochen, FRG). Thin sections ($900\text{--}1000\ \text{\AA}$) of the different segments were stained first with uranyl acetate for 1 h and then with lead citrate, and examined with Phillips EM-410 Transmission Electron Microscope (Eindhoven, Holland). The possibility that bacterial sections were transferred from their original site by sectioning was also tested in every block. No such transfers were found in more than ten blocks tested.

Antibody production

Whole cells of *A. brasilense* Cd were used to elicit antibodies in white rabbits by immunization with multiple intradermal injections as described elsewhere (Levanony *et al.*, 1987). These polyclonal antibodies after purification showed high specificity towards *A. brasilense* Cd with negligible cross reactions to other rhizosphere bacteria or to several other *Azospirillum* species (Levanony and Bashan, 1989; Levanony *et al.*, 1987).

Immuno-gold labeling for TEM

Thin sections ($900\text{--}1000\ \text{\AA}$) were placed on nickel grids. In some cases, a support film of Parlodion was also needed. The entire procedure was carried out at room temperature. All buffers and other solutions were filtered through $0.45\ \mu\text{m}$ filters (FP 030/2, Schlecher and Schuell, U.S.A.). Grids, with section down, were passed from drop to drop. Sections were first decolorized with 10% H_2O_2 for 10 min, then rinsed in distilled water. Non-specific binding was blocked for 15 minutes with 1% egg albumin (grade v, Sigma Chemical, St. Louis, Mo.) in phosphate buffered saline ($0.06\ \text{M}$ phosphate buffer supplemented with $0.15\ \text{M}$ NaCl; PBS) pH 7.2 that was supplemented with 0.05% Tween-20 (Sigma) and with $20\ \text{mM}$ NaN_3 . Excess solution was removed and the grids were transferred to specific anti-*A. brasilense* Cd primary antibody diluted 1:1000 in PBS. After 90 min at room temperature the grids were rinsed in PBS and blocking

was repeated with 1% egg albumin for 4 min. Excess solution was removed and the grids were incubated with secondary antibody goat-anti-rabbit immunoglobulin conjugated to colloidal gold (AuroProbe-EM, GAR-G15, Janssen, Belgium) diluted 1:10 in Tris-buffered saline (TBS) ($20\ \text{mM}$ tris HCl, $0.15\ \text{M}$ NaCl, pH 7.4) for 30–60 min. The grids were rinsed first in PBS, then in distilled water, and finally stained with uranyl-acetate, with the addition of lead-citrate in some cases. In a few cases a carbon coating was needed to protect the

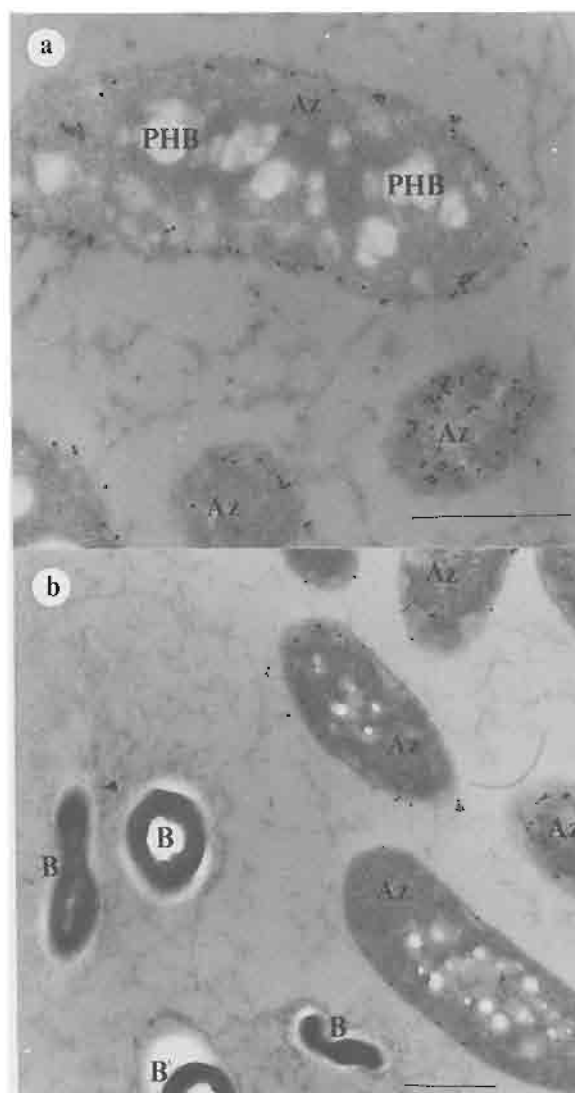


Fig. 2. TEM micrograph of colloidal-gold labeled *A. brasilense* Cd in (a) pure culture and (b) mixed culture. The bars represent $0.5\ \mu\text{m}$.

sections. The sections were examined under TEM. Various solution concentrations, buffers and incubation periods were tested, and the above procedure was found to be optimal for observing this particular plant-bacterial material.

Cytochemical controls

The following controls were tested: (i) incubation of the sections with goat anti-rabbit-gold complex, without previous incubation with primary antibodies; (ii) adsorption of the primary antibodies with an excess of antigen before application to the sections; and (iii) incubation with pre-immune serum. (Levanony *et al.*, 1987).

Quantitative evaluation of the gold labeling

The density of the gold-labeling over bacterial cells from liquid culture was compared to the labeling over bacteria present near the epidermis in sections of inoculated roots and over bacterial cells that penetrated into the cortex intercellular spaces. Since only antigenic sites present at the surface of the section interact with the specific antibody, the density of the labeling was evaluated in relation to the circumference of each bacterium on the thin section. The circumference was measured on the micrographs and the density of the labeling was calculated and statistically analyzed by Duncan's Multiple Range Test at $P \leq 0.001$.

Results

Ultrastructural identification of A. brasilense Cd in pure and mixed cultures

Pure *A. brasilense* Cd cultures, as well as cultures of mixed bacteria (originated from non-disinfected wheat seeds), were observed by TEM, using the immunogold cytochemical technique. In pure cultures of *A. brasilense* Cd, all cells were labeled with gold around their cell wall (Fig. 2a), whereas in mixed cultures, (10 mixtures) only part of the bacteria observed were labeled and identified as *A. brasilense* Cd. In addition, the non-labeled bacteria had some morphological differences compared to

A. brasilense Cd cells (Fig. 2b). The controls (both those which were not incubated with primary antibodies and those incubated with antiserum that previously had been adsorbed with the antigen) were not labeled (data not shown). Random adsorption of colloidal-gold to non-*A. brasilense* Cd materials was negligible.

Localization of Azospirillum cells along and within wheat roots by light and scanning electron microscopy

Light microscope observations of paraffin sections of the three root zones (Fig. 1c) enabled the study of the inner organization of tissues in young wheat roots, and the location of bacteria in different root tissues.

Observations of thick sections of Spurr-embedded inoculated roots showed that the intercellular spaces of the elongation zone and the root-hair zone were heavily populated with bacteria (Fig. 3). Whereas, the root tip area did not contain bacterial cells in its inner structure.

Scanning electron micrographs of roots inoculated with *A. brasilense* Cd at normal levels revealed massive colonization on the outer surface of the root tips (Fig. 4a), on the elongation zone (Fig. 4b), and on the root epidermis in the root-hair zone (Fig. 4c). Generally, the level of *A. brasilense* Cd in the solution surrounding the roots decreased with time and became very low (10^4 cfu mL⁻¹, see Fig. 1b).

Identification and localization of A. brasilense Cd outside and within wheat roots in axenic and non-axenic cultures

Examination of the three root zones, which were labeled by the immunogold technique, was carried out by TEM. Observations were made from the outer surface towards the central part of the root in each zone. In the root tip zone, bacteria were

Abbreviations Figures 2-7: Az—A. brasilense Cd; B—unidentified bacteria; C—cortex cell; CW—plant cell wall; EN—endodermal cell; EP—epidermal cell; INT—intercellular spaces; M—plant cell membrane; N—nucleus; PHB—poly-β-hydroxybutyrate; RH—root hair; V—vacuole; VS—vascular system.

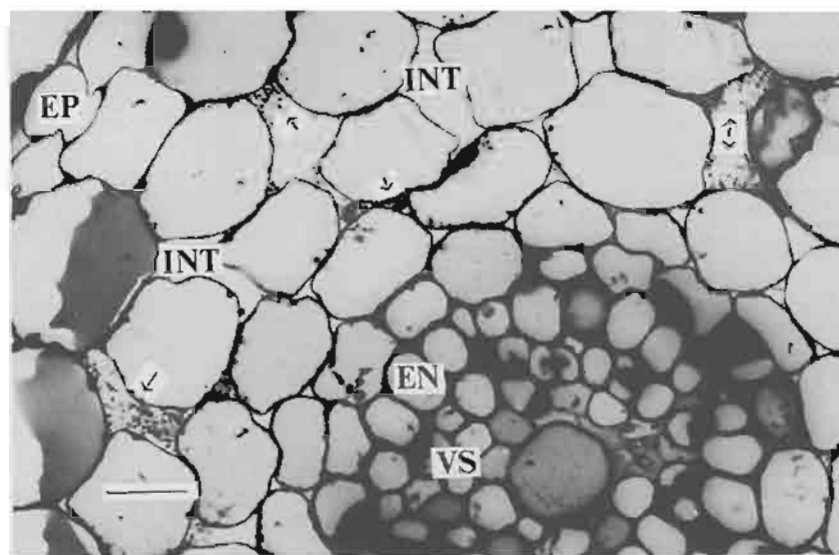


Fig. 3. Light micrograph of thick cross section of Spurr embedded roots showing the localization of *A. brasilense* within roots. Bacteria in intercellular spaces of inner layers of cortical cells in the elongation zone. Note the absence of bacteria in the endodermis and within the vascular system. Arrows indicate bacterial cells. Bar represent 20 μm .

present only on the outer surfaces of the roots. In the elongation zone, bacteria were detected on the outer surfaces (5a, 5b) and in the intercellular spaces of the cortex (Fig. 5d). In the short and the long root-hair zones, the distribution pattern of *A. brasilense* Cd was similar to that of the elongation zone. Many of the intercellular spaces of the cortex were filled with bacteria, whereas other intercellular spaces contained only a few cells of *A. brasilense* Cd (Fig. 5e). In non-axenic culture, other unidentified rhizosphere bacteria were observed near the epidermis (Fig. 5c). Although *A. brasilense* Cd cells penetrated into intercellular spaces deep in the cortex,

bacteria were not found in the root endodermis or vascular system.

The density of the gold labeling over the cells of *A. brasilense* Cd depends on the origin of the bacteria. The highest density was obtained in bacteria present in the intercellular spaces of cortical cells (Table 1).

Cell-cell interaction between A. brasilense Cd cells and plant cells

In several cases, an amorphous electron-dense material was present and formed connections between *A. brasilense* Cd cells and epidermal cells (Fig. 6a, 6b). Similar connections were observed between adjacent bacterial cells found near the epidermis layer (Fig. 6c, 6d). These connections seemed to be abundant on the non-polar side of the bacterium. However, *A. brasilense* Cd cells detected in the intercellular spaces of the root were free of any apparent connections to plant cell walls (see also Fig. 5d). This connecting material was found to be either thick or thin, and is probably strongly adherent, since it did not wash away even after the many rinsing steps required during fixation and the cytochemical labeling procedure.

Table 1. Density of gold labeling over the circumference of the bacterial cells in culture and in inoculated roots (gold particles per cm)

Bacterial cells origin	Labeling density*
Liquid culture	2.12 b ^b
Near the epidermis	3.2 b
Intercellular spaces	6.67 a

* Eight to 25 cells from different labeling experiments and sections were obtained.

^b Numbers followed by different letters differ significantly at $P \leq 0.001$ in Duncan's multiple range test.

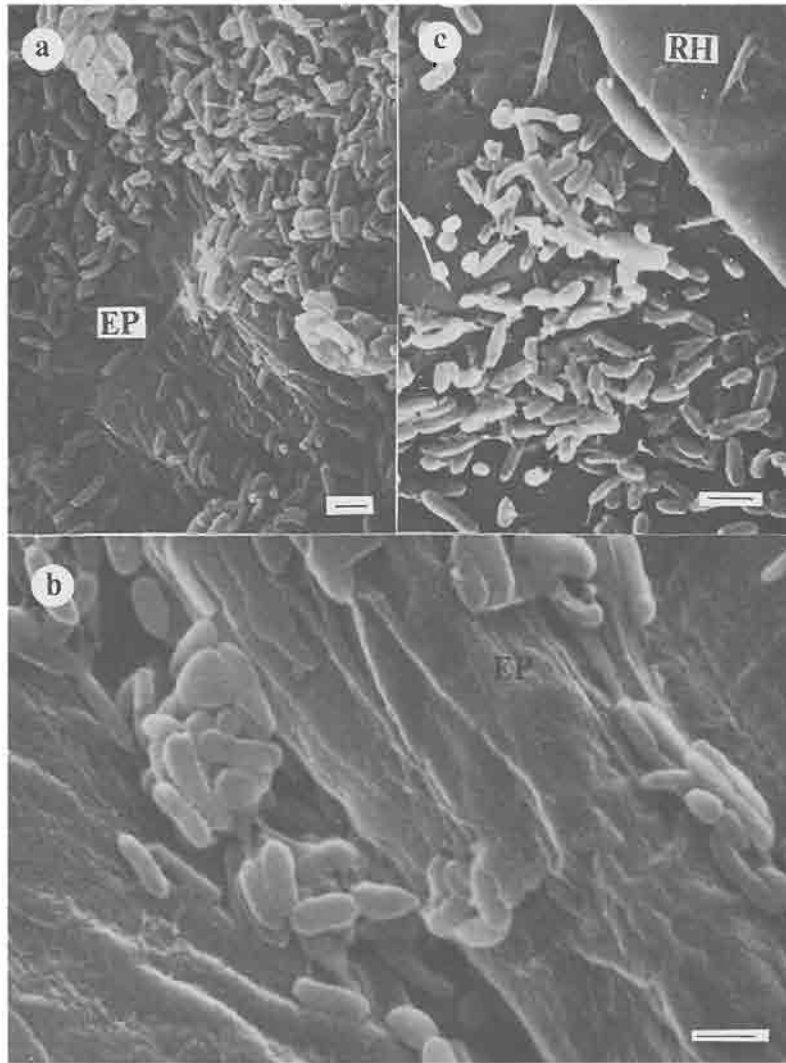


Fig. 4. SEM micrographs of *A. brasilense* colonization of wheat surfaces after inoculation with 10^6 cfu mL⁻¹, (a) Root tip zone, (b) elongation zone, and (c) root-hair zone. Note majority of the population are attached to the root and not to root hairs. Bars represent 1 μ m.

Polar and non-polar attachment of A. brasilense Cd to root epidermis

The orientation of *A. brasilense* Cd towards epidermal cells was observed by both TEM and SEM. Generally, most bacterial cells were randomly and non-polar oriented (Figs. 7a, 7b. See also Figs. 6a, 6c and 5c). However, detailed examination revealed that some *A. brasilense* Cd cells had a polar orientation (Figs. 7c, 7d).

Discussion

Azospirillum species are considered to be plant beneficial bacteria which have the potential to contribute to plant growth and yield, to adsorb to, and to proliferate on grass roots, and possibly to invade the internal parts of roots (Patriquin and Döbereiner, 1978; Patriquin *et al.*, 1983).

The interaction between rhizosphere bacterial cells and plant roots can be examined by light

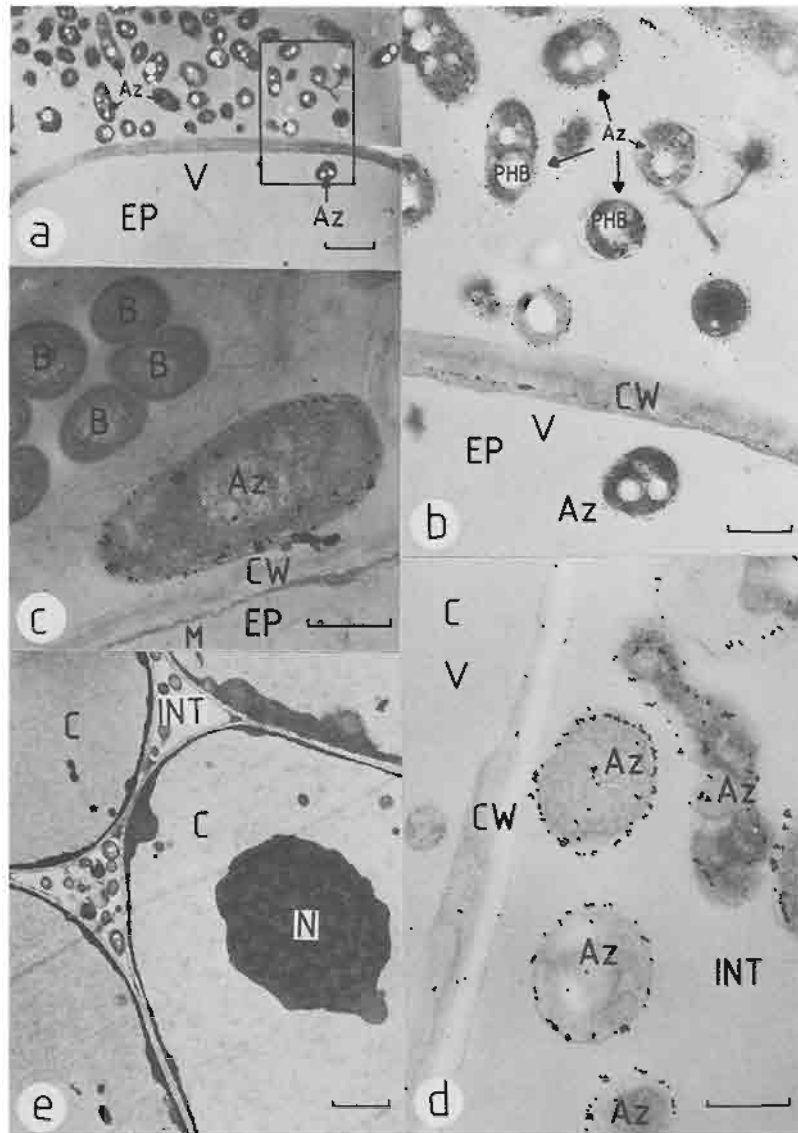


Fig. 5. TEM micrographs of gold-labeled *A. brasilense* Cd on root surface near the epidermal layer and in the intercellular spaces of the roots. (a) *A. brasilense* Cd (arrows) at root surface near an epidermal cell, (b) enlargement of insertion of Fig. 3a, (c) *A. brasilense* Cd and unidentified rhizosphere bacteria near the epidermis in non-axenic culture, (d) *A. brasilense* Cd in the intercellular spaces in the elongation zone, and (e) bacteria present in intercellular spaces of the cortex; many versus few. Bars represent 2 μm (a, e), 1 μm (b), and 0.5 μm (c, d).

microscopy. However, because roots are usually colonized by mixed populations of bacterial species and strains (Lynch, 1982), morphological identification of a particular strain is extremely difficult and occasionally speculative. Moreover, quantification of a certain rhizosphere bacterial strain, even in a semi-selective medium, is difficult due to

the presence of a naturally-occurring background bacterial population capable of growing on the medium (Bashan and Levanony, 1985).

By using immuno-gold labeling technique, specific identification of *A. brasilense* Cd was made in non-axenic plant-bacteria cultures, and it was possible to distinguish between this strain and

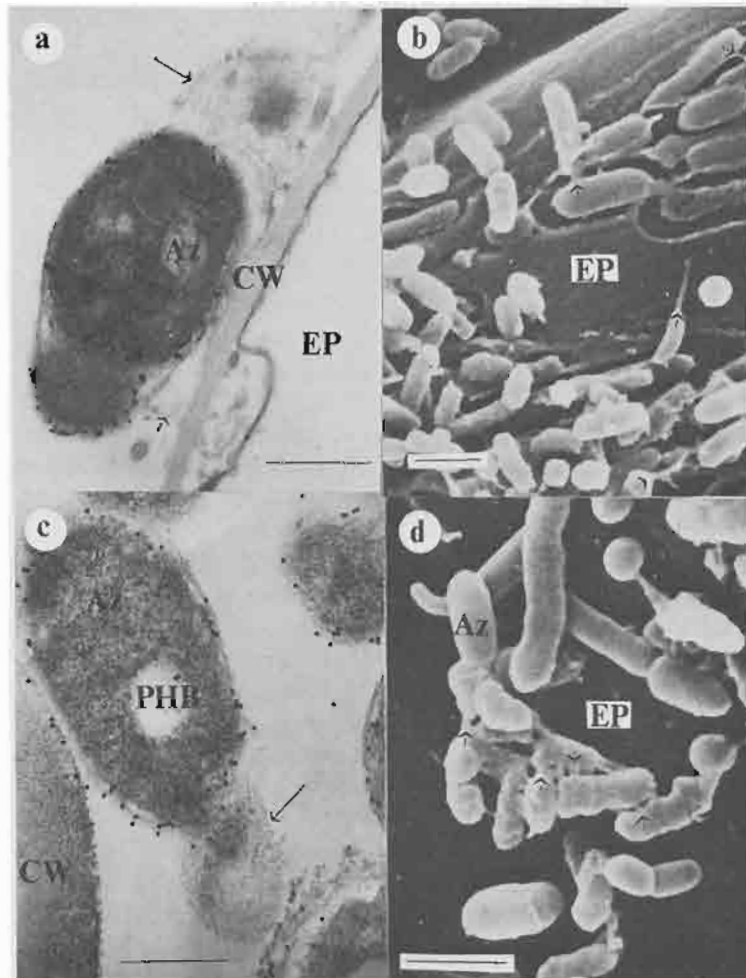


Fig. 6. Cell-cell connections between (a, b) *A. brasilense* Cd cells and plant cells, and (c, d) between adjacent bacterial cells. Arrows show the electron dense connections between the cells. The bars represent 1 μm (b, d) and 0.5 μm (a, c). (a, c) are TEM micrographs and (b, d) SEM micrographs.

other rhizosphere strains. Wheat plants, inoculated with the normal inoculum level of *A. brasilense* Cd, possessed bacterial cells adsorbed to the outer surface of the following root parts: the epidermal cells, the root-tip, the elongation and root-hair zones. This adsorption pattern differs from that reported by Umali Garcia *et al.* (1980) for axenically grown pearl-millet inoculated with *A. brasilense* Sp-7 which indicated a preferential adsorption to the root-hairs.

In the present study *A. brasilense* Cd were connected to the epidermal cells by an electron dense material. A similar substance was observed bet-

ween adjacent bacterial cells. However, such connections were not observed in the intercellular spaces. Thus, this bacterium may have two different potential interactions with root cells, one with, and one without connections, depending on the exact site of the interaction. Interaction between *Azospirillum*-like bacteria and epidermal cells of several plant species, callus or with the mucigel layer of roots were reported (Bashan and Levany, 1987; 1988a, b; Bashan *et al.*, 1986; Berg *et al.*, 1979; Döbereiner and Day, 1976; Okon and Hardy, 1983; Patriquin, 1981; Umali-Garcia *et al.*, 1980; Whallon *et al.*, 1985).

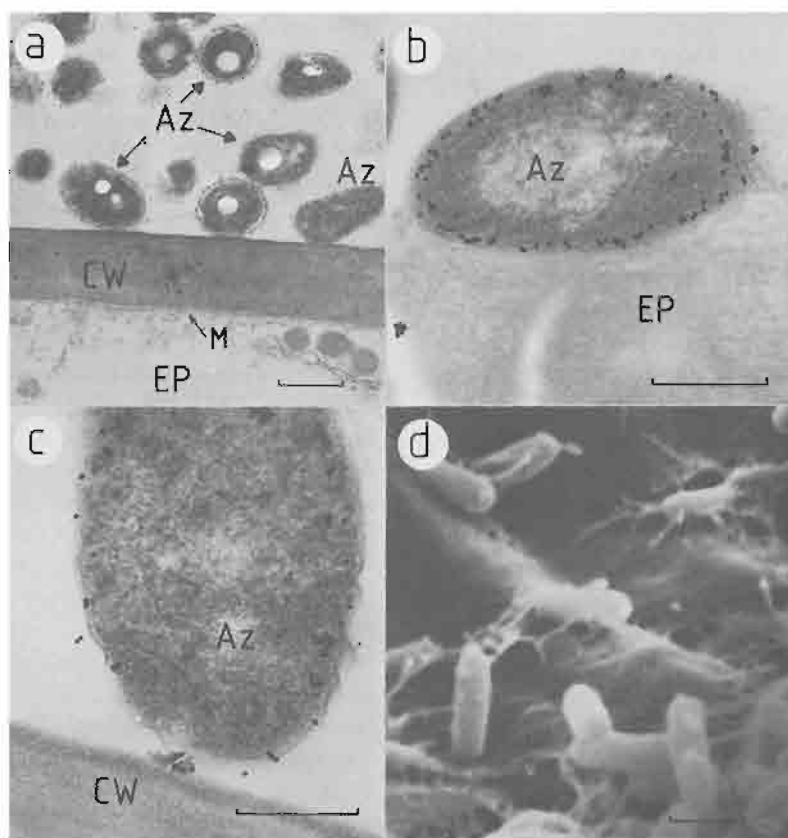


Fig. 7. Polar and non-polar attachment of *A. brasilense* Cd to epidermal cells. TEM micrographs of: (a) typical attachment of bacteria to epidermal cell, (b) intimate non-polar attachment, (c) polar attachment of bacteria to epidermal cells, and (d) SEM micrograph showing polar and non-polar attachment. Bars represent 1 μm (a, d), and 0.5 μm (b, c).

Azospirillum-like bacteria were previously observed in intercellular spaces between epidermis and the cortex and in outer cortical layer of axenically grown grasses (Patriquin, 1981; Patriquin and Döbereiner, 1978; Patriquin *et al.*, 1983) and in field-grown plants (Döbereiner and Day, 1976; Kavimandan *et al.*, 1978; Schank *et al.*, 1979). On the other hand, Reinhold *et al.* (1986) could not find Azospirillum cells within colonized roots of Kallar grass. These observations were based mainly on light microscopy, on plating methods, enrichment techniques and on non-specific tetrazolium staining. Thus, the above studies did not answer adequately the basic question of whether a massive population of Azospirillum is present or not within the plant roots. Matthews *et al.* (1983) using the peroxidase-antiperoxidase labeling method under TEM observed labeled Azospirillum in pearl-millet roots, but no visible clear association between

labeled bacteria and plant tissue was reported. By using the immunogold labeling in this study, it was possible to locate and to specifically identify *A. brasilense* Cd in intercellular spaces of cortical cells in the elongation and in the root-hair zones. The bacteria penetrated into the inner cortical layer of wheat roots, but were not detected in either the endodermal layer or in the vascular tissues. In the interior of the root tip and the meristem zones, in which the cells are tightly organized and lack intercellular spaces, no bacteria were observed. In non-axenic cultures several other rhizosphere bacteria were also found in intercellular spaces. Early reports on Azospirillum-like bacteria in the xylem vessels of field-grown maize and wheat (Kavimandan *et al.*, 1978; Patriquin and Döbereiner, 1978) were not confirmed by this study and may result from the penetration of bacteria which colonize on root surfaces into the root segments through artifi-

cial cutting wounds induced by the techniques used.

Patriquin and Döbereiner, (1978) suggested that *Azospirillum* invaded the intercellular spaces via disrupted cortical tissues where branches of lateral roots emerged from the main roots. In this study, very young wheat roots (72–96 h) were fixed immediately after their excision, and long before any lateral root emerged. Therefore, it is suggested that the invasion of *A. brasilense* Cd into the root interior is possibly by bacterial self-motility (Bashan, 1986c) without any damage (either physiologically or mechanically) to the roots. The bacteria may penetrate via the middle lamella of the epidermal layer as previously suggested by Patriquin and Döbereiner, (1978).

In conclusion, this ultrastructural study proposes that *A. brasilense* Cd, in addition to root surface colonization, could form intercellular associations with cortical cells of wheat roots.

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