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Association of *Azospirillum* with Grass Roots†

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The association between grass roots and *Azospirillum brasilense* Sp 7 was investigated by the Fahraeus slide technique, using nitrogen-free medium. Young inoculated roots of pearl millet and guinea grass produced more mucilaginous sheath (mucigel), root hairs, and lateral roots than did uninoculated sterile controls. The bacteria were found within the mucigel that accumulated on the root cap and along the root axes. Adherent bacteria were associated with granular material on root hairs and fibrillar material on undifferentiated epidermal cells. Significantly fewer numbers of azospirilla attached to millet root hairs when the roots were grown in culture medium supplemented with 5 mM potassium nitrate. Under these growth conditions, bacterial attachment to undifferentiated epidermal cells was unaffected. Aseptically collected root exudate from pearl millet contained substances which bound to azospirilla and promoted their adsorption to the root hairs. This activity was associated with nondialyzable and protease-sensitive substances in root exudate. Millet root hairs adsorbed azospirilla in significantly higher numbers than cells of *Rhizobium*, *Pseudomonas*, *Azotobacter*, *Klebsiella*, or *Escherichia*. Pectolytic activities, including pectin transeliminase and endopolygalacturonase, were detected in pure cultures of *A. brasilense* when this species was grown in a medium containing pectin. These studies describe colonization of grass root surfaces by *A. brasilense* and provide a possible explanation for the limited colonization of intercellular spaces of the outer root cortex.

Azospirillum spp. (formerly *Spirillum lipoferum* [29]) fix atmospheric nitrogen and have been isolated from the rhizospheres of a variety of tropical and subtropical nonleguminous plants (8, 18). Various grasses have responded differently to field inoculation with this soil microorganism (2, 26), and under certain conditions significant increases in the yields of plant dry matter have been obtained in the field (26) and under controlled greenhouse conditions (12, 28).

In contrast to the *Rhizobium*-legume symbiosis, the *Azospirillum*-grass interaction does not produce visible structures on roots which indicate successful infection. Studies with sugar cane (*Saccharum officinarum*) and bahia grass (*Paspalum notatum*) suggested that the majority of the N_2-fixing organisms were closely associated with roots, probably within the mucilagenous sheath or mucigel layer on the root surface (9). For these reasons, the interaction between grasses and this N_2-fixing microorganism has been described as "associative" (8). There is, however, evidence that *Azospirillum* breaches the root epidermal barrier and invades cortical and vascular tissues of the host (8, 16, 21, 22, 24, 31).

This paper describes both the adsorption and the colonization of *Azospirillum brasilense* on the root surfaces of pearl millet and guinea grass under bacteriologically defined conditions.

(Preliminary accounts of this study were presented at the Third International Symposium on Nitrogen Fixation, Madison, Wis., 1978, and at the 1979 Michigan Branch Meeting of the American Society for Microbiology.)

MATERIALS AND METHODS

Bacterial cultures and plant hosts. The organisms used and their sources were as follows: *A. brasilense* strains 13t and Sp 7, isolated by J. Dobereiner from roots of *Digitaria decumbens* in Brazil; *A. brasilense* JM 125A2, isolated by J. Milam from *Pennisetum americanum* roots in Florida; *Rhizobium trifolii* 0403 from P. Nutman; *Azotobacter vinelandii* UW10 from W. Brill; *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, and *Escherichia coli* from the culture collections of the Department of Microbiology and Cell Science, University of Florida, and the Depart-

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ment of Microbiology and Public Health, Michigan State University. *R. trifolii* 0403 was grown on yeast extract-mannitol agar (11), and all other bacteria were grown on Trypticase soy agar or broth (Difco Laboratories). *Panicum maximum* line 285 (guinea grass; from R. Smith) and *Pennisetum americanum* cv. Gahi 3 (pearl millet) were used as plant hosts. Both hosts are small-seeded species suitable for axenic culture of seedlings on glass slides (10), and both have responded to field inoculation with *A. brasilense* Sp 7 in Florida (26).

Preparation of slide cultures. Grass seeds were surface sterilized by treating them with 95% ethanol for 1 min and then with 2.6% sodium hypochlorite solution for 20 min; they were then washed in six changes of sterile deionized water. The seeds were left overnight in the last change of water, and the same procedure was repeated the following day. The seeds were then transferred to sterile water agar plates, inverted, and germinated into humid air. Seedlings with radicles approximately 2 cm long were transferred to sterile Fahraeus slide assemblies (10) containing a nitrogen-free nutrient solution (Fahraeus medium). In some experiments this medium was supplemented with 5 mM $Ca(NO_3)_2$ or KNO_3. The slide assemblies were incubated for up to 1 month in a growth chamber programmed for 14-h photoperiods and 35 to 36°C; they were under metal halide lamps which supplied photosynthetically active radiation equal to about one-half of full sunlight.

Aseptic collection of root exudate. Surface-sterilized seeds were germinated on a layer of water agar supported by a stainless steel wire mesh in sterile 1-quart (0.946-liter) Mason jars containing 50 ml of Fahraeus medium. Sterile root exudate was collected after 1 week and then centrifuged at 8,000 × *g* for 15 min and passed through a 0.45-μm filter (Millipore Corp.). Samples of the filtrate were treated in the following ways: dialysis at 4°C against phosphate-buffered saline (7), ultracentrifugation at 104,000 × *g* for 4 h, eightfold concentration by dialysis against Fahraeus medium containing 20% polyethylene glycol, and digestion with protease immobilized on carboxymethyl cellulose (Sigma Chemical Co., St. Louis, Mo.) at 22°C for 12 h with gentle shaking. After digestion, the immobilized enzyme was removed by centrifugation at 1,000 × *g*. Protein in the root exudate was measured by the method of Lowry et al. (17), using bovine serum albumin as a standard.

Adsorption studies. Sterile seedlings were grown for 2 days in Fahraeus assemblies without agar and then inoculated with 0.2 ml of a suspension containing 10^9 cells per ml, which was from a 48-h culture of *A. brasilense*. Bacterial adsorption to the grass root hairs was measured by a direct microscopic assay, using standardized inocula (7). For standardization, only adherent bacteria in physical contact with the cell walls of root hairs approximately 200 μm long were counted. In other experiments, the inoculum was preincubated in aseptically collected root exudate for 3 h, then pelleted by centrifugation, washed, and resuspended in sterile Fahraeus medium to a density of 10^9 cells per ml. These treated cells were mixed with seedlings in 10-ml sterile beakers and gently shaken for 10 to 30 min at 30°C. The roots were removed, rinsed with sterile Fahraeus medium, and examined by phase-contrast interference microscopy and scanning electron microscopy.

Scanning electron microscopy. Roots were rinsed in Fahraeus medium, fixed in 3% glutaraldehyde in 50 mM cacodylate buffer (pH 7.0), washed, postfixed with 1% osmium tetroxide in cacodylate buffer, and dehydrated in a graded series up to absolute ethanol. Roots were then dried to critical point (Bomar critical point dryer), coated with a 20- to 30-nm gold layer in a Film-Vac sputter coater, and examined with an ISI Super III scanning electron microscope at 15 kV. Transmission electron microscopy was performed as previously described (31).

Assays for pectolytic activity. *A. brasilense* was grown in 10 ml of Okon succinate medium (20) for 48 h at 37°C and then transferred to 800 ml of Okon mineral medium (20) supplemented with 200 ml of 1.5% (wt/vol) pectin, pH 7. The cells were grown at 37°C for 7 days in this pectin medium and then harvested by centrifugation. The supernatant fluid was subjected to fractional ammonium sulfate precipitation (20, 40, 60, 80, and 95% saturation). The precipitated fractions were dissolved in 2 ml of 0.1 M citrate-phosphate buffer, pH 5.2, and dialyzed against the same buffer at 4°C until free of sulfate. Cells harvested from pectin broth were washed three times with 5 ml of chilled citrate-phosphate buffer. These washings and samples (50 μl) from each dialyzed fraction were assayed for pectin lyase (transeliminase) activity by incubation with 2 ml of pectin (1 mg/ml; grade I; Sigma) in 0.1 M Tris-hydrochloride buffer, pH 8.3, followed by measurement of ultraviolet absorption at 230 nm (1). Endopolygalacturonase activity was detected by incubating 0.2 ml of the fraction precipitated between the 80 and 95% saturated ammonium sulfate solutions with 1.8 ml of 20 mM citrate buffer, pH 4.8, containing 1.3 mg of polygalacturonic acid. A boiled enzyme fraction mixed with the substrate served as a control. After 12 h, the products were loaded onto a Bio-Rad P2 column (1.7 by 30 cm) and eluted with 100 mM citrate-phosphate buffer, pH 5.2. Collected fractions were assayed for total carbohydrate by the phenol-sulfuric acid method (15).

RESULTS

Root morphology. There were few lateral roots and root hairs on pearl millet (data not shown) and guinea grass (Fig. 1A) grown axenically in nitrogen-free medium. In contrast, lateral roots and root hairs were abundant on inoculated plants (Fig. 1A and B). The enhancement of lateral root and root hair morphogenesis by the bacteria was suppressed when 5 mM $Ca(NO_3)_2$ was present (Fig. 1A). The tips of inoculated roots were surrounded by abundant mucigel, which extended through the root hair region into the crown, above where root hairs develop (Fig. 2A). Root cap cells from inoculated plants were typically sloughed off and embedded in the mucigel (Fig. 2B), whereas sterile root tips lacked abundant mucigel and their root caps remained intact (Fig. 2C).

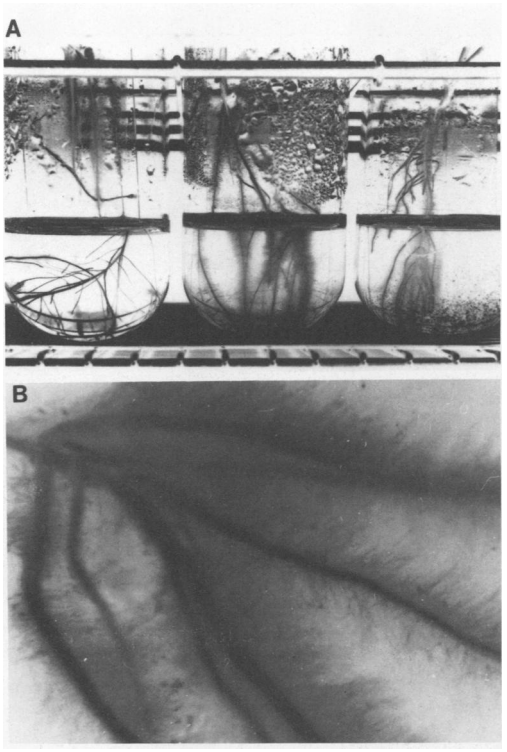

FIG. 1. *Root systems of grasses incubated for 1 month. (A) P. americanum cv. Gahi 3. Compare sterile, untreated roots (left), roots inoculated with A. brasilense (center), and roots inoculated and supplemented with 5 mM $Ca(NO_3)_2$ (right). (B) Inoculated root of P. maximum line 285 grown in nitrogen-free medium. Note the profuse root hairs and closely spaced branch roots. ×150.*

Bacterial adsorption to roots. Azospirilla from a 48-h culture attached within seconds to pearl millet and guinea grass roots previously grown for 2 days in nitrogen-free Fahraeus medium. After these short-term incubations, the adherent bacteria were associated with granular material accumulating on the surfaces of root hairs (Fig. 3A and B) and with fibrillar material accumulating on the surfaces of old epidermal cells (Fig. 3C and D). By contrast, very few azospirilla adhered to young undifferentiated epidermal surfaces near the root meristem (data not shown) and on emerging lateral roots (Fig. 3C). The observations did not change during a 1-h incubation. During a longer incubation (12 to 48 h), the bacteria colonized root epidermal surfaces covered with mucigel (Fig. 2A and C) and sloughed off epidermal tissues (Fig. 2C) and occupied the void spaces of the epidermis created by lateral root emergence (Fig. 3E). Significantly fewer azospirilla adhered to root hairs when roots were grown in Fahraeus medium supplemented with 5 mM KNO_3; for *A. brasilense* Sp 7 and *P. americanum* cv. Gahi 3, there were 3.15 ± 0.60 and 35.00 ± 10.50 adsorbed bacteria per root hair (mean ± standard devia-

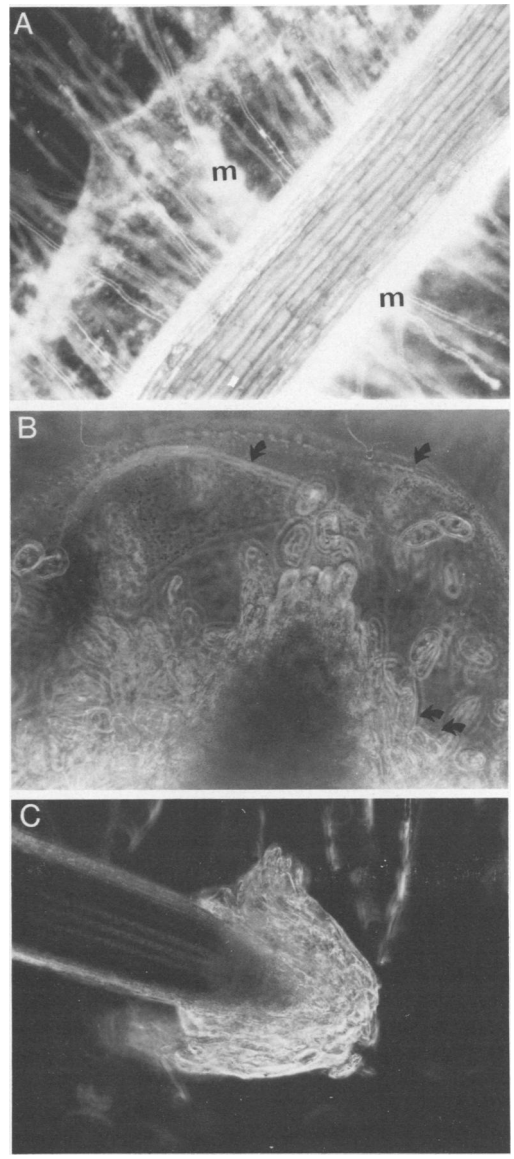

FIG. 2. *Mucigel on pearl millet roots incubated for 1 day with A. brasilense. (A) Mucigel (m) in the maturation region of the root, where root hairs develop. ×180. (B) Phase-contrast photomicrograph of root tip showing arc boundary (single arrows) formed by bacteria on mucigel. Sloughed off root cap cells (double arrow) are embedded in the mucigel. ×1,200. (C) Root tip of sterile root. Root cap is intact without mucigel. ×135.*

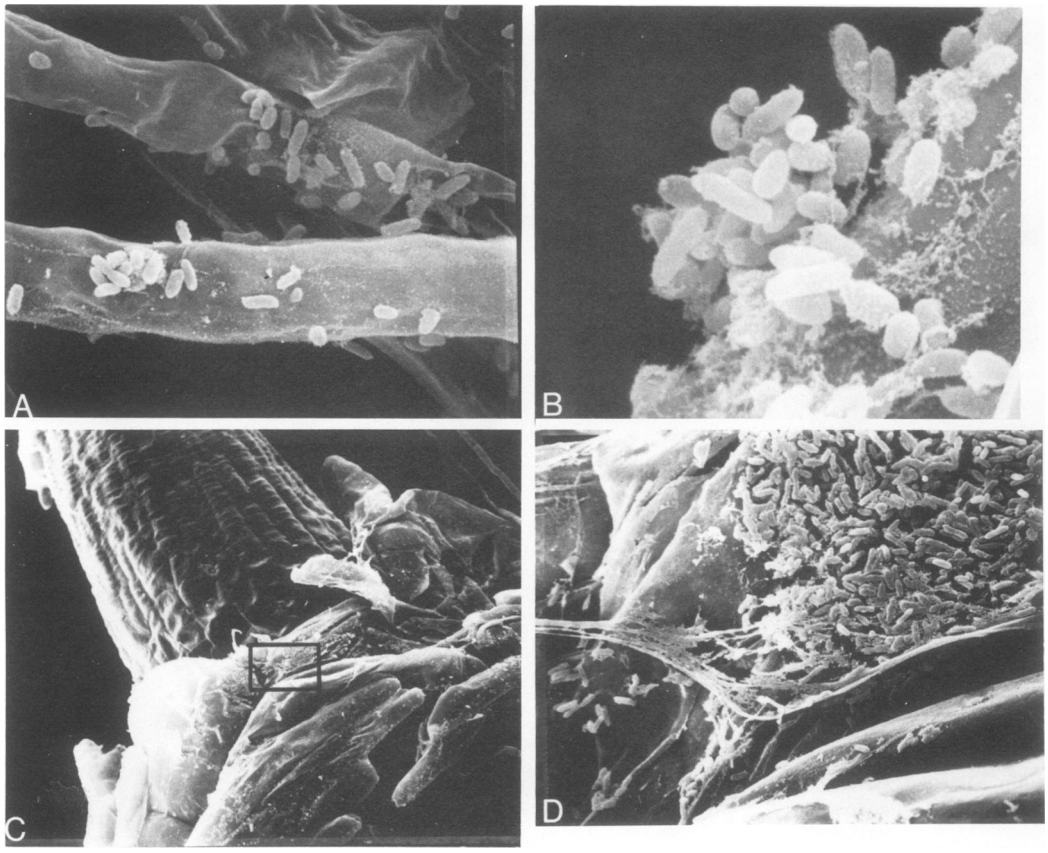

FIG. 3. *Scanning electron micrographs of A. brasilense adsorbed to epidermal cells of pearl millet. (A) A. brasilense adsorbed to mature root hairs of pearl millet grown in N-free medium. ×4,285. (B) Similar to (A), but at higher magnification. ×8,500. (C) Branch root emerging through the old epidermal cells of a primary root. Note the lack of attached azospirilla on the lateral root epidermis. ×300. (D) Enlargement of framed area in (C). Note the adherent bacteria and associated fibrillar material. ×2,000. (E) A. brasilense occupying void spaces (arrow) created by desquamation at lateral root emergence. ×3,000. (F) Lack of adherent bacteria on root hairs grown in Fahraeus medium supplemented with 5 mM KNO_3. Compare with the adsorbed bacteria associated with the granular surfaces of root hairs grown in the nitrogen-free medium (A and B) ×3,000. (G) A. brasilense adsorbed to undifferentiated epidermal cells of roots grown in N-free Fahraeus medium. ×3,000. (H) Same as (G), but the medium was supplemented with 5 mM KNO_3. ×300.*

tion; 40 200-μm root hairs per treatment) in the presence and absence, respectively, of 5 mM KNO_3. Plants grown in this modified medium appeared healthy. Under these growth conditions, there was a distinct lack of the granular topography characteristic of the root hairs grown in N-free medium (Fig. 3A, B, and F). In contrast to the results with root hairs, nitrate at 5 mM in the rooting medium did not affect adherence of azospirilla to undifferentiated epidermal cells (Fig. 3G and H).

The number of azospirilla firmly adhering to grass root hairs was influenced by the age of the bacterial culture serving as the inoculum. Azospirilla taken from 2-day-old broth cultures adhered in significantly higher numbers to pearl millet and guinea grass root hairs than did cells which were taken from cultures grown for 0.5, 3, or 5 days (Table 1). This effect of culture age on bacterial adhesion to roots was observed with a short-term incubation (1 h) but not with the standard 12-h period.

Free azospirilla incubated for 2 to 4 days in seedling slide assemblies acquired an encasement envelope layer which was surrounded with granular material having a high electron density (Fig. 4). These electron-dense granules had an apparent high affinity for the surface of the encasement envelope since they withstood the various manipulations of specimen preparation for transmission electron microscopy. These structures were not found on *A. brasilense*

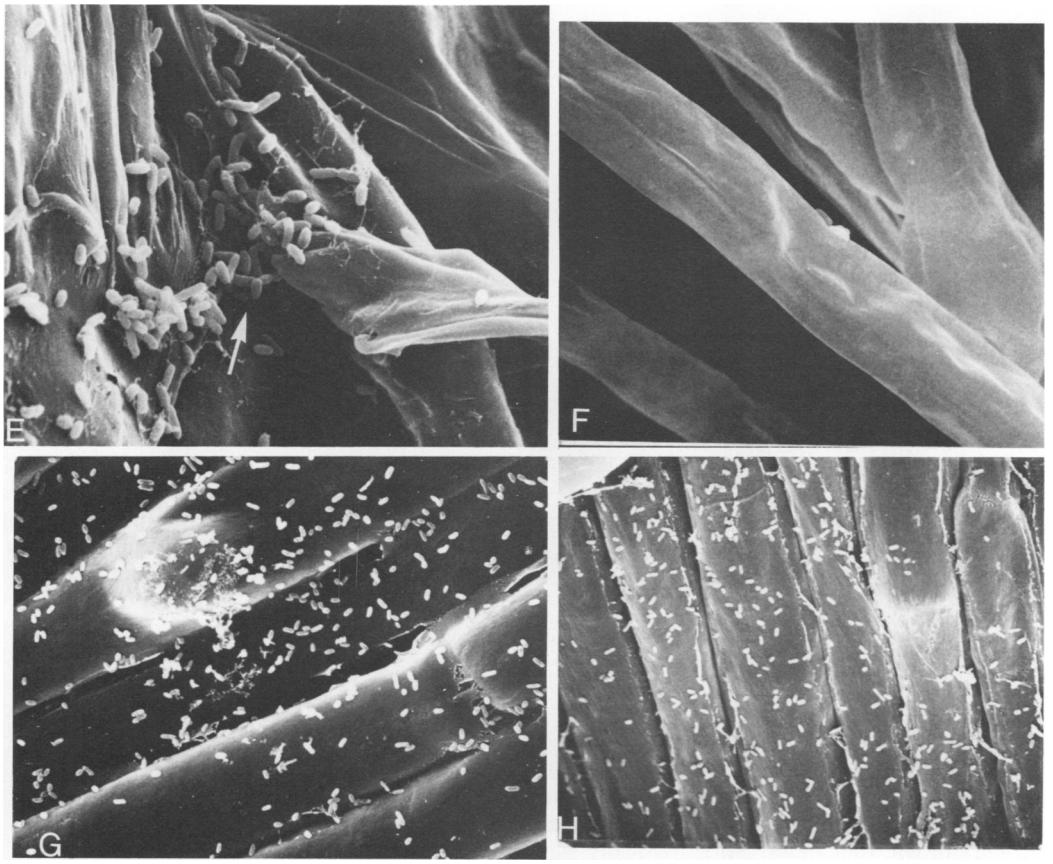

FIG. 3 (*E-H*).

TABLE 1. *Effect of age of inoculum on adsorption of A. brasilense Sp 7 to grass root hairs*[a]

Culture age (days)	No. of adsorbed bacteria per root hair[b]	
	Guinea grass	Pearl millet
0.5	19.3 ± 0.67[c]	18.2 ± 1.00
2	33.6 ± 1.65	32.6 ± 0.75
3	18.1 ± 0.69	18.8 ± 1.08
5	3.0 ± 0.38	4.8 ± 0.81

[a] Cells were grown in unshaken trypticase soy broth.
[b] For standardization, 15 roots hairs were examined per treatment, and each root hair was 200 μm long.
[c] Mean ± standard deviation.

grown in Trypticase soy broth. This observation suggested that there may be substances in the root exudate which bind to the bacteria in the vicinity of the root. Therefore we looked for evidence of such substances in root exudates and investigated what influence these substances may have on the adsorption of azospirilla to the root epidermis.

Preincubating *A. brasilense* cells with pearl millet root exudate, followed by a gentle washing of the cells, promoted adherence of the bacteria to root hair surfaces after minutes of incubation with the roots (Fig. 3A and 5A and B). This adsorption-promoting activity of the root exudate was not lost by dialysis and could be concentrated by ultracentrifugation or by dialysis of the exudate against 20% polyethylene glycol. The ability of root exudate to promote adsorption of azospirilla to root hairs was lost after storage for 2 weeks at 4°C under aseptic conditions. Protease treatment destroyed the activity of the factor(s) in root exudate responsible for promoting the bacterial adsorption (the activity was unaffected by controls without enzyme). The root exudate contained 10 μg of protein per ml. Enhancement of bacterial adsorption by root exudate was selective for root hairs and not for other epidermal cells (Fig. 5B).

Binding specificity of bacteria to grass root hairs. Quantitative measurements of bacterial adherence to pearl millet root hairs of uniform length are shown in Table 2. Three strains of *A. brasilense* and five other species of bacteria were examined, and the values were

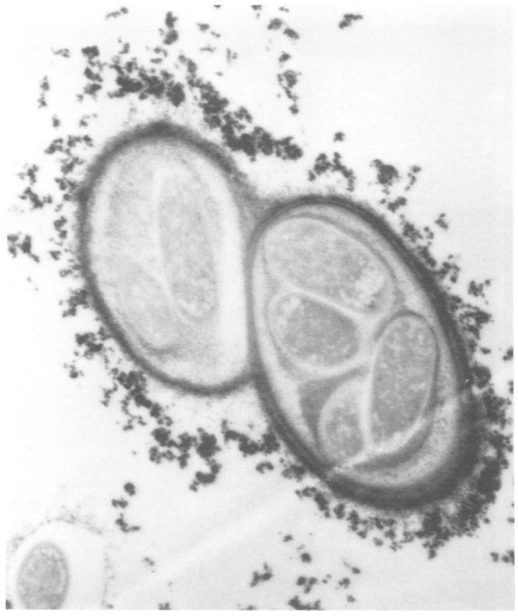

FIG. 4. *Transmission electron micrograph of A. brasilense Sp 7 grown for 4 days in a Fahraeus culture with pearl millet.* ×*16,509.*

compared statistically by the Student *t* test after the square root transformation of the mean (7). Of the various bacteria examined, the three strains of *A. brasilense* adsorbed in greatest numbers to the root hairs. No statistically significant difference was found among these three strains. *R. trifolii* 0403 and *P. fluorescens* adsorbed to the root hairs, but in significantly fewer numbers than *A. brasilense* (confidence levels of 95 and 99.5%, respectively). Very low numbers of *A. vinelandii* UW10, *E. coli*, and *K. pneumoniae* adsorbed to root hairs in this assay.

Pectolytic enzyme activity. Pectic lyase activity (transeliminase) was detected in the third wash of the pelleted cells and in the fraction of the culture supernatant fluid which precipitated at 80 to 95% ammonium sulphate saturation. This latter fraction also contained endopolygalacturonase activity, as detected by gel filtration chromatography of the reaction products after incubation with the polygalacturonic acid substrate. The elution profile showed that degradation products of various sizes were generated from incubation of the polysaccharide substrate with this enzyme fraction and that this depolymerizing activity was destroyed by boiling the enzyme preparation.

DISCUSSION

More lateral roots and root hairs developed on inoculated roots than on sterile roots. This effect on root morphogenesis was not observed when the plants were supplied with $Ca(NO_3)_2$, suggesting that the phenomenon may be related to the production of plant growth hormones by *A. brasilense* (12, 31) rather than to transfer of newly fixed nitrogen to the plant.

The accumulation of mucigel on plant roots was favored by the presence of the azospirilla. Various bacteria are known to enhance the accumulation of mucigel on roots of legumes (3), wheat (23), and several other plants grown in soil (13). Pectic acid, glucose, galactose, arabinose, glucuronic acid, and galacturonic acid have all been found in plant mucigel (4, 13, 19). This excreted mucigel on the rhizoplane may create a protected microenvironment for rapid bacterial multiplication and may sequester nutrients which are released from roots and are in the soil solution (13). It remains to be determined whether the mucigel affects the nitrogen-fixing activity of the embedded azospirilla by regulating oxygen transfer from the soil atmosphere.

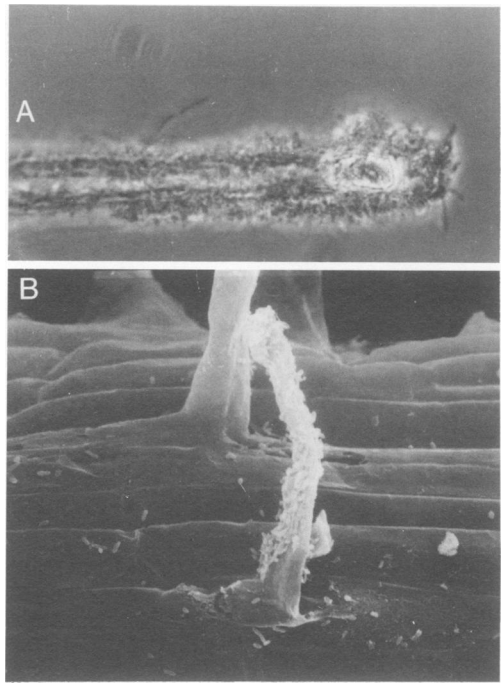

FIG. 5. *Enhancement of root hair binding by A. brasilense pretreated with pearl millet root exudate. Phase-contrast (A)* (×*1,400) and scanning electron microscope (B)* (×*1,200) photomicrographs are of cells *from similar preparations. Note that some bacteria in (A) are attached to each other end-on and that enhancement of binding by bacteria is selective for root hairs in (B). Compare the density of adherent bacteria in this figure with Fig. 3A, where the bacteria were given no prior treatment with root exudate.*

TABLE 2. *Adsorption of various bacteria to root hairs of pearl millet seedlings*

Bacterium	No. of root hairs examined[a]	No. of adsorbed cells per root hair
A. brasilense Sp 13t	16	23.7 ± 2.4[b]
A. brasilense Sp 7	18	33.0 ± 4.0
A. brasilense JM 125A2	19	32.5 ± 2.9
R. trifolii 0403	19	14.4 ± 2.0[c]
P. fluorescens	15	11.4 ± 2.9[d]
A. vinelandii UW10	15	0.6 ± 0.3[d]
K. pneumoniae	18	0.3 ± 0.2[d]
E. coli	16	0.06 ± 0.06[d]

[a] Root hairs were 200 μm long and were from two seedlings.

[b] Mean ± standard deviation.

[c] Mean less than the *A. brasilense* mean at the 95.0% confidence level.

[d] Mean less than the *A. brasilense* mean at the 99.5% confidence level.

Scanning electron microscopy revealed granular material associated with root hair surfaces and associated bacteria. The presence of similar granular material on the surfaces of clover root hairs inoculated with *Rhizobium* was noted by Dart (3). It was subsequently found that clover and *Vicia* contain proteins which bind selectively to rhizobia and can promote the adherence of these bacteria to the legume roots (7, 27). The present study has shown that pearl millet roots released protease-sensitive, nondialyzable substances which bound to azospirilla and promoted their selective adherence to root hairs. These results are compatible with the lectin recognition model proposed by Solheim (27) for the mechanism of bacterial adherence in the *Rhizobium*-legume symbiosis. According to this model, a glycoprotein lectin excreted from the legume roots binds to the rhizobia and promotes their attachment to the root surface.

Adsorption studies showed that grass roots display selectivity in the binding of bacteria to discrete regions of the root. Azospirilla were firmly attached to root hairs of grasses grown in the absence of fixed nitrogen. However, these bacteria did not attach to root hairs when the host was grown in the presence of a readily available nitrogen source. Under these latter conditions, the bacteria were associated only with mucigel at the root cap and with undifferentiated epidermal cells of roots. Presumably, mechanisms which attach *Azospirillum* to root hairs and undifferentiated epidermal cells differ in response to perturbations caused by fixed nitrogen in the rooting medium. Fixed nitrogen ions (NO_3^- and NH_4^+) in the rooting medium exerted a similar reduction in selective adherence of *R. trifolii* to clover root hairs, which was concurrent with a reduction in the level of trifoliin, a clover lectin on the root hair surface (5). Thus, as with the *Rhizobium*-clover symbiosis, the presence of nitrate affects the attachment stage of the *Azospirillum*-grass association within hours after the bacteria are inoculated.

Statistical analysis indicated that the azospirilla adhered to millet root hairs in significantly greater numbers than all of the other bacterial species examined. Thus, the grass root hairs displayed selectivity in their binding of bacteria, which favored the accumulation of azospirilla on epidermal surfaces in simple Fahraeus slide assemblies. However, *R. trifolii* and *P. fluorescens* also adhered to pearl millet root hairs. Interestingly, these bacteria share certain serological (6) and biochemical (29, 30) characteristics with *Azospirillum*. The adsorption of *Rhizobium japonicum* to cereal roots (wheat and rice) has been described previously (25). The kinetic data fit a Langmuir adsorption isotherm which predicts an adsorption-desorption equilibrium and the presence of available binding sites which occupy approximately one-third of the root surface (25). Little, if any, attachment occurred with our strains of *E. coli*, *K. pneumoniae*, and *A. vinelandii*.

Azospirilla eventually enter the root through lysed root hairs and void spaces of epidermis created by epithelial desquamation and lateral root emergence and remain intercellular within the middle lamella of the root tissue (16, 31). Mechanical injury resulting from contact with the solid support medium might provide other portals of entry for the microorganisms under natural conditions. Other workers (21, 22, 28) have isolated *Azospirillum* from the vascular elements of maize and sugar cane roots grown in solid medium. Azospirilla have also been isolated recently from stems of wheat (14) and maize (21). Thus, under certain conditions, the organism may become invasive.

The presence of azospirilla in the middle lamella (31) prompted us to investigate whether the bacteria could produce enzymes which would facilitate their colonization of this host environment. The middle lamella is largely pectic in nature (19). Pectin lyase and endopolygalacturonase activities were detected in cultures of *A. brasilense* Sp 7. These pectin-modifying enzymes may contribute to the appearance of electron-transparent zones surrounding bacteria which have colonized the middle lamellae and to the limited, invasive properties of these organisms on grass roots (31). The plant cell wall which separates the bacteria from the interior of the cortical cells may impose an inefficient exchange of carbon and nitrogen and could constitute a major limitation to the exploitation of this

association as a nitrogen-fixing system in grasses of agronomic significance.

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