Root surface colonization of non-cereal crop plants by pleomorphic Azospirillum brasilense Cd

YOAV BASHAN,1*† HANNA LEVANONY2 and ROBERT E. WHITMOYER3

1Department of Agronomy, Ohio State University, Columbus, OH 43210, USA
2Department of Plant Genetics, The Weizmann Institute of Science, Rehovot, Israel
3Electron Microscopy Laboratory, Ohio Agricultural Research and Development Center, Wooster, OH 44691, USA

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Root surface colonization by Azospirillum brasilense Cd of tomato, pepper and cotton plants under normal growth conditions and soybean plants under normal and water-stress conditions was monitored by scanning electron microscopy and bacterial counts. A. brasilense Cd was capable of efficiently colonizing the elongation and root-hair zones of all four plant species tested. In these zones, the bacteria mainly colonized the root surface (tomato, soybean), root-hairs (pepper), or both (cotton), by single cells (tomato, soybean), micro-aggregates (pepper), or a combination of the two (cotton). All inoculated plants demonstrated (i) larger amounts of mucigel-like substance on the root surface than non-inoculated plants and (ii) fibrillar material which anchored the bacterial cells to the root surface and established connections between cells within bacterial aggregates. On non-water-stressed soybean plants, most A. brasilense Cd cells in the rhizosphere occurred as vibroid forms whereas those on water-stressed plants (wilting) were cyst-like. A lower rhizosphere bacterial population was observed on water-stressed plants. When water-stress conditions were eliminated, the bacterial cells reverted to the vibroid form and a concomitant increase in the bacterial population was observed. It is suggested that cyst-like formation is a natural response for A. brasilense Cd in the rhizosphere of water-stressed plants.

Introduction

Inoculation of plants with putative beneficial rhizosphere bacteria of the genus Azospirillum has been carried out almost exclusively on a large variety of cereals (Bashan & Levanony, 1990; Patriquin et al., 1983), although a few studies have focused on the interaction of Azospirillum with other plant species such as mustard (Saha et al., 1985), tomato (Bashan & Levanony, 1988c; Bashan et al., 1989a, b), beet (Kolb & Martin, 1985), soybean (Bashan et al., 1990; Plazinski & Rolfe, 1985; Singh & Subba Rao, 1979), sweet potato (Crossman & Hill, 1987) or weeds (Bashan & Levanony, 1987). Recently, it was shown that inoculation of tomato, pepper, eggplant and cotton plants with Azospirillum brasilense Cd significantly, but inconsistently, increased plant yield (Bashan et al., 1989a).

When inoculated onto cereal roots, A. brasilense Cd multiplies and forms small aggregates, mainly in the root elongation and root-hair zones (Bashan et al., 1986). The bacteria produce holdfast fibrillar material which anchors the cells to the root surface (Levanony et al., 1989). Azospirillum cells appear in two distinct forms: the slightly vibroid form (V-form) occurring in young laboratory cultures and on plant roots (Berg et al., 1979; Tarrand et al., 1978), and the cyst form (C-form), occurring under stress or in old laboratory cultures (Bleakley et al., 1988; Eskew et al., 1977; Lamm & Neyra, 1981; Sadasivan & Neyra, 1985, 1987). The C-form may be a survival structure.

The aims of the present study were (i) to evaluate the ability of A. brasilense Cd to colonize root surfaces of several non-cereal plants and to determine colonization sites, (ii) to examine the occurrence of, and conditions for, pleomorphic A. brasilense Cd in the rhizosphere, and (iii) to evaluate whether bacterial fibrillar connections to root surfaces are a general mode of attachment of A. brasilense Cd to plant root surfaces.

Abbreviation: SEM, scanning electron micrograph.

† Present address: Department of Microbiology, Centro de Investigaciones Biologicas de Baja California Sur, La Paz, Apdo Postal 128, BCS, Mexico 23000.
Methods

Organisms. Azospirillum brasilense Cd (ATCC 29710) and the following plant species were used: tomato (Lycopersicon esculentum Mill.) cv. Na‘ama; pepper (Capsicum annuum) cv. Ma‘or; cotton (Gossypium barbadense) cv. Pima S-5; wheat (Triticum aestivum) cv. Deganit, and soybean (Glycine max) cv. Pella.

Seedling growth conditions. All seedlings except soybean were grown in 500 ml pots, using sterile, coarse-type vermiculite which had been sterilized before sowing by soaking with sterile half-strength Hoagland's nutrient solution. The pots were placed in a Conviron model EF7 growth chamber (Controlled Environment Co., Canada) [14 h light (150 μE m⁻² s⁻¹), 10 h dark at 28 ± 2 °C] for 5 d after seeding emergence. Soybean seedlings were grown in 800 ml pots in a similar manner under greenhouse conditions (27 ± 2 °C, 16 h light, 8 h darkness). Irrigation was carried out daily with sterilized water (15-20 ml per pot).

V-form bacteria had a length (L) of 1.45 ± 0.18 μm, a width (W) of 0.408 ± 0.092 μm and an average ratio (L/W) of 3.541:1 (mean of 228 measurements). C-form bacteria were defined as shorter and thicker cells with a length of 0.722 ± 0.086 μm, width of 0.59 ± 0.11 μm and an average ratio (L/W) of 1.224:1 (mean of 209 measurements).

Seedling inoculation. A. brasilense Cd was cultured in nutrient broth (Difco) medium (16 h, 250 r.p.m., 30 ± 1 °C), harvested by centrifugation, and washed twice with sterile 0.06 M-potassium phosphate buffer, pH 7.1, supplemented with 0.15 M-NaCl (PBS); the number of bacteria was then adjusted to 10⁹ c.f.u. ml⁻¹ [Bashan (1986) demonstrated a marked effect on the growth of wheat seedlings by this concentration of A. brasilense Cd]. Seedling inoculation was performed in random design with three to five replicates each, and repeated twice. A replicate consisted of either four seedlings, two electron microscopy stubs which contained three to five root segments each, two cover glasses, or two pots containing four seedlings each, according to the experiment. Significance was tested at P ≤ 0.005 in Fisher's Least Significant Difference (LSD) test or by standard error.

Bacterial counts. Root tips, elongation, differentiation and root-hair zones, and older root parts were defined as previously described (Levanony & Bashan, 1989; Levanony et al., 1989) using a stereoscopic microscope. Each root section was excised separately with a razor blade, and placed in 2.5 ml PBS. The root tip and the differentiation and elongation zones were excised together. A. brasilense Cd cells attached to the outer surface of the roots were released into the buffer by light sonication of the sample (Branson Sonifier B-12 for 3 min at 10 W). This sonication did not affect the multiplication of A. brasilense Cd (Bashan & Levanony, 1989a). The suspension was plated on BL semi-selective N-free medium (Bashan & Levanony, 1985) using a spiral plater (Spiral Systems, Cincinnati, USA), and incubated for 72 h at 30 ± 2 °C before counting. A. brasilense Cd was identified according to the descriptions of this strain made by Bashan et al. (1989b) and Eskew et al. (1977). The level of sample contamination by other rhizosphere bacteria varied and was low, ranging from 1/2 × 10⁶ to 4/4 × 10⁶ c.f.u. per root sample. No attempt was made to identify the contaminants.

Desiccation survival tests. (a) From liquid culture. Bacterial cultures (16 h, V-forms; 96 h, C-forms) were grown as described previously (Bashan, 1986; Bashan & Levanony, 1985). A 100 μl bacterial suspension was placed on a thin, acetone-cleaned, dry, microscope cover-glass and air-dried at ambient temperature (23 ± 2 °C). The cover glasses were transferred to hermetically sealed glass containers together with silica gel (to absorb moisture) for 7 d. Surviving bacterial cells were counted by immersing the cover glass in an Eppendorf microtuble fitted with nutrient broth, shaking for 3 h, and enumerating bacteria as described earlier.

(b) From soybean roots. Inoculated roots were harvested 2 d after seedling emergence for recovery of V-forms and 8 d after emergence plus water stress for recovery of C-forms. The root elongation zone was excised and the segments were dried in a forced-air oven at 30 ± 2 °C for 12 h and then transferred to the same sealed glass container as used for the liquid cultures. Bacteria were recovered from the root segments and counted as described earlier.

Mucilage extraction. This was done according to Mandimba et al. (1986), using 250 seedlings per treatment. Results are expressed as ml extracted mucilage per 250 seedlings.

Scanning electron microscopy. Roots were fixed for 4 h in 5% (v/v) glutaraldehyde in 0.2 M-cacodylate buffer, pH 7.2, under vacuum, washed twice in the same buffer and dehydrated by passage through increasing alcohol concentrations at 4 ± 1 °C. The samples were dried in a critical-point dryer (Tousimis Co., USA) in a CO₂ atmosphere. The dried samples were affixed to stubs with carbon cement, coated with platinum (10-15 nm thickness; 25 mA, 2 min, SEM Sputter Coating Unit E 5100, Polaron Equipment USA) and examined by Philips SEM 505 and ISI 40 electron microscopes at 20-30 kV.

Experimental design and statistical analysis. Experiments were performed in random design with three to five replicates each, and repeated twice. A replicate consisted of either four seedlings, two electron microscopy stubs which contained three to five root segments each, two cover glasses, or two pots containing four seedlings each, according to the experiment. Significance was tested at P ≤ 0.005 in Fisher's Least Significant Difference (LSD) test or by standard error.

Results

Root colonization of tomato, pepper and cotton plants by pleomorphic A. brasilense Cd

Observations carried out along inoculated tomato roots, followed by bacterial counts, revealed that the majority of the surface bacterial population was concentrated in the elongation and the root-hair zones (Table 1, Fig. 1a), although single cells were randomly located and enumerated in the older parts of the root system. The main distribution pattern on tomato roots was in the form of single cells (Fig. 1b, c). The bacteria were located on the bases of the root-hairs; however, the root-hairs themselves were generally free of bacteria (Fig. 1d). Some A. brasilense Cd cells had the typical vibroid shape, and others had a relatively thick fibrillar material connecting them to the roots (Fig. 1e). In order to rule out the possibility of contamination by other bacterial species with different morphology, a similar experiment with tomato roots was performed two years later under axenic conditions, with similar results. The same inoculum gave the normal bacterial morphology, i.e. lacking thick fibrillar material, when colonizing wheat roots (Bashan et al., 1986) (data not shown). A larger amount of mucilaginous substances (2-1 ml) was recovered from
Root surface colonization by *Azospirillum*

Fig. 1. Scanning electron micrographs (SEMs) of tomato root colonization by *A. brasilense* Cd. (a) Location of the bacterial population in the elongation and root-hair zones; (b) typical presence of bacteria as single cells on root surfaces; (c) enlargement of (b) showing bacteria randomly dispersed on the root surface; (d) bacteria located on the base of root-hairs; (e) vibrio shape of root-colonizing bacteria; arrows indicate thick fibrillar material connecting bacterial cells to root surface. Bars represent 1 μm (b-e) and 0.1 mm (a). Abbreviations (also used in the other electron micrographs): C, cyst-like bacteria; E, elongation zone; F, fibrillar material; RH, root-hair; RS, root surface; V, vibrio-form bacteria.
inoculated tomato plants compared to non-inoculated plants (1:3 ml).

Inoculation of pepper seedlings with *A. brasilense* Cd resulted in enlargement of the elongation and differentiation zones compared to non-inoculated pepper seedlings (1-95 ± 0-32 mm versus 0-76 ± 0-19 mm; means of 28 samples, ± SE) observed. In addition, a larger amount of mucilaginous substances was recovered from inoculated roots and root-hairs (1.8 ml versus 1.1 ml per 250 seedlings). Unlike tomato roots, colonization was mainly on the surface of root-hairs (Fig. 2b, c), although some bacteria were also found on the root surface in the root hair zone (Table 1, and Fig. 2a). The typical mode of colonization was aggregate formation, each aggregate containing several cells (Fig. 2d, e) and the microcolonies on the root-hairs were usually larger than those on the root surface (Fig. 2f). The formation of the thick fibrillar material at the poles of the bacterial cell was also observed on pepper roots (see Fig. 2 a, c, e).

Inoculation of germinating cotton seedlings with *A. brasilense* Cd resulted in the heaviest visible surface colonization of the four plant species tested. The main bacteriological population was observed, as in the other plant species, in the elongation and root hair zones (Table 1). The interaction between *A. brasilense* Cd and cotton roots produced a larger amount of mucilage-like material to the root surface (Fig. 3b, c). Microcolonies located at wide-angle cavities on the roots anchored themselves to both sides of the root cavity by a network of fibrillar material. The cells in the microcolony were also connected to each other by this fibrillar material (Fig. 3d, e). Anchoring of microcolonies to cotton roots was very common.

Effect of watering regime on morphology of *A. brasilense* Cd colonizing the roots of soybean plants

Under regular irrigation the *A. brasilense* Cd population on the root surface increased exponentially, reaching 10⁶ c.f.u. (cm root surface)⁻¹ 20 d after sowing (Fig. 4a). Nearly all the bacterial cells observed on the root surface were single vibroid (V-form) forms (Fig. 5a). *A. brasilense* Cd cells were connected to each other within the small microcolonies and to the root surface by a network of fibrillar material strands (Fig. 5b, arrows). Stopping the irrigation of soybean seedlings affected both the population size and the bacterial cell shape. During the dry period, the number of *A. brasilense* Cd cells decreased to a low level, i.e. 10⁴ c.f.u. (cm root)⁻¹, and the remaining cells were C-forms (Fig. 4c, Fig. 5c, d). Restarting irrigation of the plants resulted in the size of the bacterial population increasing 6 d later (Fig. 4c) and the bacteria observed were V-form (Fig. 4c, Fig. 5e). Twenty-six days after inoculation, *A. brasilense* Cd cells were V-forms, although a few cells kept their C-form throughout the experiment (Fig. 4c, Fig. 5f).

When no water was applied to the seeds until 8 d after sowing, C-forms appeared 3 d after sowing (Fig. 4d). In both irrigation regimes, the *A. brasilense* Cd population continued to increase for 2–3 d after the cessation of
Fig. 2. SEMs of pepper root colonization by *A. brasilense* Cd. (a) Single bacteria colonizing the root surface – note large amounts of mucilaginous substances on the root surface; (b) root hair colonized by bacteria; (c) enlargement of (b) showing connection between bacteria and root-hair surface by thick fibrillar material (arrows); (d) typical mode of colonization on root surface as small aggregates; (e) enlargement of (d) showing the cells connected to each other and to the root by fibrillar material (arrow); (f) microcolony on a root-hair – note the large amounts of mucilaginous substances and fibrillar material connecting the microcolony to the root-hair. Bars represent 1 μm (a, c-e) and 10 μm (b).
Fig. 3. SEMs of the interaction between *A. brasilense* Cd cells and cotton roots. *(a)* First type of colonization, as small aggregates randomly dispersed in the elongation zone; *(b)* second type of root colonization, as single cells connected by a dense fibrillar material (arrows); *(c)* higher magnification of a few cells in *(b)* showing the thick fibrillar material (arrows); *(d)* microcolony located at a wide-angle cavity in the root. Note the microcolony anchored to both sides of the cavity by a network of fibrillar material (arrows); *(e)* enlargement of *(d)* – note that cells in the microcolony are connected to each other by fibrillar material (arrow). Bars represent 1 μm.
watering and was composed mainly of V-form cells. Due to changes in the irrigation regimes, there were intermediate periods having V- and C-form A. brasilense Cd populations. In the absence of plants, the A. brasilense Cd population in the vermiculite decreased sharply regardless of the irrigation regime, reaching a low level [10^3 c.f.u. (g vermiculite)^{-1}] after 15 d, a decrease of 99.9% compared with the original population (Fig. 4b).

Survival post-desiccation of V-form A. brasilense Cd either from liquid culture or from soybean roots was poor (1.9 x 10^2 c.f.u. out of 10^6 c.f.u. ml^{-1} or 2.25 x 10^2 compared to 10^6 c.f.u. (g root)^{-1} on non-desiccated roots: Fig. 6). C-form bacteria survived better: 10^4 c.f.u. out of
Fig. 5. SEMs of soybean root colonization by A. brasilense Cd. (a) Typical colonization of soybean root surfaces by single cells accompanied by small aggregates; nearly all the cells are of V-form; (b) enlargement of (a) showing bacteria connected to the root surface and to adjacent bacteria by a network of fibrillar material (arrows); (c) colonization of wilting soybean roots composed of V- and C-forms of A. brasilense Cd; (d) enlargement of (c) showing the typical shape of C-form bacteria; (e) surface root colonization of wilting soybean roots 2 d after reaplication of water, showing a mixed population of V- and C-forms; no fibrillar material is visible; (f) similar to (e) but 8 d after water application, showing that although most cells are V-form, a few C-form cells remain. Bars represent 1 μm.

10^8 c.f.u. ml⁻¹ from liquid culture and 5.1 × 10^4 c.f.u. compared to 10^4 c.f.u. (g root)^⁻¹ on non-desiccated roots (Fig. 6).

Discussion
Most studies of cereal root colonization by Azospirillum have indicated that the mode of colonization is similar in all species studied, involving small aggregates or single cells concentrated mainly on the surface of the root tips and the elongation and root-hair zones, with relatively few cells attached to the root-hairs (Bashan & Levanony, 1989a, b; Bashan et al., 1986). A degree of intercellular root colonization has also been reported (Levanony et al., 1989; Patriquin et al., 1983). Although there is evidence
that *Azospirillum* is capable of colonizing and affecting the growth of crop plants other than cereals (Bashan & Levanony, 1988c; Bashan et al., 1989a, b; Kolb & Martin, 1985; Saha et al., 1985), the mode of root colonization of these plants has not been determined. This study, which is complementary to our previous one (Bashan et al., 1989a), provides additional evidence that *A. brasilense* is able to colonize the roots of crop plants belonging to four different families in addition to the Graminaceae. Unlike the uniformity of cereal root colonization between species, colonization of plant roots from other families differs from one plant species to another. Colonization characteristics (mainly root surface, mainly root-hairs, or both) varied from species to species. Nevertheless, as with many cereal roots (Bashan et al., 1986; Bashan & Levanony, 1989b; Levanony & Bashan, 1989), *A. brasilense* Cd preferentially colonized the elongation and the root-hair zones and not the older parts of the root system as suggested for sorghum (Baldani et al., 1986).

A unique feature of *Azospirillum* root colonization is the anchoring of bacterial cells to the plant surface by a network of fibrillar material. Fibriilar connections may play a role in the life cycle of *A. brasilense* Cd, whether it ison soil and sand (Bashan & Levanony, 1988a, b), wheat (Bashan et al., 1986; Levanony et al., 1989) or on several other non-cereal roots, as demonstrated in the present study. This typical characteristic of *Azospirillum* should be further evaluated in other plant-growth-promoting rhizobacteria in order to determine if it is a general feature of root colonization by beneficial bacteria.

Pleomorphism (vibroid or cyst-like forms of the bacterial cell) of *Azospirillum* in *in vitro* culture is well documented. In the description of the genus *Azospiril-

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**References**


