

Evidence for a Weak Active External Adsorption of *Azospirillum brasilense* Cd to Wheat Roots

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Azospirillum brasilense Cd, when inoculated onto wheat roots, multiplied and formed aggregates on the root surfaces and established an internal root population. Washing the roots removed most of the external but not the internal bacterial population. Killing the bacteria before or after their interaction with the roots eliminated the adsorbed bacteria from the root surface. The external adsorption of *A. brasilense* to wheat roots can be categorized as a weak active process.

INTRODUCTION

There has been intensive study during the last decade on interactions between the rhizosphere bacterium *Azospirillum* and various cereals, particularly regarding the potential contribution to plant growth and yield (Bashan, 1986*a*; Okon, 1985). *Azospirillum* spp. are known to colonize roots of many cereals and to affect plant metabolism (Döbereiner & Baldani, 1979; Baldani *et al.*, 1983; Kapulnik *et al.*, 1981; Okon & Kapulnik, 1986). The mechanism involved in this adsorption is unknown (Patriquin & Döbereiner, 1978; Patriquin *et al.*, 1983; Schank *et al.*, 1979, 1983; Umali-Garcia *et al.*, 1980). Gafni *et al.* (1986) described various modes of adsorption of *A. brasilense* to maize roots and Jain & Patriquin (1984) reported differences between various strains of *Azospirillum* in the degree of attachment to wheat root-hairs.

The purposes of the present study were to estimate the strength of the association between *A. brasilense* and wheat roots and to find out whether active metabolism of the participants is involved.

METHODS

Organisms and growth conditions. *Azospirillum brasilense* Cd (ATCC 29710) was cultured on nutrient broth (Difco) medium, as previously described (Bashan & Levanony, 1985). Bacteria were inoculated into 3-d-old wheat seedlings (*Triticum aestivum* cv. 'Deganit') grown on wet filter paper in Petri dishes (five seedlings per plate). Bashan (1986*b*) demonstrated a marked effect of inoculation on wheat seedlings at this growth stage. Inoculation was performed by placing 2 ml double-washed bacterial suspension in phosphate-buffered saline (PBS) pH 7.2 (5×10^8 c.f.u. ml⁻¹) directly on the roots. All experiments were conducted in a growth chamber (Conviron model EF7H, Controlled Environment, Canada) at 22 ± 2 °C, 10 h light and 14 h darkness.

A. brasilense Cd was identified and enumerated by the indirect and competition enzyme-linked immunosorbent assay (ELISA) developed for the detection of this strain. In the indirect ELISA, 3–4 mm pieces of roots from each plant were placed in 0.15 ml coating buffer in one well. In the competition ELISA, roots of 15 inoculated seedlings (per replicate) were homogenized with a glass homogenizer (Kontes, USA), centrifuged at 12000 *g* for 10 min and the pellet was suspended in 2 ml PBS and used as a competitor. The rest of the ELISA procedure was as described elsewhere (Levanony *et al.*, 1987). Bacterial counts were also verified by the plate count method on BL semi-selective medium (Bashan & Levanony, 1985).

Treatment of bacteria and roots. Forty-eight hours after inoculation roots of 25 seedlings per treatment were treated as follows: (i) rinsed gently for 10 s in tap water in a 1 litre container with slow stirring; (ii) washed in the same way for 2 min; (iii) soaked for 30 min in tap water and then washed for 2 min as described above. Other roots were surface disinfected with 1% (v/v) NaOCl for 3 min and washed for 2 min in tap water.

In an additional experiment, roots of inoculated seedlings were placed in 15 ml tubes (three seedlings per tube) and rinsed with 10 ml sterile PBS pH 7.2. The PBS was replaced three times with a Pasteur pipette, then the

seedlings were agitated in a vortex mixer (Vortex-Genie, Scientific Industries) at 1800 r.p.m. for 60 s. The roots were then rinsed twice with PBS and the number of *A. brasilense* Cd was determined in root homogenates by competition ELISA, and on the surface of root segments by indirect ELISA. Roots were concomitantly homogenized with a sterile glass homogenizer and decimally diluted in PBS; 0.1 ml samples were spread on semi-selective BL medium for plate counts (Bashan & Levanony, 1985).

To evaluate the involvement of metabolic activities in root-bacteria interactions, either bacteria or roots or both were treated by γ -irradiation (25 kGy from a ^{60}Co source). This irradiation killed all bacterial cells, both in the suspension, and in plant roots as tested by bacterial counts of root homogenates on BL semi-selective medium. Plant root cells were also killed by this treatment. Inoculation with dead bacterial culture (equivalent before sterilization to 10^8 c.f.u. ml $^{-1}$) or inoculation onto dead roots (killed by γ -irradiation) was done as previously described for live bacteria. In addition, roots inoculated with live bacteria were sterilized 48 h later by γ -irradiation and washed for 10 s, as previously described. Controls used in these experiments were: inoculated roots, uninoculated washed or unwashed roots, uninoculated disinfected roots and dead roots.

Scanning electron microscopy (SEM). Roots were fixed for 5 h in 5% (v/v) glutaraldehyde solution in 0.2 M cacodylate buffer pH 7.2, washed twice in the same buffer and then dehydrated with ethanol, 50% (v/v) for 30 min, 70% for 10 h, 100% for 30 min, and 100% for 60 min (all this was done at 4 °C). The samples were dried in a critical point dryer (Tousimis, USA) in freon, then in CO $_2$. The dried samples were stuck to stubs, coated with gold and examined by a Philips SEM505 scanning electron microscope at 20–30 kV.

Experimental design. All experiments were repeated three times with five replicates each. A replicate consisted of either five seedlings, six microtitre plate wells, five SEM stubs or five Petri dishes. The results presented are from representative experiments.

RESULTS AND DISCUSSION

By using the ELISA technique for the specific detection of *A. brasilense* Cd it was possible to detect a relatively large number of the associated bacteria in wheat roots. Two different types of *A. brasilense* populations were detected: an external one on the root surface and an internal one in the cortex.

The external population showed an aggregate mode of colonization and the bacteria were connected to each other and to the root surface by fibrillar material (Fig. 1 a–c). Slight rinsing of the roots released 70% of this population (Table 1), as found for maize roots (Gafni *et al.*, 1986). The external population decreased to a very low level (less than 10^4 cells per g roots, which is below the sensitivity of the ELISA technique) with longer washing (Fig. 1 d, Table 1). (The plate count method was not used because it would reveal both the internal and the external populations.) Inoculated wheat roots agitated by vortex mixing released most of the externally adsorbed bacteria; the bacteria detected by competition ELISA in the root homogenates were

Table 1. Number of *A. brasilense* Cd adsorbed to wheat root surfaces following different methods of root washings (measured 48 h after inoculation)

Root washing method	Bacterial inoculation*	No. of bacteria on root surfaces per g roots†
(a) Not washed	+	$1 \pm 0.3 \times 10^5$
	–	0
(b) Rinsed for 10 s with slow stirring	+	$3 \pm 0.6 \times 10^4$
(c) Washed for 2 min with slow stirring	+	0
	–	0
(d) Soaked for 30 min then washed as in (c)	+	0
(e) Rinsed as in (b) then vortex mixed for 60 s	+	$4 \pm 0.4 \times 10^6 \ddagger$
	+	0§

* With 10^8 c.f.u. ml $^{-1}$, 2 ml per plate.

† Measured by indirect ELISA. Zero represents bacterial numbers below the sensitivity of the method ($< 10^4$ per g roots).

‡ Inside the roots after root homogenization, measured by competition ELISA.

§ On the root surface.

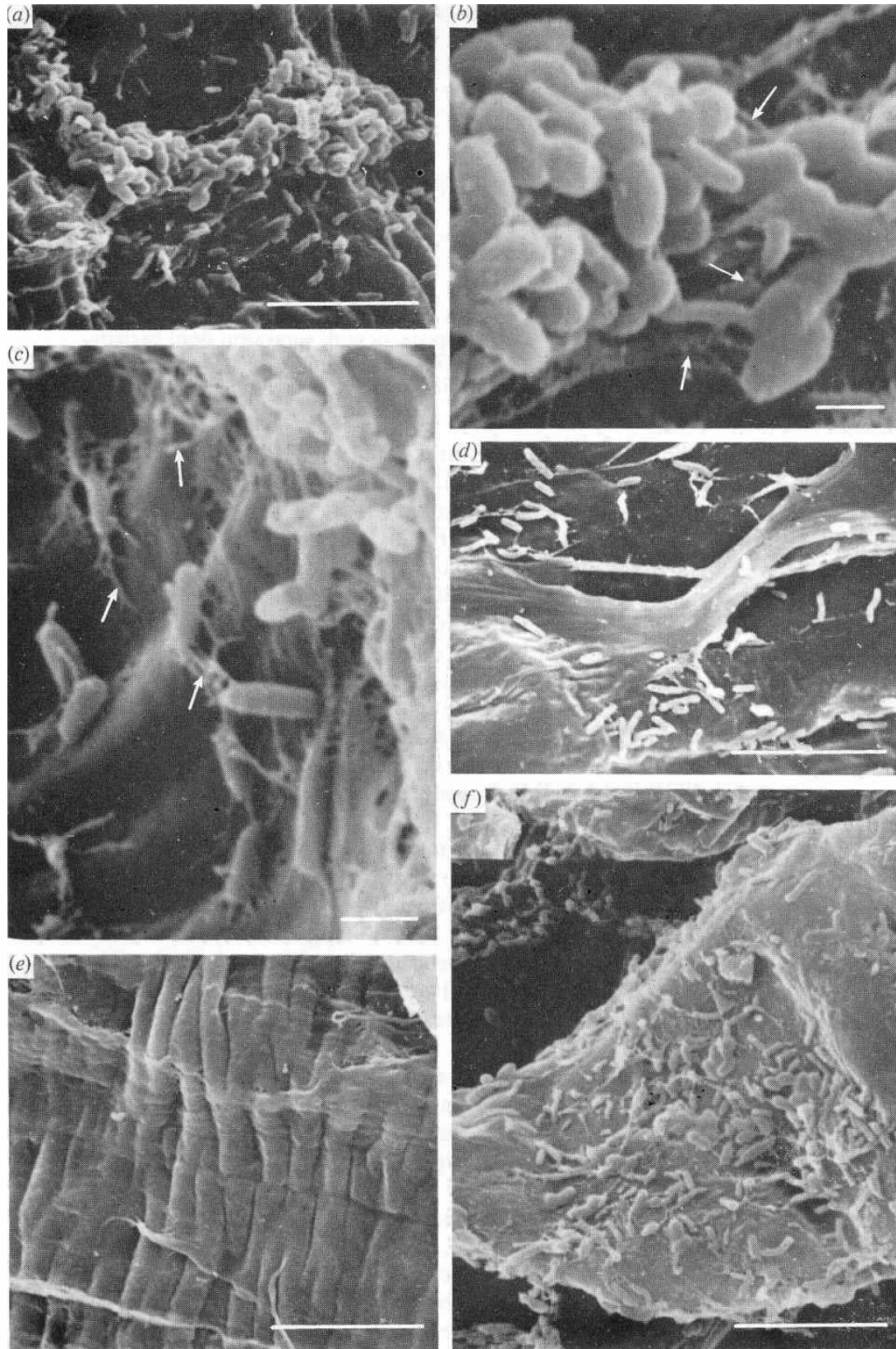


Fig. 1. Scanning electron micrographs of wheat roots inoculated with *A. brasilense* Cd. The bars represent 10 μm in (a), (d), (e) and (f), and 1 μm in (b) and (c). (a) Bacterial aggregates at the border between the elongation zone and root hair zone. (b) Higher magnification of the aggregate in (a) showing fibrillar material (arrows) connecting the bacteria to each other. (c) *A. brasilense* cells connected to the root surface by fibrillar material (arrows). (d) Random distribution of bacterial cells after very light washing; there are no bacterial aggregates. (e) Wheat root after light surface disinfection with 1% NaOCl. (f) Bacterial colonization on dead root-tip cell.

Table 2. Adsorption of *A. brasilense* Cd to wheat roots (measured 3 d after inoculation)

Root treatment*	Bacterial inoculation†	No. of bacteria per g roots	
		Competition ELISA	Counts on BL medium
None	Live	$1 \pm 0.2 \times 10^7$	$1.3 \pm 0.4 \times 10^7$
	Dead‡	0	0
Dead‡	Live	$4 \pm 0.4 \times 10^7$	$2.8 \pm 0.3 \times 10^7$
	Dead	0	0
Sterilized‡ after inoculation	Live	0	0
Disinfected§ after inoculation	Live	$2 \pm 0.4 \times 10^4$	$8.4 \pm 1.6 \times 10^4$
Disinfected§	None	0	0

* All roots were washed for 10 s, with slow stirring, after root treatment.

† With 10^8 c.f.u. ml⁻¹, 2 ml per plate, or with equivalent dead bacteria.

‡ Exposed to γ -irradiation (25 kGy).

§ Disinfected for 3 min in 1% NaOCl.

probably those which had penetrated into the inner root layers (Patriquin & Döbereiner, 1978). This internal population was relatively large: 10^6 – 10^7 c.f.u. per g root.

Disinfection of highly colonized roots eliminated most of the bacteria (only 0.2% of the original population remained alive) (Fig. 1e, Table 2). When these roots were aseptically placed on a semi-selective BL medium only a few colonies grew around them. Application of killed bacterial cells either to live or to dead roots resulted in negligible external adsorption. However, killing the roots before inoculation had no effect on the degree of bacterial adsorption (Fig. 1f, Table 2). γ -Irradiation following a massive root colonization of *A. brasilense* Cd killed both the bacteria and the plant and resulted in elimination of most of the dead bacterial cells from the roots. If dead bacteria were still attached to the roots they could be enumerated by the ELISA technique, which is capable of detecting both dead and live bacteria (Levanony *et al.*, 1987). Similarly, elimination of most of the bacterial cells after killing colonized roots could not be attributed to the light rinsing used in this procedure, because such treatment did not eliminate live bacteria from roots, as indicated above.

Adsorption of bacteria to a solid phase is known (Fletcher *et al.*, 1980) and may give the rhizosphere bacteria nutritional and favourable microspace advantages. Rhizosphere bacteria may adsorb as single cells or as aggregates, but usually are not scattered randomly (Dazzo & Hollingsworth, 1984; Shimshick & Hebert, 1979). *Azospirillum* cells were found in the intercellular spaces of the cortex of various cereals, grown either axenically (Berg *et al.*, 1979; Patriquin & Döbereiner, 1978) or under field and greenhouse conditions (Bashan, 1986a; Schank *et al.*, 1979), as well as within dead cortex cells and root hairs (Jain & Patriquin, 1984; Patriquin & Döbereiner, 1978).

This study reveals that the external adsorption of *A. brasilense* to wheat roots is rather weak: light washing removed most of the aggregates, and more prolonged washing removed nearly all the bacterial cells present on the root surface. These findings differ from those obtained for some rhizobia-legume interactions (Dazzo & Hollingsworth, 1984) and for the *Azospirillum*-maize interaction (Gafni *et al.*, 1986).

Adsorption of bacteria to plant roots can be either passive or it may depend on the active metabolism of both organisms (Fletcher *et al.*, 1980; Shimshick & Hebert, 1979). This work demonstrated that killing the bacteria, either before or after adsorption, prevented this process almost completely, indicating that active metabolism of the *Azospirillum* cells was essential for the association with the roots. Since these bacteria could colonize and adsorb to dead roots, the active metabolism of the plant may be less important. The adsorption of *A. brasilense* to the surfaces of wheat roots can thus be categorized as a weak metabolism-dependent process.

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REFERENCES

- BALDANI, V. L. D., BALDANI, J. I. & DÖBEREINER, J. (1983). Effects of *Azospirillum* inoculation on root infection and nitrogen incorporation in wheat. *Canadian Journal of Microbiology* **29**, 924–929.
- BASHAN, Y. (1986a). Enhancement of wheat root colonization and plant development by *Azospirillum brasilense* Cd. following temporary depression of the rhizosphere microflora. *Applied and Environmental Microbiology* **51**, 1067–1071.
- BASHAN, Y. (1986b). Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. *Soil Biology and Biochemistry* **18**, 297–301.
- BASHAN, Y. & LEVANONY, H. (1985). An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. *Canadian Journal of Microbiology* **31**, 947–952.
- BERG, R. H., VASIL, V. & VASIL, I. K. (1979). The biology of *Azospirillum*–sugarcane association. II. Ultrastructure. *Protoplasts* **101**, 143–163.
- DAZZO, F. B. & HOLLINGSWORTH, R. E. (1984). Trifoliin A and carbohydrate receptors as mediators of cellular recognition in *Rhizobium trifolii* clover symbiosis. *Biology of the Cell* **51**, 267–274.
- DÖBEREINER, J. & BALDANI, V. L. D. (1979). Selective infection of maize roots by streptomycin-resistant *Azospirillum lipoferum* and other bacteria. *Canadian Journal of Microbiology* **25**, 1264–1269.
- FLETCHER, M., LATHAM, M. J., LYNCH, J. M. & RUTTER, P. R. (1980). The characteristics of interfaces and their role in microbial attachment. In *Microbial Attachment to Surfaces*, pp. 67–78. Edited by R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter & B. Vincent. Chichester: Ellis Horwood.
- GAFNI, R., OKON, Y., KAPULNIK, Y. & FISCHER, M. (1986). Adsorption of *Azospirillum brasilense* to corn (*Zea mays*) roots. *Soil Biology and Biochemistry* **18**, 69–76.
- JAIN, D. K. & PATRIQUIN, D. G. (1984). Root hair deformation, bacterial attachment, and plant growth in wheat–*Azospirillum* associations. *Applied and Environmental Microbiology* **48**, 1208–1213.
- KAPULNIK, Y., OKON, Y., KIGEL, J., NUR, I. & HENIS, Y. (1981). Effects of temperature, nitrogen fertilization, and plant age on nitrogen fixation by *Setaria italica* inoculated with *Azospirillum brasilense* (strain Cd). *Plant Physiology* **68**, 340–343.
- LEVANONY, H., BASHAN, Y. & KAHANA, Z. E. (1987). Enzyme-linked immunosorbent assay for specific identification and enumeration of *Azospirillum brasilense* Cd. in cereal roots. *Applied and Environmental Microbiology* **5** (in the Press).
- OKON, Y. (1985). *Azospirillum* as a potential inoculant for agriculture. *Trends in Biotechnology* **3**, 223–228.
- OKON, Y. & KAPULNIK, Y. (1986). Development and function of *Azospirillum*-inoculated roots. *Plant and Soil* **90**, 3–16.
- PATRIQUIN, D. G. & DÖBEREINER, J. (1978). Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Canadian Journal of Microbiology* **24**, 734–742.
- PATRIQUIN, D. G., DÖBEREINER, J. & JAIN, D. K. (1983). Sites and processes of association between diazotrophs and grasses. *Canadian Journal of Microbiology* **29**, 900–915.
- SCHANK, S. C., SMITH, R. L., WEISER, G. C., ZUBERER, D. A., BOUTON, J. H., QUESENBERRY, K. H., TYLER, M. E., MILAM, J. R. & LITTELL, R. C. (1979). Fluorescent antibody technique to identify *Azospirillum brasilense* associated with roots of grasses. *Soil Biology and Biochemistry* **11**, 287–295.
- SCHANK, S. C., SMITH, R. L. & LITTELL, R. C. (1983). Establishment of associative N₂-fixing systems. *Proceedings of the Soil and Crop Science Society of Florida* **42**, 113–117.
- SHIMSHICK, E. J. & HEBERT, R. R. (1979). Binding characteristics of N₂-fixing bacteria to cereal roots. *Applied and Environmental Microbiology* **38**, 447–453.
- UMALI-GARCIA, M., HUBBELL, D. H., GASKINS, M. H. & DAZZO, F. B. (1980). Association of *Azospirillum* with grass roots. *Applied and Environmental Microbiology* **39**, 219–226.