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Active attachment of *Azospirillum brasilense* to root surface of non-cereal plants and to sand particles

H. LEVANONY and Y. BASHAN[1]

Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel and Department of Microbiology, Division of Experimental Biology, The Center of Biological Research (CIB), La Paz, P. O. Box 128, B. C. S., 23000 Mexico ([1]Corresponding author)

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Abstract

The rhizosphere bacterium *Azospirillum brasilense* Cd adsorbed strongly to light-textured and heavy-textured soils, but only slightly to quartz sand. Bacterial attachment to sand particles was mediated by a network made up of various sizes and shapes of fibrillar material. Inoculation of sand with an aggregate-deficient mutant resulted in no detectable fibrillar formation. Rinsing or agitating the sand, colonized by the wild-type and the mutant, had a greater effect on the mutant than on the parental strain. We propose that bacterial fibrils are essential for anchoring of *A. brasilense* to sand.

A. brasilense Cd was capable of efficiently colonizing the elongation and root-hair zones of tomato, pepper, cotton and soybean plants as well as of wheat plants. All inoculated plants demonstrated: (i) larger amounts of a mucigel-like substance on the root surface than non-inoculated plants, and (ii) fibrillar material which anchored the bacterial cells to the root surface. These fibrils established also connections between cells within bacterial aggregates. On non-water stressed soybean roots, most *A. brasilense* Cd cells occurred as vibroid forms. Whereas, those on roots of water-stressed plants (wilting) were cyst-like. A lower rhizosphere bacterial population was observed on water-stressed plants. When water stress conditions were eliminated, cells reverted to the vibroid form. A concomitant increase in the bacterial population was observed. We suggest that cyst-like formation is a natural response for *A. brasilense* Cd in the rhizosphere of water-stressed plants.

Introduction

The beneficial rhizosphere bacterium *Azospirillum brasilense* Cd has been applied to numerous soil types world-wide in order to inoculate crop plant roots and to enhance plant yield (Bashan and Levanony, 1990; Michiels et al., 1989). Application of *A. brasilense* Cd cells to sand revealed that part of the bacterial population is actively attached to sand particles by a network of protein bridging between individual cells and between cells and sand particles (Bashan and Levanony, 1988b).

When inoculated onto cereal roots, *A. brasilense* Cd multiplied and formed small aggregates. The aggregates are mainly found in the root elongation and the root-hair zones (Bashan et al., 1986). The bacteria produced holdfast fibrillar material which anchored the cells to the root surface (Levanony et al., 1989).

The purposes of this study were: (i) To evaluate the importance of these bridges in the life cycle of the bacterium in the sand. This will establish whether or not they are essential for *A. brasilense* Cd attachment to sand. (ii) To evaluate the ability of *A. brasilense* Cd to colonize root surfaces of several non-cereal plants and to determine colonization sites. (iii) To examine the occurrence of, and conditions for pleomorphic *A. brasilense* Cd in the rhizosphere. (iv) To evaluate whether bacterial fibrillar connections to root surfaces are a general mode of attachment of *A. brasilense* Cd to plant root surfaces.

Materials and methods

Organisms

Azospirillum brasilense Cd (ATCC 29710) (aggregating strain, agg^{+}) and a non-aggregating mutant (agg^{-}) derived from a Tn5 mutant of strain Cd (strain 29710-10b) were used. The following plant species were used: tomato (*Lycopersicon esculentum*) 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.

Sand properties, bacterial and plant growth condition and inoculation, adsorption assays, bacterial counts from sand and roots, determination of percentage adsorption and experimental design

These were previously described (Bashan and Levanony, 1988a,b; 1989a,b).

Agitation and washing procedures

Strong agitation treatment of sand-bacteria mixtures after sand colonization (Vortex mixer) was performed at 180 rpm for 60 sec before the rinsing procedure, whereas low agitation was performed at 40 rpm for 90 sec. Inoculated sand was rinsed as previously described (Bashan and Levanony, 1988b). Two rinsing durations were used; 10 sec or 2 min.

Induction of cysts in A. brasilense

Cyst-like forms of *A. brasilense* on root surfaces were induced by stopping irrigation of plants grown in vermiculite for 8 days, until plant leaves showed first signs of wilting. Then, irrigation was renewed.

Definition of bacterial V- and C-forms

Bacterial cells observed on root surfaces were defined according to their size and the ratio of length to width. V-form bacteria had a length of 1.45 ± 0.18 mm, width of 0.408 ± 0.092 mm and average ratio (L/W) of 3.554:1 (mean of 228 measurements). C-form bacteria were defined as shorter and thicker cells with a length of 0.722 ± 0.086 mm, width of 0.59 ± 0.11 mm and average (L/W) ration of 1.224:1 (mean of 209 measurements).

Results

Description of active colonization of A. brasilense *of sand*

Random dispersal of single cells or very small microcolonies characterized the population density of inoculated sand or soil particles. Bacterial cell size was smaller compared to cells which were grown in liquid medium (0.8-1.5 mm compared to 2-3 mm, respectively). Microcolonies were found in relatively small numbers and were located mainly in the shallow cavities of quartz particles. Many bacterial cells were attached to the surface of the sand particles by fibrillar ma-

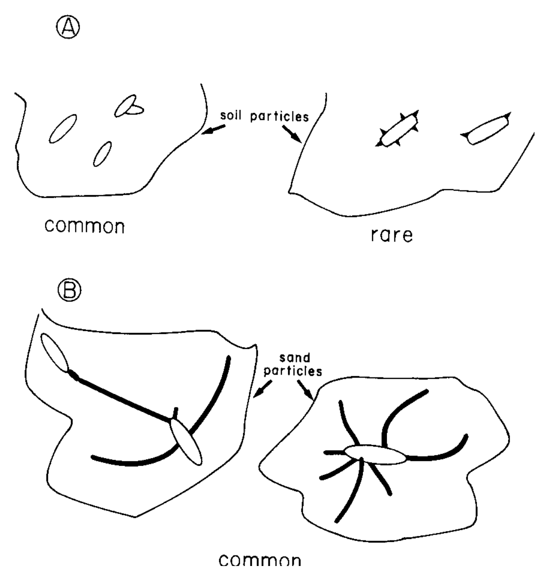

Fig. 1. Schematic presentation of *A. brasilense* agg^{+} cells colonizing sand. (**A**) Random dispersal of single cells on a soil particle. (**B**) fibrillar material connecting *A. brasilense* agg^{+} to sand.

terial (Fig. 1B). In soil, bacteria lacked any visible connection or had few very short connections to the soil (Fig. 1A). The bacterial fibrillar material was found to be either single-stranded or multistranded. It was located on any side of the bacterial cell (Fig. 1B).

Attachment of A. brasilense *agg*$^+$ *and agg*$^-$ *to sand following washing or agitation*

Bacterial attachment to sand by both strains, immediately after bacterial application, was negligible (< 0.001%). Population size of either agg$^+$ cells or agg$^-$ cells in sand was similar (>10^7 cfu mL^{-1} after 48 h). However, the percentage of attachment of the two strains to the sand differed significantly, with the agg$^-$ mutant showing lower attachment rates (Fig. 2A). Slight rinsing of the sand (after sand colonization) decreased the adsorption of both strains, but there was a greater decrease in agg$^-$ cells. Increasing the washing time almost eliminated agg$^-$ cells from the sand, while significant number of agg$^+$ cells (approx. 10^6 cfu g^{-1} sand) remained attached to the sand. Despite a decrease in the total bacterial number of agg$^+$ cells caused by

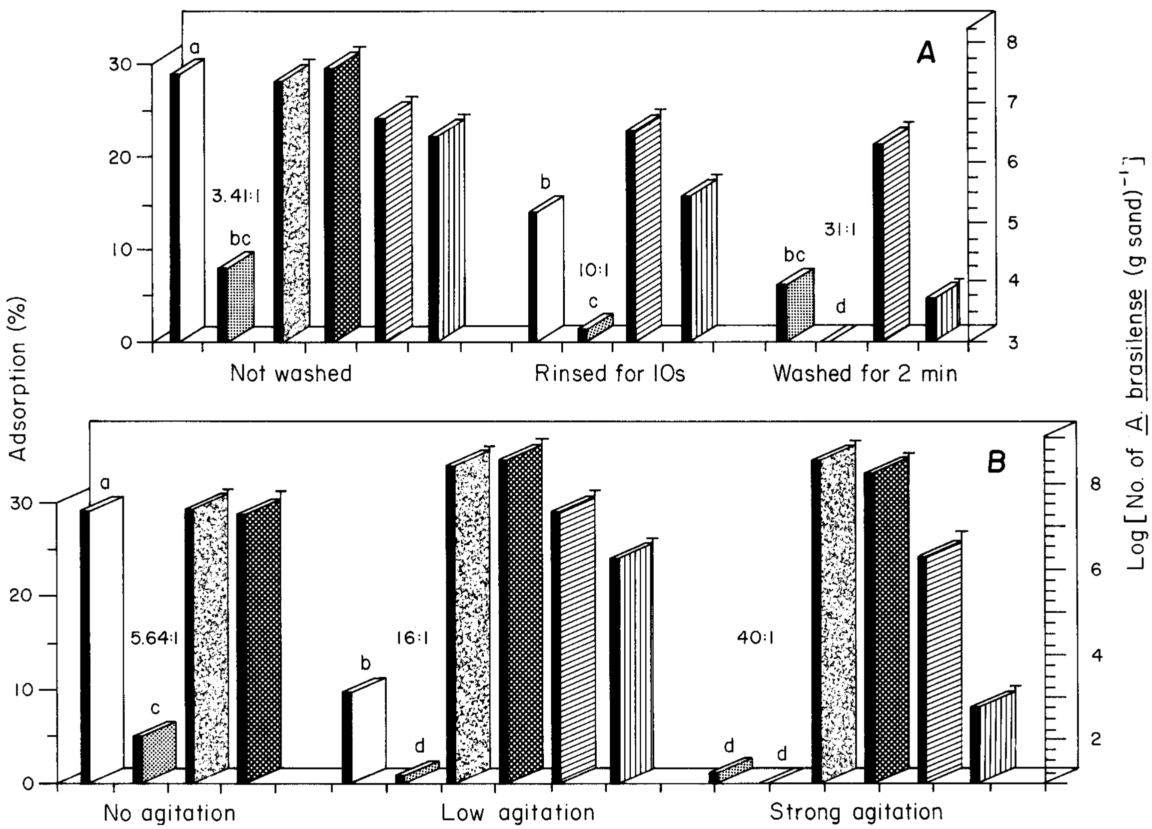

Fig. 2. (**A**) Percentage of adsorption and number of cells of *A. brasilense* (agg$^+$) and *A. brasilense* (agg-) in sand before and following washing treatments. ☐ -adsorption of agg$^+$; ▦ - adsorption of agg$^-$, ▤ - number of cells of agg$^+$ adsorbed; ▥ number of agg$^-$ cells adsorbed; total number of agg$^+$ cells (▩) and agg$^-$ cells (▦) in the sand mixture. (**B**) Percentage of adsorption and number of cells of *A. brasilense* (agg$^+$) and *A. brasilense* (agg$^-$) in sand before and after agitation. ☐ -adsorption of agg$^+$; ▦ - adsorption of agg$^-$; ▤ - number of cells of agg$^+$ adsorbed; ▥ - number of agg$^-$ cells adsorbed; total number of agg$^+$ cells (▩) and agg$^-$ cells (▦) in the sand mixture. Columns followed by a different letter (in each sub-figure, separately) differ significantly at $p \leq 0.05$. Bars represent SE. Number represent the adsorption ratio between agg$^+$ and agg$^-$ cells.

washing, the adsorption ratio between agg^+ and agg^- strains increased with increasing the washing time about 10 times (Fig. 2A).

Generally, agitation decreased bacterial adsorption. Both strains developed large populations (>10^8 cfu g^{-1} sand), and there was no significant difference between the strains. However, the adsorption ratio (between agg^+ and agg^-) increased as a result of agitation. When strong agitation was applied to sand, nearly all agg^- cells were desorbed from the sand particles (840 cells out of 10^8 cfu g^{-1} sand remained) (Fig. 2B).

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

Schematic representations of root colonization by *A. brasilense* Cd is presented in Figure 3. Colonization patterns in the different plant species followed these schemes. Modifications in colonization regarded mainly the preferred colonization sites.

Effect of watering regime on cell 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 10^6 cfu cm^{-1} root surface 20 days after sowing (Fig. 4A). Nearly all the bacterial cells observed on the root surface were single vibroid (V form) forms. 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., 10^4 cfu cm^{-1} root and the remaining cells were C-forms. Restarting irrigation of the plants resulted in the size of the bacterial population increasing 6 days later and the bacteria observed were V -form. Twenty six days after inoculation *A. brasilense* Cd cells were V -forms although a few cells kept their Gform throughout the experiment (Fig. 4C).

When no water was applied to the seeds until 8 days after sowing, C-forms appeared 3 days after sowing (Fig. 4D). In both irrigation regimes, the *A. brasilense* Cd population continued to increase for 2-3 days after the cessation of 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 the irrigation regime. *A. brasilense* Cd population reached a low level, i.e., 10^3 cfu g^{-1} vermiculite; elimination of 99.9% of the original population (Fig. 4B).

Discussion

When *Azospirillum* cells are applied to quartz sand, which holds them very loosely, they are readily detached by any water stream. Therefore, the bacterial cell should produce anchoring substances) to prevent this undesired washing below the root system.

A. brasilense Cd is known for forming large bacterial aggregates, both in liquid medium (Math et al., 1988) and on root surfaces (Bashan et al., 1986).

Recently, we have shown that *A. brasilense* Cd is actively attached to sand particles by a network of proteinaceous bridges (Bashan and Levanony 1988b). Therefore, the focus of this study has been on the role of these bacterial fibrils in sand.

Generally, desorption of bacteria from soil particles by external mechanical forces such as washing and agitation, is difficult. Bacterial cells are strongly and permanently adsorbed by the soil particles (Bashan and Levanony, 1988a; Marshall, 1980). However, attachment of *A. brasilense* Cd to quartz sand particles is relatively weak. This study presents evidence demonstrating that single and multistranded fibrillar material are present and provide detachment resistance for the cells. Such phenomenon did not occur when a mutant incapable of producing these fibrils was inoculated into the sand. Furthermore, although agitation caused partial desorption of agg^+ cells, it had an even stronger effect on agg^- cells, eliminating nearly all the bacterial cells from the sand. Therefore, it can be concluded that this network provides resistance against external physical forces applied to sand.

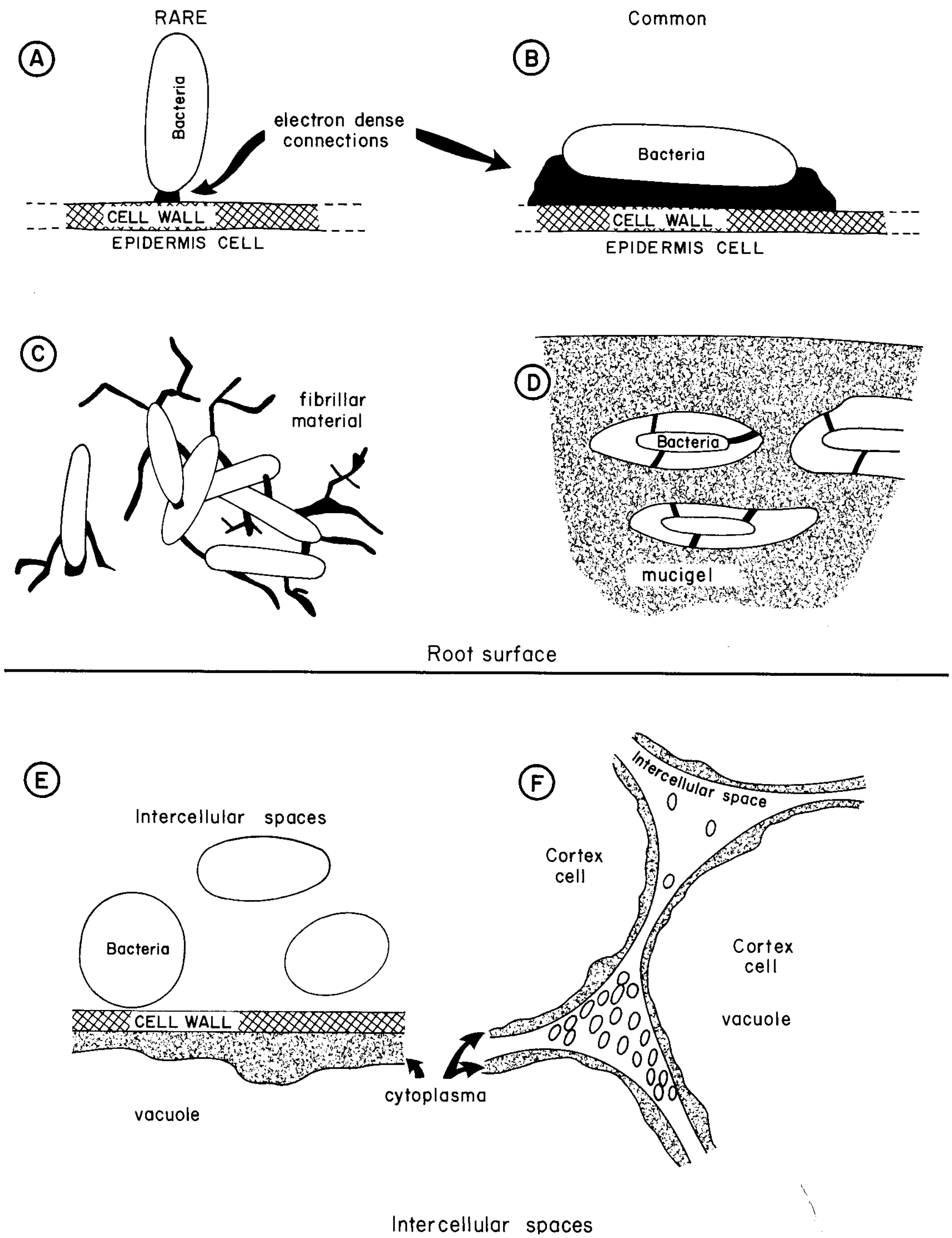

Fig. 3 Schematic representation of *A. brasilense* Cd colonization of: (**A-D**) root surface of wheat, tomato, pepper, cotton and soybean plants, showing intensive fibrillar material formation, and (**E-F**) colonization of the intercellular spaces of wheat roots showing no bacterial attachment to plant cell-walls via fibrillar connections.

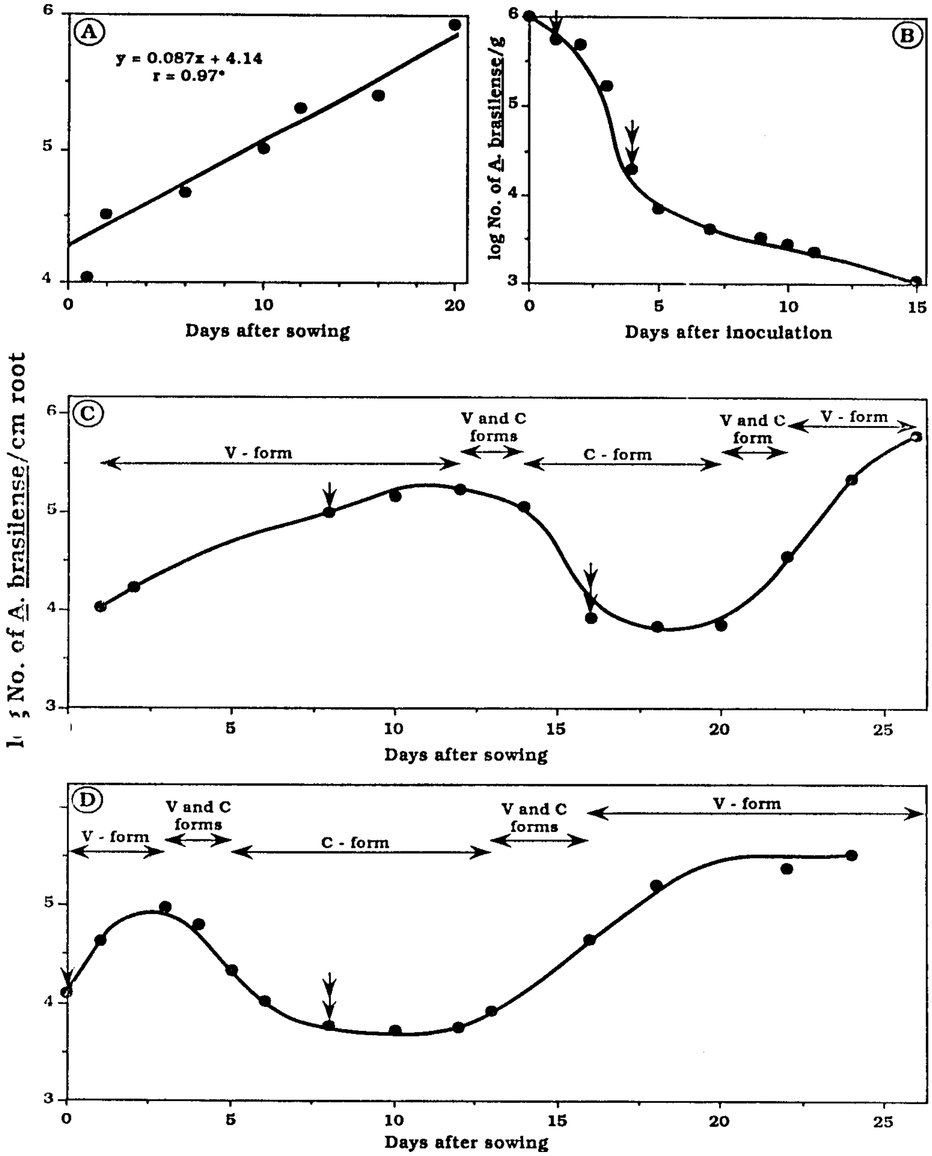

Fig. 4. Colonization of soybean roots (**A**, **C**, **D**) and vermiculite (**B**) by *A. brasilense* Cd under normal growth conditions without water limitation (**A**), and under water stress (**B-D**). ↓ - Daily irrigation was stopped; ↓ - irrigation renewed. Horizontal bars indicate the form of *A. brasilense* Cd cells found in this period of time (V-vibrio form; C-cyst form). *indicates significance of a correlation at $p \leq 0.05$. (Originally published in J. Gen. Microb. 137, 187-196, 1991.)

A unique feature of *Azospirillum* root colonization is the anchoring of bacterial cells to the plant surface by a network of fibrillar material. Fibrillar connections may play a role in the life cycle of *A. brasilense* Cd, whether it is on soil, sand (Bashan and Levanony, 1988a,b), wheat (Bashan et al., 1986; Levanony et al., 1990), or on several other non-cereal plant roots demonstrated in the present study.

Pleomorphism (vibroid or cyst-like forms of the bacterial cell) of *Azospirillum* in in vitro culture is well documented (Eskew et al., 1977;

Sadasivan and Neyra, 1987). The current study shows that this pleomorphism occurred in situ in the rhizosphere. Transition from the vegetative vibroid form to the cyst-like form and vice versa was observed following manipulation of water availability. The mechanism of this transition remains unknown.

In conclusion, our study suggests that fibrillar material plays an important role in attachment of *A. brasilense* Cd to root surface and to sand particles.

Acknowledgements

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