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CELL COLONIZATION AND INFECTION THREAD FORMATION IN SUGAR CANE ROOTS BY *ACETOBACTER DIAZOTROPHICUS*

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Summary—*Acetobacter diazotrophicus* was isolated from roots of two commercial varieties of sugar cane in Tucumán, Argentina and inoculated into stem cuttings of sugar cane. The bacterial colonization stimulated the production and development of root hairs, and changes in root hair morphology with infection-thread-like formation. The study was correlated with nitrogen fixation. The bacterized root hair cells were the site of nitrogen fixation. © 1997 Elsevier Science Ltd

INTRODUCTION

It has been suggested that sugar cane plants can benefit from the contribution of biological nitrogen fixation. Nitrogen fixing bacteria, such as *Beijerinckia, Azospirillum, Acetobacter* and *Herbaspirillum*, have been reported associated with sugar cane roots (Boddey *et al.*, 1991). The same microorganisms have also been found in Tucumán, Argentina, colonizing sugar cane roots (Bellone and Bellone, 1993).

Rhizobia are attached to roots and root hairs in legumes producing curling, followed by infection thread formation, promotion of cell division in the cortex and nodule formation. The cells of the nodule obtain the bacteria through an endocytic process and produce a peribacterial membrane. Kluepfel (1993), suggests that the invasion of other endophytic bacteria is usually through natural openings and wounds. It was observed in legumes that plants of the same species cannot be infected by the same route, suggesting that probably the plants could control the bacterial entrance (Callaham and Torrey, 1981). James *et al.* (1994) showed that *Acetobacter* spp entered into sugar cane both at emergent lateral roots and also in the root apical meristem region.

Acetobacter diazotrophicus was originally isolated from grasses and sugar cane roots in Brazil (Cavalcante and Döbereiner, 1988). We have confirmed the endophytic nature of the bacteria through microscopic studies in different sugar cane roots samples, and have analyzed the place of penetration and colonization within the root.

MATERIALS AND METHODS

Acetobacter diazotrophicus was isolated from roots of two different commercial varieties of sugar cane: NA 56-79 and TUC 77-42 in Tucumán, Argentina, using the Cavalcante and Döbereiner medium (1991). Cultures were purified in the same medium plus agar or on a potato–dextrose agar medium (Döbereiner, 1989).

Stem cuttings of sugar cane were inoculated by submerging them into a 10 day old culture with 1.8×10^8 bacteria ml^{-1}; they then were placed in pots containing sterile sand under greenhouse conditions and supplied with Hoagland and Arnon (1950) solution. Humidity was controlled by using a soil water depletion profile, to maintain the seedlings at 0.1 MPa (1bar = 0.1MPa); the root hair production was copious.

Optical microscopy was used to watch the superficial root hair colonization every 48 h from 28–60 days after inoculation. To observe the threads, the direct inmunofluorescent technique was used; polyclonal-specific antibodies were obtained by injecting whole bacterial components into rabbits, following the prescriptions of Bohlool and Schmidt (1980). Fluorochromes fluorescein isothiocyanate was coupled to the antibodies and excited by long-wave UV radiation and photographed after 2 h of exposure.

The internal colonization was studied by transmission electron microscopy, according to Hawes (1991). Root samples were fixed in glutaraldehyde 2% and 25 mM potassium phosphate buffer, pH 6.8 for 16 h at room temperature. The fixed specimens

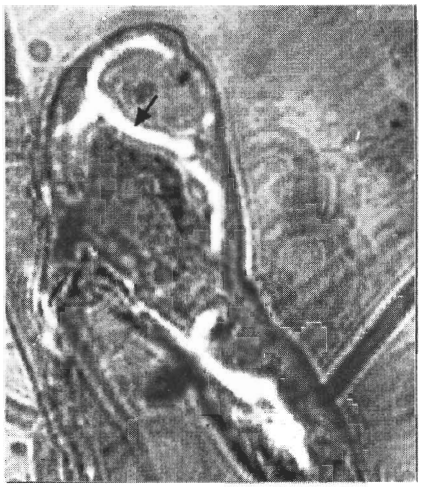

Fig. 1. Infection thread (arrow) developed in apex of root hairs of sugar cane root (4000 ×).

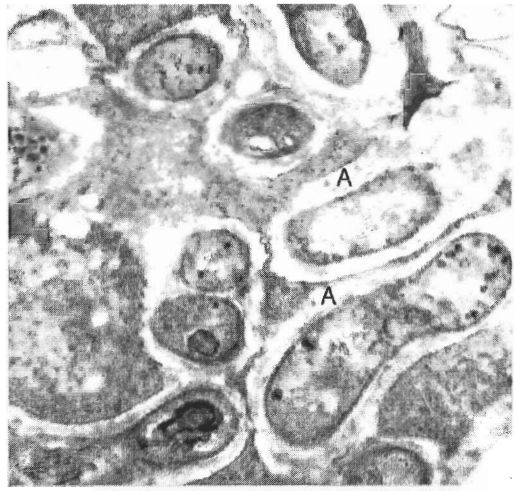

Fig. 3. Cell of sugar cane root colonized by *Acetobacter diazotrophicus*, surrounded by peribacterial membrane (27400 ×). A, Peribacterial membrane.

were then rinsed in buffer, post-fixed in 1% buffered osmium tetroxide for 2 h at 4°C, rinsed again in buffer, dehydrated in an ethanol series (30, 50, 70, 80, 90 and 100%) for 20 min in each concentration and embedded in Epon-araldite. Ultra-thin sections (40–60 nm) were cut with glass knives, picked up on uncoated grids, stained with uranyl acetate for 45 min and with lead citrate for 35 min. The samples were examined with a Zeiss microscope operating at 80 kV. Nitrogen fixation was measured by the acetylene reduction assay.

RESULTS

In some root hairs the bacterial colonization was high while in some others it was low. Those having a high colonization presented different morphologies, showing alterations of the cell wall, coincident with an infection-thread-like formation. Generally threads were located in regions near the apex of the root (Fig. 1).

During the genesis of infection-threads the nuclei of root hair cells showed movements apparently associated with the infection-thread growth. Electron microscopy suggested that the infection-thread-like formation had a different constitution from the rest of the cell and from the internal bacteria (Fig. 2). Some cells colonized by *Acetobacter*, exhibited a peribacterial membrane (Fig. 3), but some others did not (Fig. 4); however, the roots were able to reduce acetylene as indicated in Table 1.

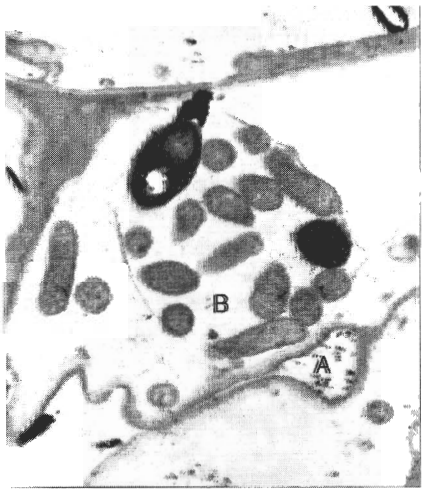

Fig. 2. Section of an infection thread in the cortex. One can see cells next to those supporting the thread are developing picnocytic process (100950 ×). A, Picnocytic process; B, Infection thread in the cortex.

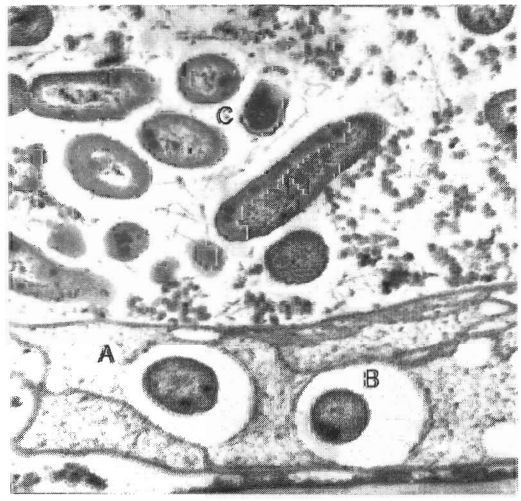

Fig. 4. Infection thread penetrating through cells colonized by *Acetobacter* without peribacterial membrane formation (18700 ×). A, Infection thread; B, *Acetobacter* within the thread; C, Bacteria colonizing the cell.

Table 1. Infection threads and acetylene reduction assay in sugar cane roots

Sugar cane varieties	Root hairs/cm^{-2} root	Infection-threads 100^{-1} root hairs	ARA (nM C_2H_4 g^{-1} root h^{-1})
NA 56-79	425 ± 78[a]	1.82	110 ± 32[a]
TUC 77-42	390 ± 51[a]	1.12	83 ± 12[a]

[a]Mean of 10 determinations.

DISCUSSION

The presence of *Acetobacter diazotrophicus* attached to root hairs and the deformations observed, have suggested that the presence of hydrolytic enzymes on the cell walls promote the deformations. On the other hand, the presence of infection threads has indicated bacterial penetration into root hairs. These two processes are similar to those of *Rhizobium* in association with legumes; however, it is not a general process as it had a very low frequency, and thus was not easily detected in other studies. Besides the way of entry suggested by James *et al.* (1994), there is a third way of entrance through root hairs; its importance in the general process of root colonization is not well known.

The fact that cells of the cortex are colonized by *Acetobacter*, and in some cases are surrounded by a peribacterial membrane, suggested that an endocytic process of certain cells of the cortex explained the cell colonization; this is compatible with the low frequency and number of cells involved in the process. Some other cells were colonized by the same kind of bacteria, but without peribacterial membrane formation.

Results indicate a possible divergence in the cellular ability of the host to be colonized by *Acetobacter,* and the acetylene reduction assay performed on the samples clearly showed that the nitrogen fixing process was functional.

Summarizing, the exact place of penetration of the root cortex is not well defined. *A. diazotrophicus* penetrated the cortex, was surrounded by the cytoplasmic membrane, and thus was incorporated into the cell. The observations suggested that the cells colonized by *Acetobacter* are the locus of nitrogen fixation and that *Acetobacter* induces the development of root hairs.

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