COLONIZATION OF STRAWBERRY (FRAGARIA ANANASSA) PLANT TISSUES BY AZOSPIRIRILLUM BRASILENSE

Beatriz C. Winik[1], María F. Guerrero Molina[2], Raúl O. Pedraza[2]
[1]Laboratorio de Microscopía Electrónica. Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán. San Miguel de Tucumán. Argentina.
[2]Facultad de Agronomía y Zootecnia. Universidad Nacional de Tucumán. San Miguel de Tucumán. Argentina.

Introduction

To the genus *Azospirillum* belong nitrogen-fixing bacteria with the ability to form beneficial interactions with a variety of higher plants (Patriquin et al., 1983). They belong to a group called PGPB (Plant Growth Promoting Bacteria), capable of affecting growth and yield of many economically important crops (Okon and Vanderleyden, 1997), being strawberry (*Fragaria ananassa*) one of them.

In contrast to the *Rhizobium*-legume symbiosis, the *Azospirillum*-plant interaction does not produce visible structures on the roots which indicate successful infection. For that reason, this interaction has been described as `associative´ (Döbereiner and Day, 1974). Generally, *Azospirillum* cells can be found everywhere along inoculated root systems (Okon and Vanderleyden, 1997).

Even when *A. brasilense* has been isolated from strawberry plants in the province of Tucumán, there are not morphological or molecular evidence of strawberry plant tissues colonization by *Azospirillum.* Therefore, the aim of this work was to evaluate by microbiological, ultraestructural and molecular methods the root colonization by *Azospirillum brasilense* and its translocation throughout stolons from inoculated mother plants to uninoculated (new born) daughter plants. This last feature represents an important agronomic advantage since strawberry plants are asexually reproduced; hence only one inoculation could allow the producers to have many plant generations already inoculated with selected bacteria and with better conditions to be planted at field.

Materials and Methods

An inoculation experiment was carried out with the purpose to evaluate the colonization and translocation of *A. brasilense*. Three commercial cultivars of strawberry (*Fragaria ananassa*, Duch): 'Camarosa', 'Milsei' and 'Selva' were inoculated with two strains of *A. brasilense*, REC3 and PEC5, isolated from sterilized roots and stolons of strawberry, respectively (Pedraza et al., 2007).

Inoculation and plant growth conditions. Three plantlets of each cultivar were inoculated with each strain of *Azospirillum*, including a set of three plants without inoculation, as control, in every case. The strawberry plantlets were inoculated with the different strains of *Azospirillum* by submerging their roots in a bacterial suspension (10^6 cfu ml^{-1}) for 20 min and then were immediately planted in disinfected pots containing sterile substrate (humus:perlome, 2:1). Plantlets were placed in a growth chamber at 28°C, 70% RH, with 16 h of photoperiod, for 7 months. The plants received 50 ml of sterile distilled water twice a week. The growing stolons were fixed in a different pot (without cutting them) in order to produce a new plant. After inoculation, samples of roots and stolons were taken to evaluate the colonization and translocation of *A. brasilense* strains by microbiological, ultrastructural and molecular tests.

MPN determinations. From all plant tissues sampled, the MPN per gram of root and stolon was determined according to Döbereiner et al. (1995), using the McCrady table for three replicates with NFb semisolid media.

Scanning electron microscopy (SEM). Samples of roots and stolons were fixed in a 3% glutaraldehyde solution buffered with 0.1M phosphate buffer pH 7.4 for 3 h at room temperature and postfixed in 1% osmium tetroxide in the same buffer overnight. Specimens were washed three times in distilled water and then treated with an aqueous solution of 2% uranyl acetate for 40 min. Afterwards, they were serially dehydrated in ethanol and acetone, critical point dried, mounted on aluminum stubs, coated with gold and examined with a ZEISS SUPRA 55VP scanning electron microscope.

Transmission electron microscopy (TEM). It was performed as described for SEM until the dehydration step and then, samples were embedded in Spurr resin. Thin sections were cut, mounted on cooper crickets, contrasted with uranyl acetate and lead citrate (Venable y Coggeshall, 1965) and examined on a ZEISS EM 109 transmission electron microscope.

DNA extraction and PCR. DNA extraction from plant tissues was carried out according to Doyle and Doyle (1987). The presence of the bacteria within the plant tissues was assessed by PCR, amplifying a fragment of the gene *nif*D, essential in the biological N_2-fixing process, using the primers and conditions prescribed by Potrich et al. (2001).

Results and Discussion

Strawberry root and stolon colonization by *A. brasilense* REC3 and PEC5 was assessed by MPN determinations, ultrastructural and molecular studies.

A. brasilense was re-isolated from the roots and stolons of all the inoculated mother plants and uninoculated daughter plants. In all cases, the MPN values of *Azospirillum* isolated from roots were higher than from stolons. The MPN ranged from $1.5 \cdot 10^3$ to $4.5 \cdot 10^6$ bacteria$\cdot g^{-1}$ of root and from $1.1 \cdot 10^1$ to $4.5 \cdot 10^4$ bacteria$\cdot g^{-1}$ of stolon.

Electron microscopy observations of inoculated strawberry roots and stolons showed clear attachment of *A. brasilense* to the root surface as well as the colonization of root and stolon inner tissues (Fig. 1B and 1D), as compared to uninoculated roots, without associated bacteria (Fig 1A and 1C). *Azospirillum* was firmly attached to the root by fibrillar material and sometimes was found associated with granular material accumulated on root surface (Fig. 1D). The anchoring of bacterial cells to the root surface by a network of fibrillar material is a typical feature of *Azospirillum*-root colonization, described by Levanony and Bashan (1991) and observed herein. Besides, two patterns of bacterial cell adsorption were observed on the surfaces of roots: single-cell randomly dispersed and small bacterial aggregates (Fig. 1D). Bacterial colonization of the stolon interior was also examined by scanning and transmission electron microscopy (Fig. 1E, 1F and 1G); the observations revealed *Azospirillum* cells gathered on groups and enclosed with a condensed capsular material in the vascular vessel area.

The presence of *A. brasilense* inside the stolons and its isolation from uninoculated daughter plant roots corroborates the translocation of the bacteria throughout stolons from inoculated mother plants to the new born plants.

Also, a molecular approach was used to study the presence of the bacteria within the plant tissues; a 710 bp fragment of the gene *nif*D was amplified by PCR from DNA samples of roots and stolons colonized by the bacteria (not shown).

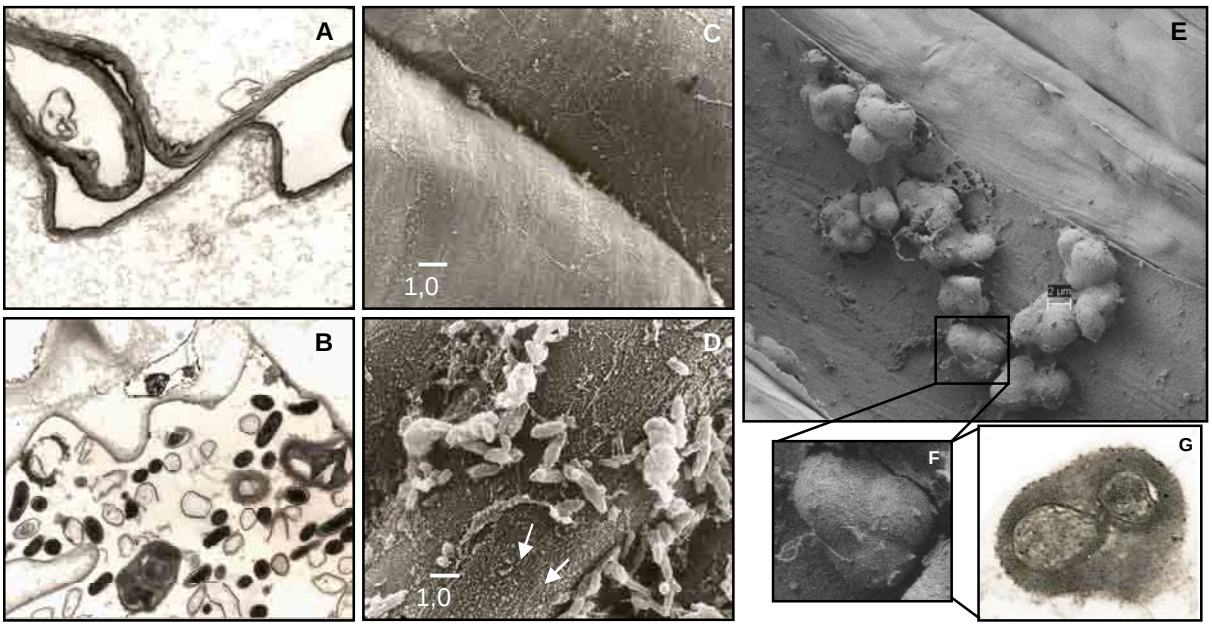

FIG 1. Strawberry root and stolon colonization by *A. brasilense REC3 and PEC5*. (A) Transmission electron micrograph of uninoculated strawberry root: `Camarosa´- Control (x10,950). Note the lack of bacteria on the inner tissues of the root. (B) Massive colonization of root cell cytoplasm inoculated with *A. brasilense REC3*: `Camarosa´-REC3 (x10,950). (C) Scanning electron microscopy of uninoculated strawberry root surface: `Milsei´-Control (x7,200). Lack of attached bacteria on the root epidermis. (D) *A. brasilense* adsorbed to epidermal cells by fibrillar material: `Milsei´-REC3 (x7,200). Note bacteria associated with the granular surfaces of the root (arrows). (E) Scanning electron micrograph of inner stolon tissue colonized by *A. brasilense*. (F) Enlargement of framed area in Fig. (E) showing a group of bacteria sheathed with a condensed material. (G)Scanning electron micrograph of bacteria immerse on a thick compacted material matrix (x18,700).

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