Interaction of *Azospirillum brasilense* and *Glomus intrarradix* in Sugar Cane Roots

Carlos H. Bellone · Carrizo de Bellone Silvia

Received: 14 April 2010 / Accepted: 18 June 2010 / Published online: 18 August 2011
© Association of Microbiologists of India 2011

Abstract Fifteen-day-old variety NA 56-79 sugar cane seedlings were inoculated with *Azospirillum brasilense* and *Glomus intrarradix*. This article aims at examining changes in sugar cane root seedlings inoculated with *Glomus intrarradix* and *Azospirillum brasilense*, the increase in microbial biomass and the acetylene reduction process as well. The internal root colonization was studied 20 days after inoculation using scanning and a transmission electron microscope. Both microorganisms entered the sugar cane root through the emergent lateral roots. The microorganisms were capable of coexisting both intra and intercellularly, producing changes in the cell wall, thus allowing colonization and interaction between the organisms. These changes increased the number of microorganisms inside the root as well as acetylene nitrogen reduction. Sugar cane plant biomass increased with joint-inoculation. The number of endophytic microorganisms and nitrogen fixing activity increased when they were colonized by *Azospirillum* and *Glomus* together.

Keywords Acetylene reduction · *Azospirillum brasilense* · *Glomus intrarradix* · Microbial biomass

Introduction

Free-living nitrogen-fixing bacteria are known to colonize root surface by adhesion; the niches protect them from oxygen

and other factors so that nitrogen fixation can occur at maximal levels what may promote the high level of nitrogen fixation observed in sugar cane plants [1–4]. It has been reported that some diazotrophs such as *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Burkholderia*, *Azospirillum brasilense*, penetrate into the sugar cane root [5–13] and can be found in intercellular spaces of the cortex where active nutrient exchange and nitrogen fixation take place [14–17]. Patrikin [16] found a high concentration of bacteria in the base of new growing roots where cells are disorganized. Nevertheless, the routes of colonization and the sites of fixation are not yet well known. The xylem vessels are the most likely places for the occurrence of biological nitrogen fixation (BNF) as they provide low pO₂ and the necessary energy for nitrogenase activity [10].

The association between plant-mycorrhiza and nitrogen-fixing bacteria plays a critical role in nutritional and plant growth processes, contributing to natural ecosystems and to several agricultural crops like sugar cane. In previous studies, bacteria were associated with fungi, what increased cellulolytic and pectinolytic activity [18–21]. The potential changes produced when both groups of microorganisms coexist in the root interior are not well known yet. This report aims at examining changes in sugar cane roots seedlings inoculated with *Glomus intrarradix* and *Azospirillum brasilense*, the increase in microbial biomass and acetylene reduction process as well.

Materials and Methods

Strains Isolation

Root samples of variety NA 56-79 commercial sugar cane were gathered (simultaneously with the bunch stage) from

C. H. Bellone (✉) · C. de Bellone Silvia
Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán, Av. Roca 1900, 4000 Tucumán, Argentina
e-mail: cbellone@yahoo.com.ar

C. de Bellone Silvia
e-mail: sicabell@yahoo.com.ar

ten locations in Tucuman, Argentina. Each sample consisted of five sub-samples (10 g of root). To obtain endophytic *Azospirillum brasilense*, the tips of 10 cm long root segments were sealed with paraffin and immersed in 1% chloramine-T for 10 min. Roots were then immersed for 10 min in 0.05 M phosphate buffer (pH 7) and washed twice in sterile water. A surface contamination assay was carried out incubating the treated segments in test tubes containing a 10 ml NFb semisolid medium supplemented with yeast extract (0.5 g l^{-1}). In all these cases, no bacterial growth was observed after 5 days of inoculation at 30°C.

The root segments were cut with sterile scissors, transferred to an *N*-free malate semisolid medium (NFb) and incubated 72 h at 30°C [22]. The positive cultures forming a typical white pellicle below the surface of the medium were streaked out on petri dishes containing an NFb solid medium supplemented with yeast extract (0.5 g l^{-1}) and Congo red (15 ml l^{-1} of a 1:400 aqueous solution). The red colonies were transferred to a new NFb semisolid medium and incubated at 30°C for 72 h. The identification of the isolates was based on Gram staining, mobility and colony morphology. The ability of bacteria to grow on various carbon substrates was assayed in an NFb medium replacing the malate source with other carbon substrates: maltose, glucose, sucrose, D-fructose, mannitol, glycerol, citrate, *N*-acetylglucosamine and myo-inositol.

The characterization was carried out by triplicate and a reference strain was used as positive control [22]. The strain of *Azospirillum* selected was developed in a liquid NFb medium enriched with 1% NH_4Cl and stirred at 120 rpm at 30°C.

The *A. brasilense* sp7 (ATC-29145) strain used as reference was kindly provided by EMBRAPA-CNPAB, Seropedica km 47. R.J. Brazil.

The Arbuscular Micorrhizal Colonization

Glomus intraradix spores were isolated from the rhizospheric soil of the same sugar cane variety by wet sieving. The spores were classified in agreement with Morton [23] and Shenck and Perez [24] and the multiplication was done on Marglobe variety tomato seedlings grown in sand that had been washed, sterilized and supplemented with a phosphorus deficient Hoagland and Arnon solution [25]. After 60 days, spores were separated by wet sieving and the suspensions were prepared according to Genderman and Nicolson [26]. The suspension of 100 spores per milliliter of sterile distilled water was used as the inoculum.

The arbuscular micorrhizic colonization was estimated by Phyllips and Hayman method [13].

Seeds and Growth Condition

Seeds of sexual origin from the crossing of NA 56-79 and Tuc 71-10 sugar cane varieties were obtained from Estacion Experimental Agro-Industrial Obispo Colombres of Tucuman. The seeds were superficially sterilized with 0.02% HgCl_2 for 2 min and then washed ten times with sterile distilled water. The seeds were placed in a Glucose–potato–agar-medium with 0.5% of yeast extract for germination. Flasks of 350 cc containing 50 cc of a mixture of sand and perlite (1:1 v/v) were sterilized at 180°C for 2 h. The flasks were then autoclaved for 1 h at 1 at. Ten days after germination of sterile seeds, the seedlings were transferred to the flasks. Hoagland and Arnon solution (pH 6–6.2) was added to restore moisture and lost nutrients. The seedlings were grown in a breeding chamber, with 14 h of light at 3000 lux (OSRAM lamp), at a diurnal temperature of $25 \pm 2^\circ\text{C}$ and of 20°C during the night. After 15 days, each plant was inoculated with a combination of 1 ml of an *Azospirillum brasilense* culture ($1.5 \times 10^7 \text{ cfu}$) and 1 ml of spore suspension of *Glomus intraradix* (100 spores/ml).

The plants were harvested and weighed 20 days after inoculation. The roots were washed six times with sterile distilled water, fixed with glutaraldehyde for 24 h and placed in Epon Araldita resin.

The external and internal microbial colonization of roots was studied using optical, scanning and transmission microscopy in the *Laboratorio de Microscopía Electrónica del NOA* (LAMENOA).

The Most Probable Number of *Azospirillum*

Seedling roots were sterilized as described before for the isolation of *Azospirillum*. The paraffined tips were removed, and the segments (10 g) were macerated with saccharose solution (4%). Serial dilutions were made and the MPN of *Azospirillum* per fresh weight gram was determined according to Döberiner [22] using Mc Crady's Table for three repetitions in an NFb semisolid medium.

Acetylene Reduction Assay (ARA)

The nitrogenase activity determined by acetylene reduction assay (ARA) was carried out by duplicate in 50 ml vials containing 25 ml of an *N*-free malate NFb semisolid medium. Isolates of sugar cane plants were grown in this medium for 48 h at 30°C. Each vial was sealed with a rubber stopper and the head space was injected with 10% (v/v) acetylene. Gas samples (0.5 ml) were removed and assayed for ethylene production with a Carle Analytical Gas Chromatograph model 311 with a flame ionization

detector and a Porapak N column (5 m × 0.5 mm). Results were analyzed by ANOVA test.

Results

The scanning electron microscopy showed the presence of *Azospirillum* and *Glomus* adhered to the lateral sugar cane root surface. The microorganisms were associated with fibril material on epidermal cells in the root-hair region (Fig. 1). Figure 2 shows the generation of a lateral root and spaces created by cell loosening that facilitate microorganisms' colonization. The external region was colonized by *Glomus intraradix* and *Azospirillum brasilense*. Light and electron micrographs of roots showed the presence of bacteria and fungi in the intercellular regions of the inner tissues (Fig. 3). These microorganisms penetrated into the sugar cane emergent lateral root which presents changes in the cell wall and where bacteria have different morphologies. The associations between bacteria and VAM contributed significantly to root hair deformations (Fig. 4). The cell wall plays an important role in this process as bacteria use the cellulose micro-fibers for intercellular passage. Consequently, cell wall stiffness is reduced due to disorganization of the microfibrils. The progression of bacterial entry beyond cell wall penetration is facilitated by the degradation of the wall via enzymes that cleave

Fig. 2 Light micrographs of sugarcane root-hair var. NA 56-79 of 10 days age. The mycorrhiza (H) and bacteria (A) colonize the internal root region. The microorganisms penetrate inside the root by degradation of the cell wall (C) (bar 30 µm)

Fig. 1 Scanning microphotograph (SEM) showing *Azospirillum brasilense* (A) and *Glomus intraradix* (H) associated by fibril material in the root-hair region (M) (bar 25 µm)

Fig. 3 Light micrographs Transverse section of sugarcane root-hair. Epidermal cell (A) in the root-hair colonized by bacteria and fungi (MVA) (bar 10 µm)

Fig. 4 SEM. Deformation (D) in sugarcane root-hair region at 10 days of the microbial inoculation with *Glomus intrarradix* and *Azospirillum brasilense* (bar 1 mm)

Fig. 6 TEM Cell wall penetration (C) by the hyphae of VAM (H) facilitated by degradation without causing damage (bar 2 μ m)

Fig. 5 Cell wall disorganization and reduced microfibril density (M) (bar 1, 2 μ m)

glycosidic bonds (Fig. 5). This entry occurs without causing severe cell damage (Fig. 6). The bacteria and fungi can be seen inside the radical cortex. In the intercellular spaces, the bacteria can be seen embedded within a fibrillar matrix.

The ETM showed that the host cell wall modifies the microsymbiont colonization and that the fungi are able to exchange biogenic elements between symbiotic species (Figs. 7, 8). Sugar cane plant biomass increases with joint-inoculation. The number of endophytic microorganisms as well as nitrogen fixing activity increase when they are colonized by *Azospirillum* and *Glomus* together (Table 1).

Discussion

Microorganisms' interactions could be more than just competitive. Because of their usual intercellular location and the fact that this microhabitat is rich in plant metabolites, a kind of nutrient chain might be established. The role of the matrix facilitates an increase in the nitrogenase synthesis through an increase in the number of bacteria. The presence of bacteria in the young roots and their subsequent introduction into the xylem and phloem vessels are favored by the cell endodermal disorganization that occurs during the primal radical growth of the future lateral roots. Cellular ripeness increases towards the apex where meristematic activity loses its capacity and the cells transform plasmatically, allowing for colonization by both types of microorganisms. The deformations observed in the

Fig. 7 Electron micrograph of sugarcane root hairs after inoculation with VAM (H) and *A. brasilense* (B). The bacteria are around the hyphae deformations (D) that present protrusions showing an intense association, the fungi exchange biogenics elements between symbiotic species (E) (bar 1 μm)

Fig. 8 Electron micrograph of sugarcane root hairs after inoculation with VAM (H) and *A. brasilense* (B). The bacteria are around the hyphae deformations (D) that present protrusions showing an intense association, the fungi exchange biogenics elements between symbiotic species (E) (bar 1 μm)

root hairs, especially in the apexes, were coincident with the presence of *Azospirillum brasilense* and *Glomus intraradix*. The colonization generated by both groups stimulates hydrolysis in cell wall hemicelluloses and generates oligosaccharides that will be used in the nutrition process [18, 27]. This suggests that the enzymes generated during settling are responsible for the morphologic changes that begin in the surface and end up in the inner regions of the cortex. At the sites of maximal root colonization, the cell wall fungi strongly diminish, what allows better interchanges of metabolites between bacteria and fungi. Following the plant growth cycle, the microorganisms can translocate into the upper organs and can reach even the aerial apical meristem of the plant (Our laboratory, unpublished data). In the lumen of xylem vessels, the bacteria colonize at random and do not show a statistical

trend related to the type of location. The presence of intracellular hyphae increases the colonization of the bacteria that occupy the intercellular spaces generated by the fungi as a consequence of the enzymatic digestion of the cell wall. Mycorrhizas are the most common vectors of endophytes and provide the usual way of colonization of plants. During this phase in the root cortex, the hyphae evidence endocytic processes with electron-dense particles and produce appendices in the hyphae that correlate with the bacterial number. The changes produced in the three symbionts (bacterium, VAM, and plant) facilitate the intimate interaction between them. These results suggest that bacteria associated with fungi utilize metabolites produced by plant cells and microsymbionts that interact with them. The increase in the nitrogen-fixing activity (ARA), and in root biomass of both microorganisms, promotes the

Table 1 Most probable number (MPN), % mycorrhization, nitrogen-fixing activity (ARA) and weight of plant in sugarcane seedling inoculated with *Azospirillum brasilense* and *Glomus intraradix*

Parameters microorganisms inoculated	MPN <i>A. brasilense</i>	% Mycorrhization	(ARA) $\text{nmol h}^{-1}/\text{plant}^1$	g/fresh weight/plant $^{-1}$
<i>Azospirillum brasilense</i>	1.2×10^3	27.30 ± 8	38 ± 12	20.9 ± 3.6
<i>Azospirillum brasilense</i> and <i>Glomus intrarradix</i>	1.6×10^5 *	63.45 ± 10 *	118.6 ± 26.3 **	41.8 ± 5.9 **

* $P < 0.05$; ** $P < 0.01$

absorption of nutrients. Endophytes can be helpful in protecting the plant from environmental stress as well as in supplying the plant with nitrogen and other growth promoting substances.

References

- Döbereiner J, Pedrosa FO (1987) Nitrogen fixing bacteria in non-legume crop plant Brock. Springer Series in Contemporary Bioscience. Science Tech Publisher, Madison, p 156
- Döbereiner J (1992) History and new perspectives of diazotrophs in association with non-leguminous plants. *Symbiosis* 13:1–13
- Lima E, Boddey RM, Döbereiner J (1987) Quantification of biological nitrogen fixation associated with sugar cane using a ^{15}N aided nitrogen balance. *Soil Biol Biochem* 19:165–170
- Urquiaga S, Cruz KHS, Boddey RM (1992) Contribution of nitrogen fixation to sugar cane: N^{15} and nitrogen balance estimate. *Soil Sci Soc Am J* 56:105–114
- Bellone CH, Bellone SC, Pedraza RO (1995) Chitinase expression in strawberry root colonized by *Azospirillum brasilense* and *V. A. Mycorrhiza*. In: Fendrik I, Del Gallo M, Vanderleyden J, Zamaroczy M (eds) *Azospirillum VI* and related microorganisms, vol 37. Springer-Verlag, Heidelberg, pp 161–165
- Boddey RM, Urquiaga S, Reis V, Döbereiner J (1991) Biological nitrogen fixation associated with sugar cane. *Plant Soil* 137:11–117
- Döbereiner J, Baldani VLD, Olivares F, Reis VM (1993) Endophytic diazotrophs: the key to BNF in gramineous plants. In: Hegazy NA, Fayez M, Monib M (eds) Nitrogen fixation with non-legumes. Cairo University, Giza, pp 395–408
- Döbereiner J, VID Baldani, Reis VM (1995) Endophytic occurrence of diazotrophs bacteria in non-leguminous crops. In: Fendrik et al (eds) *Azospirillum VI* and related microorganisms. Springer-Verlag, Heidelberg, pp 3–14
- Hurek T, Reinhold-Hurek B, Turner GL, Bergersen FJ (1994) Augmented rates of respiration and efficient nitrogen fixation at nanomolar concentrations of dissolved O_2 in hyperinduced *Azoarcus* sp. strain BH72. *J Bacteriol* 176:4726–4733
- James EK, Reis VM, Olivares FL, Baldani JI, Döbereiner J (1994) Infection of sugar cane by nitrogen fixing bacteria. *J Exp Bot* 45:757–766
- Olivares FL, Baldani VLD, Reis VM, Baldani JI, Döbereiner J (1996) Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots stem and leaves predominantly of gramineae. *Biol Fertil Soils* 21:197–200
- Pedraza RO, Diaz Ricci JC (2003) Genetic stability of *Azospirillum brasilense* after passing through the root interior of sugar cane. *Symbiosis* 34:69–83
- Phyllips JM, Hayman DS (1970) Improved procedures for clearing and staining parasitic and vesicular–arbuscular mycorrhizae fungi for rapid assessment of infection. *Trans Br Mycol Soc* 55:158–161
- Bellone CH (1996) Micorrización y Fijación de Nitrógeno en el Área Cañera de la Provincia de Tucumán. FAZ de la Universidad Nacional de Tucumán, Tesis, p 317
- Bellone CH, Bellone SC, Pedraza RO, Monzón MA (1997) Cell colonization and infection thread in sugar cane roots by *Acetobacter diazotrophicus*. *Soil Biol Biochem* 29:965–967
- Patrikin DG, Döberiner J, Jain DK (1983) Sites and processes of association between diazotrophs and grasses. *Can J Microbiol* 29:900–915
- Reis VM, Olivares FL, Döbereiner J (1994) Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. *World J Microbiol Biotechnol* 10:101–104
- Bellone CH (1983) Actividad celulolítica de tejidos no diferenciados de caña de azúcar inoculadas con dos especies de *Azospirillum*. *Rev Agron Noroeste Argent* XIX:4–8
- Bellone CH, Carrizo de Bellone S (1993) Nitrogenase, cellulase activity in sugar cane roots inoculated together with va mycorrhiza and nitrogen-fixing bacteria. In: Hegazy N, Fayez M, Monib M (eds) Nitrogen fixation with non-legumes. The American University in Cairo Press, Cairo, pp 69–75
- Bellone CH (2002) Especies de endomicorrizas en caña de azúcar en Tucumán. Manejo de sistemas microbianos para optimizar la producción agrícola y silvopastoril en algunas áreas del NOA, pp 117–121
- Paula MA, Reis VM, Döbereiner J (1991) Interaction of *Glomus clarum* with *Acetobacter diazotrophicus* in infections of sweet potato (*Ipomoea batata*), sugar cane (*Saccharum* spp.) and sweet sorghum (*Sorghum vulgare*). *Biol Fertil Soil* 11:111–115
- Döbereiner J, Baldani DVL, Baldani JI (1995) Como isolar e identificar bacterias diazotroficas de plantas não-leguminosas. MAARA EMBRAPA CNPAB, Brasília, p 60
- Morton JM, Benny GL (1990) Revised classification of arbuscular mycorrhizal fungi (*Zygomycetes*): a new order, *Glomales*. Two new suborders, *Glominiae* and *Gigasporinae*, and two new families, *Acaulosporaceae*, and *Gigasporaceae*, with an emendation of *Glomaceae*. *Mycotaxon* 37:471–491
- Shenck NC, Perez Y (1990) Manual for the identification of VA mycorrhizal fungi, 3rd edn. Synergistic Publications, Gainesville, p 286
- Hoagland DR, Arnon DL (1950) The water culture method for growing plant without soil. University of California, Berkeley, p 32
- Genderman CW, Nicolson JH (1963) Spores of mycorrhizal species extracted from soil by wet sieving and decanting. *Trans the Br Mycol Soc* 46:235–246
- Bellone CH and Monzon de Asconegui MA (1987) Electron microscopy observation of *Azospirillum* spp. cell within sugar cane (*Saccharum* spp) roots. 5th Congress International of Nitrogen Fixation. Brazil, pp 85–87