Localization and survival of *Azospirillum brasilense* Az39 in soybean leaves

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Abstract

In recent years, foliar inoculation has gained acceptance among the available methods to deliver plant beneficial micro-organisms to crops under field conditions. Colonization efficiency by such micro-organisms largely depends on their ability to survive when applied on the leaves. In this work, we evaluated the survival and localization of *Azospirillum brasilense* Az39 (Az39) in excised soybean leaves. Scanning electron microscopy and confocal laser scanning microscopy of a red fluorescent-transformed variant of Az39 were used to determine bacterial localization, while the most probable number and plate count methods were applied for bacterial quantification. Microscopic observations indicated a decrease in the number of Az39 cells on the leaf surface at 24 h after treatment, whereas midribs and cell–cell junctions of the inner leaf epidermis became highly populated zones. The presence of Az39 inside xylem vessels was corroborated at 6 h after bacterization. Az39 population did not significantly decrease throughout 24 h. We could visualize Az39 cells on the surface and in internal tissues of soybean leaves and recover them through culture methodologies. These results evidence the survival capacity of Az39 on and inside leaves and suggest a previously unnoticed endophytic potential for this well-known plant growth-promoting rhizobacteria strain.

Introduction

Nowadays, crop productivity maximization is achieved through the use of fertilizers and pesticides, usually applied at high doses, resulting in detrimental consequences on soil and environmental health. The use of plant growth-promoting rhizobacteria (PGPR) as inoculants can reduce the use of chemical fertilizers. *Azospirillum* has been one of the most studied PGPR over the last four decades due to its positive effects on plant growth

Significance and impact of the study: The plant growth-promoting rhizobacterium *Azospirillum brasilense* Az39 is widely used to formulate inoculants for nonlegume crops in Argentina. Today, seed treatment is the preferred methodology to deliver *Azospirillum* under field conditions. However, recent experiments demonstrated that foliar application might be an alternative delivery method. The combination of microscopy techniques, DsRed-tagging and standard counting procedures allowed us to confirm the survival of *A. brasilense* Az39 cells on and inside soybean leaves for at least 24 h. The potential of this strain to become a foliar inoculant is demonstrated.

Keywords

*Azospirillum brasilense*, foliar inoculation, leaf localization, PGPR, soybean.

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*Azospirillum* on soybean leaves

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and development, leading to increased yield in several crops (Cassán et al. 2020). The positive effects of this micro-organism have been related to its ability to fix atmospheric nitrogen and produce several phytohormones such as auxins (Crozier et al. 1988; Zimmer and Bothe 1988), cytokinins (Horemans et al. 1986; Cacciari et al. 1989), gibberellins (Bottini et al. 1989; Janzen et al. 1992; Piccoli and Bottini 1996), abscisic acid and ethylene (Perrig et al. 2007), as well as certain polyamines such as cadaverine (Cassán et al. 2009).

Seeds inoculation is probably the most widespread procedure to deliver beneficial micro-organisms under standard field conditions (Vendan and Thangaraju 2007; Neto et al. 2008). However, environmental hazards such as desiccation or concomitant seed treatments (i.e. fungicides) may result in significant reductions of initial bacterial titers provided to seeds and, therefore, in reduced plant colonization (Penna et al. 2011). Foliar spraying or furrow inoculation are alternative procedures to overcome these constraints and deliver PGPR to crops (Bashan et al. 2014; Ramírez-Ordorica et al. 2020). Some authors have reported positive results for foliar inoculation of Azospirillum in different crops (Zuño et al. 2016; Fukami et al. 2017; Puente et al. 2019; Correia et al. 2020).

In Argentina, the commercial use of Azospirillum brasilense Az39-based inoculants has been widely documented (Cassán and Díaz-Zorita 2016; Anriquez et al. 2019; Coniglio et al. 2019). Puente et al. (2019) observed that soybean plants inoculated at sowing with Bradyrhizobium japonicum E109 and later foliar-sprayed with A. brasilense Az39 showed higher fresh and dry shoot biomass, more nodules and higher leghemoglobin concentrations compared with those inoculated with B. japonicum E109 alone.

In a previous report, the foliar inoculation of soybean plants using an ipdC− mutant of A. brasilense Az39 impaired in indole-3-acetic acid (IAA) biosynthesis showed a reduced impact on vegetative and reproductive performance as compared to those inoculated with A. brasilense Az39 wild type; however, the presence of this bacterium in soybean leaves induced effects, independently of its IAA-biosynthetic ability (Puente et al. 2018).

Successful colonization requires compatible plant–microbe interactions. Effective colonization of plant tissues by Azospirillum is known to be a prerequisite for promoting plant growth, regardless of the mechanism of growth promotion (Bashan and de-Bashan 2010). Most bacterial colonization studies have been performed on roots and rhizosphere (Umali-Garcia et al. 1980; Bashan et al. 1991; Vande Broek and Vanderleyden 1995; Vande Broek et al. 1998); only a few studies focused on plant leaves and phyllosphere as an ecological niche for plant-beneficial bacteria (Fukami et al. 2017; Ramírez-Ordorica et al. 2020).

When delivered to the aerial part, bacteria must first establish and adhere to the leaf surface to later colonize the internal plant tissues (Yaron and Römling 2014). Scanning electron microscopy (SEM) and confocal laser-scanning microscopy (CLSM) are useful techniques to detect inoculated micro-organisms on/inside plant tissues and to evaluate colonization patterns of bacterial endophytes within specific vegetative tissues (Romano et al. 2020). The objective of this work was to evaluate the survival and localization of A. brasilense Az39 cells applied to soybean leaves, based on an excised-leaf model and the use of a red fluorescent variant of this strain.

**Results and discussion**

While the process of root colonization by Azospirillum is well-documented, little is known about the fate of this micro-organism when applied to leaves. In this work, A. brasilense Az39 (Az39) localization on the surface of soybean leaves was analysed by SEM as a preliminary approach. Bacterial cells presumably belonging to Az39 at high numbers could be visualized on soybean leaves immediately after bacterization (time 0) (Fig. 1b), while control, disinfected leaves showed no microbial colonization (Fig. 1a). After 24 h, the number of spotted bacteria decreased in the surface of bacterized leaves (Fig. 1c), and after 48 h, no cells could be visualized on the leaf surface (image not shown).

To achieve a better understanding of Az39 foliar survival and the reasons for its notorious decline on soybean leaves, a red fluorescent variant of this strain expressing a red fluorescent protein (Az39DsRed) was constructed and subsequently applied to excised soybean leaves. CLSM observations and bacterial cell counts were performed at regular intervals during 24 h after bacterization.

While quantification based on the MPN of dinitrogen-fixing bacteria showed a nearly constant population throughout 24 h in the order of $3 \times 10^5$ colony forming unit (CFU) per g in bacterized leaves, colony counts on RC-plates containing tetracycline revealed lower values and higher variability along time: bacterial counts increased from an initial value of $2.4 \times 10^7$ CFU per g up to $4.9 \times 10^4$ CFU per g at 4 h after bacterization, and then decreased, to reach at 24 h similar numbers to those soon after bacterization (Table 1). According to the model of leaf colonization by phyllobacteria proposed by Beattie and Lindow (1999), this finding could possibly be related to a phase of cell division or formation of micro-colonies that precedes the bacterial establishment in the internal leaf tissues. Regarding the bacterial population quantified by MPN in control leaves (Table 1), it is most possible that they represent diatrophic endophytic
bacteria present inside the leaves because the observations by SEM indicated the absence of superficial bacteria in this treatment (Fig. 1a).

Several authors have reported the successful in vivo visualization of azospirilla by fluorescent protein-tagging and microscopic analyses (Rothballer et al. 2003; Díaz Herrera et al. 2017; Fukami et al. 2017; Ramirez-Mata et al. 2018; Hong et al. 2019). In this study, CLSM images obtained from the adaxial and abaxial sides of the bacterized leaves at different time points (0, 2, 4, 6 and 24 h) revealed the presence of Az39DsRed cells on both sides, with an apparent tendency to redistribute with time (Fig. 2). In this sense, the greater pubescence that characterizes the abaxial side of soybean leaves could favour bacteria adherence.

Further experiments that combined epidermis peeling and CLSM allowed us to visualize in detail bacteria localization inside soybean leaves. Red fluorescent bacteria were detectable on both sides (adaxial and abaxial) of the leaf epidermis, (Fig. 3a,b). Upon closer inspection, we noticed that Az39DsRed cells frequently accumulated outside the veins (Fig. 3c) and at cell–cell junctions (Fig. 3d). Occasionally, bacterial groups were detected around stomata (Fig. 3e). Similarly, localization studies that used fluorescent proteins as markers showed that PGPR and other micro-organisms, such as phytopathogenic fungi, preferentially colonized root cells junctions (Tombolini et al. 1999; Bloemberg et al. 2000; Lagopodi et al. 2002; Bolwerk et al. 2003; Gamalero et al. 2005; Bloemberg 2007).

The internal localization of Az39DsRed was also assessed by analysing cross-sections of soybean leaves at the midrib, in the medial region of the lamina. The images obtained at 6 h after bacterization confirmed the presence of Azospirillum Az39DsRed cells inside the xylem of treated leaves (Fig. 4b,c), but not of control leaves (Fig. 4a), suggesting Azospirillum systemic movement within the plant, which in turn involves the possibility of vertical transmission through the seed. Notably, similar endophytic behaviour has been observed for A. brasilense Sp7, which was found to translocate inside Phaseolus vulgaris and accumulate in the seeds of this plant species, thus accounting for its vertical transmission (Malinich and Bauer 2018).

In this work, the combination of microscopy (SEM and CLSM) techniques using a DsRed-labeled strain and standard quantification procedures allowed us to demonstrate A. brasilense Az39 ability to colonize soybean leaves. Through these methodologies, we confirmed not only the capacity of A. brasilense Az39 to survive on soybean leaf surface but also to internalize in leaf tissues. These results establish a biological explanation for the previously documented positive effects of A. brasilense Az39 on plant leaves (Puente et al. 2019) and encourage further research on this strain as a foliar inoculant for soybean and other relevant crops.
Figure 2  Az39DsRed localization on abaxial and adaxial sides of soybean leaves at different time points. Scale bar = 50 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 3  Azospirillum brasilense Az39DsRed localization in soybean leaves. The inner epidermis of soybean leaves was examined. Both the adaxial (a) and the abaxial (b) inner epidermis demonstrated colonization. Detail of the preferred bacterial localization zones: on leaf veins (c), at cell–cell junctions (d), and around stomata (e) (white arrows). Images are a merge of the bacteria, chlorophyll and bright field channels. Scale bar = 20 μm. [Colour figure can be viewed at wileyonlinelibrary.com]
Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study were *A. brasilense* Az39 (Az39) and its fluorescent variant *A. brasilense* Az39DsRed (Az39DsRed). Both strains are deposited at the BPCV-IMYZA-INTA Culture Collection, Castelar, Buenos Aires, Argentina. The Az39DsRed variant was obtained by mobilizing the plasmid pME7134mob into Az39 by triparental mating, as described by Maroniche *et al.* (2018). Az39 wild-type was grown in Sadasivan and Neyra liquid medium (1985); Az39DsRed was grown in the same medium supplemented with 10 µg/mL of tetracycline. Incubation was carried out at 30°C under shaking (150 rev min⁻¹) for 48 h. The number of viable cells (CFU per mL) was quantified using the micro-drop method (Miles and Misra 1938) on plates containing Congo Red medium (Rodriguez Caceres 1982), modified by the addition of 20 µg mL⁻¹ of tetracycline, after incubation at 30°C for 3–6 days.

Leaf bacterization and bacteria recovery for quantification

Soybean plants were grown in a plant growth chamber with a photoperiod of 16 h light (30°C) and 8 h dark (20°C), 40–60% of relative humidity, and irrigated with a nitrogen-deficient Hoagland’s nutrient solution (Hoagland and Arnon 1950). After 15 days, the third trifoliate leaf was cut, and each leaflet (individual biological samples) was superficially disinfected with 70% ethanol (15 s), 5% sodium hypochlorite (10 min) and finally washed five times with sterilized water. These disinfected leaves were immersed for 10 min in a bacterial suspension of Az39 or Az39DsRed (at a concentration of 1 × 10⁹ CFU per mL). The excess of inoculum was allowed to drain, and samples were kept at 4–6°C until analysis.

Bacterial recovery for quantification was carried out only for Az39DsRed-inoculated leaves. To this purpose, leaves were transferred to sterile mortars containing 9 mL of sterile saline solution (SS) and crushed for 60 s to obtain the initial homogenate (dilution 10⁻¹). Serial dilutions were made from this homogenate by transferring 1 mL into tubes containing 9 mL of SS until the 10⁻⁴ dilution was obtained. Bacterial numbers were estimated by the MPN method using the semi-solid NFB medium (Dobereiner *et al.* 1995) and by direct counting of Az39DsRed colonies on agar plates containing selective RC medium (Rodriguez Cáceres 1982). Ten microlitre- aliquots of each dilution were dispensed in NFB vials to determine MPN of diazotrophs, whereas 100 µl-aliquots were deposited on RC plates and spread with a Drigalski spatula to allow *Azospirillum* colony counting. Plates and vials were incubated at 28–30°C for 4–6 days and subsequently observed to establish the number of CFU per gram of leaf fresh weight.

Localization of *A. brasilense* Az39

*Azospirillum brasilense* Az39 cells localization on the surface of soybean leaves was investigated under SEM using a FEI Quanta 250 microscope (low vacuum mode, 0–68 torr pressure, secondary electron detector) (Laboratorio Integral de Microscopia, CICYyA, INTA) at 0, 24 and 48 h after bacterization. On the other hand, Az39DsRed cell localization was assessed by confocal laser scanning microscopy (CLSM) at 0, 2, 4, 6 and 24 h after bacterization. Hand-cut slices of treated leaves were

Figure 4 Cross-sections of *Azospirillum brasilense* Az39DsRed-bacterized soybean leaves. Control leaves (a) and bacterized leaves at 6 h after treatment (b and c) are shown. Images are a merge of the bacteria, chlorophyll and bright field channels. Scale bar = 100 µm. [Colour figure can be viewed at wileyonlinelibrary.com]
mounted on glass slides with PBS 1X. Some of the leaves were previously peeled gently with a razor blade to separate the epidermis and allow visualization of the inner epidermis. The abaxial and adaxial epidermis were analysed for Az39DsRed cells on a Leica TCS-SP5 (Leica Microsystems GmbH, Wetzlar, Germany) spectral laser confocal microscope (Laboratorio Integral de Microscopia, CICVYA, INTA) using 63x (HCX PL APO CS 63 × 1.2 WATER UV) or 10× (10× × 0.4 DRY) objectives and the 543 nm HeNe laser for excitation. Fluorescence emission of DsRed was detected between 560 and 625 nm, whereas autofluorescence of chlorophyll was captured between 650 and 750 nm. The microscope power settings, detectors gain and scanning speed were adjusted to optimize contrast and resolution. Nonbacterized, surface-disinfected leaves were used as controls.

Statistical analysis

All bacterization assays were carried out in triplicate, including each three biological samples. Results from bacterial counts were subjected to ANOVA; means were compared by Duncan test (P ≤ 0.05) using INFOSTAT software (Di Rienzo 2011).

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Conflict of Interest

The authors report no conflicts of interest.

Author contributions

ML Puente (Methodology, funding acquisition, supervision and writing); M. Paneppucci (Methodology); JE García, Sabio y García, GA Maroniche; MV, Criado, R. Molina (Methodology and writing); F. Cassán (supervision and writing). All authors participated in review and editing.

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