Short Communication

Detection of *Azospirillum brasilense* by qPCR throughout a maize field trial

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A B S T R A C T

Although *Azospirillum brasilense* is used in many inoculant formulations, information of its prevalence under field conditions is still scarce. In this work, we inoculated *A. brasilense* AbV5/AbV6 (2×10^8 CFU ml^{-1}) on maize seeds and then evaluated its colonization profile throughout seventy-five days after seeding (DAS) in a field assay. The abundance of total bacteria and *A. brasilense* on the seed, root and soil rhizosphere were determined by qPCR using 16S rRNA and species-specific primers, respectively. *A. brasilense* was detected in soil at a concentration of 10^5 CFU g^{-1} of soil only until radicle emergency. From the fifth day onwards, it was detected at the roots in a concentration of 10^3 to 10^5 CFU g^{-1} of fresh root. Our results provide a sensitive approach to monitor *A. brasilense* in a field trial and reveal new information on the ecology of maize and *A. brasilense* association.

1. Introduction

Azospirillum is the most studied genera of the plant growth-promoting rhizobacteria (PGPR), being considered a model organism to understand bacterial-plant interactions (McMillan and Pereg, 2014; Pedrosa et al., 2019). It has the capacity to associate with approximately 113 species of plants, distributed within 35 botanical families, including 14 species of cereals (Pereg et al., 2016). *Azospirillum* inoculation promotes yield increase in 70% of the trials and *Azospirillum* is used in the formulation of at least 104 different inoculants fabricated in South America (Cassán and Díaz-Zorita, 2016). In Brazil, more than 9 million doses of *A. brasilense* AbV5/AbV6 were sold in 2018. These are the most commonly used strains in commercial formulation in this country (Anpii, 2019).

Azospirillum is inoculated in furrow, via soil or leaf spray, on the roots or seed surface. The latter is the most used method in agriculture, mainly due to its feasibility in application, and the reduced amount of inoculant required (Bashan and de-Bashan, 2015). The ability of inoculated PGPR to colonize plant roots depends on its exudates production, the soil characteristics and the native microbiome (de Souza et al., 2015; Sasse et al., 2018; da Costa et al., 2018). Although PGPR are commonly applied in agriculture, information about the prevalence and colonization of inoculated bacteria under field conditions is still scarce (Rilling et al., 2019). Threshold number of cells is shown to be critical to obtain positive plant responses (Bashan et al., 2014). Monitoring PGPR under field conditions by traditional microbiological methods is a challenge because the growing media are semi-selective and with low efficiency to detect bacteria from non-dominant populations. Up to now, few studies have described molecular methods to detect *Azospirillum* but qPCR appears to be the most sensitive (Couillerot et al., 2010a; Lin et al., 2011; Shime-Hattori et al., 2011; Faleiro et al., 2013; Stets et al., 2015; Priya et al., 2016; Maroniche et al., 2017). The only three published works that monitored *A. brasilense* strains using qPCR were carried out under controlled laboratory conditions and for a maximum of thirteen days after inoculation (Couillerot et al., 2010a; Faleiro et al., 2013; Stets et al., 2015). The development of specific methodologies for *A. brasilense* quantification in inoculated soil, rhizosphere, seeds or plant tissues samples are necessary not only to track this biofertilizer in its natural environment but also to evaluate its functionality. In this study, we quantified *A. brasilense* AbV5/AbV6 using qPCR method throughout seventy-five days after sowing in a field maize crop, and observed the effect of inoculation on maize yield.

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2. Material and methods

2.1. Site description

The experiment was performed in summer 2017, on an Oxisol (clayey, kaolinitic, thermic Rhodic Hapludox) under a long-term continuous no-till system in Ponta Grossa, PR, southern Brazil (25°13′ S, 50°07′ W). According to Köppen-Geiger System (Peel et al., 2007), the climate is Cfb (mesothermal, humid, subtropical), with mild summer and frequent frosts during the winter. The average altitude is 830 m with average maximum and minimum temperatures of 28 and 17 °C, respectively, and 72–304 mm of rainfall during the experiment.

2.2. Experimental design and cultural treatments

A randomized complete block design was used, with three replicates plots. Maize seeds (hybrid DKB 230 PRO3®, Dekalb) were treated with an *A. brasilense* AbV5/AbV6 commercial inoculant (2×10^8 Colony Forming Unit – CFU kg^{-1} of seed). All plots received 45 kg of N ha^{-1} as urea at sowing and extra doses (80 and 240 kg of N ha^{-1}) in V$_4$ physiological state; N treatments were only used for maize yield analyses but not for *A. brasilense* AbV5/AbV6 quantification. The effect of *A. brasilense* inoculation on maize productivity was determined by grain yield evaluation of inoculated and uninoculated plots.

2.3. Samples collection and DNA extraction

At the following maize physiological states: 1; 5; 7; 9; 11; 13; 15; 20; 25; 35; 45; 75 days after sowing (DAS), six maize plants were randomly collected from each of the three plot replicates, avoiding root damage. Therefore, eighteen plants were used to obtain each plant composite sample of inoculated treatments. Before starting experiment, twelve samples of the bulk topsoil (0–10 cm) were randomly collected and mixed to obtain a control composite sample and at 7 DAS, plants from uninoculated plots were also collected as mentioned above to form another control. The samples were transported under refrigeration for processing at the laboratory.

Rhizospheric soil and roots were processed as described by Couillerot et al. (2010b) with some modifications. Briefly, the composite sample was shaken vigorously to remove the adhered bulk soil from the roots, then, the roots were placed in a beaker, covered with sterile distilled water, and shaken for 15 min at 160 rpm in an orbital shaker (New Brunswick Scientific Classic, C25KC). Then, the roots were removed, washed with sterile distilled water, and manually ground with mortar and pestle. The remained solution was centrifuged at 5500 ×g for 10 min to recover the precipitated rhizospheric soil.

Soil and root DNA was extracted using PowerSoil DNA isolation kit and DNeasy PowerPlant Pro kit (MOBIO Laboratories, Inc.) respectively, as recommended by the manufacturer. Finally, the DNA was quantified in NanoVue™ Plus (GE Healthcare Life Sciences) and stored at −20 °C.

2.4. qPCR assay

Real-time qPCR quantifications were performed in technical triplicate and each run was repeated three times in Lightcycler® Nano Roche thermocycler (Roche Applied Science). Replicates with Cq differences of more than 0.5 cycles were not considered. Each qPCR reaction consisted of 10 µl of FastStart Essential DNA Green Master (Roche Applied Science), 1 µl of each primer (10 pmol µl^{-1}), 0.5 µl of BSA (10 mg ml^{-1} BioLabs), 20 ng µl^{-1} of DNA, and water to complete the 20 µl volume. Primers Azo-2F and Azo-2R (Stets et al., 2015) were used for *A. brasilense* species-specific quantification, and the 16S rRNA primers 341F and 534R (Bru et al., 2008) were used for all bacteria quantification. An average of 4.2 copies of the 16S rRNA gene per genome (Větrovský and Baldrian, 2013) was used to convert absolute gene copy number into CFU.

Table 1
Parameters for Azo-2 and 16S rRNA primers obtained from standard curves.

Primer	E (%)	R^2	slope	T$_m$ (°C)	LOD (CFU g^{-1} of soil)
Azo-2	100.8	0.98	−3.28	85	2×10^3–2×10^8
16S rRNA	102	0.98	−3.18	85	2×10^4–2×10^8

E: Efficiency. T$_m$: Melting temperature. LOD: Limit of detection

The cycling program included 10-min incubation at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and one cycle of 72 °C for 20 s. Melting curve analysis of the PCR products were performed to verify reaction specificity using LightCycler® Nano Software 1.1 (Roche Applied Science). Additionally, all products were analyzed by electrophoresis in 2% TBE agarose gel stained with ethidium bromide (0.5 mg ml^{-1}); for Azo-2 and 16S rRNA primers, fragments of 90 bp and 174 bp were expected, respectively.

Standard curves were constructed following Couillerot et al. (2010b) with some modifications, 300 g of uninoculated bulk soil was autoclaved three times at 120 °C for 50 min, and then, seven samples of 250 mg were placed in extraction tubes (PowerSoil DNA isolation kit – MOBIO Laboratories, Inc.) with a 10 fold dilution series ranging from 2×10^8 to 2×10^2 CFU ml^{-1} of *A. brasilense* AbV5/AbV6 from a commercial inoculant, and incubated for 1 h at 4 °C, to guarantee the interaction between soil particles and bacteria. Finally, DNA was extracted as recommended by the manufacturer. DNA was quantified in NanoVue™ Plus (GE Healthcare Life Sciences) and stored at −20 °C. The standard curves followed the technical recommendations described by Burns et al. (2005) and presented parameters (Table 1) accepted by literature (Bustin et al., 2009; D'haene et al., 2010; Svec et al., 2015).

2.5. Statistical analysis

Data were compared by the Tukey test ($P < 0.5$) using the packages Agricolae (de Mediburu, 2019) and Laercio (da Silva, 2015) in software R version 3.4.1 (R Core Team, 2013).

3. Results and discussion

It is estimated that the number of bacterial species per gram of soil varies between 2×10^3 and 8.3×10^6 (Gans et al., 2005; Schloss and Handelsman, 2006) and that approximately 10^5 CFU of *A. brasilense* is necessary for successful seed colonization (Okon and Itzigsohn, 1995). Based on the limit of detection (LOD) ranging from 2×10^3 to 2×10^8 CFU of *A. brasilense* g^{-1} of soil or root (Table 1), the qPCR quantification using Azo-2F/Azo-2R primers showed to be an effective method for tracking *A. brasilense* AbV5/AbV6 on crop fields.

3.1. Azospirillum brasilense behavior in the field conditions

Throughout the seventy-five days of maize growth evaluation, 16S rRNA gene qPCR analysis showed that the number of total bacteria remained constant in the soil and roots, around 10^7 CFU g^{-1} of soil or roots, while the *A. brasilense* AbV5/AbV6 population changed (Fig. 1).

As soon as radicle was formed, *A. brasilense* AbV5/AbV6 could be detected attached to it. At five to seven DAS, 10^5 CFU of *A. brasilense* AbV5/AbV6 were detected per gram of fresh roots and at eleven DAS, it decreased to 10^3 CFU g^{-1} of fresh root (Fig. 1). This decrease might be explained by the formation of post-embryonic shoot-borne roots or nodal roots (Hochholdinger, 2009), which make up most of the maize root system and are responsible for nutrient acquisition later in development (Hoppe et al., 1986). *Azospirillum* has an affinity for the new elongation root zone (Burdman et al., 2000; Santos et al., 2017a; Santos et al., 2017b) and it is possible that a broad portion of *A. brasilense* AbV5/AbV6 cells were in the process of migration to the new root node in formation. The formation of definitive nodal roots might have contributed to the stability of the *A. brasilense* AbV5/AbV6 population,
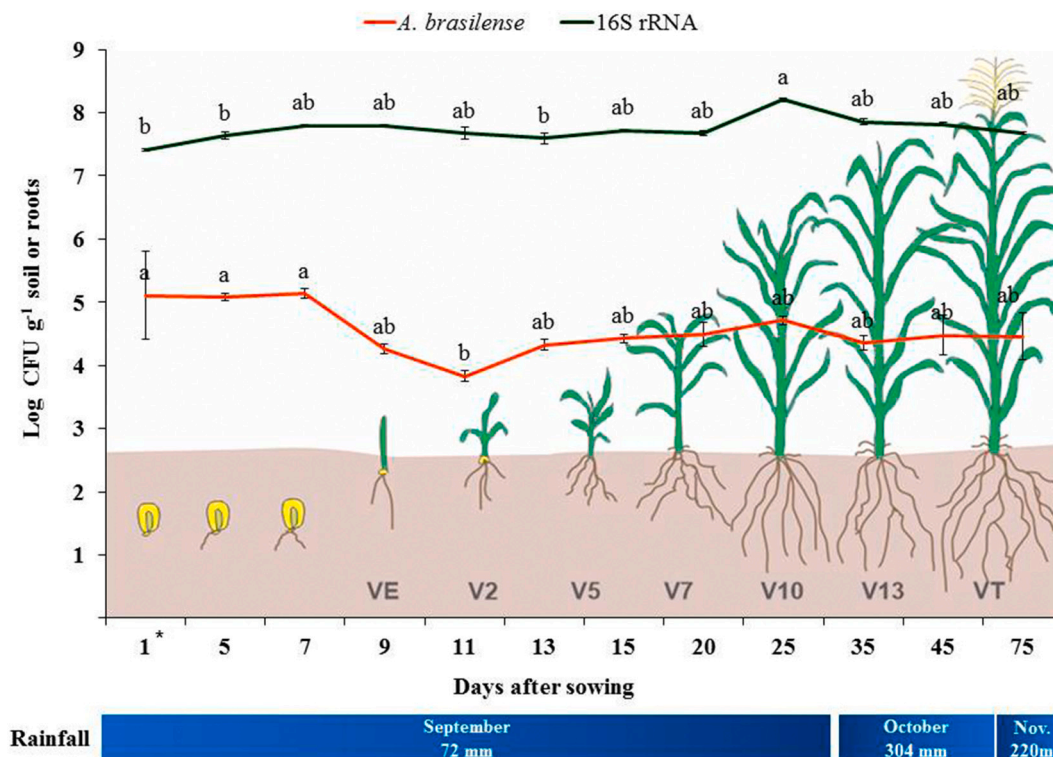
Fig. 1. Detection of total bacteria and *A. brasilense* AbV5/AbV6 in maize rhizosphere or root throughout its physiological cycle, from sowing to tasseling (VT). The indicated log CFU values were obtained from the maize root samples except at one DAS (tagged with an asterisk), in which values are from soil near to the inoculated seeds. The CFU of total bacteria was estimated by the absolute 16S rRNA gene copy number divided by an average of 4.2 copies per genome. The CFU of *A. brasilense* AbV5/AbV6 population, by itself, was estimated by the absolute number of copies of an specie-specific region of its genome, monitored by using a specific couple of primers. Data represent the mean of three separated runs and its triplicates for each day. Values followed by the same letter were not statistically different (Tukey $p \leq$ 0.05). Means of rainfall during experiment time are indicated in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
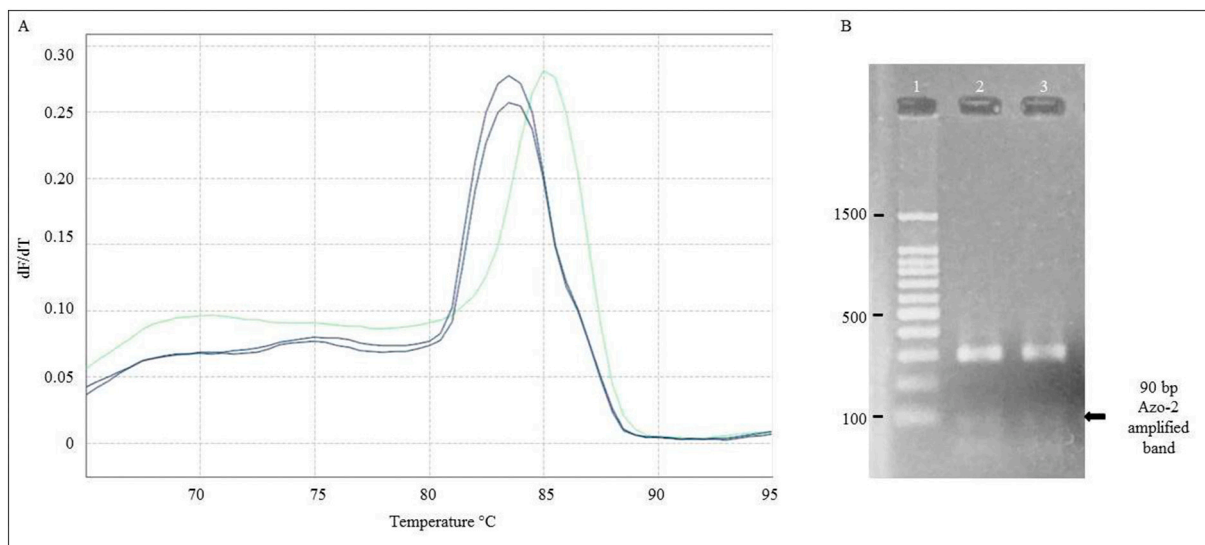

Fig. 2. *A. brasilense* detection in rhizospheric soil of inoculated maize plants collected five days after sowing. A) Melting curve of the qPCR amplification. Green line = positive control (*A. brasilense* DNA extracted from commercial inoculant); blue line = unspecific products of rhizospheric soil sample at five DAS. B) Products of qPCR observed by electrophoresis in 2% TBE agarose gel stained with ethidium bromide (0.5 mg ml^{-1}). Lane 1–1500 bp ladder (Sinapse Inc); Lanes 2 and 3 – qPCR products of rhizospheric soil sample at five DAS. Azo-2 amplified a fragment of 90 bp. Azo-2 primers may amplify unspecific products when the population of *A. brasilense* is below 10^3 CFU g^{-1} of soil or roots.

since from thirteen DAS to the last day of evaluation (75 DAS) it remained at 10^4 CFU g^{-1} of fresh root.

On the other hand, *A. brasilense* AbV5/AbV6 were only detected at the soil in the first DAS at a concentration of 10^5 CFU of *A. brasilense* AbV5/AbV6 per gram of soil (Fig. 1). From the fifth day onwards, it was not possible to detect it by qPCR indicating that the population was
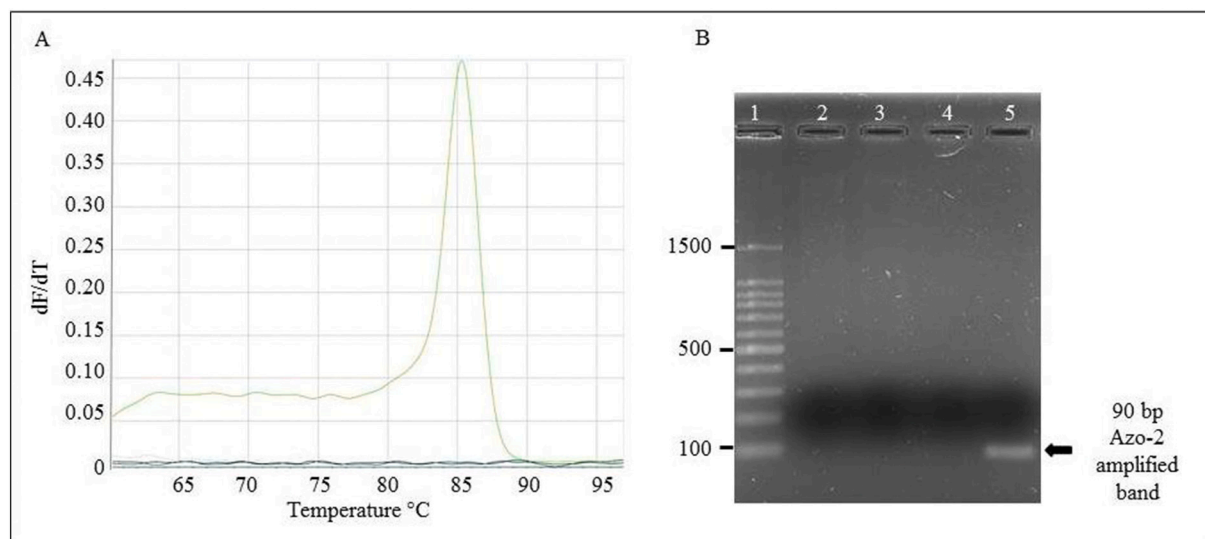
Fig. 3. *A. brasilense* detection in bulk topsoil (0–10 cm) collected before experiment and roots of uninoculated maize plants collected at seven days after sowing. A) Melting curve of the qPCR amplification. Green line = positive control (*A. brasilense* DNA extracted from commercial inoculant); blue line = no amplification of uninoculated bulk soil and maize roots. B) qPCR products observed by electrophoresis in 2% TBE agarose gel stained with ethidium bromide (0.5 mg ml^{-1}). Lane 1–1500 bp ladder (Sinapse Inc); Lane 2 – qPCR products using uninoculated bulk soil as template; Lane 3 – qPCR products using uninoculated maize roots as template. Lane 4 – NTC (no template control); Lane 5 – positive control (*A. brasilense* DNA extracted from commercial inoculant). Azo-2 amplified a fragment of 90 bp.

absent or under the limit of detection 10^3 CFU g^{-1} of soil (Fig. 2A and B). The hypothesis that *Azospirillum* do not survive well in soil was suggested in previous works (Bashan et al., 1995; Bashan and de-Bashan, 2015).

To confirm that the quantified *A. brasilense* AbV5/AbV6 population at the root and soil was the inoculated one and not soil indigenous species of *Azospirillum*, the soil before sowing and the roots from uninoculated maize plants were analyzed; no amplification was detected in both cases (Fig. 3A and B).

3.2. Azospirillum brasilense impact in maize yield

The maize grain yield average of uninoculated treatments which received 80 and 240 kg of N ha^{-1} were 12,734 and 11,977 kg ha^{-1}, respectively, while the inoculated treatments with the same amount of N reached the average of 13,865 and 13,755 kg ha^{-1}. Therefore, the gain due to inoculation was near 6% in both N treatments. The combination of *A. brasilense* AbV5/AbV6 with N fertilization intensified maize growth and yield, representing an economically viable and environmentally sustainable technique (Zeffa et al., 2019). Additionally, based on the low values of rainfall (average of 72 mm) in the first month after sowing (Fig. 1), the ability of *A. brasilense* to improve resistance to the plant in drought stress conditions was confirmed (Bashan and De Bashan, 2010; Díaz-Zorita et al., 2012).

4. Conclusions

In this study, we presented a sensitive tool to trace and determine the population size of *A. brasilense* in the environment. By using it, we showed that the *A. brasilense* inoculated on the seeds migrates to the roots right after radicle emergence. In addition, we showed that the *A. brasilense* population stayed in a concentration sufficient for generating a positive effect on maize yield. This approach is useful not only for ecological studies but also to validate inoculation practices and agricultural managements.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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