Quantification of *Azospirillum brasilense* FP2 in wheat roots by strain-specific qPCR

Maria Isabel Stets[a,b], Sylvia Alqueres[b], Emanuel Maltempi de Souza[a], Fábio de Oliveira Pedrosa[a], Michael Schmid[b], Anton Hartmann[b], and Leonardo Magalhães Cruz[a]#

Department of Biochemistry and Molecular Biology, Federal University of Parana (UFPR), Curitiba, PR, Brazil[a]; Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Department for Environmental Sciences, Research Unit Microbe-Plant Interactions, Neuherberg, Germany[b]

Running title: qPCR *Azospirillum brasilense* quantification in roots

#Adress correspondence to Leonardo M. Cruz, Department of Biochemistry and Molecular Biology, Federal University of Paraná (UFPR), P. O. BOX 19.046, 81.531-990, Curitiba, PR, Brazil; phone +55 041 3361 1657; e-mail leonardo@ufpr.br

Abstract

Azospirillum is a rhizobacterial genus containing plant growth-promoting species associated with different crops worldwide. *Azospirillum brasilense* strains exhibit a growth-promoting effect by means of phytohormone production and possibly by N_2 fixation. However, one of the most important factors for increase in crop yield by plant growth-promoting rhizobacteria is the survival of the inoculant in the rhizosphere, which is not always achieved. The objective of this study was to develop quantitative PCR protocols for

strain-specific quantification of *A. brasilense* FP2. A novel approach was applied to identify strain-specific DNA sequences based on comparison of genomic sequences within the same species. The draft-genome sequence of *A. brasilense* FP2 and Sp245 were aligned, FP2-specific regions were filtered and checked for other possible matches in public databases, Strain-specific regions were then selected to design and evaluate strain-specific primer pairs. The primer pairs AzoR2.1, AzoR2.2, AzoR5.1, AzoR5.2, and AzoR5.3 were strain-specific for *A. brasilense* FP2. These primer pairs were used to monitor quantitatively the population of *A. brasilense* in wheat roots under sterile and non-sterile growth conditions. In addition, co-inoculations with other plant-growth promoting bacteria in wheat were performed under non-sterile conditions. Results showed that *A. brasilense* FP2 inoculated to wheat roots is highly competitive and achieve high cell numbers (~10^7 CFU/g of fresh weight root) in the rhizosphere even under non-sterile conditions and when co-inoculated with other rhizobacteria, maintaining the population rather stable for at least up to 13 days after inoculation. The strategy used here can be applied for other organisms whose genome sequences are available.

Introduction

Azospirillum is one of the most important genus of plant growth-promoting rhizobacteria found worldwide under a variety of environmental and soil conditions (1) (Bashan, 2004). The diazotroph *Azospirillum brasilense* is the best studied species of the genus and is found in close association with many agriculturally important crops and exert beneficial effect on plant growth and productivity (2–4) (Fibach-Paldi, 2012; Dobbelaeere, 2003; Okon, 1994).

Nitrogen fixation(5, 6) (Sant´Anna 2011; Eckert 2001) and production of the auxin 3-indoleacetic acid (IAA) by many representatives of the genus *Azsopirillum* is related to growth promotion effects observed in inoculated plants such as increase in root length, number of root hairs and lateral roots (3) (Dobbelaere 2003).

Biotechnological use of *A. brasilense* inoculants in Latin American – and in Brazil, in particular, has increased in recent years (7) (Castro-Sowinski 2007). The strain FP2 is a spontaneous mutant of *A. brasilense* Sp7 (8) (Pedrosa and Yates, 1984). Strain Sp7 has been shown to be capable of stimulating growth of several Poaceae and increasing productivity capable of wheat and maize crops (2) (Fibach-Paldi 2012). Strain FP2 can also promote growth of wheat (9) (Camilios et al.,2014) enhance maize and wheat productivity under field conditions (unpublished). Most of the *A. brasilense* inoculants in Brazil contains strains Ab-V5 and Ab-V6, also a derivative of Sp7 strain, which were shown to increase productivity of maize and wheat under field conditions (10) (Hungria 2010) and were officially authorized for use as inoculants in these crops (10) (Hungria 2010).

However, a major problem related to *A. brasilense* inoculants is the survival of inoculated strains in the rhizosphere soil (11, 12) (Bashan 1999; Bashan 1995), which affects the inoculant performance since the effective colonization of roots is necessary for successful plant growth stimulation by *Azospirillum* (13) (Dobbelaere 2002).

To assess the diversity and taxonomy of crop plant-associated bacteria, many cultivation dependent and independent methods are currently in use (14–16) (Magnani 2013; Magnani 2010; Pisa, 2011). However, most of these methods

are not quantitative and based on the evaluation of the 16S rRNA coding gene sequences which is highly confident only at genus and species level and not specific enough to study the bacterial population dynamics at strain level, necessary for inoculant monitoring. Thus, in many cases, it is not possible to associate plant-growth promotion failure or success to the inoculated bacterial population quantitatively at strain-specific resolution, leaving the outcome of the inoculation unexplained (17) (Couillerot 2010). Furthermore, crop response to inoculation under field conditions depends heavily on a combination of the plant genotype and the bacterial strain (18–20) (Sánches 2014; Araújo 2013; Sasaki 2010) stressing the need of methodologies to evaluate the success of plant colonization accurately at high resolution. Previously, we used whole cell matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis to differentiate species of *Azospirillum* including several closely related *A. brasilense* strains (21) (Stets 2013). However, this method is not quantitative, requires growth on a culture medium and is time and labor intensive.

Quantitative PCR (qPCR) has been the method of choice to quantify rhizosphere populations because it allows high specificity, sensitivity and speed (17, 22, 23) (Couillerot and Bouffaud 2010; Couillerot and Poirier 2010; Sorensen 2009). This technique has been successfully used to quantify several bacteria associated with plants. It was successfully used for the quantification of a functionally specific subgroup of Pseudomonads in the rhizosphere (24) (Mavrodi 2007). The pathogen *Xylella fastidiosa* was quantified in citrus plants (25) (Pinheiro 2002), while the endophytic bacterium *Methylobacterium mesophilicum* was monitored by qPCR during the

Catharanthus roseus colonization (26) (Lacava 2006). In *Brassica oleracea*, the plant growth promoting *Enterobacter radicincitans* population was monitored by qPCR associated with fluorescence *in situ* hybridization (FISH) (27) (Ruppel 2006), determining not only the amount of bacteria in the colonized plants but also their location in the plant. Although these reports showed qPCR as a valuable technique to quantitatively monitor populations of unlabeled bacteria in green-house experiments, none had used strain-specific primers. The application of strain-specific primers is difficult in field experiments, where closely related indigenous bacteria may interfere in the amplification and quantification. For a strain-specific molecular monitoring, sequence characterized amplified region (SCAR) markers obtained from BOX-, ERIC-, and RAPD-PCR fragments were recently applied to design primers for a qPCR quantification of *A. brasilense* and *A. lipoferum* at strain-specific level(17, 22) (Couillerot and Bouffaud 2010; Couillerot and Poirier 2010).

The objective of this study was to develop qPCR protocols for a strain-specific quantification of the plant-growth promoting bacterium *A. brasilense* FP2 based on whole genome comparison with the closely related strain Sp245. The designed strain-specific primers were then applied for quantification to monitor the FP2-population in inoculated wheat plants under sterile and non-sterile conditions.

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Material and Methods

Bacterial strains

All *Azospirillum* strains (Table 1) were routinely grown in NFbHPN medium (28) (Machado 1991) at 30 ºC under aeration by shaking at 120 rpm. Strains from other genera were grown in DYGS medium (29) (Rodrigues 1986) containing, per 1,000 mL: 0.10% glucose, 0.20% yeast extract, 0.15% peptone, 0.50% $MgSO_4.7H_2O$, and 0.15% L-glutamic acid at pH 6.0-6.5; cultures were incubated at 30 ^oC under aeration by shaking at 120 rpm. Colony counts of all strains were performed after spreading dilutions on the respective medium plates and incubation for 72 h at 30 ºC.

Primer design

To design *Azospirillum brasilense* FP2 strain-specific primer pairs, the general strategy was used: (i) the Whole Genome Sequence (WGS) of *A. brasilense* FP2 was fragmented *in silico* from the FASTA genome sequence using in house scripts, producing 500 bp non-overlapping fragments; (ii) the genome sequence of *A. brasilense* Sp245 was used to build a local BLAST database and *A. brasilense* FP2 fragment sequences were used as queries for a BLASTn similarity search, with default parameters; (iii) fragments with no hits found were subjected to a second BLASTn (30) (Altschul 1997) search against NCBI-nt database (performed at July, 2012; GenBank release 190), using default parameters; (iv) putative strain-specific sequences, i.e. sequences without any match in the two BLAST sequence analyses, were used to design primer pair sets for *A. brasilense* FP2. In order to inspect the selected regions, the draft genome sequence of *A. brasilense* FP2 was

annotated and visually analyzed using RAST, version 2.0 (31, 32) (Overbeek 2014; Aziz 2008) and Unipro UGENE, version 1.14 (33) (Okonechnikov 2012).

The WGS of *Azospirillum brasilense* FP2 is publicly available at NCBI database under accession number APHV00000000 and assembly GCA_000404045.1, with total sequence length of 6,885,108 bp, 413 contigs (N50 of 29,432 bp), GC content of 68.1 %, and genome coverage of 25X. The WGS of *Azospirillum brasilense* Sp245 is available at NCBI database under accession numbers from HE577327 to HE577333 (1 chromosome and 6 plasmids) and assembly GCA_000237365.1, with total sequence length 7,530,241 bp (total assembly gap length of 6,000 bp), 67 contigs (N50 186,382 bp), and GC content of 68.6 %.

Primer design was performed using Primer Express 3.0 (Applied Biosystems, Foster City, CA) based on (i) an amplicon size inferior to 200 bp and primers lengths ranging from 18 to 22 bp; (ii) high melting temperature of primers (Tm approximately 60 ºC) and low Tm difference between primers (ΔTm< 2 ºC); and (iii) lack of predicted hairpin loops, duplexes and primer-dimer formation.

Primer selection and evaluation

The designed primer pairs were synthesized by Eurofins (Ebersberg, Germany) and qualitatively analyzed by conventional PCR with about 30 ng of genomic DNA, 10 pmol of each primer, 1 U Taq DNA polymerase (Taq Dream Invitrogen Inc.), Taq DNA polymerase buffer, 200 mmol/µL of desoxyribonucleotide and sterile ultra-pure water to a final volume of 10 µL. The cycling programme included a 10 min initial denaturation, incubation at 95

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ºC, followed by 25 cycles consisting of denaturation at 95 ºC for 15 s, annealing at 60 ºC for 60 s followed by 72 ºC for 30 s and final elongation of 10 min at 70 °C. A primer pair was considered strain-specific if: (i) successful amplification occurred using DNA of the target strain as template; (ii) the absence of cross-amplification with non-target strains; and (iii) the absence of amplification in the control tube reaction, where no DNA was added. Genomic DNA from 14 strains of 10 species and 4 genera (Table 1) were used as templates for PCR reactions. A second step was performed under quantitative PCR conditions to check primer specificity (melting curves) and amplification efficiency, as described below.

Quantitative PCR conditions

Quantitative PCR (qPCR) was performed in a total reaction volume of 25 µL containing 12.5 µL Power SYBR® Green PCR Master Mix (Applied Biosystems), 6.25 µL of a primer mix (final concentration of 1 µmol) and 6.25 µL of 2.5 ng/µL diluted template DNA. MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems) and an ABI Prism 7500 (Applied Biosystems) were used. The cycling programme included a 10 min incubation at 95 ºC followed by 40 cycles consisting of 95 ºC for 15 s, 60 ºC for 60 s followed by 72 ºC for 30 s and additional 72 °C for 10 min. Amplification specificity was verified by melting curve analysis of the PCR products performed using the ABI Prism 7500 Sequence Detection version 1.2.3 software (Applied Biosystems).

Primer efficiency determination

Genomic DNA from *A. brasilense* FP2 was used to prepare ten-fold dilution series (in triplicates). Sterile water was used as negative control. The cycle threshold (Ct), was automatically determined for each sample by the ABI Prism 7500 Sequence Detection version 1.2.3 (Applied Biosystems) software. A standard curve was generated by plotting the Ct number against the logarithm of bacterial DNA concentration (data not shown) and used to calculate the amplification efficiency (E) (Table 2).

Generation of standard curves for qPCR quantification of *A. brasilense* FP2 in wheat roots

The standard curves for quantification of *A. brasilense* FP2 in wheat was constructed according to (22) (Couillerot and Poirier 2010), with the following modifications. Wheat plantlets were grown under axenic condition as described below for 7 days, roots were collected and crushed using mortar and pestle in liquid nitrogen. A volume of 100 µL of *A. brasilense* FP2 culture (dilutions from 10^2 to 10^9 CFU) was added to 100 mg of crushed roots, mixed and left 1 h of incubation at room temperature. The whole mixture was used for DNA extraction with the Fast DNA Spin kit (MPbio, USA) according to manufacturer's instructions; qPCR was performed as described above. The standard curve was generated by plotting Ct number versus CFU added per tube. No bacteria were added to the negative control.

DNA preparation

Genomic DNA from bacterial cultures and wheat roots was extracted using Fast DNA Spin kit (MPbio, USA) according to manufacturer's instructions. DNA concentrations were assessed by O.D. measurements at 260 nm with a NanoDrop device (Nanodrop technologies, Wilmington, DE, USA).

qPCR quantification of *Azospirillum brasilense* FP2 on the wheat roots

For the sterile experiments, seeds of wheat (*Triticum aestivum*), cultivar Schöndorfer, were surface sterilized using a protocol described by (25) (Pinheiro 2002). Afterward, seeds were germinated on nutrient agar plates (Analytical Fluka) for 3 days, transferred to glass tubes containing 16 mL of Hoagland solution and quartz beads of approximately 3 mm of diameter and then incubated in a greenhouse with 14 h light / 10 h dark cycle at 23 ºC and humidity above 50%.

For the experiments under non-sterile conditions, seeds were germinated as previously described without surface sterilization, Commercial gardener soil (type ED-73, Bayerische Gärtnereigenossenschaft) was suspended in Hoagland medium at a final concentration of 1% (w/v), filtered and this suspension was used as inoculum in glass tubes containing quartz beads. The negative control consisted of non-inoculated plants. Different experiments were conducted to evaluate plants inoculated with *A. brasilense* FP2 or co-inoculated in the presence of other wheat-associated diazotrophs (in the same amount), namely *A. brasilense* NH, *H. seropedicae* Z67, *G. diazotrophicus* DSM 5601 and *A. lipoferum* DSM 1691. The control consisted

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of *A. brasilense* FP2 inoculated plants. All microorganisms were grown until about 10^9 CFU/mL and the cells were washed once with 1X PBS buffer (Applichem, Denmark). In all experiments, approximately 10^7 CFU/plant were inoculated in the plant growing medium and incubated for 14 days. The experiments were performed in biological and technical triplicates and samples were collected every 2 days.

Determination of Colony Forming Units (CFU)

To determine the number of Colony Forming Units (CFU), the roots were crushed using a mortar, serially diluted (10^{-1} to 10^{-7}) in saline (NaCl 0.9%), plated on NFbHPN medium and the colonies were counted.

Experimental design and statistical analysis

Experiments in a growth chamber followed a randomized block design. Colony counts were expressed as CFU per g of fresh root, and qPCR quantification data was converted to CFU equivalents per g of fresh root. The data were subjected to analysis of variance and means compared by Tukey test at 1% error probability, using The SAEG 8.0 program (Sistema para Análise Estatísticas, Universidade Federal de Viçosa, Viçosa, Brazil).

Results

Primer design and evaluation of amplification efficiency

For the strain-specific primer design, strain-specific genomic regions were selected from the comparison between Whole Genome Sequence (WGS) of *Azospirillum brasilense* FP2 and *A. brasilense* Sp245, the closest strain with

genome sequence available so far, using the procedures detailed in Material and Methods section. The genome sequence comparison was based on BLAST analysis of 500bp sequence fragments of *A. brasilense* FP2 against genome sequence of *A. brasilense* Sp245 local database, in a first round, and against NCBInt database, in a second round. Although this analysis is database-dependent and do not guarantee to select strain-specific genomic regions, in practice, the genome comparison between two very closely related strains (i. e., with very high genomic synteny) allows for the selection of genomic regions not likely to match sequences of more distantly related organism, as shown by BLAST results against a comprehensive database. Sequences with no hits found in BLAST analysis against Sp245 genome sequence did not show significant hits against NCBInt database too. Using this methodology, six coding and intergenic regions from *A. brasilense* FP2 genome were selected and a total of 10 primer pairs were designed and tested for cross-amplification against 13 different bacterial DNAs, including four *A. brasilense* strains, six other *Azospirillum* spp. and two *Roseomonas* spp. strains (Figure 1 shows the most relevant primer pairs).

Five out of ten primer pairs were strain-specific for *A. brasilense* FP2, namely AzoR2.1, AzoR2.2, AzoR5.1, AzoR5.2, and AzoR5.3 (Table 2). For one of the primer pairs, Azo-2, amplicons were generated for all four strains of *Azospirillum brasilense* tested (FP2, NH, Sp245, and Sp7), but no amplification was observed for *Roseomonas* genomospecies 6 CCUG 33010, *Roseomonas fauriae* KACC 11694, *Burkholderia tropica* Ppe5, and *Burkholderia brasilense* M171 (data not shown.

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Genome sequences from strains FP2 and Sp245 of *A. brasilense* share high degree of synteny, however strain-specific primer pairs were designed from two FP2 contig sequences that did not aligned along the chromosome or any plasmid sequences from Sp245 strain (Supplementary data 1A). On the contrary, the primer pair Azo-2 was designed from a contig sequence of FP2 that aligns to Sp245 chromosome sequence (Supplementary data 1B), although the alignment in the region of primer binding did not show perfect match (data not shown). Automatic annotation of *A. brasilense* FP2 draft genome sequence revealed that the amplicon from the primer pair Azo-2 was predicted to be located at the end of a CDS (coding sequence) for a hypothetical protein. On the other hand, amplicons from strain-specific primer pairs were predicted to be located in a non-coding region (AzoR2.1 and AzoR2.2) or fall into a CDS for TniQ domain containing protein (AzoR5.1, AzoR5.2, and AzoR5.3; Supplementary data 2). Interestingly, the regions surrounding amplicons for strain-specific primers contain some CDSs related to phage and mobile elements.

The efficiency of all strain-specific primer pairs obtained in this study was tested by constructing a standard curve with increasing concentrations of *A. brasilense* FP2 DNA (Table 2). The primers AzoR5.1 and AzoR5.2 were discarded for further analysis based in the lowest efficiency rate and primers AzoR2.1, AzoR2.2 and AzoR5.3 were used to quantify *A. brasilense* FP2.

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qPCR quantification of *Azospirillum brasilense* FP2 on wheat roots

In order to test the ability of the strain-specific primer pairs to quantify *A. brasilense* FP2 in the rhizosphere, a growth chamber experiment was conducted with wheat plants inoculated with *A. brasilense* FP2 under sterile and non-sterile conditions.

To monitor the population of *A. brasilense* FP2 in wheat roots, three strain-specific primer pairs with the highest amplification efficiency (AzoR2.1, AzoR2.2 and AzoR5.3) were selected. The primer pair Azo-2, was used to quantify the total *A. brasilense* population and a universal 16S rRNA coding gene targeted primer pair (Doumit Camilios Neto, personal communication) was used for total bacteria quantification (Table 2).

Initially, a standard curve was constructed from a fixed amount of crushed plant-root tissues mixed with each serially diluted total DNA of *A. brasilense* FP2 (see Material and Methods). The inclusion of plant material in standard curve was based on the observation of Couillerot et al. (17) (Couillerot and Bouffaud 2010) that the presence of root extract decreased the detection limit for quantification of *A. lipoferum* CRT1 on maize. The inclusion of root extract allow for integration of plant background DNA into the technical sensitivity limit of the final standard curve, thereby making quantification closer to reality. The equation for the qPCR-quantification standard curve was used to estimate the amount of bacteria in wheat roots inoculated with *A. brasilense* FP2. The detection limit of the technique was 10^4 CFU/g of wheat root (Supplementary data 3). In the first attempt to monitor population of *A. brasilense* FP2, wheat

was inoculated and cultivated under sterile conditions. In non-inoculated plants, strain *A. brasilense* FP2 or any other bacteria were not detected by qPCR or plate counting techniques. Figure 2A shows the results of bacteria number in wheat inoculated under sterile conditions using primer pair Azo-2 (specific for *Azospirillum* spp.) and the primer pairs AzoR2.1, AzoR2.2 and AzoR5.3 (specific for strain FP2). There was no statistical difference between the measurements using the three strain-specific primer pairs or between species and strain-specific primer pairs. A large number of bacteria was observed in the first days after inoculation (roughly 10^7 to 10^8 CFU/g of wheat root; Figure 2B). The *A. brasilense* FP2 quantification was also analyzed by plate counting method in NFbHPN medium. Higher variability was observed for plate counting method in the first days. However, no statistical differences were observed in sampling points by the cell quantification using either, qPCR or plate counting methods, from day 7 (Figure 2B). The results also revealed an increase in fresh weight of roots and shoots of plants inoculated with *A. brasilense* FP2 (Figures 2C-D). This stimulation due to inoculation was most evident in the roots. In the second attempt, wheat was inoculated and cultivated under non-sterile conditions. The results showed no statistical difference for quantification of *A. brasilense* FP2 by qPCR methodology using three different strain-specific primer pairs (AzoR2.1, AzoR2.2 and AzoR5.3). Similar number of bacteria was observed when the strain-specific primer pairs or species-specific primer pair Azo-2 were used. As expected, the universal primer pair for 16S rRNA coding genes (estimate the total number of bacteria) showed higher numbers of cells per gram of wheat roots, although statistical differences were not achieved for all sampling points. Except at day 1, the

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differences in cell numbers comparing universal primer pair for 16S rRNA coding genes and species-/strains-specific primer pairs were 2-5 fold (Figure 3A). No statistical differences were also observed for the most sampling points when comparing cell counting techniques, althoug plate counting has shown higher variability (Figure 3B). These results suggests the population of the inoculated bacteria is high and stable for at least 13 days after inoculation and that the diversity of total bacteria and *Azospirillum* genus is limited in the rhizosphere of wheat plants and reflects at rather low diversity of bacteria in the soil used for cultivation. The number of CFU in the soil was evaluated by plate counting using DYGS media, reaching values of 10^3 to 10^4 CFU and confirmed the occurrence of a low diversity in the soil used for cultivation of wheat and inoculation experiments.

The population of *A. brasilense* FP2 was stable, even when the rhizobacteria *Azospirillum brasilense* NH, *Herbaspirillum seropedicae* Z67, *Gluconacetobacter diazotrophicus* DSM 5601 and *Azospirillum lipoferum* DSM 1691 were co-inoculated in wheat plants in non-sterile conditions, leaving the FP2 counts above 10^7 CFU/g of root fresh weight. No statistical difference in the *A. brasilense* FP2 numbers were achieved for qPCR quantification comparing strain-specific primer pairs and the primer pair Azo-2 (Figure 4A) or for the most sampling points comparing results from qPCR and plate count techniques (Figure 4B). The differences in cell numbers comparing universal primer pair for 16S rRNA coding genes and species-specific primer pairs ranged from $2.2x10^9$ (3 days after inoculation) to $3.9x10^7$ (13 days after inoculation; Figure 4A). However, the number of total bacteria, including the other inoculants was significantly higher until 7 days after

inoculation, but roughly dropped to a similar level of *A. brasilense* and strain FP2 after that sampling point. This results reinforce the finding that *A. brasilense* FP2 maintain a stable population in the rhizosphere/roots of the plants during the period of colonization and further indicate that strain FP2 is highly competitive, a desirable characteristic for inoculant production. When strain-specific primer pairs for strain FP2 (AzoR2.1, AzoR2.2 and AzoR5.3), developed in this work, were used with DNA from inoculated plants with other rhizobacteria strains under non-sterile conditions, there were no amplification product, confirming the primer specificity for the detection of *A. brasilense* FP2 (data no shown).

Taken together, results from all inoculation experiments shows that the number of *A. brasilense* FP2 cells was stable and not below 10^7 CFU/g of fresh weight root, indicating that this bacterium is competitive, maintaining its population at high level, even in the presence of competing rhizobacteria (Supplementary data 4).

Discussion

Inoculants containing *Azospirillum* have been tested under field conditions with important crops and *A. brasilense* strains, including strain FP2, were recognized as very effective in promoting plant growth and some of them being authorized for the production of commercial inoculants in Brazil (10) (Hungria 2010). Despite the importance of these plant-growth promoting bacteria (PGPB), no rapid method was available to monitor this strain during the experiments.

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A nested PCR method was proposed for the detection of *Azospirillum lipoferum* CRT1 in the rhizosphere by Baudoin et al. (34) (Baudoin and Couillerot 2010). However, the primers, designed from 16S and 23S rRNA intergenic region coding genes fragments, proved not to be specific enough to develop strain-specific qPCR quantification. Several optimizations regarding specificity and efficiency were then applied to design strain-specific qPCR-primers to detect bacterial strains based on sequence characterized amplified regions (SCAR) markers (17, 34) (Couillerot and Bouffaud 2010; Baudoin and Couillerot 2010). In this study, we developed a strain-specific qPCR protocol to quantify the PGPB *Azospirillum brasilense* strain FP2 inoculated to roots of wheat plants based on comparative genome analysis. To achieve this goal, we designed strain-specific primers by *in silico* comparison of 500 pb fragments of a draft *A. brasilense* FP2 genome with the *A. brasilense* Sp245 genome. Unique FP2 strain fragments were also used to search NCBI non-redundant database for similarity. The strain-specific fragments identified so far were used for the primer design. Many authors have reported different methods to design taxon-specific primers, usually based on experimental approaches. Konstantinov et al. (35) (Konstantinov 2005) isolated specific genomic fragments from the type strain and related strains by digesting the genomic DNA with restriction enzyme and then make a subtractive hybridization with closest strains to eliminate shared DNA fragments. The unique fragments were extracted from the gel, cloned, sequenced and used to design specific primers to detect *Lactobacillus sobrius* 001T. Fujimoto et al. (36) (Fujimoto 2008) and Maruo et al. (37) (Mauro 2006) developed a PCR-based method for the identification and quantification of *Lactobacillus casei*

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strain Shirota and *Lactobacillus lactis* subsp. cremoris FC, respectively, using strain-specific primers derived from RAPD analysis. The authors evaluated the survival of these strains through the gastrointestinal tract to monitor the cell numbers before and after the administration of fermented milk containing this strain by qPCR with the strain-specific primers. Pereira et al. (38) (Pereira 2014) developed a qPCR method for quantification of PGPB *H. seropedicae* in the rhizosphere of maize seedlings. Primer pairs were designed from genome sequence of *H. seropedicae* SmR1 (39) (Pedrosa and Monteiro 2011) and tested against 12 different species. Although the selected genome regions did not match with any other sequences in NCBI database, the primers were not evaluated against other *H. seropedicae* strains, not allowing any conclusion about their strain-specificity. In the last decade, whole-genome sequencing has become a rapid and cost effective way to provide comprehensive information about an organism (40) (Havlak 2004). Although the achievement of a complete genome is a demanding process, the draft genome sequencing can be obtained with a high breadth of coverage. In the present work, we have shown that the direct comparison of genomic sequence of closely related organisms is a rapid and reliable approach to detect specific DNA regions to be used as strain-specific genetic markers for the use of strain-specific quantitative detection of bacteria colonizing roots and the rhizosphere. The rationale of this approach relies on three facts: (i) genome sequence provides the genetic information to a highest resolution; (ii) divergent regions between two genome sequences of very close organisms (i. e., strains of the same species) are most likely to diverge from more distant *taxa*; and (iii) the absence of sequence similarity between the possible

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genomic strain-specific regions and sequences in large public databases covering most of the taxa from different environments can be broadly accepted as the absence of these regions in other organisms.

The use of strain-specific primers developed in this work to monitor population of *A. brasilense* FP2 inoculated in wheat showed that the bacteria colonize the roots of the plant at 10^7 to 10^8 CFU/g of root in the first days after inoculation, maintaining the population relatively stable until 13 days after inoculation and exerting plant-growth promotion effect. Although, for some experiments, the plant-growth promotion effect was not evident during the period analyzed, this effect is frequently observed in later development stages of the plants (41) (Kloepper 1980). Couillerot et al. (17) (Couillerot and Bouffaud 2010) also observed a high number of bacteria (10^5 to 10^7 CFU/g of maize root), either by qPCR or plate counting, 1 to 3 days after inoculation of *A. brasilesne* UAP-154 and CFN-535 inoculated in maize.

In conclusion, five strain-specific primer pairs, AzoR2.1, AzoR2.2, AzoR5.1, AzoR5.2 and AzoR5.3, for *A. brasilense* strain FP2 were successfully designed and tested to monitor the population fluctuation of this strain after inoculation to wheat roots under sterile and non-sterile conditions. We demonstrated, that *A. brasilense* FP2 maintained a high number of cells in association with plant roots within 2 weeks after inoculation. Thus, in our work we showed that the strain-specific primer pairs designed by using available genome sequence information could be effectively applied to quantitatively monitor population of a PGPB in the rhizosphere of the inoculated plants. The described design strategy of strain-specific primers is theoretically possible for any microorganism with available whole genome sequence in the database.

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The qPCR methodology developed in this work is a generally applicable tool to monitor the population dynamics of inoculated bacteria in crop plants with potential application in field experiments. Furthermore, this technique could also be applied for the quality control of commercially available inoculants, where rigid controls of contamination and number of inoculant cells have to guarantee the efficiency of the final product.

Acknowledgments

We are grateful to Roseli Prado, Valter Baura and Marilza Lamour for technical assistance. This work was supported by CNPq, INCT da Fixação de Nitrogênio/MCT, Fundação Araucária and CAPES.

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Figure 1. Specificity of primer pairs designed to amplify *Azospirillum brasilense* FP2. Lanes correspond to: (L) DNA Ladder, (1) *A. brasilense* FP2, (2) *A. brasilense* NH, (3) *A. brasilense* SP7, (4) *A. brasilense* Sp245, (5) *A. lipoferum* DSM 1691, (6) *A. rugosum* DSM 19657, (7) *A. canadense* LMG 23617, (8) *A. amazonense* DSM 2787, (9) *A. irakense* DSM 1158a, (10) *Roseomonas* genomospecies 6 CCUG 33010, (11) *Roseomonas fauriae* KACC 11694, and (12) negative control (no template DNA). The 16S rRNA coding gene primer pair was used as positive amplification control. Primer pairs AzoR2.1, AzoR2.2, AzoR5.1, AzoR5.2, and AzoR5.3 produced amplicons only when *A. brasilense* FP2 DNA was used as template and were considered strain-specific primers; primer pair AzoR6.1 produced cross-species amplicons and was not able to amplify all *A. brasilense* strains tested (i. e., no amplification for strain Sp7 was observed), being discarded for further analyses.

Figure 2. Enumeration *Azospirillum brasilense* FP2 in inoculated wheat roots under sterile conditions. **(A)** The species-specific primer pair Azo-2 was used for *A. brasilense* enumeration and strain-specific primer pairs AzoR2.1, AzoR2.2, and AzoR5.3 were used for *A. brasilense* FP2 enumeration. No statistical difference was observed. **(B)** Comparison of *A. brasilense* FP2 enumeration by qPCR and plate counting methods. Values for qPCR are mean of three experiments using strain-specific primer pairs. Asterisks indicate statistical difference ($p<0.01$). **(C-D)** *A. brasilense* FP2 plant growth promotion effect observed for root fresh weight and shoots, respectively. Asterisks indicate statistical difference ($p<0.01$).

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Figure 3. Enumeration of *Azospirillum brasilense* FP2 associated with wheat roots under non-sterile conditions by qPCR. **(A)** The species-specific primer pair Azo-2 was used for *A. brasilense*; strain-specific primer pairs AzoR2.1, AzoR2.2, and AzoR5.3 were used for the *A. brasilense* FP2; and primer pair for 16S rRNA coding gene was used for total bacteria. No statistical difference was observed. **(B)** Quantification of *A. brasilense* FP2 associated with wheat roots by qPCR and plate counting methods. Values for qPCR are mean of three strain-specific primer pairs. Asterisks indicate statistical difference ($p<0.01$).

Figure 4. Quantification of *Azospirillum brasilense* FP2 associated with wheat roots under non-sterile conditions co-inoculated with *Azospirillum brasilense* NH, *Herbaspirillum seropedicae* Z67, *Gluconacetobacter diazotrophicus* DSM 5601 and *Azospirillum lipoferum* DSM 1691 by qPCR method. **(A)** The species-specific primer pair Azo-2 was used for *A. brasilense* quantification; strain-specific primer pairs AzoR2.1, AzoR2.2, and AzoR5.3 were used for *A. brasilense* FP2 quantification; and primer pair for 16S rRNA coding gene was used for the total bacteria quantification. For each day, different letters indicate statistical difference at $p<0.01$. **(B)** Quantification of *Azospirillum brasilense* FP2 associated with wheat roots by qPCR and plate counts methods. Values for qPCR are mean of three strain-specific primer pairs. Asterisks indicate statistical difference ($p<0.01$).

698 *Table 1 Bacteria strains used in this study.*

Microorganism	Reference
Azospirillum amazonense DSM 2787	Helmholtz Zentrum München, strain collection
Azospirillum brasilense FP2	Pedrosa andYates, 1984
Azospirillum brasilense NH	Helmholtz Zentrum München, strain collection
Azospirillum brasilense Sp245	Helmholtz Zentrum München, strain collection
Azospirillum brasilense Sp7	Helmholtz Zentrum München, strain collection
Azospirillum canadense LMG 23617	Helmholtz Zentrum München, strain collection
Azospirillum irakense DSM 11586a	Helmholtz Zentrum München, strain collection
Azospirillum lipoferum DSM 1691	Helmholtz Zentrum München, strain collection
Azospirillum rugosum DSM 19657	Helmholtz Zentrum München, strain collection
Burkholderia brasiliensis M171	Helmholtz Zentrum München, strain collection
Burkholderia tropica PPe5	HelmholtzZentrumMünchen, strain collection
Gluconacetobacter diazotrophicus DSM 5601	Helmholtz Zentrum München,

	strain collection
Roseomonas genomospecies 6 CCUG 33010	Helmholtz Zentrum München, strain collection
Roseomonas fauriae KACC 11694	Helmholtz Zentrum München, strain collection

699 *Table 2 Primer characteristics and qPCR evaluated parameters.*

Primer	Sequence[a]	Length (mers)	GC%	R^2	Slope	Efficiency[b]	
						(E)	(%E)
16S rRNA gene[c]	F: TCGCTAGTAATCGCGGATCA	20	50%	0.9995	3.3	2.01	101.3
	R: TGTGACGGGCGGTGTGTA	18	61%				
Azo-2	F: GCGCGGGAAGTCCTGAAT	18	61%	0.9934	3.4	1.97	96.8
	R: CCCTTCACCATCCAGTCGAT	20	55%				
AzoR2.1	F: CGCCACCATGCGATCAA	17	59%	0.9980	3.3	2.01	101.3
	R: GCATGCCCAGTACTGCAAGTC	21	57%				
AzoR2.2	F: CCTTCACCTGGACGGTTCAG	20	60%	0.9982	3.5	1.94	94.0
	R: CGCGGCCAGCAGACTT	16	69%				
AzoR5.1	F: GATCACTGGACTCGGCTGTCA	21	57%	0.9977	3.7	1.88	87.6
	R: ATCGACCGTTCTCAGCGTCTA	21	52%				
AzoR5.2	F: TCACTGGACTCGGCTGTCAA	20	55%	0.9996	3.6	1.89	88.8
	R: ATATCGACCGTTCTCAGCGTCTA	23	48%				
AzoR5.3	F: AATTCTTTCCGTTGGCTTTCAA	22	36%	0.9995	3.4	1.97	96.8
	R: GCTTGCCGACCGGAGTATC	19	63%				

[a]F, forward primer; R, reverse primer; [b]the efficiency was calculated using the equation $E = 10^{-1/slope} - 1$ and %E = (L – 1) * 100; [c]Primer "F" bind region from 1,267 to 1,286 bp and primer "R" bind region from 1,319 to 1,336 bp of 16S rRNA gene sequence from *Azospirillum brasilense* Sp7 (GenBank accession number X79739).

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