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1 Quantification of Azospirillum brasilense FP2 in wheat roots

- by strain-specific qPCR
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16 Abstract

- 17 Azospirillum is a rhizobacterial genus containing plant growth-promoting
- 18 species associated with different crops worldwide. Azospirillum brasilense
- 19 strains exhibit a growth-promoting effect by means of phytohormone
- 20 production and possibly by N₂ fixation. However, one of the most important
- 21 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.
- 23 The objective of this study was to develop quantitative PCR protocols for

24 strain-specific quantification of A. brasilense FP2. A novel approach was applied to identify strain-specific DNA sequences based on comparison of 25 genomic sequences within the same species. The draft-genome sequence of 26 A. brasilense FP2 and Sp245 were aligned, FP2-specific regions were filtered 27 and checked for other possible matches in public databases, Strain-specific 28 29 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 30 strain-specific for A. brasilense FP2. These primer pairs were used to monitor 31 guantitatively the population of *A. brasilense* in wheat roots under sterile and 32 non-sterile growth conditions. In addition, co-inoculations with other plant-33 34 growth promoting bacteria in wheat were performed under non-sterile conditions. Results showed that A. brasilense FP2 inoculated to wheat roots 35 is highly competitive and achieve high cell numbers (~10⁷ CFU/g of fresh 36 weight root) in the rhizosphere even under non-sterile conditions and when 37 co-inoculated with other rhizobacteria, maintaining the population rather 38 39 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. 40

41 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 53 Brazil, in particular, has increased in recent years (7) (Castro-Sowinski 2007). 54 55 The strain FP2 is a spontaneous mutant of A. brasilense Sp7 (8) (Pedrosa 56 and Yates, 1984). Strain Sp7 has been shown to be capable of stimulating 57 growth of several Poaceae and increasing productivity capable of wheat and maize crops (2) (Fibach-Paldi 2012). Strain FP2 can also promote growth of 58 wheat (9) (Camilios et al., 2014) enhance maize and wheat productivity under 59 field conditions (unpublished). Most of the A. brasilense inoculants in Brazil 60 contains strains Ab-V5 and Ab-V6, also a derivative of Sp7 strain, which were 61 shown to increase productivity of maize and wheat under field conditions (10) 62 (Hungria 2010) and were officially authorized for use as inoculants in these 63 64 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).

70 To assess the diversity and taxonomy of crop plant-associated bacteria, many

- cultivation dependent and independent methods are currently in use (14–16)
- 72 (Magnani 2013; Magnani 2010; Pisa, 2011). However, most of these methods

73 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 74 not specific enough to study the bacterial population dynamics at strain level, 75 76 necessary for inoculant monitoring. Thus, in many cases, it is not possible to associate plant-growth promotion failure or success to the inoculated bacterial 77 78 population quantitatively at strain-specific resolution, leaving the outcome of the inoculation unexplained (17) (Couillerot 2010). Furthermore, crop 79 response to inoculation under field conditions depends heavily on a 80 81 combination of the plant genotype and the bacterial strain (18–20) (Sánches 2014; Araújo 2013; Sasaki 2010) stressing the need of methodologies to 82 83 evaluate the success of plant colonization accurately at high resolution. Previously, we used whole cell matrix-assisted laser desorption ionization 84 time-of-flight mass spectrometry (MALDI-TOF MS) analysis to differentiate 85 species of Azospirillum including several closely related A. brasilense strains 86 (21) (Stets 2013). However, this method is not quantitative, requires growth 87 on a culture medium and is time and labor intensive. 88 Quantitative PCR (gPCR) has been the method of choice to quantify 89 90 rhizosphere populations because it allows high specificity, sensitivity and 91 speed (17, 22, 23) (Couillerot and Bouffaud 2010; Couillerot and Poirier 2010; Sorensen 2009). This technique has been successfully used to quantify 92 several bacteria associated with plants. It was successfully used for the 93 94 quantification of a functionally specific subgroup of Pseudomonads in the rhizosphere (24) (Mavrodi 2007). The pathogen Xylella fastidiosa was 95 quantified in citrus plants (25) (Pinheiro 2002), while the endophytic bacterium 96 Methylobacterium mesophilicum was monitored by qPCR during the 97

98 Catharanthus roseus colonization (26) (Lacava 2006). In Brassica oleracea, the plant growth promoting Enterobacter radicincitans population was 99 monitored by qPCR associated with fluorescence in situ hybridization (FISH) 100 (27) (Ruppel 2006), determining not only the amount of bacteria in the 101 colonized plants but also their location in the plant. Although these reports 102 103 showed qPCR as a valuable technique to quantitatively monitor populations of 104 unlabeled bacteria in green-house experiments, none had used strain-specific primers. The application of strain-specific primers is difficult in field 105 106 experiments, where closely related indigenous bacteria may interfere in the 107 amplification and quantification. For a strain-specific molecular monitoring, 108 sequence characterized amplified region (SCAR) markers obtained from BOX-, ERIC-, and RAPD-PCR fragments were recently applied to design 109 primers for a gPCR quantification of A. brasilense and A. lipoferum at strain-110 111 specific level(17, 22) (Couillerot and Bouffaud 2010; Couillerot and Poirier 112 2010). The objective of this study was to develop qPCR protocols for a strain-specific 113 guantification of the plant-growth promoting bacterium A. brasilense FP2 114 115 based on whole genome comparison with the closely related strain Sp245. 116 The designed strain-specific primers were then applied for quantification to

- 117 monitor the FP2-population in inoculated wheat plants under sterile and non-
- 118 sterile conditions.

AEN

119 Material and Methods

120 Bacterial strains

- 121 All Azospirillum strains (Table 1) were routinely grown in NFbHPN medium
- 122 (28) (Machado 1991) at 30 °C under aeration by shaking at 120 rpm. Strains
- from other genera were grown in DYGS medium (29) (Rodrigues 1986)
- 124 containing, per 1,000 mL: 0.10% glucose, 0.20% yeast extract, 0.15%
- 125 peptone, 0.50% MgSO₄.7H₂O, and 0.15% L-glutamic acid at pH 6.0-6.5;
- 126 cultures were incubated at 30 °C under aeration by shaking at 120 rpm.
- 127 Colony counts of all strains were performed after spreading dilutions on the
- respective medium plates and incubation for 72 h at 30 °C.

129 **Primer design**

- 130 To design *Azospirillum brasilense* FP2 strain-specific primer pairs, the general
- 131 strategy was used: (i) the Whole Genome Sequence (WGS) of *A. brasilense*
- 132 FP2 was fragmented *in silico* from the FASTA genome sequence using in
- house scripts, producing 500 bp non-overlapping fragments; (ii) the genome
- 134 sequence of *A. brasilense* Sp245 was used to build a local BLAST database
- 135 and A. brasilense FP2 fragment sequences were used as queries for a
- 136 BLASTn similarity search, with default parameters; (iii) fragments with no hits
- 137 found were subjected to a second BLASTn (30) (Altschul 1997) search
- against NCBI-nt database (performed at July, 2012; GenBank release 190),
- 139 using default parameters; (iv) putative strain-specific sequences, i.e.
- 140 sequences without any match in the two BLAST sequence analyses, were
- 141 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

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- annotated and visually analyzed using RAST, version 2.0 (31, 32) (Overbeek
- 144 2014; Aziz 2008) and Unipro UGENE, version 1.14 (33) (Okonechnikov
- 145 2012).
- 146 The WGS of Azospirillum brasilense FP2 is publicly available at NCBI
- 147 database under accession number APHV00000000 and assembly
- 148 GCA_000404045.1, with total sequence length of 6,885,108 bp, 413 contigs
- 149 (N50 of 29,432 bp), GC content of 68.1 %, and genome coverage of 25X. The
- 150 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
- 153 7,530,241 bp (total assembly gap length of 6,000 bp), 67 contigs (N50
- 154 186,382 bp), and GC content of 68.6 %.
- 155 Primer design was performed using Primer Express 3.0 (Applied Biosystems,
- 156 Foster City, CA) based on (i) an amplicon size inferior to 200 bp and primers
- 157 lengths ranging from 18 to 22 bp; (ii) high melting temperature of primers (Tm
- approximately 60 °C) and low Tm difference between primers (Δ Tm< 2 °C);
- and (iii) lack of predicted hairpin loops, duplexes and primer-dimer formation.

160 **Primer selection and evaluation**

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

°C, followed by 25 cycles consisting of denaturation at 95 °C for 15 s, 167 annealing at 60 °C for 60 s followed by 72 °C for 30 s and final elongation of 168 10 min at 70 °C. A primer pair was considered strain-specific if: (i) successful 169 amplification occurred using DNA of the target strain as template; (ii) the 170 absence of cross-amplification with non-target strains; and (iii) the absence of 171 172 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 173 templates for PCR reactions. A second step was performed under quantitative 174 PCR conditions to check primer specificity (melting curves) and amplification 175 efficiency, as described below. 176

Quantitative PCR conditions

Quantitative PCR (gPCR) was performed in a total reaction volume of 25 µL 178 containing 12.5 µL Power SYBR® Green PCR Master Mix (Applied 179 Biosystems), 6.25 µL of a primer mix (final concentration of 1 µmol) and 6.25 180 µL of 2.5 ng/µL diluted template DNA. MicroAmp Optical 96-Well Reaction 181 182 Plate (Applied Biosystems) and an ABI Prism 7500 (Applied Biosystems) were used. The cycling programme included a 10 min incubation at 95 °C 183 followed by 40 cycles consisting of 95 °C for 15 s, 60 °C for 60 s followed by 184 185 72 °C for 30 s and additional 72 °C for 10 min. Amplification specificity was 186 verified by melting curve analysis of the PCR products performed using the ABI Prism 7500 Sequence Detection version 1.2.3 software (Applied 187 Biosystems). 188

189 **Primer efficiency determination**

190	Genomic DNA from A. brasilense FP2 was used to prepare ten-fold dilution
191	series (in triplicates). Sterile water was used as negative control. The cycle
192	threshold (Ct), was automatically determined for each sample by the ABI
193	Prism 7500 Sequence Detection version 1.2.3 (Applied Biosystems) software.
194	A standard curve was generated by plotting the Ct number against the
195	logarithm of bacterial DNA concentration (data not shown) and used to
196	calculate the amplification efficiency (E) (Table 2).
197	Generation of standard curves for qPCR quantification of <i>A</i> .
198	brasilense FP2 in wheat roots
199	The standard curves for quantification of A. brasilense FP2 in wheat was
200	constructed according to (22) (Couillerot and Poirier 2010), with the following
201	modifications. Wheat plantlets were grown under axenic condition as
202	described below for 7 days, roots were collected and crushed using mortar
203	and pestle in liquid nitrogen. A volume of 100 μ L of A. brasilense FP2 culture
204	
204	(dilutions from 10 ² to 10 ⁹ CFU) was added to 100 mg of crushed roots, mixed
204	(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

- 207 manufacturer's instructions; qPCR was performed as described above. The
- 208 standard curve was generated by plotting Ct number versus CFU added per
- 209 tube. No bacteria were added to the negative control.

210 **DNA preparation**

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

215 gPCR quantification of Azospirillum brasilense FP2 on the

216 wheat roots

217 For the sterile experiments, seeds of wheat (*Triticum aestivum*), cultivar

218 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 °C and

- humidity above 50%.
- 224 For the experiments under non-sterile conditions, seeds were germinated as
- 225 previously described without surface sterilization, Commercial gardener soil
- 226 (type ED-73, Bayerische Gärtnereigenossenschaft) was suspended in
- 227 Hoagland medium at a final concentration of 1% (w/v), filtered and this
- suspension was used as inoculum in glass tubes containing quartz beads.
- 229 The negative control consisted of non-inoculated plants. Different experiments
- 230 were conducted to evaluate plants inoculated with A. brasilense FP2 or co-
- 231 inoculated in the presence of other wheat-associated diazotrophs (in the
- same amount), namely A. brasilense NH, H. seropedicae Z67, G.
- 233 diazotrophicus DSM 5601 and A. lipoferum DSM 1691. The control consisted

234 of A. brasilense FP2 inoculated plants. All microorganisms were grown until

- about 10⁹ CFU/mL and the cells were washed once with 1X PBS buffer
- 236 (Applichem, Denmark). In all experiments, approximately 10⁷ CFU/plant were
- 237 inoculated in the plant growing medium and incubated for 14 days. The

238 experiments were performed in biological and technical triplicates and

samples were collected every 2 days.

240 Determination of Colony Forming Units (CFU)

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

244 Experimental design and statistical analysis

- 245 Experiments in a growth chamber followed a randomized block design.
- 246 Colony counts were expressed as CFU per g of fresh root, and qPCR
- 247 quantification data was converted to CFU equivalents per g of fresh root. The
- 248 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
- 250 Análise Estatísticas, Universidade Federal de Viçosa, Viçosa, Brazil).

251 **Results**

252 **Primer design and evaluation of amplification efficiency**

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

256 genome sequence available so far, using the procedures detailed in Material and Methods section. The genome sequence comparison was based on 257 BLAST analysis of 500bp sequence fragments of A. brasilense FP2 against 258 259 genome sequence of A. brasilense Sp245 local database, in a first round, and against NCBInt database, in a second round. Although this analysis is 260 261 database-dependent and do not guarantee to select strain-specific genomic regions, in practice, the genome comparison between two very closely related 262 strains (i. e., with very high genomic synteny) allows for the selection of 263 264 genomic regions not likely to match sequences of more distantly related organism, as shown by BLAST results against a comprehensive database. 265 266 Sequences with no hits found in BLAST analysis against Sp245 genome sequence did not show significant hits against NCBInt database too. Using 267 this methodology, six coding and intergenic regions from A. brasilense FP2 268 genome were selected and a total of 10 primer pairs were designed and 269 270 tested for cross-amplification against 13 different bacterial DNAs, including 271 four A. brasilense strains, six other Azospirillum spp. and two Roseomonas spp. strains (Figure 1 shows the most relevant primer pairs). 272 273 Five out of ten primer pairs were strain-specific for A. brasilense FP2, namely 274 AzoR2.1, AzoR2.2, AzoR5.1, AzoR5.2, and AzoR5.3 (Table 2). For one of the 275 primer pairs, Azo-2, amplicons were generated for all four strains of Azospirillum brasilense tested (FP2, NH, Sp245, and Sp7), but no 276 277 amplification was observed for Roseomonas genomospecies 6 CCUG 33010, Roseomonas fauriae KACC 11694, Burkholderia tropica Ppe5, and 278 279 Burkholderia brasilense M171 (data not shown.

280 Genome sequences from strains FP2 and Sp245 of A. brasilense share high degree of synteny, however strain-specific primer pairs were designed from 281 two FP2 contig sequences that did not aligned along the chromosome or any 282 plasmid sequences from Sp245 strain (Supplementary data 1A). On the 283 contrary, the primer pair Azo-2 was designed from a contig sequence of FP2 284 285 that aligns to Sp245 chromosome sequence (Supplementary data 1B), although the alignment in the region of primer binding did not show perfect 286 match (data not shown). Automatic annotation of A. brasilense FP2 draft 287 288 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 289 290 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 291 AzoR2.2) or fall into a CDS for TniQ domain containing protein (AzoR5.1, 292 293 AzoR5.2, and AzoR5.3; Supplementary data 2). Interestingly, the regions 294 surrounding amplicons for strain-specific primers contain some CDSs related to phage and mobile elements. 295 The efficiency of all strain-specific primer pairs obtained in this study was 296 297 tested by constructing a standard curve with increasing concentrations of A. 298 brasilense FP2 DNA (Table 2). The primers AzoR5.1 and AzoR5.2 were 299 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.

301 **qPCR quantification of** *Azospirillum brasilense* **FP2 on wheat**

302 **roots**

303 In order to test the ability of the strain-specific primer pairs to quantify A.

304 *brasilense* FP2 in the rhizosphere, a growth chamber experiment was

305 conducted with wheat plants inoculated with A. brasilense FP2 under sterile

306 and non-sterile conditions.

307 To monitor the population of A. brasilense FP2 in wheat roots, three strain-

308 specific primer pairs with the highest amplification efficiency (AzoR2.1,

309 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

311 gene targeted primer pair (Doumit Camilios Neto, personal communication)

312 was used for total bacteria quantification (Table 2).

313 Initially, a standard curve was constructed from a fixed amount of crushed 314 plant-root tissues mixed with each serially diluted total DNA of A. brasilense 315 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 316 317 Bouffaud 2010) that the presence of root extract decreased the detection limit 318 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 319 of the final standard curve, thereby making quantification closer to reality. The 320 equation for the qPCR-quantification standard curve was used to estimate the 321 322 amount of bacteria in wheat roots inoculated with A. brasilense FP2. The detection limit of the technique was 10⁴ CFU/g of wheat root (Supplementary 323 data 3). In the first attempt to monitor population of A. brasilense FP2, wheat 324

325 was inoculated and cultivated under sterile conditions. In non-inoculated plants, strain A. brasilense FP2 or any other bacteria were not detected by 326 qPCR or plate counting techniques. Figure 2A shows the results of bacteria 327 number in wheat inoculated under sterile conditions using primer pair Azo-2 328 (specific for Azospirillum spp.) and the primer pairs AzoR2.1, AzoR2.2 and 329 330 AzoR5.3 (specific for strain FP2). There was no statistical difference between the measurements using the three strain-specific primer pairs or between 331 species and strain-specific primer pairs. A large number of bacteria was 332 observed in the first days after inoculation (roughly 10⁷ to 10⁸ CFU/g of wheat 333 root; Figure 2B). The A. brasilense FP2 quantification was also analyzed by 334 335 plate counting method in NFbHPN medium. Higher variability was observed for plate counting method in the first days. However, no statistical differences 336 were observed in sampling points by the cell quantification using either, qPCR 337 or plate counting methods, from day 7 (Figure 2B). The results also revealed 338 339 an increase in fresh weight of roots and shoots of plants inoculated with A. 340 brasilense FP2 (Figures 2C-D). This stimulation due to inoculation was most evident in the roots. In the second attempt, wheat was inoculated and 341 342 cultivated under non-sterile conditions. The results showed no statistical difference for quantification of A. brasilense FP2 by gPCR methodology using 343 three different strain-specific primer pairs (AzoR2.1, AzoR2.2 and AzoR5.3). 344 345 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 346 primer pair for 16S rRNA coding genes (estimate the total number of bacteria) 347 showed higher numbers of cells per gram of wheat roots, although statistical 348 349 differences were not achieved for all sampling points. Except at day 1, the

350 differences in cell numbers comparing universal primer pair for 16S rRNA coding genes and species-/strains-specific primer pairs were 2-5 fold (Figure 351 3A). No statistical differences were also observed for the most sampling 352 points when comparing cell counting techniques, althoug plate counting has 353 shown higher variability (Figure 3B). These results suggests the population of 354 355 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 356 rhizosphere of wheat plants and reflects at rather low diversity of bacteria in 357 the soil used for cultivation. The number of CFU in the soil was evaluated by 358 plate counting using DYGS media, reaching values of 10³ to 10⁴ CFU and 359 360 confirmed the occurrence of a low diversity in the soil used for cultivation of wheat and inoculation experiments. 361 362 The population of A. brasilense FP2 was stable, even when the rhizobacteria Azospirillum brasilense NH, Herbaspirillum seropedicae Z67, 363 Gluconacetobacter diazotrophicus DSM 5601 and Azospirillum lipoferum 364 DSM 1691 were co-inoculated in wheat plants in non-sterile conditions, 365 leaving the FP2 counts above 10⁷ CFU/g of root fresh weight. No statistical 366 367 difference in the A. brasilense FP2 numbers were achieved for gPCR quantification comparing strain-specific primer pairs and the primer pair Azo-2 368 (Figure 4A) or for the most sampling points comparing results from qPCR and 369 plate count techniques (Figure 4B). The differences in cell numbers 370

371 comparing universal primer pair for 16S rRNA coding genes and species-

specific primer pairs ranged from 2.2×10^9 (3 days after inoculation) to 3.9×10^7

373 (13 days after inoculation; Figure 4A). However, the number of total bacteria,

including the other inoculants was significantly higher until 7 days after

375 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. 376 brasilense FP2 maintain a stable population in the rhizosphere/roots of the 377 plants during the period of colonization and further indicate that strain FP2 is 378 highly competitive, a desirable characteristic for inoculant production. When 379 380 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 381 rhizobacteria strains under non-sterile conditions, there were no amplification 382 product, confirming the primer specificity for the detection of A. brasilense 383 FP2 (data no shown). 384

Taken together, results from all inoculation experiments shows that the number of *A. brasilense* FP2 cells was stable and not below 10⁷ 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).

390 **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.

398 A nested PCR method was proposed for the detection of Azospirillum lipoferum CRT1 in the rhizosphere by Baudoin et al. (34) (Baudoin and 399 Couillerot 2010). However, the primers, designed from 16S and 23S rRNA 400 401 intergenic region coding genes fragments, proved not to be specific enough to develop strain-specific qPCR quantification. Several optimizations regarding 402 403 specificity and efficiency were then applied to design strain-specific qPCRprimers to detect bacterial strains based on sequence characterized amplified 404 regions (SCAR) markers (17, 34) (Couillerot and Bouffaud 2010; Baudoin and 405 406 Couillerot 2010). In this study, we developed a strain-specific qPCR protocol to guantify the PGPB Azospirillum brasilense strain FP2 inoculated to roots of 407 408 wheat plants based on comparative genome analysis. To achieve this goal, we designed strain-specific primers by in silico comparison of 500 pb 409 fragments of a draft A. brasilense FP2 genome with the A. brasilense Sp245 410 411 genome. Unique FP2 strain fragments were also used to search NCBI non-412 redundant database for similarity. The strain-specific fragments identified so 413 far were used for the primer design. Many authors have reported different methods to design taxon-specific primers, usually based on experimental 414 415 approaches. Konstantinov et al. (35) (Konstantinov 2005) isolated specific 416 genomic fragments from the type strain and related strains by digesting the genomic DNA with restriction enzyme and then make a subtractive 417 418 hybridization with closest strains to eliminate shared DNA fragments. The 419 unique fragments were extracted from the gel, cloned, sequenced and used to design specific primers to detect Lactobacillus sobrius 001T. Fujimoto et al. 420 (36) (Fujimoto 2008) and Maruo et al. (37) (Mauro 2006) developed a PCR-421 422 based method for the identification and quantification of Lactobacillus casei

423 strain Shirota and Lactobacillus lactis subsp. cremoris FC, respectively, using strain-specific primers derived from RAPD analysis. The authors evaluated 424 the survival of these strains through the gastrointestinal tract to monitor the 425 426 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 427 428 2014) developed a qPCR method for quantification of PGPB H. seropedicae in the rhizosphere of maize seedlings. Primer pairs were designed from 429 genome sequence of H. seropedicae SmR1 (39) (Pedrosa and Monteiro 430 431 2011) and tested against 12 different species. Although the selected genome regions did not match with any other sequences in NCBI database, the 432 433 primers were not evaluated against other H. seropedicae strains, not allowing any conclusion about their strain-specificity. In the last decade, whole-genome 434 sequencing has become a rapid and cost effective way to provide 435 comprehensive information about an organism (40) (Havlak 2004). Although 436 437 the achievement of a complete genome is a demanding process, the draft 438 genome sequencing can be obtained with a high breadth of coverage. In the present work, we have shown that the direct comparison of genomic 439 440 sequence of closely related organisms is a rapid and reliable approach to 441 detect specific DNA regions to be used as strain-specific genetic markers for the use of strain-specific quantitative detection of bacteria colonizing roots 442 443 and the rhizosphere. The rationale of this approach relies on three facts: (i) genome sequence provides the genetic information to a highest resolution; (ii) 444 divergent regions between two genome sequences of very close organisms (i. 445 e., strains of the same species) are most likely to diverge from more distant 446 447 taxa; and (iii) the absence of sequence similarity between the possible

448 genomic strain-specific regions and sequences in large public databases covering most of the taxa from different environments can be broadly 449 accepted as the absence of these regions in other organisms. 450 451 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 452 the roots of the plant at 10⁷ to 10⁸ CFU/g of root in the first days after 453 inoculation, maintaining the population relatively stable until 13 days after 454 455 inoculation and exerting plant-growth promotion effect. Although, for some 456 experiments, the plant-growth promotion effect was not evident during the 457 period analyzed, this effect is frequently observed in later development stages of the plants (41) (Kloepper 1980). Couillerot et al. (17) (Couillerot and 458 Bouffaud 2010) also observed a high number of bacteria (10⁵ to 10⁷ CFU/g of 459 460 maize root), either by gPCR or plate counting, 1 to 3 days after inoculation of A. brasilesne UAP-154 and CFN-535 inoculated in maize. 461 462 In conclusion, five strain-specific primer pairs, AzoR2.1, AzoR2.2, AzoR5.1, 463 AzoR5.2 and AzoR5.3, for A. brasilense strain FP2 were successfully 464 designed and tested to monitor the population fluctuation of this strain after inoculation to wheat roots under sterile and non-sterile conditions. We 465 demonstrated, that A. brasilense FP2 maintained a high number of cells in 466 association with plant roots within 2 weeks after inoculation. Thus, in our work 467 468 we showed that the strain-specific primer pairs designed by using available genome sequence information could be effectively applied to quantitatively 469 470 monitor population of a PGPB in the rhizosphere of the inoculated plants. The described design strategy of strain-specific primers is theoretically possible for 471 472 any microorganism with available whole genome sequence in the database.

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.

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653	Figure 1. Specificity of primer pairs designed to amplify Azospirillum
654	brasilense FP2. Lanes correspond to: (L) DNA Ladder, (1) A. brasilense FP2,
655	(2) A. brasilense NH, (3) A. brasilense SP7, (4) A. brasilense Sp245, (5) A.
656	lipoferum DSM 1691, (6) A. rugosum DSM 19657, (7) A. canadense LMG
657	23617, (8) A. amazonense DSM 2787, (9) A. irakense DSM 1158a, (10)
658	Roseomonas genomospecies 6 CCUG 33010, (11) Roseomonas fauriae
659	KACC 11694, and (12) negative control (no template DNA). The 16S rRNA
660	coding gene primer pair was used as positive amplification control. Primer
661	pairs AzoR2.1, AzoR2.2, AzoR5.1, AzoR5.2, and AzoR5.3 produced
662	amplicons only when A. brasilense FP2 DNA was used as template and were
663	considered strain-specific primers; primer pair AzoR6.1 produced cross-
664	species amplicons and was not able to amplify all A. brasilense strains tested
665	(i. e., no amplification for strain Sp7 was observed), being discarded for
665 666	(i. e., no amplification for strain Sp7 was observed), being discarded for further analyses.
666	further analyses.
666 667	further analyses. Figure 2. Enumeration <i>Azospirillum brasilense</i> FP2 in inoculated wheat roots
666 667 668	further analyses. Figure 2. Enumeration <i>Azospirillum brasilense</i> FP2 in inoculated wheat roots under sterile conditions. (A) The species-specific primer pair Azo-2 was used
666 667 668 669	further analyses. Figure 2. Enumeration <i>Azospirillum brasilense</i> FP2 in inoculated wheat roots under sterile conditions. (A) The species-specific primer pair Azo-2 was used for <i>A. brasilense</i> enumeration and strain-specific primer pairs AzoR2.1,
666 667 668 669 670	further analyses. Figure 2. Enumeration <i>Azospirillum brasilense</i> FP2 in inoculated wheat roots under sterile conditions. (A) The species-specific primer pair Azo-2 was used for <i>A. brasilense</i> enumeration and strain-specific primer pairs AzoR2.1, AzoR2.2, and AzoR5.3 were used for <i>A. brasilense</i> FP2 enumeration. No
666 667 668 669 670 671	further analyses. Figure 2. Enumeration <i>Azospirillum brasilense</i> FP2 in inoculated wheat roots under sterile conditions. (A) The species-specific primer pair Azo-2 was used for <i>A. brasilense</i> enumeration and strain-specific primer pairs AzoR2.1, AzoR2.2, and AzoR5.3 were used for <i>A. brasilense</i> FP2 enumeration. No statistical difference was observed. (B) Comparison of <i>A. brasilense</i> FP2
666 667 668 669 670 671 672	further analyses. Figure 2. Enumeration <i>Azospirillum brasilense</i> FP2 in inoculated wheat roots under sterile conditions. (A) The species-specific primer pair Azo-2 was used for <i>A. brasilense</i> enumeration and strain-specific primer pairs AzoR2.1, AzoR2.2, and AzoR5.3 were used for <i>A. brasilense</i> FP2 enumeration. No statistical difference was observed. (B) Comparison of <i>A. brasilense</i> FP2 enumeration by qPCR and plate counting methods. Values for qPCR are
666 667 668 669 670 671 672 673	further analyses. Figure 2. Enumeration <i>Azospirillum brasilense</i> FP2 in inoculated wheat roots under sterile conditions. (A) The species-specific primer pair Azo-2 was used for <i>A. brasilense</i> enumeration and strain-specific primer pairs AzoR2.1, AzoR2.2, and AzoR5.3 were used for <i>A. brasilense</i> FP2 enumeration. No statistical difference was observed. (B) Comparison of <i>A. brasilense</i> FP2 enumeration by qPCR and plate counting methods. Values for qPCR are mean of three experiments using strain-specific primer pairs. Asterisks

677 Figure 3. Enumeration of Azospirillum brasilense FP2 associated with wheat roots under non-sterile conditions by qPCR. (A) The species-specific primer 678 pair Azo-2 was used for A. brasilense; strain-specific primer pairs AzoR2.1, 679 AzoR2.2, and AzoR5.3 were used for the A. brasilense FP2; and primer pair 680 for 16S rRNA coding gene was used for total bacteria. No statistical difference 681 682 was observed. (B) Quantification of A. brasilense FP2 associated with wheat roots by qPCR and plate counting methods. Values for qPCR are mean of 683 three strain-specific primer pairs. Asterisks indicate statistical difference 684 685 (p<0.01). Figure 4. Quantification of Azospirillum brasilense FP2 associated with wheat 686 roots under non-sterile conditions co-inoculated with Azospirillum brasilense 687 NH, Herbaspirillum seropedicae Z67, Gluconacetobacter diazotrophicus DSM 688 689 5601 and Azospirillum lipoferum DSM 1691 by qPCR method. (A) The species-specific primer pair Azo-2 was used for A. brasilense quantification; 690 strain-specific primer pairs AzoR2.1, AzoR2.2, and AzoR5.3 were used for A. 691 brasilense FP2 quantification; and primer pair for 16S rRNA coding gene was 692 used for the total bacteria quantification. For each day, different letters 693 694 indicate statistical difference at p<0.01. (B) Quantification of Azospirillum 695 brasilense FP2 associated with wheat roots by qPCR and plate counts methods. Values for qPCR are mean of three strain-specific primer pairs. 696

697 Asterisks indicate statistical difference (p<0.01).

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⁶⁹⁸ Table 1 Bacteria strains used in this study.

Microorganism	Reference
	Helmholtz Zentrum München,
Azospirillum amazonense DSM 2787	strain collection
Azospirillum brasilense FP2	Pedrosa andYates, 1984
	Helmholtz Zentrum München,
Azospirillum brasilense NH	strain collection
Azoopirillum brooilopoo Sp245	Helmholtz Zentrum München,
Azospirillum brasilense Sp245	strain collection
Azospirillum brasilense Sp7	Helmholtz Zentrum München,
Azospiniiuni brasilense opr	strain collection
Azospirillum canadense LMG 23617	Helmholtz Zentrum München,
	strain collection
Azospirillum irakense DSM 11586a	Helmholtz Zentrum München,
Azospinium nakense bom 11000a	strain collection
Azospirillum lipoferum DSM 1691	Helmholtz Zentrum München,
	strain collection
Azospirillum rugosum DSM 19657	Helmholtz Zentrum München,
Azospinium rugosum Dom 19001	strain collection
Burkholderia brasiliensis M171	Helmholtz Zentrum München,
	strain collection
Burkholderia tropica PPe5	Helmholtz ZentrumMünchen,
שנותוטועכוומ נוטאונמ דרכט	strain collection
Gluconacetobacter diazotrophicus DSM 5601	Helmholtz Zentrum München,

	strain collection
Roseomonas genomospecies 6 CCUG 33010	Helmholtz Zentrum München,
Roseomonas genomospecies o CCOG 550 To	strain collection
Roseomonas fauriae KACC 11694	Helmholtz Zentrum München,
Roseomonas launae NACC 11094	strain collection

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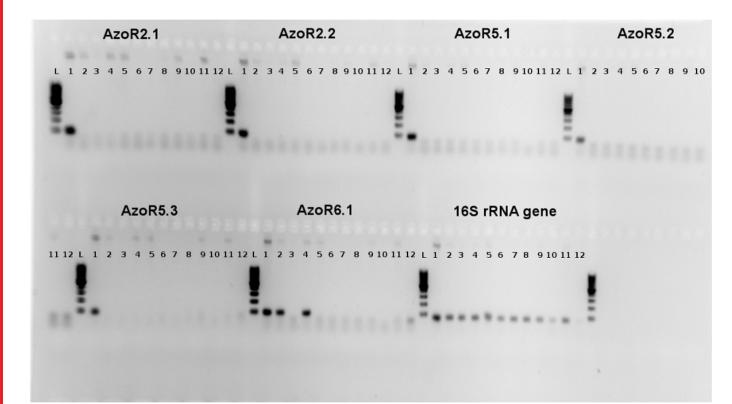
699 7	Table 2 Primer characteristics and qPCR evaluated parameters.
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Primer	Sequence ^a	Length (mers)	GC%	R²	Slope	Efficiency ^b	
						(E)	(%E)
16S rRNA gene ^c	F: TCGCTAGTAATCGCGGATCA	20	50%	0.9995	3.3	2.01	101.3
	R: TGTGACGGGCGGTGTGTA	18	61%	0.9995	5.5	2.01	101.
	F: GCGCGGGAAGTCCTGAAT	18	61%	0.9934	3.4	1.97	96.8
Azo-2	R: CCCTTCACCATCCAGTCGAT	20	55%				
A . DO 4	F: CGCCACCATGCGATCAA	17	59%	0.9980	3.3	2.01	101.3
AzoR2.1	R: GCATGCCCAGTACTGCAAGTC	21	57%				
AzoR2.2	F: CCTTCACCTGGACGGTTCAG	20	60%	0.9982	3.5	1.94	94.0
AZORZ.Z	R: CGCGGCCAGCAGACTT	16	69%				
	F: GATCACTGGACTCGGCTGTCA	21	57%	0.9977	3.7	1.88	87.6
AzoR5.1	R: ATCGACCGTTCTCAGCGTCTA	21	52%				
A . DE 0	F: TCACTGGACTCGGCTGTCAA	20	55%	0.9996		1.89	88.8
AzoR5.2	R: ATATCGACCGTTCTCAGCGTCTA	23	48%		3.6		
	F: AATTCTTTCCGTTGGCTTTCAA	22	36%	0.0005		4.07	
AzoR5.3	R: GCTTGCCGACCGGAGTATC	19	63%	0.9995	3.4	1.97	96.8

Applied and Environmental Microbiology ^aF, forward primer; R, reverse primer; ^bthe efficiency was calculated using the equation $E = 10^{-1/slope} - 1$ and %E = (L - 1) * 100; ^cPrimer "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).

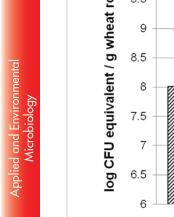
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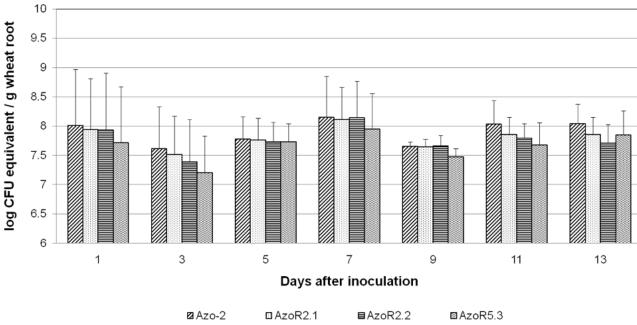
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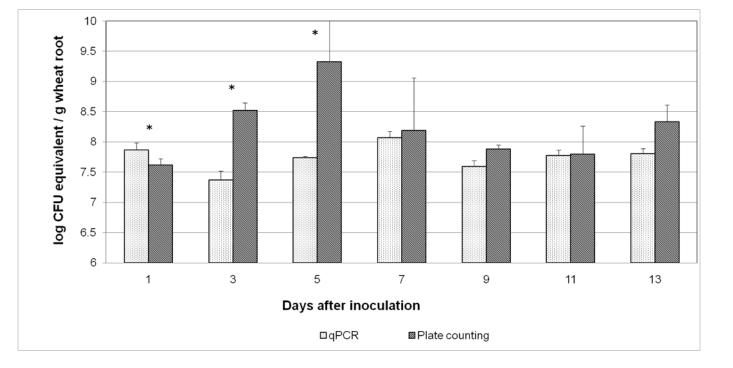
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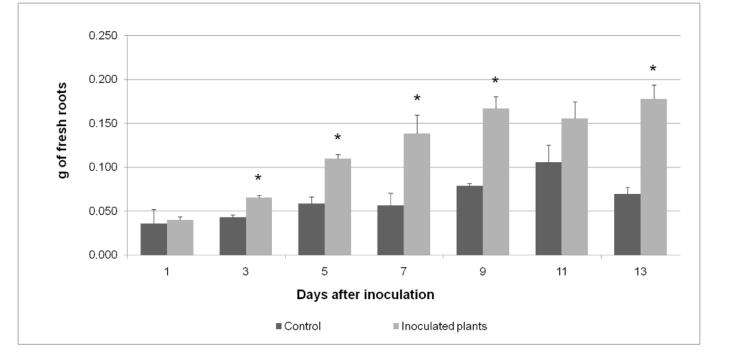
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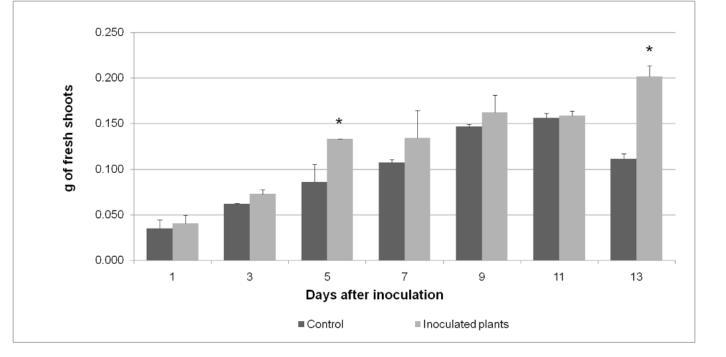




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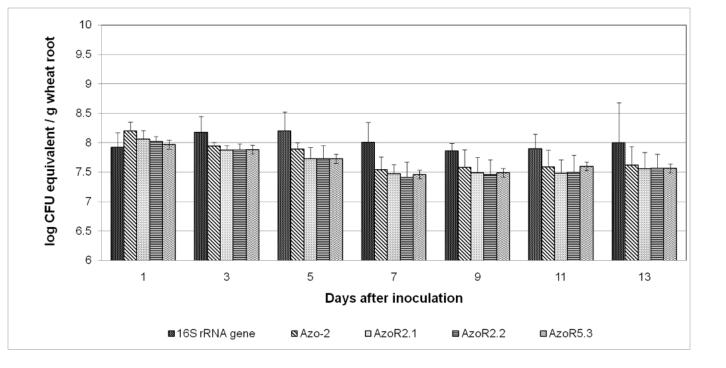






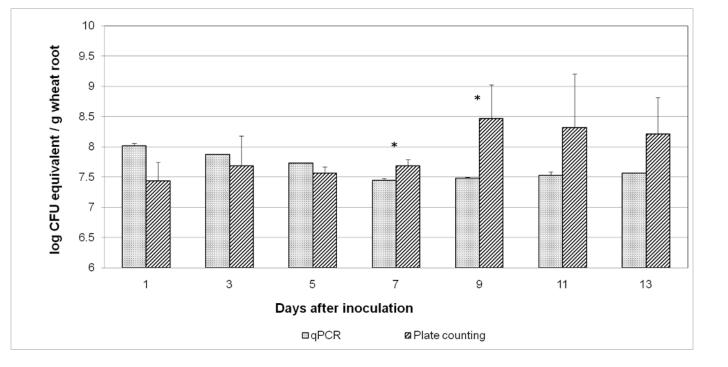
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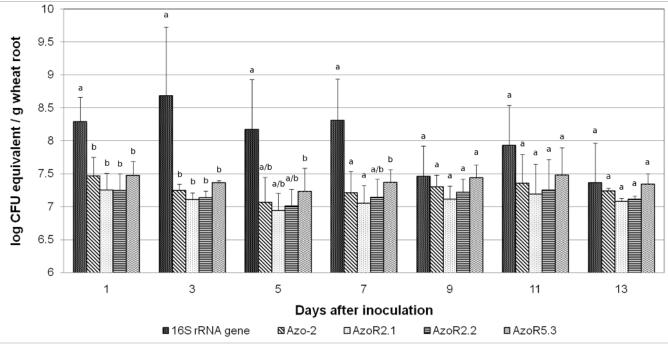
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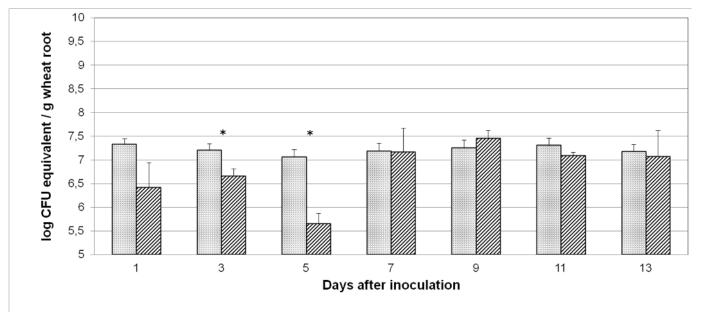
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