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PII: S0981-9428(17)30225-5

DOI: 10.1016/j.plaphy.2017.07.003

Reference: PLAPHY 4928

To appear in: *Plant Physiology and Biochemistry*

Received Date: 6 March 2017

Revised Date: 20 June 2017

Accepted Date: 3 July 2017

Please cite this article as: A.R. Schenberger Santos, R.M. Etto, R.W. Furmam, D. Leandro de Freitas, K. Freire d'Eça Nogueira Santos, E. Maltempi de Souza, Fá. de Oliveira Pedrosa, Ricardo.Antô. Ayub, M.B. Reynaud Steffens, C.W. Galvão, Labeled *Azospirillum brasilense* wild type and excretion-ammonium strains in association with barley roots, *Plant Physiology et Biochemistry* (2017), doi: 10.1016/j.plaphy.2017.07.003.

Labeled *Azospirillum brasilense* wild type and excretion-ammonium strains in association with barley roots

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Abstract

Soil bacteria colonization in plants is a complex process, which involves interaction between many bacterial characters and plant responses. In this work, we labeled *Azospirillum brasilense* FP2 (wild type) and HM053 (excretion-ammonium) strains by insertion of the reporter gene *gusA*-kanamycin into the dinitrogenase reductase coding gene, *nifH,* and evaluated bacteria colonization in barley (*Hordeum vulgare*). In addition, we determined inoculation effect based on growth promotion parameters. We report an uncommon endophytic behavior of *A. brasilense* Sp7 derivative inside the root hair cells of barley and highlight the promising use of *A. brasilense* HM053 as plant growth-promoting bacterium.

Key words

nifH fusion, biofilm, plant growth-promotion, nitrogen fixation.

1. Introduction

Plant and soil bacteria participate in several molecular signaling events that establish specific symbiotic, endophytic, or associative relationships. Such relationships differ according to plant genotypes, soil types, bacterial strains and abilities to improve plant growth (Philippot et al., 2013). *Azospirillum sp*. is one of the most studied genera of plant growth-promoting bacteria (PGPB) at present due its capacity to colonize many plant species (Cassán and Diaz-Zorita, 2016). Plant inoculation with strains of *Azospirillum brasilense* induces primary root elongation of economically important grasses, and improves plant growth and productivity. The plant growth-promotion by Azospirilla is mainly associated with its ability to produce and secrete phytohormones (indole-3-acetic acid, cytokinins, and gibberellins) and nitric oxide (Fibach-Paldi et al., 2012). However, recently, Pankievicz et al. (2015) showed that *Setaria viridis* inoculated with the ammonium-excreting *A. brasilense* mutant strain HM053 fixed ~12 231 parts per trillion N_2 on a dry root mass basis, which are sufficient to provide the plant's daily N demand. It indicates that, under suitable conditions, *S. viridis* can obtain sufficient nitrogen via biological nitrogen fixation to promote plant growth.

Reporter gene *gusA,* encoding for the β-glucuronidase enzyme, is an interesting tool to understand colonization mechanisms in plants (Jefferson et al., 1987). The *gusA* fusion with *nifH* - the structural gene encoding dinitrogenase reductase subunit of nitrogenase enzyme - allows the identification and tracking of bacteria during the association, besides the detection of nitrogenase expression in the host plant. Although the interaction between *A. brasilense* and maize or wheat plants have been well studied, the association of *A. brasilense* with barley remains poorly understood (Santa et al., 2004). Barley is an experimental model for *Poaceae* (gramineus plant) adapted to climate change and cultivated throughout the world (Dawson et al., 2015). Since barley does not form nitrogen-fixing symbiotic structures, such as root nodules, the use of labeled bacteria helps to understand plant–bacteria interaction. In this work, *A. brasilense* wild-type (FP2) and excretion-ammonium (HM053) strains containing the chromosomal P*nifH-gusA* fusion were created and used to evaluate the bacterial colonization and growth promotion in barley.

2. Materials and Methods

2.1. Bacterial strains and media

Escherichia coli strains were grown at 37^{o}C in LB medium. *A. brasilense* FP2 (Sp7 ATCC 29145 Nif^{+} Sm^{r} Nal^{r}) and its derivative HM053 strain, that is resistant to ethylenediamine (EDA^{r}) and able to excrete ammonium (Machado et al., 1991), were grown at 30^{o}C in Nfb lactate medium supplemented with 50mM of phosphate solution and 20 mM of NH_4Cl (NFbHPN) (Pedrosa and Yates, 1984). Both *A. brasilense* strains were used to construct mutants which carry a chromosomal P*nifH-gusA* fusion.

2.2. Construction of A. brasilense strains containing the chromosomal P*nifH- gusA fusion*

The plasmid containing the P*nifH-gusA* fusion was constructed using the plasmids pSUP202::*nifHDK* of *A. brasilense* (Cb^r Cm^r Tc^r; Souza, E. M.) and pWM6 (Metcalf and Wanner, 1993). The plasmid containing the structural genes of nitrogenase has two sites for the enzyme *SacI* inserted into the *nifH* gene and pWM6 plasmid releases the promoterless *gusA*-kanamycin (*gusA-Km*) cassette when treated with the same enzyme. Therefore, the two plasmids were cleaved with *SacI* enzyme, ligated and inserted into *E. coli* DH5α [F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ^-] competent cells. A colony with the construction in the desired orientation, named pnifHDKgusA (Table S1) was selected by restriction analysis. The P*nifH-gusA* fusion was integrated into the chromosome of *A. brasilense* by homologous recombination after biparental conjugation between the donor *E. coli* S17.1-lambda pir [*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7 (*lambda *pir)*] containing the plasmid pnifHDKgusA and the recipients *A. brasilense* FP2 and HM053 strains. The conjugation was performed as follows: when the cells reached the log phase, 5 µL of the *E. coli* culture was set on the 50 µL drop of *A. brasilense* which were placed on a LB:NFbHPN lactate (1:1) solid plate. After 24h of incubation at 30°C, the cell mass was resuspended in 500 µL of liquid NfbHPN lactate and platted on a NfbHPN solid media containing streptomycin (Sm, 80 µg ml^{-1}), nalidixic acid (Nal, 10 µg ml^{-1}) and kanamycin (Km, 50 µg mL^{-1}). The antibiotic resistance profile allowed the identification of the transconjugants originated from double- and single- recombination, DR and SR, respectively. Since SR transconjugants also incorporated pSUP202 vector into the chromosome, they are also tetracycline (Tc, 10 µg mL^{-1}) resistant. To confirm the presence of the P*nif-gusA* fusion into the transconjugants chromosome, selected colonies were grown on NFbHP solid media with or without ammonia (20 mM) plus Sm, Nal, Km, glutamate (1 mM) and 5-bromo-4-chloro-3-indolyl-L-D-glucuronide (X-gluc, 30 µg mL^{-1}). PCR analysis using *A. brasilense* genomic DNA as template and primers which anneal to the *gusA* and *nifH* genes, gusA-F (5' CCGTAATGAGTGACCGCATC 3') and nifH-R (5' CTCCTGCTGCACTCATTATCC 3'), respectively, were also performed following these conditions: 1 cycle of 95°C for 5 min; 25 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min and 1 cycle of 72°C for 10 min (Fig. S1).

2.3. A. brasilense inoculation in barley for histochemical and plant growth analysis

Surface-sterilized barley (*Hordeum vulgare* L. CAUÊ) seeds were germinated on a sterilized Germitest paper roll using the Between Paper (BP) method during 48 h at BOD Incubator at 30°C. *A. brasilense* FP2 and HM053 strains and their P*nifH-gusA* mutant derivatives were incubated at 30°C for 17 h or until reach an OD_{600}~1.0 (10^9 CFU ml^{-1}). The culture was centrifuged and washed three times with phosphate buffer (100 mM, pH 6.8).

For histochemical analysis, seedlings were transferred to tubes containing sterilized polypropylene beads and Hoagland's solution without nitrogen, and inoculated with *A. brasilense* at 10^6 CFU ml^{-1}. Hoagland's solution with nitrogen (2.0 mM of KNO_3 and 0.5 mM of NH_4NO_3) was used only in the positive control. Microscope analyses were performed using intact plant roots after

3, 7 and 12 days of growth in Conviron growth chamber (Conviron, Inc., Winnipeg, Manitoba, Canada) set to 30°C with 12 h photoperiod. For histochemical detection of GUS activity, the roots were incubated for 30 min to 2 h with 50 mM sodium cacodylate buffer (pH 7.5) containing 0.5 mg ml^{-1} of X-gluc at 45°C, and were visualized by bright field microscopy. Images were captured on ZEISS Axiophot Microscope.

For plant growth analysis, seedlings were transferred to plastic pots containing sterilized vermiculite and were grown in controlled conditions with 16 h photoperiod. The vermiculite was kept wet by using Hoagland's solution with nitrogen (positive control) or without nitrogen (negative control and inoculated with *A. brasilense*). The following parameters were measured after 14, 21 and 35 days of growth: stem length (mm); longest root length (mm); total root length average (mm) and total root length normalized (mm). Root fresh weight (g) and total fresh weight (g) were measured only after 35 days. Data were submitted for analysis of variance (ANOVA) and means were compared by the Duncan test ($P \leq 0.05$) in the R program (R Core Team, 2007) with the Agricolae package. Biometric parameters were ordinated by principal component analysis (PCA) on the correlation matrix, in the R program (R Core Team, 2007) with the Vegan package.

3. Results and Discussion

To monitor *A. brasilense* wild-type (FP2) and excretion-ammonium (HM053) strains' colonization pattern into the barley plant, the pnifHDKgusA plasmid was constructed. It contains the reporter *gusA* gene under control of the *A. brasilense nifH* gene promoter. After plasmid insertion into *A. brasilense* FP2 and HM053 strains by biparental conjugation, double- and single-recombinant (DR and SR, respectively) mutant strains were obtained and could be identified based on their antibiotic resistance profiles (Table S1). Only the SR transconjugants could grow in the presence of tetracycline, as the entire pnifHDKgusA vector was inserted into their genome. DR transconjugants just contain *gusA-Km* cassette, therefore, they are sensitive to it. All tested mutants showed capability to express the protein GUS, confirming the insertion of *gusA-Km* cassette (Table S2). The FP2 transconjugants showed GUS activity only under nitrogen-fixing conditions, confirming that the expression of the *nif* genes in this strain is regulated by the nitrogen fixed levels. On the other hand, HM053 transconjugants expressed *gusA* in the presence and absence of ammonia, confirming its Nif^c phenotype (Vitorino et al., 2001). The nitrogenase activity of SR derivatives showed that, despite the *gusA* insertion, the structural genes of nitrogenase are intact. In contrast their DR derivatives did not show any nitrogenase activity (data not shown), due to deletion and replacement of *nifH* gene by *gusA* gene in these mutants (Fig. S1).

By generating P*nifH-gusA* single and double recombinants, it was possible to monitor $nifH^+$ and $nifH^-$ bacteria, respectively, in association with barley, and the response of the plant to this association. For that, plants were grown in hydroponic culture and inoculated with FP2 and HM053 SR and DR. Since the *gusA* gene was placed under control of *nifH* promoter, it was expected to detect labeled cells where nitrogen fixation was being elicited (Fig. 1). Three days after inoculation,

an increased number of *A. brasilense* cell aggregates were found attached to the surface of the root, mainly at lateral branching point (Fig. 1a). However, 7 days after inoculation clumps of *A. brasilense* were visualized at the entire root system (Fig. 1b). Twelve days after inoculation, the colonization zones focused in young areas, such as zone of elongation and differentiation (Fig. 1c). The colonization pattern in barley roots did not change either when we compared different strains (FP2 and HM053) or recombinants (SR and DR) of the same strain (Fig. S2).

A. brasilense also colonized primary and secondary roots in the interior of hairs with intact cell walls (Fig. 1d). Surface colonization by labeled *Azospirillum* was well demonstrated in wheat and sorghum (Ramos et al., 2002). Studies using random reporter gene insertion observed *A. brasilense* strain Sp245 in the interior of root hairs (Assmus et al., 1995; Schloter and Hartmann, 1998), however, Sp7 and Wa3 strains were restricted to the root hair zone. While these works did not report the endophytic *A. brasilense* Sp7 strain in the interior of root hairs, here the two strains - FP2 and HM053 - derived from Sp7 strain (Pedrosa and Yates, 1984) were able to penetrate and express nitrogenase in the root hairs of barley, which is an evidence of the low levels of nitrogen and oxygen in this cell, otherwise nitrogenase would be inhibited (Dixon and Kahn, 2004).

A. brasilense distribution is characteristic of an early-endophytic bacterium (Mercado-Blanco and Prieto, 2012) and the results showed here support this idea. The exact process that allow *A. brasilense* to associate with plant tissues are poorly understood (McMillan and Pereg, 2014). There is molecular evidence for strain specificity considering the effect of inoculation (Chamam et al., 2013); nevertheless, a wide review of *Azospirillum* associations showed this bacteria as a general PGPB (Pereg et al., 2015). This data highlights the importance of more colonization studies to better understand *A. brasilense* interactions.

Barley plants were also cultivated in vermiculite substrate and inoculated with *A. brasilense* FP2, HM053, or their transconjugant derivatives for biometric analysis. Noninoculated plants with N (2.0 mM of KNO_3 and 0.5 mM of NH_4NO_3) and without nitrogen were also analyzed. No statistical difference in root growth was detected among barley plants 14 or 21 days (Table S3 and S4) after inoculation with the *A. brasilense* strains. However, 35 days after inoculation, plants treated with strains FP2, HM053, or their SR derivatives strains presented longer roots ($P \leq 0.05$) than the noninoculated controls or the ones inoculated with the DR mutant strains (Table 1). PCA analysis confirmed that the increase of root and stem length, and root and plant weight, are positively correlated ($P \leq 0.05$) to the inoculation of *A. brasilense* FP2, HM053, or their SR derivatives strains (Fig. 2).

The broadly favorable results obtained by *Azospirillum* inoculation in plants is well characterized (Barbieri and Galli, 1993; Hungria et al., 2010; Santa et al., 2008, 2004) and occur due to the fact that *Azospirillum* fix nitrogen and is able to produce phytohormones, such as indol-3-acetic acid (IAA) (Meza et al., 2015). *A. brasilense* synthesize IAA by the tryptophan-dependent way (Duca et al., 2014). The tryptophan synthesis requires a great quantity of ammonium (Güneş et al., 2014). Therefore, the absence of nitrogenase activity showed by the *A. brasilense* double-

recombinant P*nifH-gusA* mutants not only compromised nitrogen fixation, but also might had reduced the production of tryptophan and then the bacteria's ability to produce IAA.

4. Conclusion

In summary, the results of this study show that *A. brasilense* FP2 and HM053 P*nifH-gusA* mutants are capable of colonizing and expressing *nif* genes in the root surface and in the interior of root hairs of barley. Furthermore, in vitro essays showed that inoculation of $nifH^+$ strains (FP2, HM053, FP2-SR, and HM053-SR) increased barley biometrical parameters, whereas $nifH^-$ strains (FP2-DR and HM053-DR) did not. Our results confirmed *A. brasilense* HM053 as a biofertilizer that can be used with other efforts to improve barley yield and strengthened the importance of nitrogen fixation to plant growth-promotion in *A. brasilense*. Further analysis might be necessary to evaluate the contribution of other factors like phytohormone production for *A. brasilense* plant-growth promotion in barley.

5. Acknowledgements

We would like to thank the Brazilian Program of National Institutes of Science and Technology-INCT/Brazilian Research Council-CNPq/MCT, CNPq, CAPES and Fundação Araucária of the Paraná State for the financial support. We thank Valter Antônio de Baura, Maria Isabel Stets, Luiza Maria de Araújo and Ligia Dombroski dos Santos for technical support.

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Figure 1: Bright field microscopy of *Azospirillum brasilense* HM053-SR on roots of barley. Three (A), seven (B) and twelve (C) days after inoculation. *A. brasilense* structure and inside barley root hair cells (D).

Figure 2: Ordination of the barley trials based upon the biometric parameters. Only the parameters with P <0.05 for significance after 999 permutations are displayed. Each vector points to the direction of increase for a given variable and its length indicates the strength of the correlation between the variable and the ordination scores. **Legend: C N-:** Non-inoculated control without N; **C N+:** Non-inoculated control with N; **FP2 WT:** FP2 wild-type; **FP2 SR:** *nifH-gusA* single recombinant FP2; **FP2 DR:** *nifH-gusA* mutant FP2; **HM SR:** *nifH-gusA* single recombinant HM053; **HM DR:** *nifH-gusA* double recombinant HM53. **cl:** stem length; **rlg:** longest root length average; **alr:** total root length average; **sr10:** total root length normalized; **rfw:** root fresh weight; **tfw:** total fresh weight.

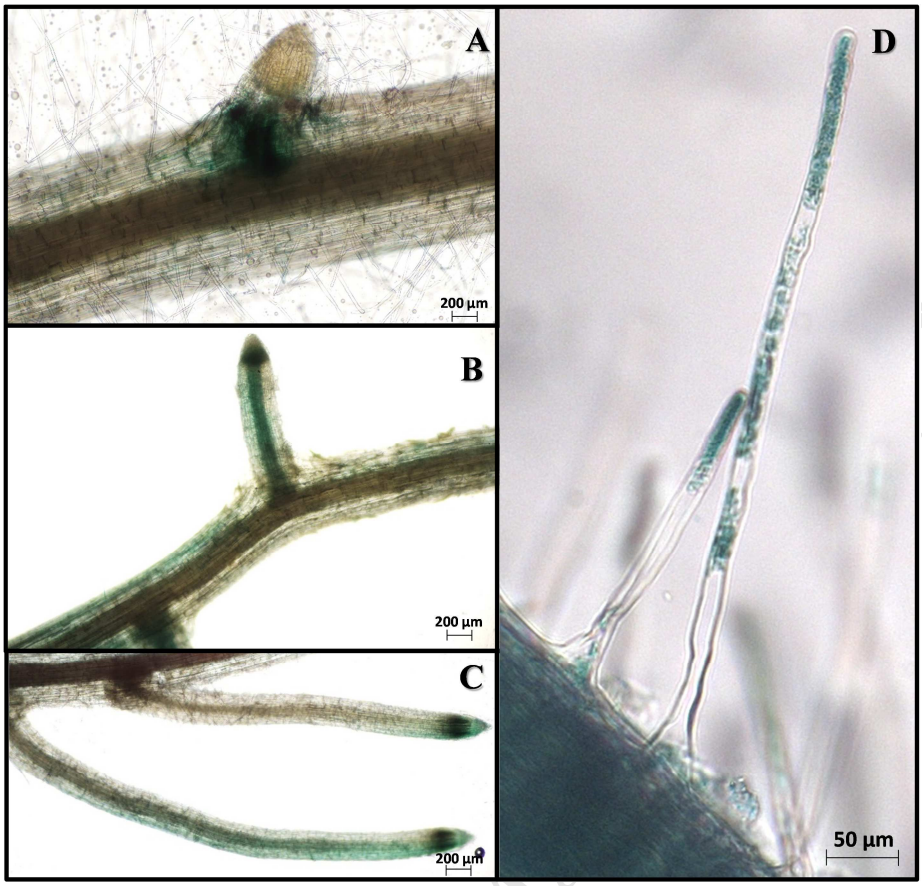
Table 1: Effects of *A. brasilense* FP2, HM053 and their derivatives transconjugants in barley growth after 35 days inoculation.

Treatment	Stem (mm)	Longer Root length (mm)	Root length average (mm)	Normalized Root length (mm)	Root fresh weight (mg)	Total fresh weight (mg)
FP2	197.3 a	286.4 a	180.2 a	125.3 a	47.77 a	225.6 ab
FP2-SR	205 a	257.3 ab	165 ab	133 a	44.71 ab	220.3 ab
HM053	195.9 ab	257.3 ab	179.4 a	117.9 ab	38.48 bc	200.5 bc
HM053-SR	216.9 a	269 ab	180.6 a	133.86 a	35.12 cd	248.9 a
C N-	194.9 ab	188.8 cd	122.3 c	91.29 c	32.69 cde	189.6 bc
C N+	209.3 a	222.22 bc	128.5 c	83.08 c	25.66 ef	182 c
FP2-DR	175.1 bc	184.2 cd	144 bc	101.6 bc	27.51 def	147.8 d
HM053-DR	171 c	171 d	130.1 c	86.19 c	2342 f	144.6 d

Data represent the means of nine replicates. Means values followed by the same letter are not statistically different (Duncan, $P \leq 0.05$).

300 **Figure 1**

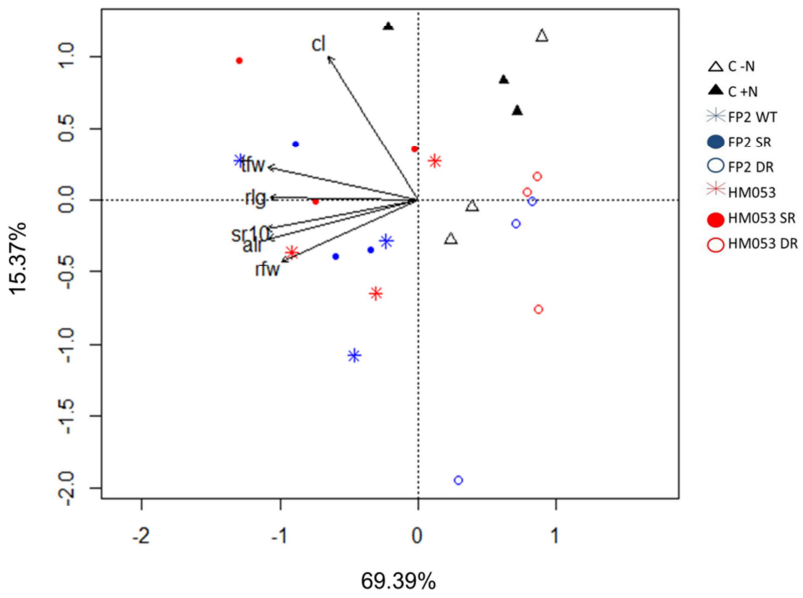
301



302

303

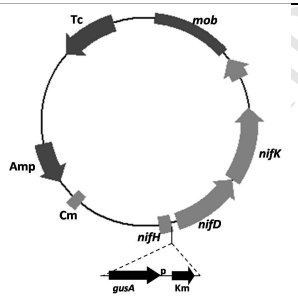
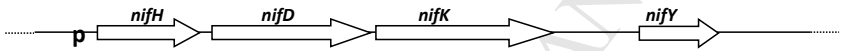
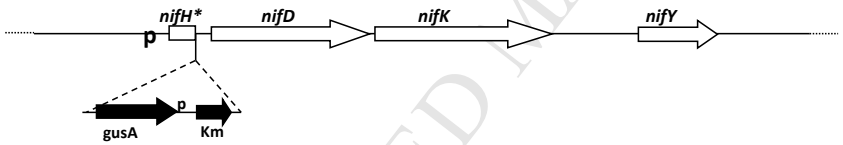
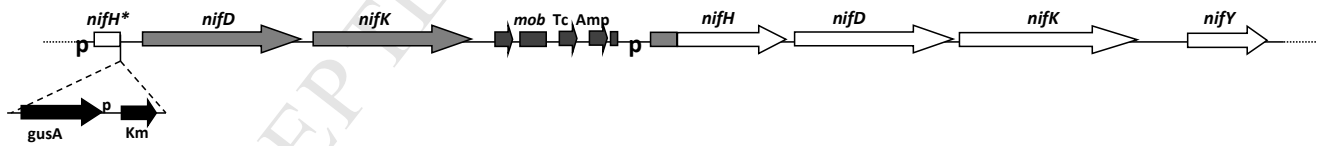
304 **Figure 2**



305

Supplemental material

Table S1- Map of the pnifHDKgusA plasmid and organization of the operon *nifHDKY* of *A. brasilense* FP2 or HM053 before and after double or single crossing over. The genotype of each construction or strain is also shown.

Plasmid or *Azospirillum brasilense* strain	Maps	Genotype
Plasmid pnifHDKgusA (pSUP2012:: *nifHDK*, containing *Sac*I gusA-Km cassete inside *nifH* gene)		*nif* $^{-}$, *gusA*$^{+}$, Tc^{r}, Amp^{r}, Cm^{r}
A. brasilense FP2 or HM053 chromosomal DNA		*nif* $^{+}$, *gusA*$^{-}$, Sm^{r}
A. brasilense FP2 or HM053 chromosomal DNA after DOUBLE crossing over		*nif* $^{-}$, *gusA*$^{+}$, Sm^{r}, Km^{r}
A. brasilense FP2 or HM053 chromosomal DNA after SINGLE crossing over		*nif* $^{+}$, *gusA*$^{+}$, Sm^{r}, Km^{r}, Tc^{r}, Amp^{r}

Legend: Amp, ampicillin resistance gene; Tc, tetracycline resistance gene; Cm, chloramphenicol resistance gene; Km, kanamycin resistance gene; *gusA*, promoterless beta-glucuronidase gene; *mob*, mob gene; p, promoter; open and closed arrow identified as *nifH, nifD, nifK and nifY* are chromosomal or plasmidial genes, respectively. * inactive *nifH* due to a *SacI* deletion.

Table S2: GUS activity of *A. brasilense* FP2, HM053 and their single- and double- recombinants (SR and DR, respectively) in absence (-N) or in the presence (+N) of 20mM of ammonium chloride.

***Azospirillum brasilense* strains**	**GusA activity**	
	-N	+N
FP2	-	-
FP2-SR, FP2-DR	+	-
HM053	-	-
HM053-SR, HM053-DR	+	+

Legend: +, positive GusA activity; -, negative GusA activity.

Table S3: Effects of *A. brasilense* FP2, HM053 and their derivatives transconjugants in barley growth after 14 days inoculation.

Treatment	Stem (mm)	Longer Root length (mm)	Root length average (mm)	Normalized Root length (mm)
C N-	157.4 a	259.3a	172 a	104.2 a
C N+	150.4 ab	246.8 a	157.4 ab	93.04 a
FP2	142.6 bc	264.9 a	157.8 ab	95.33 a
FP2-SR	145 abc	243.9 a	140.7 b	93.6 a
FP2-DR	142.1 bc	169.9 b	110.8 c	76.26 b
HM053	143 bc	238.1 a	151.5 ab	102.8 a
HM053-SR	135.7 cd	256.8 a	146.3 b	97.67 a
HM053-DR	129 d	157.4 b	94.75 c	67.94 b

Data represent the means of nine replicates. Means values followed by the same letter are not statistically different (Duncan, $P \leq 0.05$).

Table S4: Effects of *A. brasilense* FP2, HM053 and their derivatives transconjugants in barley growth after 21 days inoculation.

Treatment	Stem (mm)	Longer Root length (mm)	Root length average (mm)	Normalized Root length (mm)
C N-	176 b	236 abc	139.6 b	88.85 a
C N+	172.7 b	216.3 bc	153.1 b	93.49 a
FP2	170.2 b	147.8 d	136.9 ab	102.4 a
FP2-SR	173.6 b	152.6 d	151.5 ab	92.51 a
FP2-DR	148.6 c	185.4 cd	165.5 ab	33.1 b
HM053	178 b	240.2 ab	145.9 ab	99.56 a
HM053-SR	200.9 a	270.9 a	175 a	105.3 a
HM053-DR	143.6 c	162.5 d	157.8 ab	31.57 b

Data represent the means of nine replicates. Means values followed by the same letter are not statistically different (Duncan, $P \leq 0.05$).

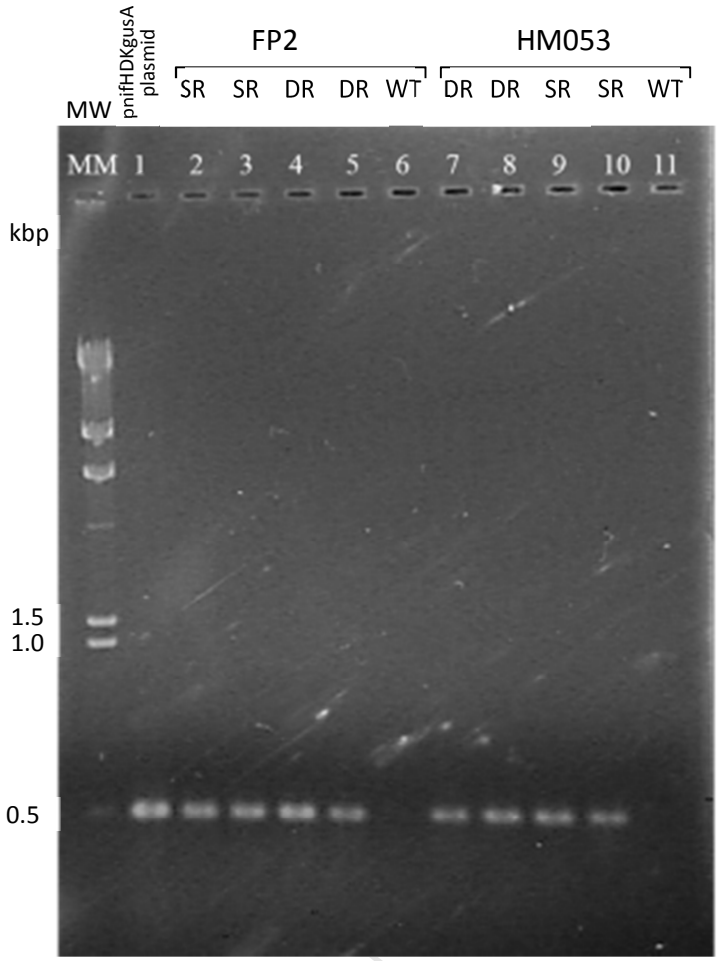


Fig. S1: Confirmation of *gusA* gene insertion into *nifH* gene of *A. brasilense* FP2 and HM053 single and double recombinants by PCR. PCR products were visualized after agarose gel electrophoresis TAE 1% and ethidium bromide staining. **Legend: WT:** wild type; **SR:** single recombinant; **DR:** double recombinant. The bacterial recombinants SR and DR were analyzed in duplicates.

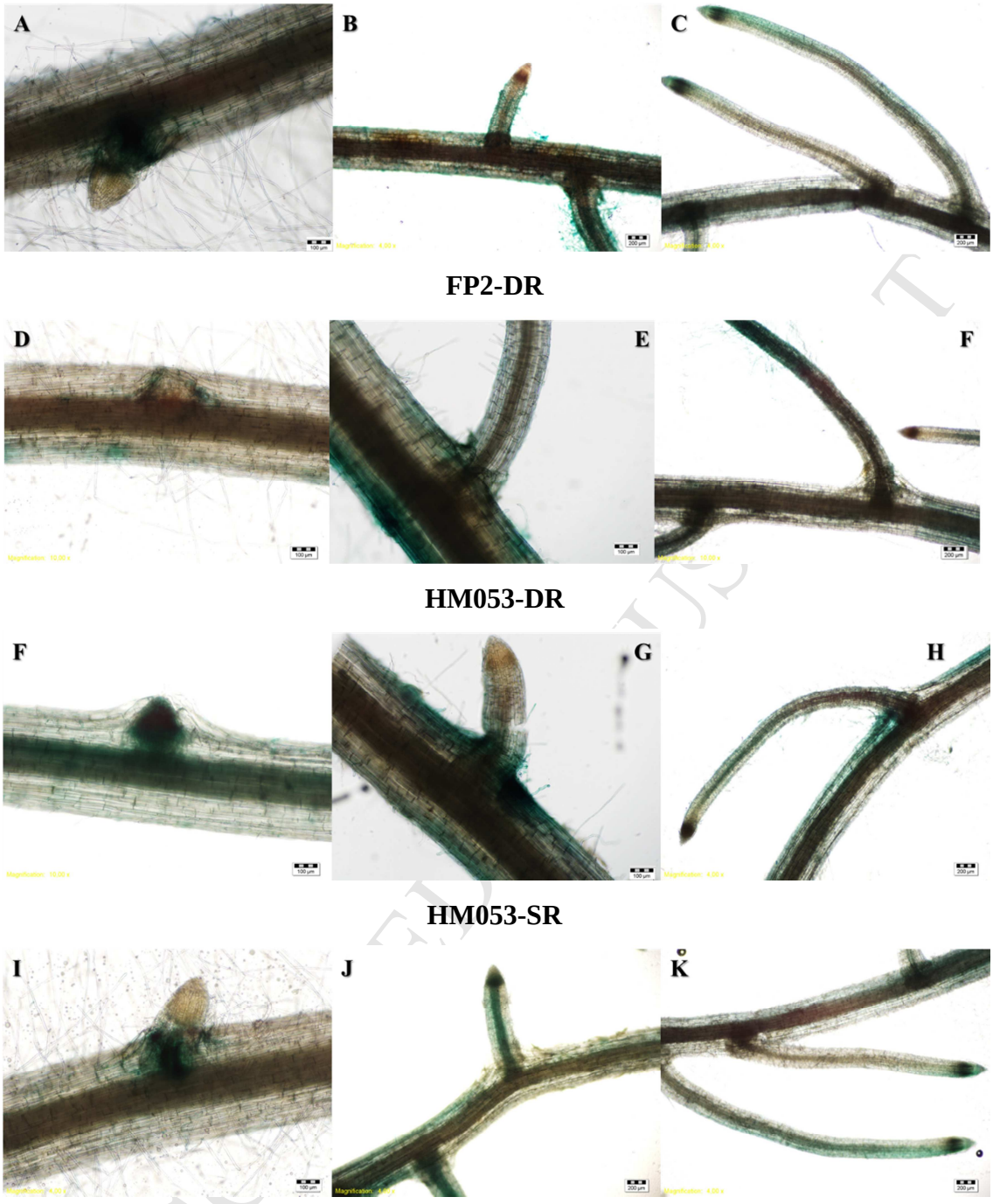


Fig. S2: Barley roots inoculated with: FP2-SR followed by 3, 7 and 12 days (**A**, **B** and **C**, respectively), FP2-DR (**D**, **E** and **F**), HM053-DR (**F**, **G** and **H**), HM053-SR (**I**, **J** and **K**).

Labeled *Azospirillum brasilense* wild type and excretion-ammonium strains in association with barley roots

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Highlights

- *A. brasilense* strains containing the insertion of the reporter gene *gusA* into *nifH* gene were created.
- *A. brasilense* wild type and excretion-ammonium strains colonized and expressed the nitrogenase enzyme inside barley root hair cells.
- The excretion-ammonium strain of *A. brasilense* was characterized as a promising plant growth-promoting bacteria in barley.

Labeled *Azospirillum brasilense* wild type and excretion-ammonium strains in association with barley roots

Author Contributions

Conceived and designed the experiments: C.W. Galvão; R.M. Etto; R. A. Ayub; F.O. Pedrosa; E.M. Souza; M.B.R. Steffens.

Performed the experiments: A. R. S. Santos; R. W. Furmam; D. L. Freitas; K. F. E. N. Santos; C.W. Galvão; R.M. Etto.

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Wrote the paper: C.W. Galvão; R.M. Etto; A. R. S. Santos.

Provided initial infrastructure support: C.W. Galvão; R.M. Etto; R. A. Ayub; F.O. Pedrosa; E.M. Souza; M.B.R. Steffens.

Infrastructure support, supervised all aspects of the project and reviewed and edited the manuscript: F.O. Pedrosa; E.M. Souza; M.B.R. Steffens.