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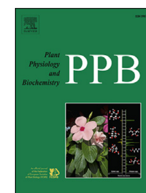

Short communication

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.
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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 N2 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

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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 °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 °C in Nfb lactate medium supplemented with 50 mM 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 PnifH- 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 pnifHDK-gusA (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 24 h 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

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