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ORIGINAL PAPER

# Hydrogen-uptake genes improve symbiotic efficiency in common beans (*Phaseolus vulgaris* L.)

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**Abstract** Hydrogen-uptake (Hup) activity is implicated in the mitigation of energy losses associated with the biological nitrogen fixation process, and has been related to productivity increases in some legume hosts. However, in common bean (*Phaseolus vulgaris* L.) the expression of hydrogenase is rare. In this study an 18-kb *hup* gene cluster from *Rhizobium leguminosarum* bv. viciae encoding a NiFe hydrogenase was successfully transferred to three common bean rhizobial strains lacking hydrogenase activity ($Hup^-$) but symbiotically very effective and used in commercial inoculants in Brazil: one strain originally from Colombia (*Rhizobium tropici* CIAT 899), and two strains from Brazil (*R. tropici* H 12 and *Rhizobium freirei* PRF 81). The inclusion of $NiCl_2$ in the nutrient solution did not increase hydrogenase activity, indicating that common bean plants allow efficient nickel provision for hydrogenase synthesis in the bacteroids. The symbiotic performance—evaluated by nodulation, plant growth, N accumulation and seed production—of wild-type and $Hup^+$ derivative strains was compared in experiments performed with cultivar Carioca under greenhouse conditions, in sterile

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substrate and in non-sterile soil. Statistically significant increases in one or more parameters were observed for all three $Hup^+$ derivatives when compared to the respective wild-type strain. Differences were found mainly with the Brazilian strains, reaching impressive increases in nodule efficiency and seed total N content. The results highlight the potential of using *Rhizobium* $Hup^+$ strains for the design of more energy-efficient inoculants for the common bean crop.

**Keywords** Biofertilizer · Inoculant · Nitrogenase efficiency · Plant-growth promoting bacteria

## Introduction

Biological nitrogen fixation (BNF) is a key biological process for Earth's N balance. Reduction of atmospheric nitrogen ($N_2$) to ammonia is mediated by the enzyme nitrogenase, in a process that produces hydrogen gas ($H_2$) as an energy-rich obligate by-product that consumes at least 25% of the energy supplied to the nitrogenase. The $H_2$ produced usually diffuses into the surrounding soil, implying in loss of energy that otherwise could be directed to the reduction of $N_2$, thereby affecting the energy efficiency of BNF, and with a great impact on the symbioses of rhizobia with several legumes (Hungria and Neves 1987; Neves and Hungria 1987; Hungria and Ruschel 1989; Baginsky et al. 2005; Imperial et al. 2006; Annan et al. 2012). Some rhizobial strains possess genes coding for another enzyme, a hydrogen-uptake NiFe hydrogenase—Hup—that recycles part of the energy (ATP) spent in the BNF process through the oxidation of the released $H_2$ (Ruiz-Argüeso et al. 1979; Hungria and Neves 1987; Neves and Hungria 1987; Dong and Layzell 2001; McLearn and Dong 2002). Soybean (*Glycine max* (L.) Merr.), lupines (*Lupinus* spp.), and cowpea (*Vigna unguiculata* L.) are among the most well-known legumes capable of forming symbiotic relationships with rhizobia that possess functional hydrogenase-uptake genes (Hungria et al. 1989; Souza et al. 1999; Baginsky et al. 2005; Brito et al. 2005; Annan et al. 2012).

After hydrogen-uptake capacity was demonstrated in legume nodules, symbioses with rhizobia lacking hydrogenase-uptake systems have been viewed as energetically inefficient. However, the superiority of symbiotic performance in rhizobia possessing functional hydrogenases (called $H_2$-uptake positive or $Hup^+$ strains) over those lacking the enzyme ($Hup^-$) reported by some authors (e.g. Schubert and Evans 1976; Albrecht et al. 1979; Hanus et al. 1981; Hungria et al. 1989; Baginsky et al. 2005), has not been conclusive according to others (Drevon et al. 1982; Golding and Dong 2010).

Partnership between rhizobia and common bean (*Phaseolus vulgaris* L.) is crucial, as this legume represents a basic protein source in many countries of South and Central America, Asia and Africa. However, there are concerns about the capacity of BNF to support common bean plant growth and yield; in general, limitations have been attributed to plant breeding programs focused on N-fertilizer, to highly competitive but poorly effective indigenous population of rhizobia in soils, and to high susceptibility to diseases and environmental stresses (Graham 1981; Buttery et al. 1987; Hungria and Vargas 2000; Dwivedi et al. 2015). Nevertheless, increases in nodulation, BNF and grain yield have been observed when selected elite commercial strains are used as inoculants in Brazil (Hungria et al. 2000, 2003, 2013), even when the strains are $Hup^-$ (Ormeño-Orrillo et al. 2012). Besides, it is worth mentioning that it has been long suggested that inoculation with $Hup^+$ rhizobial strains could help to optimize the BNF process with common beans (Hungria and Neves 1987; Neves and Hungria 1987; Hungria and Ruschel 1989).

In this study, $Hup^+$ derivatives of three very effective but $Hup^-$ *Rhizobium* strains used in commercial inoculants for the common bean crop in Brazil were obtained. Afterwards, the symbiotic performance of wild-type and engineered strains was compared in cultivar Carioca, considered as a host genotype with high capacity for BNF.

## Materials and methods

### Rhizobial strains and generation of hydrogen-recycling derivative strains

Three strains used in commercial inoculants for the common bean crop in Brazil [*Rhizobium tropici* CIAT 899 (= SEMIA 4077) and H 12 (= SEMIA 4088), and *Rhizobium freirei* PRF 81 (= SEMIA 4080) (Hungria et al. 2000, 2003)] were selected as targets to improve

their nitrogen fixation efficiency by incorporating the ability to recycle hydrogen evolved by nitrogenase. Analysis of genome sequences (available for two out of the three strains (Ormeño-Orrillo et al. 2012) revealed the presence of DNA regions encoding versions of hydrogenase gene clusters inactivated by relevant deletions. Consistent with this, these strains do no synthesize a functional $H_2$-uptake hydrogenase in bean bacteroids (our unpublished results and Fig. 1a). Also, Southern hybridization using a *Rhizobium leguminosarum* bv. viciae (*Rlv*) UPM791 *hupS* probe on *Eco*RI-digested genomic DNAs from these strains allowed the detection of faint, single bands on each strain corresponding to high molecular weight restriction fragments (20–23 kb, data not shown). To generate strains with $H_2$-recycling ability, a Tn5 derivative mini-transposon (TnHB100) containing an

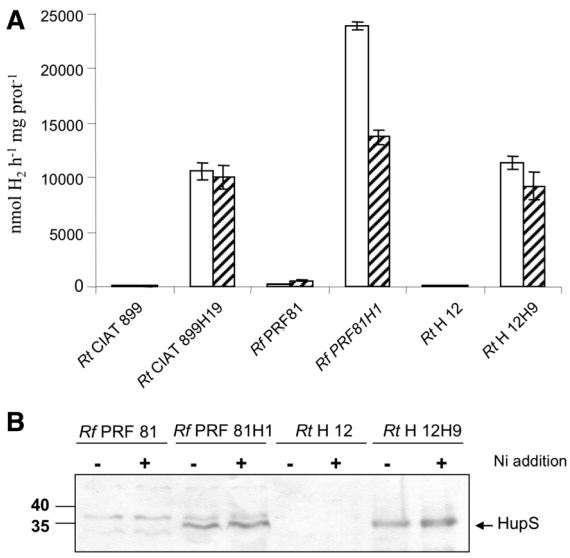


**Fig. 1** Expression of hydrogenase activity in bacteroids induced by *Rhizobium* strains in common bean nodules. **A** hydrogenase activity was measured in suspensions of bacteroids obtained from plants grown with regular nutrient solutions (white bars), or in the same solutions supplemented with $Ni^{2+}$ (85 μM $NiCl_2$, shaded bars). Data are the average of two replicates, and bars indicate standard error; **B** Immunological detection of hydrogenase small subunit in bacteroid crude extracts of the indicated *R. tropici (Rt) or R. freirei* (*Rf*) strains. Bacteroids were obtained from common bean plants grown as described in panel A. 30 μg of protein obtained from bacteroids of the indicated strains were loaded onto each lane. Immunoblots were revealed with antisera raised against *R. leguminosarum* HupS (diluted 1:100). The position of relevant molecular weight markers (kDa) is shown at the left of the figure. The horizontal arrow at the right denotes the position of HupS-immunoreactive band

18-kb $H_2$-uptake (hup) gene cluster from *Rhizobium leguminosarum* bv. viciae strain UPM791 was transferred by mating into each rhizobial strain as described before (Báscones et al. 2000). Basically, the mini-transposon was introduced into the different rhizobial strains by conjugation using *Escherichia coli* S17.1 (pTnHB100) as the donor strain and spectinomycin-resistant transconjugants were checked for the presence of the hydrogenase gene cluster by Southern analysis. Transconjugants arose at a frequency of approximately $10^{-8}$ per recipient cell; at least 50% of the spectinomycin-resistant transconjugants showed an additional signal, corresponding to a ca. 4-kb *Eco*RI restriction fragment following hybridization to the *hupS* probe (data not shown). Using this procedure, *R. tropici* CIAT 899H19 and PRF 81H1, and *R. freirei* H 12H9 derivatives incorporating *Rlv* UPM791 hydrogenase gene cluster were generated. Bacteroid suspensions from these TnHB100-containing strains were evaluated for expression of hydrogenase activity using a hydrogen electrode, as previously reported (Báscones et al. 2000). Three transconjugants per recipient strain were tested and found to display similar levels of hydrogenase activity, as expected from a transposon in which hydrogenase genes have their own symbiotic NifA-dependent promoter. By the end of the screening one derivative strain was chosen for each one of the three parental strains to proceed with the greenhouse experiments. The expression of hydrogenase was further confirmed by immunological detection of the small subunit of the enzyme in bacteroid cell crude extracts using antiserum raised against *R. leguminosarum* HupS, as previously described by Brito et al. (1994).

We determined the insertion sites for two of these derivative strains: in the case of *R. tropici* H12H9, TnHB100 was located within a gene putatively encoding a cellulose synthase and 100% identical to *R. tropici* CIAT899H19_PC05720; in *R. freirei* PRF 81H1, the insertion was on gene locus RHSP_05501, putatively encoding a dihydroxyacetone kinase. None of these two insertions are in regions whose mutations could be rationally linked to increases in productivity.

All wild-type and derivative strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054), in Londrina, State of Paraná, Brazil, and at the Centro de

Biotecnología y Genómica de Plantas (C.B.G.P.), Universidad Politécnica de Madrid, Spain.

### Greenhouse experiments in sterile substrate

Wild-type and $Hup^+$ derivative strains were individually grown in modified-yeast mannitol medium (YM) (Hungria et al. 2016), for 5 days, in the dark, at 28 °C. Cultures were adjusted to $10^9$ cells $mL^{-1}$ by optical density, based on growth curves previously obtained for each strain. Seeds of common bean cultivar Carioca, a good $N_2$-fixing genotype (e.g. Hungria et al. 2000), were surface sterilized and three seeds were sown in each sterilized Leonard jar containing sterile substrate composed of sand and vermiculite (1:2, v/v), as described before (Hungria et al. 2016). Each seed received 1 mL of the corresponding rhizobial culture. Two treatments were included as controls: non-inoculated plants without and with N [applied as 80 mg of N ($KNO_3$) $plant^{-1}$ $week^{-1}$]. All jars received sterile N-free nutrient solution (Hungria and Araujo 1994) whenever necessary.

The experiment was performed under greenhouse conditions, with photoperiod of 12/12 h (day/night) and temperature averages of 28.1/21.3 °C (day/night). After 5 days, plants were thinned to two per pot. The experiment was performed in a complete randomized block design with eight replicates.

At flowering, 38 days after emergence (DAE), plants were harvested. Shoots were separated from roots and placed in a forced-air dryer at 65 °C until constant weight was obtained (approximately 72 h). Roots were carefully washed and also placed in the dryer. After 72 h in an air-forced drier at 50 °C nodules were removed from roots and dried again. Nodulation (nodule number and dry weight), shoot dry weight and shoot N concentration (Kjeldahl-based analyzer, Tecator, Sweden) were determined.

### Greenhouse experiments in pots containing non-sterile soil

Rhizobial inoculants and seed surface sterilization were performed as described in the previous experiment with sterile substrate. The experiment was performed in pots of 5-kg capacity containing 4 kg of soil. The indigenous population of common bean rhizobia in the soil was estimated as $10^4$ cells $g^{-1}$ of soil by the most probable number (MPN) counting technique with common bean plants of cultivar Carioca, and the statistical tables of Hungria and Araujo (1994). Soil chemical properties were evaluated as described by Hungria et al. (2013), and the main values were as follows: pH in $CaCl_2$ of 5.3; N, 0.18 g $kg^{-1}$; C, 2.35 g $kg^{-1}$, P, 8.2 mg $kg^{-1}$.

Five seeds were sown per pot and each received 1 mL of inoculant containing $10^9$ cells $mL^{-1}$ of the corresponding strain. As in the experiment with sterile substrate, non-inoculated controls with and without N-mineral (the same amount as under sterile conditions) were included. The experiment was performed in a complete randomized block design with eight replicates. After 5 days, plants were thinned to two plants per pot. Growth conditions, including light, temperature and nutrient solution were as described in the greenhouse experiment with sterile substrate.

At late maturity stage, plants were harvested to evaluate seed production parameters. Pods were collected, opened and the number of seeds per plant was evaluated. After drying (72 h in an air-forced drier at 50 °C), seed dry weight was determined, following the evaluation of seed N concentration (Kjeldahl-based analyzer, Tecator, Sweden).

### Statistical analysis of the greenhouse data

Prior to the statistical analyses data were checked for normality of errors and homogeneity of variances. One-way general linear model ANOVA was used, and when significant difference among treatments was detected, Tukey's Test was employed for a posteriori multiple comparison of the treatment means. Differences were considered significant at $p < 0.05$ and $p < 0.01$. Statistical analyses were performed with the software SAS® 9.4 (SAS Institute, North Caroline, USA).

## Results

A mini-transposon (TnHB100) containing an 18-kb $H_2$-uptake (hup) gene cluster from *R. leguminosarum* bv. viciae (*Rlv*) strain UPM791 was successfully transferred into three common bean elite rhizobial strains used in commercial inoculants in Brazil, *R. tropici* strains CIAT 899 and H 12, and *R. freirei* PRF 81. All three $Hup^+$ derivatives exhibited high levels of hydrogenase activity in common bean bacteroids,

suggesting efficient recycling of the $H_2$ evolved by nitrogenase in nodules (Fig. 1a).

*R. leguminosarum* bv. phaseoli is also a symbiont of common bean, and as in the genetically closely related *R. leguminosarum* bv. viciae it has been shown that nickel availability to the host plant pea (*Pisum sativum* L.) limits hydrogenase, by affecting the processing of the hydrogenase structural subunits (Brito et al. 1994), we investigated the effects of including $NiCl_2$ in the nutrient solution. We did not observe any increase in the level of activity of the hydrogenase, indicating that common bean plants allow efficient nickel provision to bacteroids (Fig. 1a). In one case (*R. tropici* PRF 81H1), the addition of nickel was detrimental for activity, suggesting higher sensitivity to nickel toxicity in this strain.

By using antiserum raised against Rlv HupS (Brito et al. 1994) we verified that immunoblots of extracts from bacteroids from the engineered strains, and not those from wild-type strains, showed immunereactive bands of an estimated size compatible with that of the hydrogenase small subunit (ca. 35 kDa, Fig. 1b).

In order to determine the effect of hydrogen recycling in the symbiotic performance, wild-type and $Hup^+$-derivative strains were compared in assays carried out with sterile substrate and also with non-sterile soil as substrate. In the first experiment, performed with sterile substrate, significant increases in parameters related to $N_2$-fixation were associated to the $Hup^+$ trait in one or more of the engineered strains. Differences were observed in nodule number (NN) for *R. tropici* H 12H9, nodule dry weight (NDW) for *R. tropici* CIAT 899H19, shoot N concentration (SNC) for CIAT 899H19 and *R. freirei* PRF 81H1, shoot total N content (STNC) for PRF 81H1 and nodule efficiency (NE) for H 12H9 and PRF 81H1 (Table 1). Remarkable responses were observed mainly with plants inoculated with the Brazilian elite strains H 12H9 and PRF 81H1, with increases in NE of 27.5 and 23.2%, respectively, in comparison to the respective wild-type strains. Noticeable also was the increase in NN of 45.2% with strain *R. tropici* H 12H9 (Table 1).

In the experiment performed using as substrate non-sterile soil, the $Hup^+$ trait also resulted in statistically significant increases in seed production parameters in plants inoculated with the Brazilian strains *R. tropici* H 12H9 and *R. freirei* PRF 81H1, but not with the Colombian strain CIAT 899H19 (Table 2). With both Brazilian strains significant increases were observed in seed dry weight (SeDW) and seed total N content (SeTNC). Impressively, *R. tropici* H 12H9 $Hup^+$ strain increased SeTNC by 27.1% as compared to the wild type (Table 2).

## Discussion

Brazil is amongst the world's greatest producers and consumers of common bean, and in the country the grains represent the main source of protein for the lower income population; nevertheless, National mean yield is very low when compared to the genetic potential of the cultivars released (SEAB 2017), and a similar situation is reported in other countries. As N is usually the most limiting nutrient in soils used to cultivate common bean in South and Central America, Africa and Asia, improvements in the contribution of BNF could result in great impact and increased grain yields. However, under field conditions it has long been a general idea that the legume establishes inefficient $N_2$-fixing symbioses (Graham 1981; Buttery et al. 1987; van Kessel and Hartley 2000). Besides, it has also been shown that it is feasible to successfully survey elite strains with higher capacity of BNF and competitiveness, greatly improving grain yield (Hungria et al. 2000, 2003; Mulas et al. 2011; Vanlauwe et al. 2019). Intriguingly, although the hydrogen-uptake property has been subject of intensive studies and promises of increased yields in the 1970s and 1980s (e.g. Schubert and Evans 1976; Hungria and Neves 1987), it has fallen into a relative ostracism in the following decades. Yet, several studies have shown that effective hydrogen-uptake rhizobial strains can improve the efficiency of the BNF, plant performance and yield (e.g. Schubert and Evans 1976; Albrecht et al. 1981; Hanus et al. 1981; Hungria and Neves 1987; Hungria et al. 1989; Baginsky et al. 2005).

The hydrogen-uptake activity is variable among rhizobia (Bedmar and Phillips 1984; Bedmar et al. 1984; Golding and Dong 2010; Imperial et al., 2006) and, apparently, it is less frequently observed in rhizobia nodulating temperate legumes (Drevon et al. 1982; Golding and Dong 2010). In addition, although hydrogenase is a bacterial enzyme, it has been noted that the host plant may exert an influence on its expression as reported for common bean (Navarro et al. 1993), lupines (Murillo et al. 1989), lotus (*Lotus*

**Table 1** Effect of $Hup^+$ trait on symbiotic performance of three elite inoculant rhizobial strains with *Phaseolus vulgaris* cv. Carioca grown in sterile substrate

| Strains | Nodulation | | Plant growth and N content | | | Nodule efficiency |
|---|---|---|---|---|---|---|
| | NN (n° $pl^{-1}$) | NDW (g $pl^{-1}$) | SDW (g $pl^{-1}$) | SNC (g $kg^{-1}$) | STNC (mg N $pl^{-1}$) | NE (mg N g $nod^{-1}$) |
| CIAT 899 wt | 498 | 0.648 | 9.63 | 30.31 | 29.23 | 47.55 |
| CIAT 899 $HUP^+$ | 534 | 0.601 | 9.34 | 30.89 | 28.85 | 48.45 |
| $p^a$ | 0.172 | 0.032 | 0.710 | 0.001 | 0.238 | 0.063 |
| Increase (%)[b] | $7.2^{n.s.}$ | − 7.3* | $-3.0^{n.s.}$ | 1.9** | $-1.3^{n.s.}$ | $1.9^{n.s.}$ |
| H 12 wt | 457 | 0.642 | 9.50 | 30.49 | 28.82 | 48.18 |
| H 12 $HUP^+$ | 665 | 0.646 | 10.88 | 30.27 | 32.90 | 61.44 |
| $p^a$ | 0.028 | 0.513 | 0.481 | 0.451 | 0.971 | 0.032 |
| Increase (%)[b] | 45.2* | $0.76^{n.s.}$ | $14.5^{n.s.}$ | $-0.73^{n.s.}$ | $14.2^{n.s.}$ | 27.5* |
| PRF 81 wt | 535 | 0.591 | 10.00 | 32.61 | 32.61 | 56.79 |
| PRF 81 $HUP^+$ | 548 | 0.554 | 10.60 | 34.30 | 36.36 | 69.99 |
| $p^a$ | 0.153 | 0.473 | 0.201 | 0.008 | 0.006 | 0.009 |
| Increase (%)[b] | $2.3^{n.s.}$ | $-6.2^{n.s.}$ | $6.0^{n.s.}$ | 5.2** | 11.5** | 23.2** |
| Control (-I)[c] | 0 | 0 | 0.34 | 18.62 | 0.63 | |
| Control (-I $^+$N)[c] | 0 | 0 | 17.26 | 25.62 | 44.22 | |

Experiments were performed under controlled greenhouse conditions in Leonard jars containing sterile substrate. Plants were harvested at flowering stage (38 days after emergence)

Parameter abreviations: *NN* nodule number, *NDW* nodule dry weight, *SDW* shoot dry weight; *SNC* shoot N concentration; *STNC* shoot total N content; *NE* nodule efficiency (TSN/NDW)

[a]Data are the means of eight replicates; NN data were transformed to log (x + 1) for the analysis; test F

[b]Percentages of increase due to $Hup^+$ strains as regard to respective wild-type strains: *n.s.* statistically non-significant, *significant at 5% and **significant at 1% level using test "t"

[c]Non-inoculated control, with or without N-mineral (80 mg N $pl^{-1}$ $week^{-1}$)

*corniculatus* L.; Monza et al. 1997), pea (Bedmar and Phillips 1984) and lentil (*Lens culinaris*; Brito et al. 2008) symbioses.

In a pioneer study of hydrogenases of common bean microsymbionts, both CIAT 899 and CFN 299 were positive for the hybridization with a probe containing genes of *B. japonicum*, but both had $Hup^-$ phenotypes in nodules of 30-day-old plants (van Berkum et al. 1994). The sequencing of the genomes of *R. tropici* CIAT 899 and strain PRF 81 (Ormeño-Orrillo et al. 2012), this last one now reclassified as *R. freirei* (Dall'Agnol et al. 2013), as well as of strain CFN 299 (Ormeño-Orrillo et al. 2016), now reclassified as *R. leucaenae* (Ribeiro et al. 2012), and also of *R. tropici* H 12 (data not published) has shown that all harbor truncated genes in the hydrogenase operons. These genetic defects are consistent with the lack of hydrogenase activity observed in the wild-type strains in the present study.

In *R. leguminosarum*, *hup* and *hyp* genes are organized as *hupSLCDEFGHIJKhypABFCDEX* and result in a functional hydrogenase (Brito et al. 2005; Imperial et al. 2006). A method of transferring the capacity of oxidation of $H_2$ from *R. leguminosarum* to other rhizobial strains by the introduction of a mini-transposon has been developed (Báscones et al. 2000), and in this study it was used to transfer these genes to three very effective but $Hup^-$ strains used in Brazilian commercial inoculants, CIAT 899, PRF 81 and H 12. The $Hup^+$ phenotype was confirmed, allowing a proper comparison of $Hup^-$ and $Hup^+$ symbiotic performances. In all three strains the introduction of the Hup mini-tranposon TnHB100 resulted in high levels of hydrogenase activity in bacteroids, and the

**Table 2** Effect of $Hup^+$ trait of three elite inoculant strains on seed production and N content parameters of *Phaseolus vulgaris* cv. Carioca grown in non-sterile soil

| Strain | Seed production parameter | | | |
|---|---|---|---|---|
| | SeN (no. $pl^{-1}$) | SeDW (g $pl^{-1}$) | SeNC (g $kg^{-1}$) | SeTNC (g $pl^{-1}$) |
| CIAT 899 wt | 83.8 | 12.58 | 27.70 | 34.81 |
| CIAT 899 $HUP^+$ | 90.4 | 14.83 | 24.54 | 36.78 |
| $p^a$ | 0.213 | 0.296 | 0.762 | 0.224 |
| Increase (%)[b] | $7.9^{n.s.}$ | $17.9^{n.s.}$ | $-11.4^{n.s.}$ | $5.6^{n.s.}$ |
| H 12 wt | 73.8 | 12.47 | 29.60 | 36.21 |
| H 12 $HUP^+$ | 91.8 | 15.18 | 30.17 | 46.9601 |
| $p^a$ | 0.226 | 0.012 | 0.821 | 0.048 |
| Increase (%)[b] | $24.4^{n.s.}$ | 21.8* | $1.9^{n.s.}$ | 27.1* |
| PRF 81 wt | 74.4 | 12.98 | 30.25 | 38.84 |
| PRF 81 $HUP^+$ | 81.0 | 13.43 | 32.10 | 42.40 |
| $p^a$ | 0.151 | 0.047 | 0.952 | 0.016 |
| Increase (%)[b] | $8.9^{n.s.}$ | 3.5* | $6.1^{n.s.}$ | 9.2* |
| Control (-I)[c] | 38.0 | 5.03 | 24.10 | 12.12 |
| Control (-I $^+$N)[c] | 124.0 | 18.90 | 32.20 | 60.86 |

Plants were harvested at physiological maturity

Parameter abbreviations: *SeN* seed number, *SeDW* seed dry weight, *SeNC* seed N concentration, *SeTNC* seed total N content

[a]Data are means of eight replicates; test F

[b]Percentages of increase due to $Hup^+$ strains as regard to respective wild-type strains: *n.s.* statistically non-significant, *significant at 5% and **significant at 1% level using teste "t"

[c]Non-inoculated control, with or without N-mineral (80 mg N $pl^{-1}$ $week^{-1}$)

activity was not increased in the presence of extra levels of nickel in the plant nutrient solution. Similar results have been previously observed in bacteroids induced by *R. etli* CFN 42 in common bean of cultivar Negro Jamapa (Brito et al. 2000). In contrast, *R. leguminosarum* bacteroids induced in pea nodules require high levels of nickel in the nutrient solution to attain maximum levels of hydrogenase activity and enzyme processing levels (Brito et al. 1994). These data indicate that *P. vulgaris* plants are able to efficiently provide nickel to bacteroids for hydrogenase synthesis.

When symbiotic parameters were compared in the wild-type and $Hup^+$ derivative strains, the results have shown that the $Hup^+$ trait, in general, resulted in significant increases in BNF and seed production parameters. CIAT 899 was isolated in Colombia, while H 12 and PRF 81 are indigenous to Brazil, and the results were more prominent with the Brazilian strains. Outstanding increases were shown in nodule number and nodule efficiency, of 45.2 and 27.5%, respectively, with the H 12-derivative $Hup^+$ strain. Our results with common bean are in agreement with studies comparing $Hup^+$ and $Hup^-$ strains in cowpea (Souza et al. 1999; Baginsky et al. 2005) and soybean (Hungria et al. 1989). Most importantly, parameters related to seed production, including seed dry weight and seed total N content were also significantly improved by the $Hup^+$ trait in plants inoculated with the two Brazilian derivative strains.

The synthesis of inorganic N-fertilizers requires large quantities of fossil fuel and its application results in the release of nitrous oxide from soil as well in contamination of water reservoirs; therefore, improving BNF efficiency and reducing the use of N-fertilizers would also help to alleviate the environmental N footprint by decreasing the need for N fertilizers (Dong et al. 2003; Golding and Dong 2010; Hungria et al. 2013; Sá et al. 2017). In this context, improving rhizobial strains for use as inoculants with legume crops by means of properly engineering $Hup^+$ rhizobial strains might result in superior symbiotic performance (Kent et al. 1998; Golding and Dong 2010). In the engineering process described here, strains have incorporated an 18-kb hydrogenase gene cluster obtained from the symbiotic plasmid of *R. leguminosarum* UPM791, along with a resistance marker (spectinomycin). Removal of this marker is feasible (Báscones et al. 2000), thus resulting in modified strains containing only rhizobial DNA. Our results highlight the biotechnological potential of using $Hup^+$ strains for the improvement of BNF with common bean.

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**Authors' contributions** Conceived and designed the experiments: TR-A, JI, JMP, MH. Performed the experiments: ART, BB, JI, JMP, TR-A, MH. Analyzed the data: ART, BB, JI, JMP, TR-A, MH. Contributed reagents/materials/analysis tools: TR-A, MH. Wrote the paper: ART, JI, JMP, IAC, TR-A, MH. All authors read and approved the final manuscript.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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