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Symbiotic effectiveness of *Bradyrhizobium ingae* in promoting growth of *Inga edulis* Mart. seedlings

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ABSTRACT: *Inga edulis* Mart. is a leguminous tree adapted to acidic and low-fertility soils that establishes symbioses with nitrogen (N)-fixing bacteria. The identification of effective bacteria in biological N fixation may bolster the use of *I. edulis* in degraded or modified areas and agroforestry systems. Therefore, the aims of this study were evaluation of the symbiotic effectiveness of eight strains of the *Bradyrhizobium* genus native to Roraima in *Inga edulis* plants, and *in vitro* evaluation of the ability of the eight strains of *Bradyrhizobium* to develop plant growth-promoting characteristics. Determination of symbiotic effectiveness was carried out via three experiments: the first in a greenhouse in pots with a sterile substrate; the second in a greenhouse in pots containing non-sterile soil; and the third in a nursery in bags with a non-sterile substrate. Twelve treatments were evaluated: inoculation with eight strains of *Bradyrhizobium ingae* (ERR 490, ERR 492, ERR 493, ERR 494^T, ERR 496, ERR 497, ERR 498, and ERR 569); inoculation with two strains indicated for *Inga marginata*, BR 6609 and BR 6610 (positive controls); no inoculation but with mineral N; and neither inoculation nor mineral N. All of the experiments were conducted in a completely randomized design with four replicates. The first experiment was conducted for 60 days, and the other experiments were conducted for 100 days. For all of the experiments, the number of nodules, nodule dry matter, root dry matter, shoot dry matter, number of leaflets, plant height, stem diameter, total N in the shoots, root/shoot dry matter ratio, Dickson's quality index, relative effectiveness, and the Pearson correlation between the variables under study were evaluated. The strains were also evaluated by their ability to solubilize calcium and aluminum phosphates and to produce indolic compounds. The results showed that *B. ingae* strains were effective in biological N fixation, especially the ERR 493, ERR 498, and ERR 569 strains. These strains increased the production of shoot dry matter and total N and exhibited relative effectiveness higher than 100 % in all of the experiments. The *B. ingae* strains were also able to solubilize calcium and aluminum phosphates, despite their synthesis of indolic compounds. Thus, the strains of *B. ingae* can be used for inoculation in the production of *I. edulis* seedlings.

Keywords: biological nitrogen fixation, phosphate solubilization, indolic compounds.

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INTRODUCTION

Species of the *Inga* genus are leguminous trees belonging to the Leguminosae family and Mimosoideae subfamily, which includes approximately 300 species in neotropical regions (Pennington, 1997); 131 of these species occur in Brazil (Garcia and Fernandes, 2015). *Inga* spp. have the potential for use in low-nitrogen (N) input agriculture because they establish symbioses with N-fixing bacteria, yield high biomass, and are tolerant of acidic soils (Hands, 1998).

Inga edulis Mart. is a species that has been used by Latin American farmers aiming to provide shade for coffee (*Coffea arabica* L.) and cacao (*Theobroma cacao* L.) plants (Leblanc et al., 2006). This leguminous plant is also commonly intercropped with *Terminalia amazonia* (J. F. Gmel.) Excell in degraded areas with the goal of promoting the development of this timber species (Nichols et al., 2001). The symbiotic effectiveness between N-fixing bacteria and leguminous trees is critical for improving sustainable production systems. For example, plants that establish symbiosis with N-fixing bacteria such as *Inga* spp. are extremely important for organic coffee cultivation systems in Mexico, where the application of N via chemical fertilizers is not allowed (Grossman et al., 2006).

Inga edulis occurs in diverse phytogeographical domains in Brazil, including the Amazon, *Caatinga*, *Cerrado*, and Atlantic Forest (Possette and Rodrigues, 2010; Souza et al., 2011; Garcia and Fernandes, 2015). Despite its wide occurrence and its potential for use in agroforestry systems and in recovery of degraded or altered areas, there are no bacterial strains recommended for these leguminous plants. Studies in this field can lead to the selection of effective N-fixing bacteria, thereby increasing N input in more sustainable systems, resulting in increased soil fertility. In Brazil, there are only two bacterial strains currently recommended for inoculation of the *I. marginata* species (Franco and Faria, 1997; Brasil, 2011). Therefore, it is necessary to select strains for other *Inga* species.

Recently, strains of N-fixing bacteria were isolated from *Inga laurina* (Sw.) Willd. nodules collected in the state of Roraima, Brazil. These strains were characterized and described as a new *Bradyrhizobium* species named *B. ingae* (Silva et al., 2014). Although these strains were capable of nodulating *I. edulis*, their symbiotic effectiveness was not evaluated. In addition to capacity for N fixation in these bacterial strains, it is necessary to identify other important processes in them that could promote plant growth, including phytohormone production and phosphate solubilization (Antoun et al., 1998; Boiero et al., 2007; Kuss et al., 2007; Oliveira-Longatti et al., 2014). Therefore, the main hypothesis of our study was that *B. ingae* strains isolated from *I. laurina* in Roraima are effective in biological N fixation and can be used for *I. edulis* seedling production. In addition, another hypothesis is that *B. ingae* strains have mechanisms for promoting plant growth, such as the ability to solubilize phosphates and produce phytohormones. Along with biological N fixation, these growth-promoting mechanisms could aid the development of *I. edulis* seedlings and other plant species.

The objectives of this study were to evaluate the symbiotic effectiveness of eight *B. ingae* strains native to Roraima in *I. edulis* plants and to evaluate the ability of these strains to develop characteristics for promoting plant growth *in vitro*.

MATERIALS AND METHODS

Origin of strains

The bacterial strains used in this study were isolated from *I. laurina* (Sw.) Willd. nodules collected under natural savannah conditions in the state of Roraima at the Monte Cristo Experimental Station that belongs to *Embrapa* Roraima and in an area within the city of Boa Vista. The geographic coordinates of these sampling areas were 2° 50’ 21” N, 60° 40’ 32,25” W and 2° 57’ 00” N, 60° 42’ 25” W, respectively.

These strains were genotypically and biochemically identified as a new species named *B. ingae* (Silva et al., 2014). The strains described included ERR 490, ERR 492, ERR 493, ERR 494^T, ERR 496, ERR 497, ERR 498, and ERR 569. Although such strains were isolated from *I. laurina* plants, they were capable of nodulating *I. edulis* Mart. However, their symbiotic effectiveness was not evaluated.

Treatments used in the symbiotic effectiveness experiments

The following twelve treatments were used in the symbiotic effectiveness experiments: inoculation with eight strains of *B. ingae* (ERR 490, ERR 492, ERR 493, ERR 494^T, ERR 496, ERR 497, ERR 498, and ERR 569); inoculation with two strains of *Bradyrhizobium* spp. recommended in Brazil by the Ministry of Agriculture, Livestock and Food Supply (Ministério de Agricultura, Pecuária e Abastecimento - MAPA) for *I. marginata* (BR 6609 and BR 6610); no inoculation or added mineral N (Control -N); and no inoculation but with added mineral N (10 mg of N per plant was added weekly in the form of ammonium nitrate) (Control +N). A completely randomized design was used for all of the experiments, with four replicates, for a total of 48 plots per experiment.

Symbiotic effectiveness was evaluated in three experiments: in pots with a sterile substrate in a greenhouse; in pots containing non-sterile soil in a greenhouse; and in bags with non-sterile soil in a nursery.

Inga edulis seeds collected at the Água Boa Experimental Station of Embrapa Roraima were used for all the experiments. This experimental station is located in Boa Vista, Roraima, Brazil, at the coordinates 02° 39' 00" N and 60° 49' 40" W. The seeds were disinfected with 92 % ethyl alcohol for 30 s, followed by 2 % sodium hypochlorite for 1 min, and then washed six times with sterile distilled water.

The strains were cultured in liquid medium 79 (pH 6.8) (Fred and Waskman, 1928) and incubated with shaking at 28 °C for 72 h. The optical density (OD) was adjusted to 0.8 at 630 nm, which represented approximately 10^8 cells mL^{-1}. One milliliter of bacterial culture was used to inoculate each plant 15 days after emergence.

Experiment 1 – Symbiotic effectiveness in sterile substrate in a greenhouse

The first experiment was conducted in a greenhouse under controlled temperature (28 °C) and light (50 % shade) in Leonard pots (Vincent, 1970). A 2:1 mixture of medium texture sand and vermiculite was used in the top part of the pots as substrate. In the bottom part, Hoagland's nutrient solution (pH 7.0) was used (Hoagland and Arnon, 1950) as modified by Guimarães et al. (2012) with low N concentration (5.25 mg L^{-1}). The pots were autoclaved (120 °C, 128 atm) twice for 1 h each time. One seed inoculated with 1 mL of bacterial medium was planted in each pot. Each pot received 800 mL of nutrient solution per week; a 4-fold dilution was used for 30 days, and a 2-fold dilution was used for the remainder of the experiment (60 days). The Control +N treatment received 10 mg of N per plot per week in the form of ammonium nitrate.

Experiment 2 – Symbiotic effectiveness in non-sterile soil in a greenhouse

The second experiment was conducted in a greenhouse using polyethylene pots with 2.41 dm^3 capacities. These pots contained non-sterile soil and washed medium-texture sand in a 1:1 ratio. A *Latossolo Amarelo Distrófico* (Oxisol) was collected from the 0.00-0.20 m layer of the Embrapa Roraima station at the coordinates 02° 42' 30" N and 47° 38' 00" W. The soil used in the experiment had the following chemical properties: pH(H_2O) of 5.2; 5.0 g kg^{-1} of organic matter; 12 mg dm^{-3} of P; 0.05 cmol$_c$ dm^{-3} of K^+; 0.7 cmol$_c$ dm^{-3} of Ca^{2+}; 0.7 cmol$_c$ dm^{-3} of Mg^{2+}; 1.8 cmol$_c$ dm^{-3} of H+Al; and 0.1 cmol$_c$ dm^{-3} of Al^{3+}.

Two seeds were planted per pot. Plants were thinned after germination to maintain one plant per pot. Pots were irrigated with 100 mL of distilled water as necessary to maintain adequate soil moisture.

The experiment was irrigated with sterile Hoagland and Arnon (1950) nutrient solution as modified by Guimarães et al. (2012). For each treatment, a total of 100 mL per week of nutrient solution was applied per pot; for the first 30 days a 4-fold dilution was applied, followed by a 2-fold dilution for another 30 days, and an undiluted solution until the end of the experiment. The experiment was conducted for 100 days.

Experiment 3 – Symbiotic effectiveness in non-sterile substrate in a nursery

The third experiment was conducted under nursery conditions, using 3 L polyethylene bags. A mixture (1:1:1) of soil with clay texture (*Latossolo Vermelho-Amarelo*), soil with sandy texture (*Latossolo Amarelo*), and a coarse texture sand was used. The *Latossolo Vermelho-Amarelo* and the *Latossolo Amarelo*, both classified as Oxisol, were collected at the Monte Cristo and Água Boa experimental stations of Embrapa Roraima, respectively. After mixing the soils, this substrate had the following chemical properties: pH(H_2O) of 5.9; 6.7 g kg^{-1} of organic matter; 2.57 mg dm^{-3} of P; 0.04 $cmol_c$ dm^{-3} of K^+; 0.5 $cmol_c$ dm^{-3} of Ca^{2+}, 0.17 $cmol_c$ mg^{-3} of Mg^{2+}; 1.7 $cmol_c$ dm^{-3} of H+Al; and 0.08 $cmol_c$ dm^{-3} of Al^{3+}.

Two seeds were planted per bag. After thinning, one seed remained in each bag. Irrigation was performed daily using an automated sprinkler system. This experiment was conducted for 100 days. Nutrient solution was applied according to the procedure used for the greenhouse experiment with non-sterile soil.

Variables evaluated and statistical analyses

One replicate of each treatment was removed to observe nodule formation 45 days after sowing in each experiment. In addition, at the end of each experiment, plants were collected to analyze the following variables: number of nodules (NN); nodule dry matter (NDM); shoot dry matter (SDM); root dry matter (RDM); number of leaflets (NL); plant height (PH); stem diameter (SD); and total N in the shoots (TN). The RDM/SDM ratio and Dickson's quality index (DQI) (Dickson et al., 1960) were calculated. The latter was calculated using the following equation:

DQI = TDM (g)/PH (cm)/SD (mm) + SDM (g)/RDM (g)

where TDM refers to the total dry matter (SDM+RDM). Moreover, relative effectiveness (RE) was determined using the formula RE = inoculated SDM × 100/SDM with N, where RE is the relative effectiveness, inoculated SDM is the shoot dry matter of plants inoculated with the tested strains and SDM with N is the shoot dry matter with N and without inoculation (Control +N) (Bergensen et al., 1971). The Shapiro-Wilks normality test at a 5 % significance level was applied to all the variables. The NN, NDM, and NL data did not follow normal distributions and were transformed to $(x+1)^{0.5}$ before analysis of variance. The means were compared using the Scott-Knott test (Scott and Knott, 1974) at a significance level of 5 %. Statistical analyses were conducted using R-3.2.2 software (R Core Team, 2013) through the statistical package ExpDes.pt version 1.1.2 (Ferreira et al., 2013).

Pearson's correlation (r)

Pearson's correlation (r) was also calculated for the variables analyzed in the three symbiotic effectiveness experiments using R-3.2.2 software.

Phosphate solubilization

To investigate phosphate solubilization, two experiments were conducted. One experiment aimed to evaluate the ability of the selected strains to solubilize calcium phosphate ($CaHPO_4$). The other experiment aimed to check the ability of strains to solubilize aluminum phosphate ($AlPO_4$). An NBRIP medium was used (Nautiyal, 1999) with a modified source of phosphate. A total of 2.6 g of $CaHPO_4$ with pH adjusted to 7.0 was used for

the $CaHPO_4$-solubilization experiments. A total of 2.36 g of $AlPO_4$ with pH adjusted to 4.5 was used for the $AlPO_4$-solubilization experiments. In solid media, 10 g of agar per liter was added.

To evaluate the ability of bacterial strains to solubilize calcium and aluminum phosphates in Petri dishes, bacteria were cultured in liquid medium 79 for 72 h, and the OD was adjusted to 0.5-0.7 at 630 nm. After that, 10 µL of bacterial suspension was inoculated at three equidistant points in a Petri dish containing media with precipitated phosphates. The experiment was conducted in a completely randomized design with three replicates. Two strains were included as positive controls for phosphate solubilization: $BR11001^T$ (*Azospirillum brasilense*) and $BR11175^T$ (*Herbaspirillum seropedicae*). The media with inoculated strains were incubated at 28 °C for 18 days. Every six days, the diameter of the solubilization halo (translucent areas surrounding the colonies) was measured using a digital caliper (three measurements per colony). A solubilization index (SI) was obtained from these measurements for each strain using the following formula: SI = halo diameter (mm)/colony diameter (mm) (Berraquero et al., 1976). Based on the SI, the strains were classified according to their ability to solubilize phosphates as low (SI < 2), medium (2 ≤ SI < 4) or high (SI > 4).

To determine the solubilization of insoluble phosphates in liquid media, a 125-mL Erlenmeyer flask containing 30 mL of NBRIP medium and precipitated P was inoculated with approximately 1 mg of cells cultured on solid medium 79. For the medium containing P precipitated with Al, $AlPO_4$ corresponding to 12 mg L^{-1} of P was used. The experiment was set up as a completely randomized design with three replicates. Two strains were included: $BR11001^T$ (*Azospirillum brasilense*) and $BR11175^T$ (*Herbaspirillum seropedicae*).

For determining solubilized P, erlenmeyer flasks containing the media inoculated with the selected strains were incubated at 28 °C under constant shaking at 150 rpm for four days. At the end of this period, the pH was determined, and the material was centrifuged at 10,000 rpm for 5 min. Next, 5 mL of the bacterial supernatant was transferred to a 50-mL disposable cup, and 10 mL of diluted ammonium molybdate solution and approximately 30 mg of ascorbic acid were added to each cup (Embrapa, 2009). After incubation for 1 h, samples were read using an SP2000-UV spectrophotometer at 660 nm. The P concentration was estimated using a standard curve previously prepared with 0, 0.1, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 4.5, 5.0, and 6.0 mg L^{-1} of P as KH_2PO_4. Statistical analyses of the solubilization experiments were conducted as described in the experiments of effectiveness.

Production of indole compounds

With the aim of quantifying the production of indoles, *B. ingae* strains were cultured in liquid medium 79 for 72 h, and the OD was adjusted to 0.6-0.8 at 630 nm. Aliquots of the bacterial suspensions (500 µL) were inoculated in 6 mL of medium 79 (without tryptophan, and supplemented with 100 mg L^{-1} tryptophan) with three replicates. They were then incubated in the dark at 28 °C for 72 h under constant agitation at 120 rpm.

To quantify the indole compounds produced after this period, cultures were centrifuged at 10,000 rpm for 10 min. Three milliliters of the supernatant were transferred to flasks, and 2 mL of Salkowski reagent was added (Sarwar and Kremer, 1995). This mixture was kept in the dark for 20 min for color development; an intense pink color is obtained when there are large amounts of indole compounds. Color intensity was determined using a SP2000-UV spectrophotometer at 535 nm. The concentration of indole compounds was estimated using a standard curve previously prepared with an uninoculated sterile culture medium and known quantities of indole acetic acid (IAA), 0, 10, 25, 50, 75, and 100 µg AIA mL^{-1} (Sigma Aldrich, I3750). Statistical analysis of the production of indolic compounds was conducted as previously described in the symbiotic effectiveness section.

RESULTS

Experiment 1 – Symbiotic effectiveness in sterile substrate in a greenhouse

In the first experiment in sterile substrate, all of the *B. ingae* strains induced the formation of more than 200 nodules per plant (Table 1) and there were no significant differences between the strains recommended for *I. marginata* (BR 6609 and BR 6610). With respect to NDM, treatments ERR 490 and ERR 494^T did not differ from BR 6609; however, these two treatments produced significantly higher NDM than the other treatments. There were no significant differences for RDM and NL. For treatments inoculated with ERR 492, ERR 493, ERR 494^T, ERR 496, and ERR 569 strains, PH was significantly higher than in the other treatments (Table 1). SD was significantly higher in four strains, ERR 492, ERR 493, ERR 494^T, and ERR 569. For SDM, with the exception of ERR496 and ERR 497, all of the *B. ingae* strains showed significant differences compared to the recommended strains (BR 6609 and BR 6610) and the Controls –N and +N. For the TN variable, the Control -N was significantly lower than the other treatments. The RDM/SDM ratio (Figure 1a) was significantly higher in the Control -N treatment than in the other treatments. There were no significant differences in the DQI among the treatments (Figure 1a). A total of seven strains (ERR 490, ERR 492, ERR 493, ERR 494^T, ERR 496, ERR 498, and ERR 569) had RE values higher than 100 % (Figure 2).

Experiment 2 – Symbiotic effectiveness in non-sterile soil in a greenhouse

In the second experiment conducted in a greenhouse, three strains (ERR 492, ERR 494^T, and ERR 498) showed significantly higher NN than the others (Table 2). For NDM, the ERR 492, ERR 493, ERR 494^T, ERR 498, ERR 496, ERR 497, ERR 569, and BR 6610 strains showed significant differences (Table 2). Of the 10 inoculated treatments, five treatments exhibited significant increases in RDM above 5.52 g per plant. The best result for RDM was achieved in the treatment with the ERR 496 strain, resulting in an increase of 6.68 g per plant, followed by the treatments with ERR 497, ERR 569, and BR 6610 and the Control +N treatment. For PH, SD, NL, and SDM, there were no significant differences

Table 1. *Inga edulis* Mart. response to inoculation with *Bradyrhizobium ingae* strains under greenhouse conditions in pots with sterile substrate for 60 days (Experiment 1)

Treatment	NN	NDM	RDM	PH	SD	NL	SDM	TN
	no.	mg		cm	mm	no.	g	mg
ERR 490	367.67 a	656.0 a	2.44	32.00 b	5.21 b	37.67	6.09 a	178.34 a
ERR 492	279.67 a	495.0 b	2.81	36.33 a	6.43 a	37.00	6.11 a	181.11 a
ERR 493	296.67 a	492.4 b	2.78	41.83 a	6.27 a	35.33	7.34 a	203.03 a
ERR 494^T	331.67 a	633.2 a	3.32	39.67 a	6.49 a	35.00	7.77 a	212.72 a
ERR 496	234.67 a	552.6 b	2.60	39.67 a	5.36 b	34.67	5.53 b	194.29 a
ERR 497	220.00 a	328.7 c	1.87	29.33 b	5.64 b	34.00	4.56 b	153.54 a
ERR 498	337.00 a	507.3 b	2.82	31.00 b	5.76 b	33.33	5.97 a	201.38 a
ERR 569	332.00 a	474.3 b	2.67	35.67 a	7.14 a	32.33	6.90 a	214.32 a
BR 6609	279.00 a	690.7 a	2.29	32.50 b	5.81 b	43.00	4.91 b	161.12 a
BR 6610	144.67 a	443.1 b	1.93	31.17 b	5.00 b	41.33	4.99 b	150.83 a
Control -N[(1)]	0.00	0.00	2.48	22.50 b	4.49 b	39.00	3.08 b	45.48 b
Control +N	3.67	31.3	3.42	31.33 b	5.91 b	30.00	5.43 b	139.90 a
CV (%)	21.8	2.62	18.69	16.73	12.09	15.4	18.03	22.78

NN: number of nodules; NDM: nodule dry matter; RDM: root dry matter; PH: plant height; SD: stem diameter; NL: number of leaflets; SDM: shoot dry matter; TN: total N in the shoots. Values in the same column followed by the same letter were not significantly different according to the Scott-Knott test at 5 % probability. [(1)] The Control -N and Control +N treatments were not included in the statistical analyses for the NN and NDM variables. CV: coefficient of variation.

among the treatments evaluated. For TN, the ERR 492, ERR 493, ERR 494^T, ERR 496, ERR 497, and ERR 498 strains showed significant differences. There were no significant differences in the RDM/SDM ratio and DQI (Figure 1b). Eight strains showed RE values greater than 100 % (ERR 492, ERR 493, ERR 494^T, ERR 496, ERR 497, ERR 498, ERR 569, and BR 6609) (Figure 2).

Experiment 3 – Symbiotic effectiveness in non-sterile substrate in a nursery

The ERR 492, ERR 493, ERR 497, ERR 498, and ERR 569 strains showed significant differences in NN (Table 3). Almost all of the *B. ingae* strains, except for ERR 490, induced the production of a greater quantity of matter and showed significant differences for NDM. There were no significant differences for RDM and SD among the treatments. For PH, the ERR 492, ERR 493, ERR 496, ERR 497, ERR 498, ERR 569, BR 6610, and the Control +N treatment were significantly different. For NL, the ERR 493, ERR 494^T, ERR 498, and ERR 569 strains showed significantly higher values than the other remaining strains. The

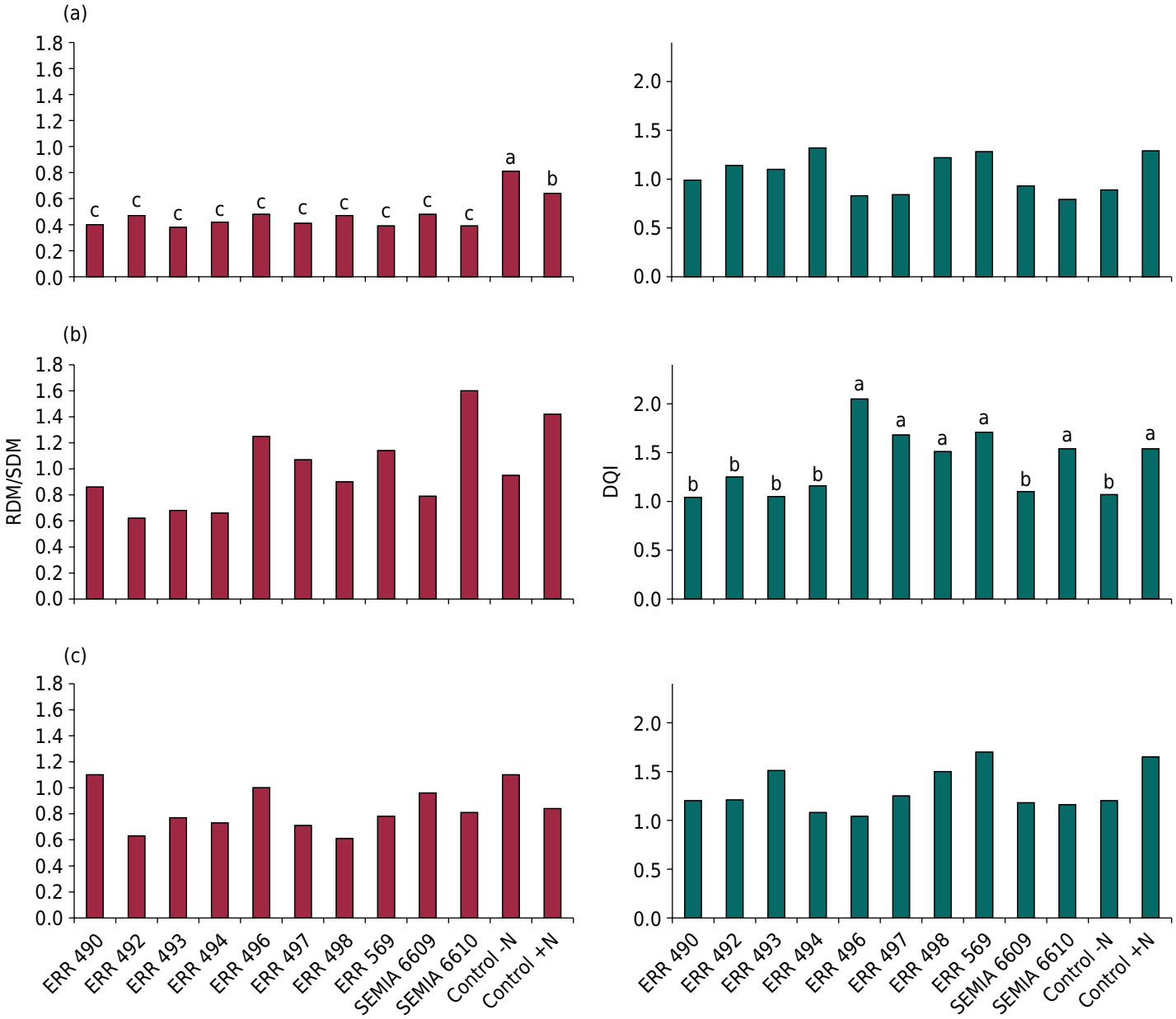

Figure 1. Effects of *Bradyrhizobium ingae* inoculation on the relationship of root/shoot dry matter ratio (RDM/SDM) and Dickson's quality index (DQI) of *Inga edulis* Mart. seedlings. (a) Experiment 1 - sterile substrate in a greenhouse; (b) Experiment 2 - non-sterile soil in a greenhouse; and (c) Experiment 3 - non-sterile substrate in a nursery. Values followed by the same letter were not significantly different according to the Scott-Knott test at 5 % probability.

ERR 492, ERR 493, ERR 497, ERR 498, and ERR 569 strains and the Control +N treatment exhibited significantly different values for SDM. A total of four strains (ERR 493, ERR 497, ERR 498, and ERR 569) showed significant differences for TN, with higher values than the Control +N treatment. There were no significant differences among the treatments for the RDM/SDM ratio and DQI (Figure 1c). Three strains (ERR 493, ERR 498, and ERR 569) showed RE values higher than 100 % (Figure 2).

Pearson's correlation (r)

Pearson's correlations among the variables assessed in all the effectiveness experiments are presented in the table 4. In the first experiment, the Pearson's correlation was positive and significant for NN with NDM, PH, SD, SDM, and TN; NDM with PH, SD, SDM, and TN; RDM with SD, SDM, and TN; PH with SD, SDM, and TN; SD with SDM and TN;

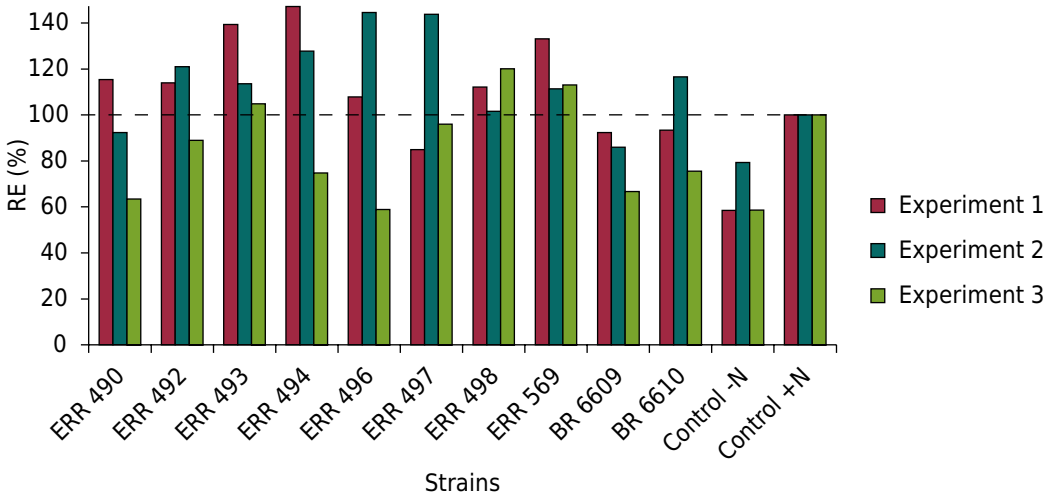

Figure 2. Relative effectiveness of *Bradyrhizobium ingae* strains inoculated in *Inga edulis* Mart. plants. Experiment 1 - sterile substrate in a greenhouse; Experiment 2 - non-sterile soil in a greenhouse; and Experiment 3 - non-sterile substrate in a nursery.

Table 2. *Inga edulis* Mart. response to inoculation with *Bradyrhizobium ingae* strains under greenhouse conditions in pots with non-sterile soil for 100 days (Experiment 2)

Treatment	NN	NDM	RDM	PH	SD	NL	SDM	TN
	no.	mg		cm	mm	no.	g	mg
ERR 490	15.75 c	57.05 b	3.45 b	29.13	4.97	34.25	3.92	74.6 b
ERR 492	104.25 a	333.43 a	3.19 b	26.00	4.70	33.75	5.67	168.7 a
ERR 493	56.75 b	292.43 a	3.23 b	30.50	4.82	32.75	4.81	127.0 a
ERR 494^{T}	91.50 a	372.03 a	3.60 b	28.63	4.36	35.50	5.52	151.4 a
ERR 496	39.75 b	285.85 a	6.68 a	25.00	4.98	38.00	6.12	141.3 a
ERR 497	56.50 b	389.41 a	5.70 a	26.25	4.53	50.00	5.87	146.6 a
ERR 498	86.50 a	401.15 a	4.19 b	21.63	4.42	35.75	4.70	134.4 a
ERR 569	49.25 b	246.28 a	5.60 a	26.00	4.84	29.00	4.86	99.5 b
BR 6609	31.50 b	110.35 b	3.20 b	22.38	4.39	26.75	3.86	90.1 b
BR 6610	39.25 b	217.17 a	5.52 a	27.25	4.66	31.25	4.95	126.0 b
Control -N	2.50 c	7.55 b	3.40 b	23.75	4.26	25.00	3.52	71.3 b
Control +N	8.75 c	31.85 b	6.34 a	28.50	4.48	29.00	4.57	102.3 b
CV (%)	12.38	5.43	37.06	17.03	8.64	12.73	28.86	7.12

Number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), plant height (PH), stem diameter (SD), number of leaflets (NL), shoot dry matter (SDM), and total N in the shoots (TN). Values in the same column followed by the same letter were not significantly different according to the Scott-Knott test at 5 % probability. CV: coefficient of variation.

and SDM with TN. Significant correlations were found between the following variables in the second experiment: NN with NDM, SDM, and TN; NDM with RDM, SDM, and TN; RDM with NL, SDM, and TN; PH with SD and SDM; NL with RDM and SDM; and SDM with TN. In the third experiment, the following significant correlations were observed (Table 4): NN with NDM, PH, SD, NL, SDM, and TN; NDM with RDM, PH, SD, NL, SDM, and TN; RDM with SD, SDM, and TN; PH with SD, SDM, and TN; SD with NL, SDM, and TN; NL with SDM and TN; and SDM with TN.

Phosphate solubilization

Only two of the eight *B. ingae* strains showed a solubilization halo in solid media containing $CaHPO_4$ (Table 5), ERR 490 and ERR 496, with a SI of 1.57 and 1.20, respectively, which are classified as low. Of the strains recommended for *I. marginata*, BR 6609 (*Bradyrhizobium* sp.) had a SI of 4.10 (high). The control strains BR 11175^T (*Herbaspirillum seropedicae*) and BR 11001^T (*Azospirillum brasilense*) had a SI of 3.23 and 2.18 (both medium), respectively (Table 5). None of the strains tested produced a solubilization halo in solid medium containing $AlPO_4$. However, in liquid medium, four strains of *B. ingae* and *Bradyrhizobium* spp. were able to solubilize $AlPO_4$: ERR 490 (0.34 mg L^{-1} of P), ERR 492 (0.06 mg L^{-1} of P), ERR 493 (0.03 mg L^{-1} of P), and BR 6609 (0.53 mg L^{-1} of P), corresponding to 2.83, 0.5, 0.25, and 4.42 % solubilization of the total P added to the medium, respectively (Table 5). The BR 11001^T and BR 11175^T strains used as positive controls showed 6.25 (0.75 mg L^{-1} of P) and 3.66 % (0.44 mg L^{-1} of P), respectively.

Production of indole compounds

Four of the eight *B. ingae* strains, ERR 490, ERR 492, ERR 493, and ERR 494^T, exhibited indole compound production in the presence of tryptophan, with production values ranging from 1.48 to 4.82 µg mL^{-1} (Table 5). In addition to the *B. ingae* strains, the BR 6610 strain was also capable of synthesizing 4.23 µg mL^{-1} of indole compounds, and the positive control, BR 11175^T, synthesized 2.50 µg mL^{-1}. In the absence of tryptophan, three strains still exhibited this characteristic: ERR 492, ERR 494^T, and ERR 496, with respective values of 5.77, 7.57, and 2.62 µg mL^{-1}. Of the recommended

Table 3. *Inga edulis* Mart. response to inoculation with *Bradyrhizobium ingae* strains under nursery conditions in bags with non-sterile substrate for 100 days (Experiment 3)

Treatment	NN	NDM	RDM	PH	SD	NL	SDM	TN
	no.	mg		cm	mm	no.	g	mg
ERR 490	16.00 c	84.73 b	3.84	22.88 b	4.89	24.75 b	3.54 b	74.9 b
ERR 492	82.50 a	267.90 a	3.13	23.88 a	4.75	30.00 b	4.94 a	141.0 b
ERR 493	85.25 a	384.53 a	4.16	23.50 a	4.73	34.75 a	5.83 a	173.0 a
ERR 494^T	44.75 b	266.23 a	2.90	21.00 b	4.60	36.50 a	4.17 b	126.0 b
ERR 496	60.00 b	338.53 a	3.21	24.13 a	4.57	29.75 b	3.30 b	108.8 b
ERR 497	74.50 a	326.53 a	3.71	25.25 a	4.43	31.50 b	5.40 a	173.0 a
ERR 498	97.25 a	320.75 a	4.11	28.00 a	4.35	37.50 a	6.69 a	199.5 a
ERR 569	97.75 a	430.73 a	4.94	26.75 a	4.26	33.00 a	6.34 a	222.7 a
BR 6609	6.00 c	142.88 b	3.58	22.50 b	4.12	30.00 b	3.77 b	81.9 b
BR 6610	53.50 b	203.03 b	3.39	25.25 a	4.03	35.75 a	4.18 b	114.3 b
Controle -N	4.00 c	68.88 b	3.51	19.50 b	5.09	27.50 b	3.26 b	68.2 b
Controle +N	9.25 c	175.05 b	4.78	25.25 a	4.90	42.00 a	5.63 a	120.7 b
CV (%)	22.51	31.48	28.64	10.57	10.59	9.12	24.32	28.96

Number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), plant height (PH), stem diameter (SD), number of leaflets (NL), shoot dry matter (SDM), and total N in the shoots (TN). Values in the same column followed by the same letter were not significantly different according to the Scott-Knott test at 5 % probability. CV: coefficient of variation.

strains for *I. marginata*, only the BR 6609 strain was able to synthesize 2.30 µg mL^{-1} of indole compounds in the absence of tryptophan (Table 5). The positive control strains BR 111175^{T} and BR 11001^{T} synthesized 49.02 and 3.94 µg mL^{-1} of indole compounds, respectively.

DISCUSSION

In this study, the ability of strains of *B. ingae*, a recently-described species (Silva et al., 2014) for promoting growth in *I. edulis* seedlings, was investigated using three experiments: pots with sterile substrate in a greenhouse, pots with non-sterile soil in a greenhouse, and non-sterile substrate in a nursery setting. Generally, nodules were present on the plants 45 days after sowing in all experiments. These observations differ from those found in other species belonging to the genus *Inga*. For example, *I. oerstediana* and *I. jinicuil* exhibited nodulation at 150 and 130 days after sowing, respectively (van Kessel and Roskoski, 1983; Grossman et al., 2006). The rapid establishment of symbiosis between plant and bacteria might be due to the rapid growth of *I. edulis*, which is a desirable characteristic for use in agroforestry systems. It is also noteworthy that *I. edulis* is a plant adapted to acidic soils with low fertility (Hands, 1998), which are common properties of savanna soils of Roraima (Benedetti et al., 2011). Moreover, inoculation with native strains that are adapted to these soils could have promoted the rapid development of

Table 4. Matrix of Pearson correlation (*r*) of variables utilized in the three experiments of symbiotic effectiveness of *Bradyrhizobium ingae* inoculated in *Inga edulis* Mart. seedlings

	NN	NDM	RDM	PH	SD	NL	SDM	TN
NN	1.00							
	1.00							
	1.00							
NDM	0.75***	1.00						
	0.79***	1.00						
	0.70***	1.00						
RDM	0.33ns	0.25ns	1.00					
	-0.13***	0.14***	1.00					
	0.27ns	0.39**	1.00					
PH	0.45**	0.52**	0.30ns	1.00				
	0.20ns	0.08ns	-0.03ns	1.00				
	0.51***	0.36*	0.17ns	1.00				
SD	0.62***	0.41*	0.53**	0.59***	1.00			
	-0.03ns	0.11ns	0.21ns	0.37*	1.00			
	0.39**	0.30*	0.33*	0.56***	1.00			
NL	0.11ns	0.01ns	0.00ns	-0.16ns	0.05ns	1.00		
	0.17ns	0.25ns	0.12***	0.17ns	0.26ns	1.00		
	0.34*	0.31*	0.16ns	0.14ns	0.35*	1.00		
SDM	0.74***	0.63***	0.64***	0.76***	0.75***	-0.02ns	1.00	
	0.52***	0.70***	0.23***	0.14**	0.21ns	0.36***	1.00	
	0.69***	0.67***	0.50***	0.46**	0.43**	0.43**	1.00	
TN	0.69***	0.67***	0.37*	0.68***	0.72***	0.10ns	0.78***	1.00
	0.63***	0.75***	-0.04***	0.13ns	0.05ns	0.26ns	0.83***	1.00
	0.75***	0.71***	0.43**	0.49***	0.45**	0.43**	0.92***	1.00

Number of nodules (NN), nodule dry matter (NDM), root dry matter (RDM), plant height (PH), stem diameter (SD), number of leaflets (NL), shoot dry matter (SDM), and total N in the shoots (TN). Correlation coefficients: the first line is for Experiment 1 – sterile substrate in a greenhouse; the second line, Experiment 2 – non-sterile soil in a greenhouse; and the third line, Experiment 3 – non-sterile substrate in a nursery. *, ** and ***: statistical significance at $\rho<0.05$; $\rho<0.01$ and $\rho<0.001$, respectively; ns: not significant.

symbiosis. Differences in symbiotic effectiveness between inoculated and uninoculated treatments were observed later in the experimental cycle, at 60 days for sterile conditions and 100 days for non-sterile conditions, when the experiments were collected.

After conducting these three experiments, it was observed that *B. ingae* strains were effective in promoting *I. edulis* seedling development in both sterile and non-sterile substrates (Tables 1, 2, and 3). It was verified that NN was higher in sterile substrate (Table 1). Values for NDM obtained in sterile substrate were also higher than those obtained in non-sterile substrates. The decreases in NN and NDM in non-sterile conditions could be due to the presence, even if in low amounts, of organic matter and, consequently, N in the soil. Furthermore, competition between bacteria and other native microorganisms in the soil may reduce the bacterial population present in the inoculant. Thus, during the process of selecting bacteria for the inoculation of legume plants, after determining the strains which are effective in N-fixing under sterile conditions, it also becomes necessary to evaluate their competition with native bacteria in non-sterile soils (Stowers and Elkan, 1980; Franco and Faria, 1997). In some variables, although there were increases in RDM, PH, SD, and NL when plants were inoculated with bacterial strains, these differences were not significant in all of the experiments. The lack of significant differences for some variables in different experiments might be correlated with genetic variability in the native tree species. However, there were significant differences for NN, NDM, SDM, and TN. SDM did not exhibit significant differences in pots filled with non-sterile soils, but there was an increase due to inoculation. For example, inoculation with the ERR 496 strain led to the production of 6.12 g of SDM, whereas 3.52 g was produced in the Control -N treatment (Table 2).

For the RDM/SDM ratio, there were only significant differences in the first experiment conducted in pots with sterile substrate (Figure 1a). In this case, the Control -N treatment had the highest value, followed by the Control +N treatment. The RDM/SDM ratio is one of the parameters used to assess the stability of forest seedlings, and very small values can compromise the establishment of seedlings in the field (Ferraz and Engel, 2011). However, this ratio must be evaluated with caution in inoculation experiments because the Control -N treatment (without inoculation and without N) has reduced shoot development, resulting in a higher value for the RDM/SDM ratio. The DQI (Table 1) must also be

Table 5. Phosphate solubilization and production of indole compounds by *Bradyrhizobium ingae* strains

Strain	$CaHPO_4$ solubilization	$AlPO_4$ solubilization			Production of indole compounds	
	SI	Soluble P	pH	% of soluble P	Trp+	Trp-
		mg L^{-1}			μg mL^{-1}	
ERR 490	1.57 (low)	0.34	3.85	2.83	2.48 b	-
ERR 492	-	0.06	4.20	0.50	4.82 a	5.77 b
ERR 493	-	0.03	4.27	0.25	2.55 b	-
ERR 494^T	-	0		0	1.48 b	7.57 b
ERR 496	1.20 (low)	0		0	-	2.62 c
ERR 497	-	0		0	-	-
ERR 498	-	0		0	-	-
ERR 569	-	0		0	-	-
BR 6609	4.10 (high)	0.53	3.78	4.42	-	2.30 c
BR 6610	-	0		0	4.23 a	-
BR 11175^T	3.23 (medium)	0.44	4.22	3.66	2.50 b	49.02 a
BR 11001^T	2.18 (medium)	0.75	3.48	6.25	-	3.94 c
CV (%)	-	28.09	-		13.68	35.03

SI: solubilization index, low (SI < 2), medium (2 ≤ SI < 4) or high (SI > 4). Means followed by the same letter were not significantly different according to the Scott- Knott test at 5 % probability. CV: coefficient of variation.

evaluated with caution because there are no reference values for quality in the literature for different native tree species (Ferraz and Engel, 2011), which hinders analysis of the DQI. However, the values found in this study for the three experiments were higher than those presented by Góes et al. (2015) for *I. laurina* seedlings. Those authors obtained DQI values ranging from 0.36 to 0.43 after inoculation with N-fixing bacteria, whereas they obtained a value of 0.97 for the treatment that received N fertilizer. These results indicate that *B. ingae* strains were effective in promoting *I. edulis* seedling development because they did not produce results different from the Control +N treatment.

Variation in the RE of *B. ingae* strains was detected in our study. Three of these strains (ERR 493, ERR 498, and ERR 569) had values higher than 100 % in all three experiments (Figure 1), indicating that these strains were capable of effectively supplying N to the plants. Few studies have calculated the RE in leguminous trees. For example, Marques et al. (2001) found an RE of 56.4 % for the combined inoculation of rhizobia strains and mycorrhizal fungi in *Centrolobium tomentosum*. In a study of *I. oerstediana* conducted by Grossman et al. (2006), none of their investigated strains exhibited RE over 100 %; all of the strains showed lower biomass production compared to the treatments with N and without inoculation in both non-sterile and sterile conditions. However, the BR 6609 and BR 6610 strains exhibited RE of 81 % when evaluated under sterile conditions after inoculation of *I. marginata* (Franco and Faria, 1997). Currently, these two strains are recommended for inoculation of this species (Brasil, 2011). Therefore, *B. ingae* strains also have potential to be recommended for inoculation of *I. edulis* seedlings.

Correlation analysis of the variables utilized in all of the three experiments of symbiotic effectiveness (Table 4) showed that NDM, NN, SDM, and TN had the highest values and were significant, with positive correlations with the other variables. In this sense, these variables must be considered for evaluation of the effectiveness of N-fixing bacteria in *Inga* plants. Although NN showed a positive correlation with several of the variables studied, NDM should be used for selection purposes (Norris and Date, 1976) because ineffective or inactive nodules can form, resulting in a higher number of nodules but with low effectiveness. Nodule dry matter has a direct correlation with plant dry matter production and N concentration (Döbereiner, 1966; Stowers and Elkan, 1980). This observation was confirmed in the three experiments, with values higher than 0.60 (Table 4). The variables total N and total dry matter production are important for the selection of high-performing strains for biological N fixation in legumes. Therefore, the use of bacterial strains effective in biological fixation results in increased N content and, consequently, increased shoot development and is an alternative for improving soil fertility. Plants of the genus *Inga* have been used to provide N to recover degraded areas and also in agroforestry systems (Grossman et al., 2006). *Inga* spp. have also been used to provide shade on coffee bean farms in Mexico. In addition to providing shade, the use of *I. jinicuil* was found to be a good source of N input in this production system (Roskoski, 1981). After evaluating the results obtained in this study, three strains, ERR 493, ERR 498, and ERR 569, were selected as being the most effective in biological N fixation in *I. edulis*. These strains stood out for their TN contents and, as a consequence, higher SDM production, and RE values that were greater than 100 %.

It is advantageous that the selected strains also have other mechanisms for promoting plant growth not limited to biological N fixation, because they can contribute to the production of higher quality seedlings. Thus, their $CaHPO_4$ and $AlPO_4$ solubilization abilities and their ability to produce indole compounds were evaluated. It was observed that *B. ingae* strains were able to solubilize $CaHPO_4$ in solid medium and $AlPO_4$ in liquid medium. The formation of a solubilization halo was not detected in solid medium containing $AlPO_4$. This result differed from other $AlPO_4$ solubilization studies in which several authors detected solubilization halos in solid media containing P precipitated with Al (Hara and Oliveira, 2005; Marra et al., 2011, 2012; Oliveira-Longatti et al., 2014). However, it is important to note that the media and the sources of P were different from these studies. Such

differences might explain the absence of a solubilization halo in the *B. ingae* strains tested. The absence of a solubilization halo in solid media cannot be used as the only criterion in selecting solubilizing organisms, mainly because bacteria can solubilize phosphates (Ca, Al, or Fe) without forming a visible solubilization halo (Bashan et al., 2013). These results are in agreement with Souchie et al. (2005), who detected a low incidence of Al-solubilizing organisms in solid media but observed that all the strains were capable of solubilizing $AlPO_4$ in liquid media. In this sense, available P must also be quantified. Although there was no halo formation in solid media, three *B. ingae* strains (ERR 490, ERR 492, and ERR 493) were capable of solubilizing $AlPO_4$ in liquid media (Table 5).

The *B. ingae* strains were also capable of producing indole compounds with or without tryptophan (Table 5). The results obtained for synthesis of indole compounds by *B. ingae* strains were similar to results found for other *Bradyrhizobium* spp. native to the Amazon, with maximum values of 10 μg mL^{-1} (Oliveira-Longatti et al., 2014). Of the *B. ingae* strains, ERR 494^T and ERR 492 exhibited significant amounts in a tryptophan-independent pathway. It is highly advantageous to select bacteria that can synthesize indole compounds via tryptophan-independent pathways because the amino acid content of soils is generally low.

Biological N fixation by *Bradyrhizobium* is well documented. However, some studies have reported that the strains belonging to this genus share characteristics of plant growth-promoting rhizobacteria, such as phytohormone production, phosphate solubilization, and production of siderophores (Antoun et al., 1998; Boiero et al., 2007; Oliveira-Longatti et al., 2014). *B. ingae* strains showed plant growth-promoting mechanisms that may improve the development of *Inga* seedlings; they could also be tested as growth promoters on non-legumes. The most effective strains for biological N fixation were ERR 493, ERR 498, and ERR 569. In addition to its strong N-fixation ability, the ERR 493 strain was able to solubilize $AlPO_4$ and produce indole compounds in the absence of tryptophan. Thus, this particular strain has great potential for promoting the growth and development of *I. edulis* seedlings. Such characteristics are extremely important for the production of high-quality seedlings of leguminous trees because they aid in the survival of these seedlings after transplanting in the field.

CONCLUSIONS

The *Bradyrhizobium ingae* strains evaluated in this study were able to effectively nodulate *Inga edulis* Mart. plants.

The ERR493, ERR 498, and ERR 569 strains were most effective in biological N fixation.

In addition to biological N fixation, *B. ingae* strains have other mechanisms that promote plant growth, such as their abilities to solubilize calcium and aluminum phosphates and synthesize indole compounds.

Bradyrhizobium ingae strains can be used as an inoculant during *I. edulis* seedling production.

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