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Growth promotion of common bean and genetic diversity of bacteria from Amazon pastureland

Linnajara de Vasconcelos Martins Ferreira[1], Fernanda de Carvalho[2], Júlia Fonseca Colombo Andrade[2], Fatima Maria de Souza Moreira[2]*

[1]Federal Institute of Pará – Campus Marabá Rural, BR 155 km 25, PA 26 de Março, C.P. 41 – 68508-979 – Marabá, PA – Brazil.
[2]Federal University of Lavras – Dept. of Soil Science – Sector of Biology, Microbiology and Biological Processes, C.P. 3037 – 37200-000 – Lavras, MG – Brazil.
*Corresponding author <fmoreira@dcs.ufla.br>

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ABSTRACT: A significant number of bacterial species, particularly in the rhizosphere, may benefit plant growth and development. This group of bacteria is known as plant growth-promoting rhizobacteria (PGPR). This study identified genetically isolates of common bean nodules used to trap bacteria from Amazon pastureland and investigated their capacity of nodulating and promoting growth of common bean when inoculated or co-inoculated with CIAT899 strain (*Rhizobium tropici*). Two experiments were carried out in a greenhouse, in axenic conditions, using the common bean cultivar Talismã. In the first experiment, 56 PGPR strains were evaluated individually regarding growth promotion and nodulation. In the second experiment, inoculation of seven PGPR strains previously selected in the first experiment was evaluated in three forms of N supply: Co-inoculation with CIAT 899 in the presence of low N-mineral concentration; individual inoculation in the presence of high N-mineral concentration; and individual inoculation in the presence of low N-mineral concentration. The 16S rRNA gene sequencing showed predominance of *Pseudomonas* genus, identified in 35 % of the sequenced strains. Other genera were identified: *Rhizobium*, *Burkholderia*, *Xanthomonas* and *Bacillus*. Inoculation of the seven strains with CIAT 899 promoted distinct plant growth in different forms of N supply. In addition, N-mineral supply can be replaced by co-inoculation with strains of *Pseudomonas* sp. (UFLA 02-281 and UFLA 02-293) and *Bacillus* sp. (UFLA 02-298) identified in this study.
Keywords: *Phaseolus vulgaris*, co-inoculation, endophytic bacteria, rhizobia

Introduction

In the search for promising isolates that promote plant growth, the Amazon region stands out for its high diversity of organisms in the soil, including microorganisms. This fact was observed in the diversity of rhizobia genera and strains found in western Amazon (Guimarães et al., 2012; Jaramillo et al., 2013). Besides, in the Amazon region, expressive area of the soil is used for pastureland, where recent studies have found a high bacterial diversity (Carvalho et al., 2016; Soares et al., 2016).

Studies show that bacterial strains isolated from this region have the potential to act as plant growth promoters in the biological nitrogen fixing (BNF) process (Ferreira et al., 2012) or in other processes, such as inhibition of phytopathogenic fungal growth, solubilization of phosphates (Marra et al., 2012), production of indole-3-acetic acid (IAA) (Silva et al., 2012; Oliveira-Longatti et al., 2013) and having adaptation to abiotic stresses (Medeiros et al., 2011; Soares et al., 2014).

Co-inoculation of *Rhizobium* and plant growth-promoting rhizobacteria (PGPR) in legumes have received more attention in recent years (Tilak et al., 2006; Mishra et al., 2011; Samavat et al., 2012). This combination brings positive effects on the cultivation of legume species, since it provides growth and nutrient absorption, as it happens in lentil plants with the co-inoculation of *Pseudomonas* sp. with *Rhizobium leguminosarum* (Mishra et al., 2011), increases of nodulation in some crops, such as in pigeon pea [(*Cajanus cajan* (L.) Mill sp.)], with the co-inoculation of *Pseudomonas fluorescens* with *Rhizobium* sp. (Tilak et al., 2006). Besides, this combination presents positive effects on grain yield, as it happens in bean crop, with the co-inoculation of *Pseudomonas* and *Rhizobium* (Samavat et al., 2012).

In Brazil, three *Rhizobium* strains are authorized as inoculants for the common bean: CIAT 899, PRF 81, and H 12. Results show that bean plants can benefit from BNF in the field, with no need of N fertilizers application. The challenge is to apply appropriate management of this symbiosis to increase its efficiency in N supply to plants. Co-inoculation of *Rhizobium* and PGPR may be a strategy to improve efficiency of these strains.

The objective of this study was to genetically identify isolates from nodules of common bean inoculated with Amazon pasture land soil, and to verify their ability to nodulate and promote growth of bean plants co-inoculated or not with CIAT 899.

Materials and Methods

Strains origin

The strains used in this study (56) were isolated from inside the nodules of the common bean inoculated with soils from the Amazon region under pasture systems (Moreira et al., 2009).

16S rRNA gene sequencing of bacterial strains

Bacterial strains were grown in medium 79 (Fred and Waksman, 1928). DNA of 56 bacterial strains was extracted by the alkaline lysis method (Niemann et al.,

1997). The 16S rRNA gene was partially amplified with final reaction volume of 50 µL. The following concentrations were: 5 µL DNA, 5 µL 10X buffer for each PCR, 5 µL dNTP Mix (0.2 mM of each dNTP), 5 µL $MgCl_2$ (2.5 mM), 1 µL of each primer (10 mmol L^{-1}) (27F primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R primer (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991), 0.4 µL Taq DNA polymerase (5 U μL^{-1}), and sterile. Amplification reaction occurred following initial denaturation (94 °C for 5 min), 40 denaturation cycles (94 °C for 40 s), annealing (55 °C for 40 s), extension (72 °C for 1.5 min) and final extension (72 °C for 7 min). Reaction was performed in thermocycler. PCR products were separated in 1 % agarose gel and visualized under UV light.

PCR products were sequenced using the oligonucleotide primer 27F and 1492R. Sequence quality was evaluated using the BioNumerics software (version 7.1). Additionally, the sequences were subjected to BLAST to compare with similar sequences deposited in the GenBank database (National Center for Biotechnology Information - NCBI) (http://www.ncbi.nlm.nih.gov) and were deposited under the accession numbers KU613374 to KU613408.

Experiment 1: Authentication and promotion of vegetative growth of common bean cultivar Talismã plants: *in vitro* assay

The experiment was carried out in a greenhouse from June to July 2014 and was conducted for 40 days. Plants were cultivated in sterilized 500 mL long neck bottles. The experimental design was completely randomized, with three replications and 59 treatments. Treatments consisted of inoculations with 56 bacterial strains isolated from bean nodules (Table 1); one reference strain approved by MAPA (Brazilian Ministry of Livestock Agriculture and Supply) as inoculum for bean crops; and two negative controls without inoculation, with high and low N-mineral concentrations. The experiment used the following stock solutions of N-mineral sources: $Ca(NO_3)_2.4H_2O$ mol L^{-1} : 236.6 g L^{-1}; KNO_3 mol L^{-1} : 101.111 g L^{-1} and $NH_4H_2PO_4$ mol L^{-1}: 132.14 g L^{-1}. From these stock solutions, high N concentration solution received respectively: 4; 0.6 and 0.1 mL L^{-1}, while low N concentration solution received: 0.1; 0.125 and 0.25 mL L^{-1}.

In the inoculated treatments, and in the control without inoculation and with low N concentration, Hoagland (Hoagland and Arnon, 1950) nutrient solution with low N concentration (5.25 mg L^{-1}) was applied. In the control with high N-mineral concentrations, complete Hoagland solution was used, with 52.5 mg L^{-1} nitrogen. Subsequently, all bottles were autoclaved for 60 min at a pressure of 1.5 kg cm^2, at 121 °C.

Long neck bottles containing 500 mL Hoagland nutrient solution were covered with aluminum foil, and two filter paper strips (2 cm in width and length equal to the bottle height) were put inside each bottle to support roots and promote contact of the plant with the nutrient solution. Before sowing, bean seeds (cv. Talismã) were surface disinfected using 98 % ethanol (30 s), 2 % sodium hypochlorite (2 min) and then subjected to successive rinses in autoclaved distilled water (Guimarães et al., 2012). After disinfection, seeds were placed in sterile Petri dishes containing moistened filter paper and cotton, where they remained for 48 h in a growth chamber at 28 °C, for rootlets emergence.

For inoculum preparation, bacterial strains were cultivated in liquid culture medium 79 (Fred and Waksman, 1928), stirred at 110 rpm, at 28 °C, for 3 days. At sowing, 1 mL of the inoculant (10^9 Cells mL^{-1}) was added to the pre-germinated seeds in the inoculated treatments. In the controls without inoculation, 1 mL of the culture medium without inoculum was applied.

Thirty-five days (pre-flowering stage) after the experiment beginning, plants were harvested to determine the following traits: number of nodules (NN); nodule dry matter (NDM); shoot dry matter (SDM); root dry matter (RDM); and total dry matter (TDM). To determine NN, nodules were detached from roots and counted. To determine NDM, SDM and RDM, nodules, shoot and roots were placed in paper bags and allowed to dry in forced air circulation oven at 60 °C until constant weight. Data were subjected to analysis of variance, using the statistical software SISVAR version 5.1 (Ferreira, 2011). Treatments were compared by the Scott-Knott test at 5 % probability. NN and NDM values had been previously transformed into square root of (Y + 0.5).

Experiment 2: Inoculation and co-inoculation of bean with plant growth-promoting bacteria

To evaluate plant growth promotion potential subjected to inoculation and co-inoculation with bean inoculant rhizobia strain (CIAT 899), seven strains were selected (UFLA 02-274, UFLA 02-276, UFLA 02-281, UFLA 02-282, UFLA 02-298, UFLA 02-290, UFLA 02-293) from those that stood out for shoot and root dry matter, as observed in experiment 1, as well as, from those that had already been identified by sequencing, at the time the experiment was carried out.

The experiment was carried out in Oct and Nov, 2014, in autoclaved Leonard jars (Vincent, 1970), containing 700 mL Hoagland solution (Hoagland and Arnon, 1950) in the bottom and a mixture of sand and vermiculite (1:2) in the upper part. The experiment was conducted for 50 days. The experimental design was completely randomized in a factorial design (8 × 3) with three replications. Factor 1 was the individual inoculation of seven strains, plus one treatment without inoculation. Factor 2 was three forms of N supply: low (5.25 mg L^{-1}) and high (52.5 mg L^{-1}) N-mineral concentration in the nutrient solution and co-inoculation with CIAT899 with low N-mineral concentration.

Bean seeds (cv. Talismã) were surface disinfected as described in experiment 1. In each jar, four seeds were sown and inoculated with 1 mL of the culture in liquid medium 79 (Fred and Waksman, 1928) with strains at log growth stage (10^9 cells mL^{-1}). In treatments

Table 1 – Number of nodules (NN), nodules dry matter (NDM), shoot dry matter (SDM), root dry matter (RDM), and total dry matter (TDM) of bean plants inoculated with strains isolated from the Amazon pastureland.

Treatment	NN	NDM	SDM	RDM	TDM
	NN per plant		g per plant		
UFLA 02-305	0.00 c	0.00 b	0.10 c	0.09 c	0.19 c
UFLA 02-306	0.00 c	0.00 b	0.12 c	0.16 b	0.28 c
UFLA 02-307	0.00 c	0.00 b	0.15 c	0.19 b	0.34 c
UFLA 02-308	0.00 c	0.00 b	0.16 c	0.15 b	0.31 c
UFLA 02-309	0.00 c	0.00 b	0.16 c	0.19 b	0.36 c
UFLA 02-310	0.00 c	0.00 b	0.16 c	0.18 b	0.35 c
UFLA 02-311	0.00 c	0.00 b	0.16 c	0.12 b	0.29 c
UFLA 02-272	0.00 c	0.00 b	0.16 c	0.23 a	0.40 c
UFLA 02-295	0.00 c	0.00 b	0.16 c	0.23 a	0.40 c
UFLA 02-312	0.00 c	0.00 b	0.17 c	0.17 b	0.34 c
UFLA 02-313	0.00 c	0.00 b	0.17 c	0.22 a	0.38 c
UFLA 02-314	0.00 c	0.00 b	0.17 c	0.23 a	0.40 c
UFLA 02-315	0.00 c	0.00 b	0.18 c	0.20 b	0.38 c
UFLA 02-316	0.00 c	0.00 b	0.18 c	0.16 b	0.33 c
UFLA 02-273	0.00 c	0.00 b	0.19 c	0.23 a	0.42 c
UFLA 02- 267	0.00 c	0.00 b	0.19 c	0.21 b	0.39 c
UFLA 02-317	0.00 c	0.00 b	0.19 c	0.20 b	0.40 c
UFLA 02-318	0.00 c	0.00 b	0.19 c	0.28 a	0.48 b
UFLA 02-281	0.00 c	0.00 b	0.20 c	0.23 a	0.43 c
UFLA 02-289	0.00 c	0.00 b	0.20 c	0.18 b	0.38 c
UFLA 02-269	0.00 c	0.00 b	0.20 c	0.20 b	0.40 c
UFLA 02-319	0.00 c	0.00 b	0.21 c	0.25 a	0.45 b
UFLA 02-290	0.00 c	0.00 b	0.21 c	0.23 a	0.43 c
UFLA 02-272	0.00 c	0.00 b	0.21 c	0.21 b	0.41 c
UFLA 02-320	0.00 c	0.00 b	0.21 c	0.14 b	0.35 c
UFLA 02-283	0.00 c	0.00 b	0.21 c	0.31 a	0.52 b
UFLA 02-293	0.00 c	0.00 b	0.21 c	0.22 a	0.41 c
UFLA 02-271	0.00 c	0.00 b	0.21 c	0.19 b	0.40 c
UFLA 02-284	0.00 c	0.00 b	0.21 c	0.26 a	0.47 b
UFLA 02-277	0.00 c	0.00 b	0.21 c	0.25 a	0.46 b
UFLA 02-295	0.00 c	0.00 b	0.22 c	0.19 b	0.42 c
UFLA 02-280	15.21 b	0.014 a	0.22 c	0.19 b	0.46 b
UFLA 02-270	0.00 c	0.00 b	0.22 c	0.24 a	0.46 b
UFLA 02-278	0.00 c	0.00 b	0.22 c	0.24 a	0.47 b
UFLA 02-287	12.04 b	0.013 a	0.23 c	0.19 b	0.42 c
UFLA 02-302	0.00 c	0.00 b	0.23 c	0.21 b	0.44 b
UFLA 02-211	0.00 c	0.00 b	0.23 c	0.27 a	0.50 b
UFLA 02-303	0.00 c	0.00 b	0.24 c	0.22 a	0.46 b
UFLA 02-276	0.00 c	0.00 b	0.24 c	0.23 a	0.48 b
UFLA 02-292	0.00 c	0.00 b	0.23 c	0.24 a	0.50 b
UFLA 02-301	0.00 c	0.00 b	0.25 b	0.25 a	0.50 b
UFLA 02-15	0.00 c	0.00 b	0.25 b	0.23 a	0.49 b
UFLA 02-296	0.00 c	0.00 b	0.25 b	0.28 a	0.51 b
UFLA 02-274	0.00 c	0.00 b	0.25 b	0.23 a	0.47 b
UFLA 02-299	0.00 c	0.00 b	0.26 b	0.21 a	0.47 b
UFLA 02-298	0.00 c	0.00 b	0.26 b	0.23 a	0.50 b
UFLA 02-300	0.00 c	0.00 b	0.27 b	0.29 a	0.56 b
UFLA 02-09	11.45 b	0.017 a	0.21 c	0.21 b	0.42 c
UFLA 02-286	0.00 c	0.00 b	0.27 b	0.22 a	0.49 b
UFLA 02-282	0.00 c	0.00 b	0.28 b	0.24 a	0.51 b
UFLA 02-268	0.00 c	0.00 b	0.20 c	0.23 a	0.51 b
UFLA 02-279	18.66 a	0.016 a	0.29 b	0.21 b	0.50 b
UFLA 02-285	11.35 b	0.015 a	0.28 b	0.21 b	0.49 b
UFLA 02-275	0.00 c	0.00 b	0.31 b	0.15 b	0.46 b
UFLA 02-288	0.00 c	0.00 b	0.31 b	0.26 a	0.57 a
UFLA 02-297	0.00 c	0.00 b	0.34 b	0.22 a	0.56 b
Control 52.5 mg L^{-1}	0.00 c	0.00 b	0.55 a	0.36 a	0.92 a
Control 5.25 mg L^{-1}	0.00 c	0.00 b	0.16 c	0.17 b	0.33 c
CV %	48.74	0.35	28.51	22.95	21.35

CV = coefficient of variation. Means followed by the same letters in the columns do not differ by the Scott-Knott test at 5 % probability.

without inoculation, only 1 mL autoclaved liquid culture medium 79 was added.

After sowing and inoculating, jars were covered with a layer of sand paraffin (10 kg sand, 1 L chloroform, and 10 g paraffin) to prevent contamination. Thinning was carried out five days after emergence, leaving one plant per jar. During the experiment, the nutrient solution was prepared, autoclaved, and periodically replaced in jars, according to the plant absorption rate.

At 45 days after sowing, corresponding to the flowering stage, plants were harvested to determine the following traits: number of nodules (NN); nodules dry matter (NDM); shoot dry matter (SDM); root dry matter (RDM); total dry matter (TDM); and N accumulation in the shoots (NAS). To determine NDM and SDM, the same procedures described for the authentication experiment were used. After weighing, N accumulated in the shoots (NAS) was calculated by multiplying the weight of dry shoots by the N content, and measured by the semi micro-Kjeldahl method described by Liao (1981).

Data were subjected to analysis of variance, using the statistical program SISVAR, version 5.1 (Ferreira, 2011). Treatments were compared by the Scott-Knott test at 5 % probability. NDM and NN values had been previously transformed into square root of (Y + 0.5).

In order to confirm the cultural and genetic characteristics of the inoculated strains, 10 nodules were selected from each co-inoculation treatment for re-isolation. For surface disinfection, nodules were first immersed in 95 % ethanol. Next, in H_2O_2 for 3 min, and after that, they were rinsed 10 times with sterile distilled water (Guimarães et al., 2012). Subsequently, nodules were macerated in plates containing culture medium 79 (Fred and Waksman, 1928) and the material was spread to obtain isolated colonies. Afterward, sequencing was carried out as described before.

Results

Of the 56 strains studied, 35 were successfully identified. A comparison of the 16S rRNA gene partial sequencing of strains evaluated with sequences deposited in the GenBank revealed that strains belong to *Rhizobium*, *Burkholderia*, *Xanthomonas*, *Brevibacillus*, and *Bacillus* genera, with predominance of *Pseudomonas* (Table 2).

Table 2 – Identification of strains isolated from nodules of common bean grown in soil of pasture land from Amazon based on the most similar sequences found in GenBank (NCBI).

Strains	Sequence length	Most similar sequences found in GenBank (NCBI)		
		Similarity	Accession number	Species
		%		
UFLA 02-267	612	99	KF295815	*Burkholderia fungorum*
UFLA 02-268	714	100	FJ534682	*Burkholderia* sp.
UFLA 02-288	783	100	HG794272	*Burkholderia fungorum*
UFLA 02-294	1340	99	CP010027	*Burkholderia fungorum*
UFLA 03-304	1308	99	CP010027	*Burkholderia fungorum*
UFLA 02-302	1274	99	CP010027	*Burkholderia fungorum*
UFLA 02-270	1244	100	LC005608	*Brevibacillus* sp.
UFLA 02-295	1260	99	LC005608	*Brevibacillus* sp.
UFLA 02-296	904	99	KT580607	*Brevibacillus* sp.
UFLA 02-299	1305	99	LC005608	*Brevibacillus* sp.
UFLA 02-290*	985	100	NR116873	*Bacillus magaterium*
UFLA 02-298*	1001	99	AB533761	*Bacillus* sp.
UFLA 02-15	792	100	AY822513	*Bacterium RSD-1-2*
UFLA 02-289	358	100	KC894165	*Pseudomonas* sp.
UFLA 02-291	738	100	KT154837	*Pseudomonas* sp.
UFLA 02-293*	1329	100	CP011789	*Pseudomonas putida*
UFLA 02-297	1324	99	KJ748611	*Pseudomonas putida*
UFLA 02-303	498	99	KP279906	*Pseudomonas* sp.
UFLA 02-286	601	100	KU350602	*Pseudomonas* sp.
UFLA 02-281*	685	99	LC270240	*Pseudomonas* sp.
UFLA 02-282*	738	100	LN847264	*Pseudomonas* sp.
UFLA 02-283	584	100	JX827617	*Pseudomonas* sp.
UFLA 02-284	498	100	KP067092	*Pseudomonas* sp.
UFLA 02-269	681	100	KC879711	*Pseudomonas koreensis*
UFLA 02-273	501	100	EU853182	*Pseudomonas* sp.
UFLA 02-275	374	100	KM08802	*Pseudomonas* sp.
UFLA 02-276*	369	100	GQ868350	*Pseudomonas* sp.
UFLA 02-277	601	100	KF053343	*Pseudomonas* sp.
UFLA 02-278	403	100	KF767887	*Pseudomonas putida*
UFLA 02-279	530	100	KP128829	*Rhizobium* sp.
UFLA 02-280	988	99	KJ632048	*Rhizobium* sp.
UFLA 02-09	453	99	KC113610	*Rhizobium etli*
UFLA 02-285	651	99	KJ513849	*Rhizobium* sp.
UFLA 02-287	704	100	KJ734011	*Rhizobium* sp.
UFLA 02-274*	649	100	KF923840	*Xanthomonas* sp.

*Strains evaluated for growth promotion potential when subjected to inoculation and co-inoculation with CIAT 899 strain.

In the individual inoculation experiment (experiment 1), treatments influenced all the evaluated parameters ($p \leq 0.05$) (Table 1). There was no nodulation in the control groups, indicating no contamination in the experiment.

Of the 56 strains that were inoculated, only strains UFLA 02-09, UFLA 02-287, UFLA 02-279, UFLA 02-280 and UFLA 02-285 nodulated bean plants and belonged to the *Rhizobium* genus (Table 2). All strains showed SDM production lower ($p \geq 0.05$) when compared to the control with high N-mineral concentration (Table 1). Among the evaluated strains, 14 stood out for presenting SDM production superior to that of the control with low N concentration ($p \leq 0.05$) (Table 1). Of these strains, only two are nodulating strains (UFLA 02-279 and UFLA 02-285).

In relation to RDM, 55 % of the strains had better performance, with gains similar ($p \leq 0.05$) to that of the control with high N concentration (Table 1).

Seven strains studied in the inoculation and co-inoculation tests (experiment 2) are non-nodulating species. However, they showed good results for plant growth promotion, as observed in experiment 1. These strains belong to the genera: *Pseudomonas* (UFLA 02-276, UFLA 02-281, UFLA 02-282, UFLA 02-293), *Bacillus* (UFLA 02-290 and UFLA 02-298) and *Xanthomonas* (UFLA 02-274).

There was significant interaction between inoculation of the seven strains and forms of N supply for all variables studied, except for NDM (Tables 3 and 4), since only the forms of N supply presented effects.

Table 3 – Number of nodules, nodules dry matter, shoot dry matter, root dry matter, and total dry matter of bean plants inoculated with strains isolated from Amazon pastureland in different forms of N supply.

Strains	Species	CIAT 899	High N concentration (52.5 mg L^{-1})	Low N concentration (5.25 mg L^{-1})
		Number of nodules (NN per plant)		
UFLA 02-282	*Pseudomonas* sp.	42.33 dA	0.00 aB	0.00 aB
UFLA 02-276	*Pseudomonas* sp.	44.33 dA	0.00 aB	0.00 aB
UFLA 02-290	*Bacillus magaterium*	120.33 bA	0.00 aB	0.00 aB
UFLA 02-281	*Pseudomonas* sp.	90.00 cA	0.00 aB	0.00 aB
UFLA 02-298	*Bacillus* sp.	187.00 aA	0.00 aB	0.00 aB
UFLA 02-293	*Pseudomonas putida*	176.33 aA	0.00 aB	0.00 aB
UFLA 02-274	*Xanthomonas* sp.	85.66 cA	0.00 aB	0.00 aB
Control		86.00 cA	0.00 aB	0.00 aB
		Shoot dry matter (g per plant)		
UFLA 02-282	*Pseudomonas* sp.	0.34 bB	2.48 aA	0.26 bB
UFLA 02-276	*Pseudomonas* sp.	0.39 bA	0.48 cA	0.27 bA
UFLA 02-290	*Bacillus magaterium*	0.45 bB	1.41 bA	0.33 bB
UFLA 02-281	*Pseudomonas* sp.	1.06 aA	1.17 bA	0.53 bB
UFLA 02-298	*Bacillus* sp.	1.37 aB	2.08 aA	0.87 aB
UFLA 02-293	*Pseudomonas putida*	1.67 aA	2.13 aA	0.49 bB
UFLA 02-274	*Xanthomonas* sp.	0.40 bB	1.50 bA	0.29 bB
Control		0.19 bB	0.92 bA	0.17 bB
		Root dry matter (g per plant)		
UFLA 02-282	*Pseudomonas* sp.	0.26 aB	1.35 aA	0.32 aB
UFLA 02-276	*Pseudomonas* sp.	0.32 aA	0.48 cA	0.34 aA
UFLA 02-290	*Bacillus magaterium*	0.44 aA	0.57 cA	0.41 aA
UFLA 02-281	*Pseudomonas* sp.	0.42 aB	1.43 aA	0.58 aB
UFLA 02-298	*Bacillus* sp.	0.49 aB	1.05 bA	0.64 aB
UFLA 02-293	*Pseudomonas putida*	0.59 aB	1.05 bA	0.50 aB
UFLA 02-274	*Xanthomonas* sp.	0.34 aB	0.80 cA	0.38 aB
Control		0.50 aA	0.36 cB	0.37 aB
		Total dry matter (g per plant)		
UFLA 02-282	*Pseudomonas* sp.	0.60 cB	3.84 aA	0.58 bB
UFLA 02-276	*Pseudomonas* sp.	0.71 cA	0.96 cA	0.61 bA
UFLA 02-290	*Bacillus magaterium*	0.77 cA	0.87 cA	1.99 aA
UFLA 02-281	*Pseudomonas* sp.	1.48 bB	2.61 bA	1.11 bB
UFLA 02-298	*Bacillus* sp.	1.87 bB	3.13 bA	1.51 aB
UFLA 02-293	*Pseudomonas putida*	2.75 aA	2.95 bA	1.00 bB
UFLA 02-274	*Xanthomonas* sp.	0.75 cB	2.30 bA	0.67 bB
Control		0.69 cB	1.30 bA	0.54 bB
		Nitrogen accumulation in shoots (mg per plant)		
UFLA 02-282	*Pseudomonas* sp.	11.52 bB	96.57 aA	8.00 aC
UFLA 02-276	*Pseudomonas* sp.	18.13 bB	26.98 dA	6.79 aC
UFLA 02-290	*Bacillus magaterium*	16.86 bB	73.26 bA	6.28 aC
UFLA 02-281	*Pseudomonas* sp.	18.86 bB	47.63 cA	13.31 aC
UFLA 02-298	*Bacillus* sp.	41.77 aB	59.52 bA	12.96 aC
UFLA 02-293	*Pseudomonas putida*	50.35 aB	78.20 bA	17.81 aC
UFLA 02-274	*Xanthomonas* sp.	10.44 bB	46.11 cA	6.55 aB
Control		15.80 bB	26.86 dA	7. 95 aC

Means followed by the same lowercase letter in the columns between the seven strains and control by uppercase letters in the lines between the forms of N supply do not differ by the Scott-Knott test at 5 % probability.

Bean inoculant strain (CIAT 899) efficiently nodulated this species, and the uninoculated controls showed no nodulation, indicating that the experiment was not contaminated and that the experimental conditions were favorable to nodulation. In the treatments individually inoculated with the seven previously selected strains, no bean nodulation was observed in the presence of low and high N-mineral concentration in the nutrient solution, as expected.

Co-inoculation of UFLA 02-293 (*Pseudomonas* sp.), UFLA 02-290 and UFLA 02-298 (*Bacillus* sp.) with CIAT 899 resulted in higher NN (176, 120 and 187 nodules per

Table 4 – Nodules dry matter (NDM) of bean plants inoculated with strains isolated from Amazon pastureland in different forms of N supply.

Strains	Species	Dry matter of nodules
		mg per plant
UFLA 02-282	*Pseudomonas* sp.	16^{NS}
UFLA 02-276	*Pseudomonas* sp.	12
UFLA 02-290	*Bacillus magaterium*	55
UFLA 02-281	*Pseudomonas* sp.	23
UFLA 02-298	*Bacillus* sp.	27
UFLA 02-293	*Pseudomonas putida*	35
UFLA 02-274	*Xanthomonas* sp.	16
Control		25
Forms of N supply		
CIAT 899		79 a
High N concentration (52.5 mg L^{-1})		0 b
Low N concentration (5.25 mg L^{-1})		0 b

Means followed by the same lowercase letters in the rows do not differ according to the Scott-Knott test at 5 % probability.

plant, respectively) in relation ($p \leq 0.05$) to individual inoculation with CIAT 899 (86 nodules per plant) (Table 3). Co-inoculation of CIAT 899 with UFLA 02-276 and UFLA 02-282 reduced NN (Table 3).

For NDM, effects were observed only for N supply ($p \geq 0.05$) (Table 4). Co-inoculation of the seven strains with CIAT 899 provided the best results for this variable.

The best results for SDM production ($p \leq 0.05$) were observed in the treatments inoculated with the UFLA 02-293, UFLA 02-298 and UFLA 02-282 in the presence of high N-mineral concentration (Table 3), in the co-inoculation of CIAT 899 with these strains along together with the UFLA 02-281, and in the individual inoculation of UFLA 02-298 in the presence of low N concentration (Table 3). The co-inoculation of CIAT 899 with UFLA 02-281 and UFLA 02-293 showed that the production of shoot dry matter was higher than in treatments cultivated with low N concentration ($p \leq 0.05$), and similar to treatments cultivated with high N concentration ($p \geq 0.05$) (Table 3).

Inoculation of seven strains in the presence of low N concentration and co-inoculation of these strains with CIAT 899 did not affect root dry matter production ($p \geq 0.05$) (Table 3). However, in the presence of high N concentration, inoculation with UFLA 02-282, UFLA 02-281, UFLA 02-298 and UFLA 02-293 promoted higher RDM production ($p \leq 0.05$) and these strains were allocated in groups superior to the control, which was not inoculated with any strain (Table 3). Forms of N supply in the treatments inoculated with UFLA 02-276 and 02-290 UFLA did not influence RDM production. Higher RDM production was observed for the other strains inoculated in the presence of high N concentration (Table 3).

Co-inoculation of UFLA 02-281, UFLA 02-298 and UFLA 02-293 strains with CIAT 899 provided the highest TDM production ($p \leq 0.05$) (Table 3). However, in the presence of high N concentration, only UFLA 02-282 stood out ($p \leq 0.05$) (Table 3). The same form of N supply showed inhibitory effect ($p \leq 0.05$) (Table 3) for inoculation of UFLA 02-276 and UFLA 02-290 strains. The highest TDM production in the presence of low N concentration was verified by the inoculation with UFLA 02-290 and UFLA 02-298 ($p \leq 0.05$) (Table 3). Treatments with high N concentration were superior to the other forms of N supply, except for the treatments with co-inoculation of CIAT 899 with UFLA 02-276, UFLA 02-290 and UFLA 02-293, with no difference between them ($p \leq 0.05$) (Table 3). Therefore, when inoculated with CIAT 899, these strains were effective in promoting bean growth, since inoculation with only CIAT 899 promoted TDM similar to the control.

Treatments with low N concentration showed that inoculation of the seven strains did not influence NAS. However, NAS increased when plants were cultivated in high N concentrations inoculated with UFLA 02-282, UFLA 02-290, UFLA 02-281, UFLA 02-298 UFLA 02-274 and UFLA 02-293, or with the co-inoculation of CIAT 899 with UFLA 02-298 and UFLA 02-293. Treatments inoculated in the presence of high N concentration promoted higher NAS. Co-inoculation results were higher than in treatments with low N concentration (Table 3).

Results of sequencing re-isolated strains (Table 5) suggested that four are endophytes nodules. Re-isolation of UFLA 02-274 (*Xanthomonas* sp.) and UFLA 02-290 (*Bacillus* sp.) (Table 2) was not successful. Thus, their identities were not possible to confirm. UFLA 02-298 was not successfully amplified and was only evaluated by phenotypic characterization in culture medium. CIAT 899 was also observed, confirming that nodulation was promoted by the bean inoculant strain.

Discussion

The 16S rRNA gene sequencing, with the identification of six distinct genera, demonstrate a significant incidence of possible endophytes nodules, since most genera are not recognized as legume symbiont.

Among the studied strains, bacteria of the genus *Rhizobium* were the only ones to nodulate bean plants. Other studies report nodulation of this bean species by *R. tropici* (Martínez-Romero et al., 1991), *R. etli* (Segovia et al.,1993; Wang et al., 1999), *R. gallicum* (Amarger et al., 1997), *R. giardinii* (Amarger et al., 1997), *R. yanglingense* (Tan et al., 2001) and by other genera, such as *Sinorhizobium* sp. (Toledo et al., 2003), *Mesorhizobium* sp. (Chen et al., 1991) and *Burkholderia* sp. (Ferreira et al., 2012).

PGPR can be free-living, associative or endophytic. Endophytic PGPR are able to colonize plant roots and, in the case of legume species, it is capable of cohabiting with BNF within the nodules. According to Kan et al. (2007), these bacteria possibly penetrate into the plant tissue, together with nodulating strains, during infection

Table 5 – Genetic identification of Re-isolated strains from nodules of common bean obtained from co-inoculation experiment based on the most similar sequences found in GenBank (NCBI).

Re-isolated strains	Sequence length	Most similar sequences found in GenBank (NCBI)		
		Similarity	Accession number	Species
		%		
UFLA 02-276	403	100	GQ868350	*Pseudomonas* sp.
UFLA 02-276	1291	100	GQ868350	*Pseudomonas* sp.
UFLA 02-276	812	99	GQ868350	*Pseudomonas* sp.
UFLA 02-276	1014	100	GQ868350	*Pseudomonas* sp.
UFLA 02-281	476	100	LC270240	*Pseudomonas* sp.
UFLA 02-281	614	100	LC270240	*Pseudomonas* sp.
UFLA 02-281	944	100	LC270240	*Pseudomonas* sp.
UFLA 02-281	960	99	LC270240	*Pseudomonas* sp.
UFLA 02-281	1220	100	LC270240	*Pseudomonas* sp.
UFLA 02-293	654	100	CP011789	*Pseudomonas putida*
UFLA 02-282	895	100	LN847264	*Pseudomonas putida*
UFLA 02-282	1327	99	LN847264	*Pseudomonas putida*
CIAT899 Control	88	100	KF773126	*Rhizobium tropici*
CIAT 899 Control	627	100	KF773126	*Rhizobium tropici*
CIAT 899 T3	840	100	KF773126	*Rhizobium tropici*
CIAT 899 T3	564	100	KF773126	*Rhizobium tropici*
CIAT 899 T1R1	802	99	KT356836	*Rhizobium* sp.
CIAT 899 T1R2	1221	99	NR102511	*Rhizobium* sp.
CIAT 899 T1R3	899	100	KP205042	*Rhizobium* sp.
CIAT 899 T1R3	1166	100	LN833444	*Rhizobium* sp.
CIAT 899 T3R3	951	99	KP760691	*Rhizobium* sp.
CIAT 899 T18R3	1013	100	KF773126	*Rhizobium tropici*
CIAT 899 T18R4	865	99	KF773126	*Rhizobium tropici*
CIAT 899 T18R1	1223	100	NR102511	*Rhizobium sp.*
CIAT 899 T18R2	654	100	KF773126	*Rhizobium tropici*

and nodules formation. The genera *Agrobacterium*, *Pseudomonas*, *Enterobacter*, *Pantoea*, *Bacillus* and *Paenibacillus* are more frequently reported as nodule endophytes (Kan et al., 2007; Li et al., 2008; Shiraishi et al., 2010; Costa et al., 2016). In this study, *Bacillus* (UFLA 02-298) and *Paenibacillus* (UFLA 02-276, UFLA 02-293, UFLA 02-281 and UFLA 02-282) were detected as possible endophytes nodules.

Similar to pasturelands, a significant genetic diversity of nodulating and non nodulating PGPR genera were also found in soils under agricultural systems in western Amazon by Guimarães et al. (2012) (*Bradyrhizobium*, *Rhizobium*, *Burkholderia* and *Achromobacter*) and agroforestry systems by Jaramillo et al. (2013) (*Bradyrhizobium*, *Rhizobium*, *Ochrobactrum*, *Paenibacillus*, *Bosea*, *Bacillus*, *Enterobacter*, *Stenotrophomonas*) using *Vigna unguiculata* (L.) Walp as trap plant. These results show that high symbiotic and genetic diversity of bacterial strains from different land use systems in the western Amazon as a potential PGPR source.

In the first experiment, although strains belonging to the genera *Pseudomonas*, *Xanthomonas*, *Burkholderia*, and *Bacillus* (Table 2) did not nodulate common bean plants, they promoted plant growth in low N concentration. This result was probably caused due to phytoestimulation by other biological processes, such as phytohormones production (Costa et al., 2016). *Azospirillum*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Paenibacillus*, *Pseudomonas* and *Burkholderia* strains are often described as potential plant growth promoters due to the action in different biological processes, especially in phosphate solubilization and phytohormones synthesis (particularly IAA) (Samavat et al., 2012; Oliveira-Longatti et al., 2013; 2014; Costa et al., 2016).

The ability to fix N may be affected by several biotic and abiotic factors (Tsai, 1993; Ali et al., 2009). The interaction of *Rhizobium* with other microorganisms in the soil is one of the factors that affects this process and both stimulation and inhibition of nodulation and plant growth may occur, depending on the interaction between symbionts and growth-promoting bacterial strains. In the present study, this fact was presented by nodulation stimulation by *Pseudomonas* sp. (UFLA 02-293), *Bacillus* sp. (UFLA 02-290) and *Pseudomonas* sp. (UFLA 02-298) strains, and nodulation inhibition by *Pseudomonas* sp. (UFLA 02-282 and UFLA 276) strains. However, not all strains that stimulated nodulation in co-inoculated treatments with CIAT 899 promoted growth or increased SDM, RDM, and TDM production.

In RDM, SDM and TDM production, NAS was different for strains depending on N supply, except for RDM in the co-inoculated treatments and in the presence of low N concentration. Notably, the highest increase in SDM was obtained by the combination of *Bacillus* sp. (UFLA 02-298) and *Paenibacillus* sp. (UFLA 02-293, UFLA 02-281 and UFLA 02-282) co-inoculated with *Rhizobium tropici* CIAT 899. Individual inoculations of PGPR in the presence of low N concentration did not influence NAS. This result was expected since they are not nodulating strains, nor N_2-fixing strains; however, they influence plant growth by other processes (Costa et al., 2016). Nevertheless, co-inoculation of PGPR with *Bacillus* sp. (UFLA 02-298) and *Paenibacillus* sp. (02-293) together with *Rhizobium tropici* (CIAT 899) increased NAS.

In the literature, PGPR of the genus *Pseudomonas* are reported as growth promoters, as verified by Samatava et al. (2012). This genus also showed relevant performance when co-inoculated with *Rhizobium tropici* CIAT 899. In other studies, the contribution of the co-inoculation of *Paenibacillus* strains (Rodrigues et al., 2012) and *Enterobacter* (Costa et al., 2016) with *Bradyrhizobium* was observed on the dry matter yield of cowpea and soybean plants, respectively.

Inoculation of CIAT 899 with *Pseudomonas* sp. and *Bacillus* sp. can be an effective strategy to produce bio-fertilizers for beans. The results help establish an inoculum or a combination of inoculum for bean yield improvement, as well as the understanding of their performance under low and high N-minerals concentrations.

Conclusions

16S rRNA gene sequencing showed the predominance of *Pseudomonas* genus as bean plants nodules endophytic strains. Other genera were identified: *Rhizobium*, *Burkholderia*, *Xanthomonas* and *Bacillus*. Thus, high incidence of possible endophytic strains was found in the nodules, since most of these genera are not known as legume plants symbiont.

This result also demonstrates a significant genetic diversity of bacterial strains under pasture system by the presence of strains of *Bacillus* and *Pseudomonas* genera, showing potential to be used as plant growth promoters. Inoculation of seven strains with CIAT 899 promoted varied plant growth in the different forms of N supply. N-mineral supply may be replaced by the co-inoculation of CIAT 899 with UFLA 02-281, UFLA 02-286 and UFLA 02-293, which are plant growth promoting strains. Tests under field conditions are necessary to validate the promising results on a large scale.

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