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# Genetic Variability and Symbiotic Efficiency of *Erythrina velutina* Willd. Root Nodule Bacteria from the Semi-Arid Region in Northeastern Brazil

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**ABSTRACT:** Legume-rhizobia symbiosis is a cross-kingdom association that results in large amounts of nitrogen incorporated in food webs. For the Brazilian semi-arid region, data on genetic variability and symbiotic efficiency of Papilionoidae rhizobial communities are very scarce. The aim of this study was to evaluate the genetic variability and the symbiotic efficiency of eight rhizobial isolates obtained from “mulungu” (*Erythrina velutina* Willd.) nodules. For 16S rRNA gene sequencing, the genomic DNA was extracted using a commercial kit, amplified with universal primers, and subjected to sequencing reactions. For the isolate ESA 71, PCR amplifications for *nodC* and *nodA* genes were attempted. Rhizobial efficiency was assessed by two greenhouse experiments. The first assay was carried out under gnotobiotic conditions, with sterile sand as a substrate; the second experiment was conducted in a non-sterile soil. For both experiments, the inoculation treatments consisted of a single inoculation of each isolate, in addition to a treatment with *Bradyrhizobium elkanii* BR 5609 as a reference strain. Furthermore, two non-inoculated control treatments, supplied and not supplied with mineral N, were also evaluated. Bacterial identification indicated that both  $\alpha$  and  $\beta$ -rhizobia could be found in “mulungu” root nodules. Three isolates were classified within the *Rhizobium* genus, four bacteria belonged to *Bradyrhizobium* and one isolate clustered with *Burkholderia*. Positive amplification of an intragenic fragment of the *nodA* gene using a primer set to  $\beta$ -rhizobia could be found for ESA 71 (*Burkholderia*). All bacterial isolates were effective in colonizing “mulungu” roots. In the first experiment, all inoculated treatments and N fertilization increased the N concentration in “mulungu” shoot tissues. For total N in the shoots, the isolates ESA 70, ESA 72, and ESA 75 stood out. In the non-sterile substrate experiment, the isolates ESA 70, ESA 71, ESA 72, and ESA 75, together with the reference strains, induced increases in the shoot N concentration and total accumulation compared to the absolute control. The results indicate that “mulungu” is able to establish associations with efficient  $\alpha$  and  $\beta$ -rhizobia in Brazilian semi-arid soils.

**Keywords:** biological nitrogen fixation, “mulungu”, tree legumes, “Caatinga”, rhizobia.

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## INTRODUCTION

Biological nitrogen fixation (BNF) is a natural process carried out by a restricted group of prokaryotes that contain the nitrogenase enzymatic complex. These enzymes are able to reduce atmospheric N<sub>2</sub> to ammonium compounds, allowing introduction of N in food webs (Boyd and Peters, 2013). Among these bacteria, there is a group known collectively as “rhizobia” which contain several species able to establish symbiotic associations with a wide range of legumes, especially those belonging to the Faboideae (Papilionoideae) and Mimosoideae sub-families (Sprent, 2009). Legume-rhizobia association is very important in nature and is responsible for incorporation of large amounts of N in terrestrial environments (Freitas et al., 2010; Boyd and Peters, 2013).

The dry tropical forest known as “Caatinga” is the typical phytogeography in the Brazilian Northeastern semi-arid region. This phytogeographic domain is characterized by a high incidence of sunlight, a short and irregular rainy season (up to 800 mm yr<sup>-1</sup>), and high average temperatures (Albuquerque et al., 2012). These conditions have led to the selection of highly adapted plant species, together with their associated microbial community (Albuquerque et al., 2012; Fernandes-Júnior et al., 2015).

Fabaceae (Leguminosae) is the largest family in the Brazilian “Caatinga” encompassing around 617 species within 127 genera (Zappi et al., 2015). Among these plants, several are able to establish efficient symbiotic associations with rhizobia, and N fixation rates by the native legumes are around 13 kg ha<sup>-1</sup> yr<sup>-1</sup> of N in some “Caatinga” areas (Freitas et al., 2010; Souza et al., 2012), indicating the presence of an active diazotrophic community, able to fix N under harsh environmental conditions.

Research efforts in recent years have been applied to evaluate the diversity of bacterial isolates obtained from native legumes to improve our understanding of rhizobia diversity in the “Caatinga” (Teixeira et al., 2010; Freitas et al., 2014; Martins et al., 2015; Menezes et al., 2016). The results indicated large diversity among the rhizobia inhabiting the root nodules of the legume species already evaluated. Plants belonging to the *Mimosa* genus are the best studied among “Caatinga” native legumes, and advances in the taxonomy of their rhizobia have shown the prevalence of β-rhizobia, mainly *Paraburkholderia* spp. (formerly *Burkholderia*) (Reis Júnior et al., 2010; Martins et al., 2015). Regarding Papilionoidae tree legumes, results achieved around the world have indicated the prevalence of α-rhizobia inside their root nodules, although β-rhizobia have also already been observed with lower frequency (Rasolomampianina et al., 2005; Elliott et al., 2007). Studies aiming to isolate and classify the rhizobial community from Papilionoidae trees from the “Caatinga” are still scarce.

*Erythrina velutina* Willd., known as “mulungu”, in the Papilionoidae subfamily and Phaseoleae tribe, is a native tree legume from the Brazilian “Caatinga” that has several uses for people from the Brazilian semi-arid region. The use of its wood/timber and its application as an herb, according to folklore, are noteworthy (Ribeiro et al., 2006; Queiroz, 2009). In addition to this traditional knowledge, systematic studies carried out in the last decade have also indicated the potential of *E. velutina* as a source of alkaloids with several pharmacological properties, notable for their anticonvulsant and anxiolytic effects (Ribeiro et al., 2006; Ozawa et al., 2008), signaling its biotechnological potential.

“Mulungu” is able to associate effectively with rhizobial isolates. Recently, bacteria were isolated from “mulungu” nodules and evaluated as to their metabolic characteristics and genetic profile using the Box-PCR marker (Menezes et al., 2016). The results indicated highly variable physiological characteristics among the isolates and distinguish Box-PCR profiles showing low similarity with known *Bradyrhizobium*, *Rhizobium*, *Ensifer*, and *Paraburkholderia* reference strains. In spite of its efficient association, up to now, *E. velutina* does not have bacterial isolates authorized for use as commercial inoculants in Brazil.

Studies evaluating the diversity and phylogenetic position of rhizobia from “mulungu” can aid in achieving a better understanding of rhizobial ecology in the semi-arid region.

In addition, selection of symbiotically efficient indigenous rhizobia for this species may reveal the presence of bacterial candidates to be used in commercial inoculants. Prospection of these bacteria can help in producing healthy “mulungu” seedlings directed to several uses.

We hypothesized that, among the bacteria evaluated in the present study, there are isolates with symbiotic efficiency and that these rhizobia are taxonomically different, based on their 16S rRNA sequences. The aim of this study was evaluation of the genetic variability of rhizobial isolates from *Erythrina velutina* root nodules by means of 16S rRNA gene sequences analysis, as well as evaluation of their symbiotic performance under gnotobiotic conditions and in non-sterilized soil experiments.

## MATERIALS AND METHODS

### Bacterial isolates and DNA extraction

The bacterial isolates evaluated in the present study were obtained from a trap-host experiment using different soil samples from the “Caatinga” in the lower-middle region of the São Francisco River Valley as a substrate for plant growth. These bacteria were previously authenticated and phenotypically characterized by Menezes et al. (2016). For this study, the nodulating fast-growing isolates ESA 68, ESA 69, ESA 70, and ESA 71 and the slow-growing isolates ESA 72, ESA 73, ESA 74, and ESA 75 were evaluated. These bacterial isolates were deposited in the Culture Collection of Microorganisms of Interest to Agriculture of *Embrapa Semiárido* (CMISA) at -80 °C.

For DNA extraction, the bacterial isolates were grown in YM medium without bromothymol blue as a pH indicator (Vincent, 1970) for three days for the fast-growing rhizobia and six days for the slow-growing ones in an orbital shaker at room temperature. After the time of incubation, the DNA of the bacterial isolates was extracted with the commercial kit HiYield™ Genomic DNA Mini Kit (Real Biotech Corporation, Taipei, Taiwan), following manufacturer instructions. DNA integrity was verified by horizontal electrophoresis in a 0.8 % (w/v) agarose gel in TBE 0.5X buffer stained with Gel Red (Biotium) and visualized with UV light in a gel image register system (Vilber Lourmat, France). The DNA samples were stored at -20 °C until PCR manipulation.

### DNA manipulation, sequencing, and bacterial identification

The 16S rRNA amplifications were conducted using the universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Weisburg et al., 1991). The PCR reactions were adjusted to 35 µL (1X reaction buffer, 2.0 mmol L<sup>-1</sup> of MgCl<sub>2</sub>, 0.25 mmol L<sup>-1</sup> of each dNTP, 1 U of Taq DNA polymerase, and 0.25 µmol L<sup>-1</sup> of each primer) and were conducted in a Veriti 96-well thermocycler (Applied Biosystems, Foster City, CA, USA) applying a 4 min initial denaturation step at 94 °C, followed by 35 cycles of denaturation (94 °C for 1 min), annealing (60 °C for 45 s), and extension (72 °C for 2 min) and a final extension step of 5 min. Amplification was verified in agarose gel as described above. Purification of PCR products were carried out using the HiYield™ PCR DNA Mini Kit (Real Biotech Corp, Taipei, Taiwan) commercial kit, following manufacturer instructions. Sequencing reactions were made using the 27F primer in a 3730 xl genetic analyser (Applied Biosystems, Foster City, CA, USA) at Macrogen (Seoul, South Korea).

The quality of the sequences was verified using the Sequence Scanner Software v. 2.0 (Applied Biosystems). Good quality sequences (around 1000 bases) were used for bacterial identification through comparison with the sequences available at the GenBank database (National Center for Biotechnology Information) by means of the Basic Local Alignment Search Tool (BLASTn) (<http://www.ncbi.nlm.nih.gov/Genbank/>) (Altschul et al., 1990). For construction of the phylogenetic tree, the closest 16S rRNA sequences and others belonging to type strains were taken from the GenBank. The sequences were deposited in the GenBank database under accession numbers KU513773 to KU513780.

For sequence comparison, the alignment of our sequences and those obtained from GenBank were carried out. The ClustalW algorithm was used for alignment, and the Neighbor-Joining method and the Jukes-Cantor model were applied for phylogenetic tree construction. Phylogeny was tested by the bootstrap method with 1,000 replications. The sequence alignments and tree construction were made with the aid of MEGA 6.0 software (Tamura et al., 2013).

For a better understanding of the symbiotic characteristics of ESA 71, amplifications of intragenic fragments from *nodC* and *nodA* genes were carried out. PCR for amplification of *nodC* gene from  $\beta$ -rhizobia was carried out using the primers NodCforB (CTCAATGTACACARNGCRTA) and NodCrevB (GAYATGGARTAYTGGYT) (Elliott et al., 2007). For amplification of the *nodA* gene from  $\beta$ -rhizobia, the primers NodAforB (CRGTGGARGGTBYGYTGGGA) and NodArevB (TCAYARCTCDGGBCCGTTBCG) (Klonowska et al., 2012) were applied. For the PCR of the *nodC* gene for  $\alpha$ -rhizobia, the primers NodCF (AYGTHGTYGAYGACGGTTC) and NodCR(I) (CGYGACAGCCANTCKCTATTG) were used (Laguerre et al., 2001). Reactions for the *nodA* gene amplification from  $\alpha$ -rhizobia were made using the primer pair NodA-1 (TGCRGTGGAARNTRNNCTGGGAAA) and NodA-2 (GGNCCGTCRTCRAAWGTCARGTA) (Haukka et al., 1998). For these reactions, together with the bacterial isolate ESA 71, a *Paraburkholderia sabiae* strain (BR3407<sup>T</sup>) was used as a reference. This reference strain was kindly provided by the curators of the Johanna Döbereiner Diazotrophic Bacteria Culture Collection (CCBD) of *Embrapa Agrobiologia* (Seropédica, RJ, Brazil).

The *nodC* and *nodA* PCRs were carried out with the same mixes used for 16S rRNA, changing the primer pairs. The thermal programs had 4 min of an initial denaturation step at 94 °C, followed by 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 45 s) and 5 min of a final extension step. For the NodA-1 and NodA-2 primers, the annealing temperature adopted was 49 °C. The PCR products were subjected to agarose gel (1.5 % w/v) horizontal electrophoresis (120 V for 1.5 h), and visualized in a UV chamber.

### **Symbiotic efficiency of bacterial isolates on sterile and non-sterile substrates**

The symbiotic performance of bacterial isolates was evaluated under greenhouse conditions in two experiments. In the first experiment, the bacterial isolates were tested in an experiment under gnotobiotic conditions. For this assay, “mulungu” seeds were first surface disinfected with 96° GL ethanol for 30 s, sodium hypochlorite 2.5 % (v/v) for five minutes, followed by eight washes in distilled autoclaved water (DAW) (Vincent, 1970). The experiment was set up in polystyrene pots (500 mL). The substrate used was washed sand autoclaved (120 °C and 1.5 atm for 1 h) twice with no less than 72 h between the sterilizations. The pots were also disinfected by washing with sodium hypochlorite 2.5 % (v/v), followed by three washes with DAW. Pots were carefully filled with the sterile sand and three seeds per pot were sown soon after filling.

For inoculation, bacteria were grown in YM medium, as described above, up to the end of the exponential growth phase (around 10<sup>9</sup> cells per mL). Soon after sowing, 1 mL of the culture broth was inoculated over each seed. The pots were supplied with 100 mL of DAW daily until cotyledon drop (at 12 days after sowing - DAS). At 15 DAS, the plants were thinned and one plant was left per pot. From 15 DAS to the end of experiment, the plants received around 150 mL of DAW daily, and 200 mL of a N-free nutrient solution, described by Norris and T'Mannetje (1964), was applied once a week. The assay was conducted up to 89 DAS, at which time the experiments were harvested. The roots were separated from the shoots and washed carefully under running tap water; and nodules were detached and counted. The roots, shoots, and nodules of each plant were separated into paper bags and left to dry in an air flow chamber at 65 °C for seven days and then weighed for determination of the variables shoot dry matter (SDM), root dry matter (RDM), and nodule dry matter (NDM). Shoots were also ground and sieved (2 mm) for determination of shoot nitrogen concentration (SNC) by the semi-micro Kjeldahl method

(Liao, 1981). These values were used for calculation of total nitrogen content (TNC) through the multiplication of SNC (mg N g<sup>-1</sup> plant) by SDM.

The second experiment was carried out with non-sterilized soil as a substrate. The soil sampled was an *Argissolo Vermelho-Amarelo* (Ultisol) from the Bebedouro Experimental Field of *Embrapa Semiárido* (Petrolina, PE). The samples were taken from the surface layer (0.0-0.2 m) and the chemical properties of the soil assessed were: pH(H<sub>2</sub>O) 6.3; electrical conductivity 0.11 dS m<sup>-1</sup>; organic matter 12.3 g kg<sup>-1</sup>; P 44.62 mg kg<sup>-1</sup> (Mehlich-1); K 140 mg dm<sup>-3</sup> (Mehlich-1); Ca<sup>2+</sup> 2.0 cmol<sub>c</sub> dm<sup>-3</sup> (1 mol L<sup>-1</sup> KCl); Mg<sup>2+</sup> 0.4 cmol<sub>c</sub> dm<sup>-3</sup> (1 mol L<sup>-1</sup> KCl); Al<sup>3+</sup> 0.05 cmol<sub>c</sub> dm<sup>-3</sup> (1 mol L<sup>-1</sup> KCl); H+Al 0.66 cmol<sub>c</sub> dm<sup>-3</sup>; cation exchange capacity 3.44 cmol<sub>c</sub> dm<sup>-3</sup>; base saturation 81 %. The experiment was implemented in 500 mL polystyrene pots filled with the soil samples collected.

Disinfection of the seed surface, bacterial growth and inoculation, implementation of the experiment, and plant thinnings were carried out as described above. The plants were supplied with tap water as described above. The experiment was harvested at 89 DAS. Plant manipulation and the variables evaluated were as described for the first experiment.

Both experiments were set up in a completely randomized design, with three replications for the first experiment and four replications for the second experiment. The control treatments evaluated were one without inoculation and N application (absolute control) and other one with a supply of 500 mg of N (NH<sub>4</sub>NO<sub>3</sub>) split into two application, at 20 and 50 DAS. The inoculation treatments were single inoculation of each bacterial isolate from *Embrapa Semiárido* and, as a reference strain, the inoculation of *Bradyrhizobium elkanii* strain BR 5609 (SEMIA 6100), authorized for use as an inoculant for *Erythrina verna*. As in the case of BR3407<sup>T</sup>, the reference strain BR 5609 was kindly provided by the CCBBD curators.

Normal data distribution of the greenhouse experiments was verified by the Shapiro-Wilk test. Data were evaluated by Anova using the transformation (x+1)<sup>0.5</sup>, and the Scott-Knott means range test (p<0.05) was applied. Statistical analysis was carried out using the Sisvar 5.0 software (Ferreira, 2011).

## RESULTS

### Genetic diversity of bacterial isolates

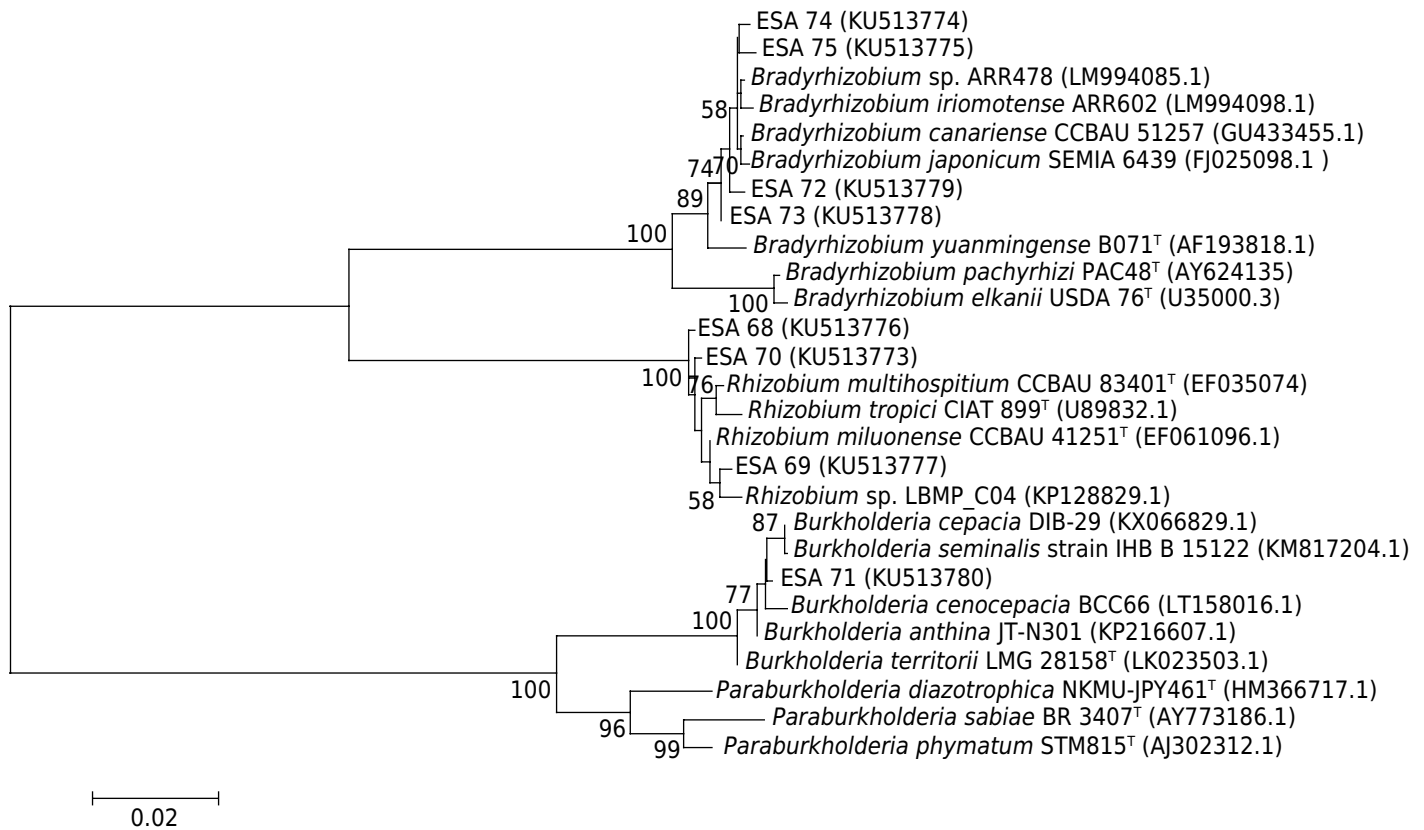
Comparison of the 16S rRNA sequences obtained from the *Erythrina velutina* bacterial isolates indicated that this host is nodulated predominantly by α-proteobacteria, but there is also β-rhizobia inside its root nodules. The isolates ESA 72, ESA 73, ESA 74, and ESA 75 were classified as belonging to the *Bradyrhizobium* genus. These four isolates were related to the *Bradyrhizobium japonicum* genetic cluster and distant *Bradyrhizobium elkanii/Bradyrhizobium pachyrhizi* group (Figure 1). The bacteria ESA 68, ESA 69, and ESA 70, were classified as *Rhizobium* sp. The isolate ESA 69 showed more than 99 % similarity to a *Rhizobium* sp. isolate and was also close to the strain type of *Rhizobium miluonense*. 16S rRNA sequences of the bacterial isolates ESA 68 and ESA 70 showed low similarity (below 98 %) to the other *Rhizobium* sequences evaluated and were distant from the *R. miluonense*, *R. multihospitium*, or *R. tropici* genetic clusters.

ESA 71 was most closely related to the β-proteobacterium *Burkholderia* sp. The *Burkholderia* isolate exhibited a high relationship (more than 99 %) to strains of the *Burkholderia cepacea* complex (Bcc). This isolate also showed low similarity (below 97 %) to nodulating bacteria belonging to the *Paraburkholderia* genus.

### Amplification of intragenic fragments of nod genes from ESA 71

Amplification of the *nodC* and *nodA* genes from ESA 71 showed patterns different from those observed in the reference strain BR 3407<sup>T</sup>. The PCR using the primers to amplify





**Figure 1.** Neighbor-joining phylogenetic tree based on the partial 16S rRNA gene sequence for eight bacteria from *Erythrina velutina* and 15 other reference strains (1000 bp). Numbers in the nodes represents the bootstrap value from 1000 replications. Bootstraps values lower than 50 % are not shown.

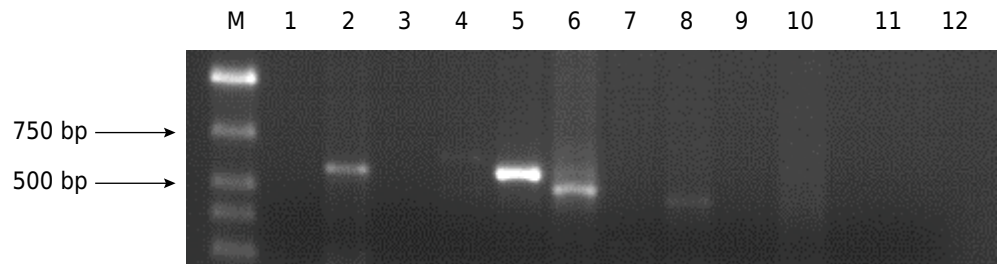
the *nodA* from  $\beta$ -rhizobia showed positive amplification for both bacteria. For ESA 71, the amplicon obtained applying NodAforB and NodArevB primers had around 550 bp, while for BR 3407<sup>T</sup>, the amplicon size was around 480 bp, sizes within the range of molecular weight for  $\beta$ -rhizobia *nodA* amplification (Figure 2). For *nodC* amplification with the primers for  $\beta$ -rhizobia, positive amplification was only observed for the reference strain, and no bands were observed for the ESA 71 isolate.

For the reactions using the primers to amplify the intragenic fragments of *nodC* and *nodA* of  $\alpha$ -rhizobia, no amplifications could be observed for either gene for ESA 71 and BR 3407<sup>T</sup>.

### Symbiotic efficiency under gnotobiotic conditions

All bacterial isolates and the *Bradyrhizobium elkanii* strain BR 5609 were able to nodulate the host *E. velutina* under the gnotobiotic conditions tested. Comparing all inoculations and the control treatments, statistical differences were not found for the variables shoot dry matter (SDM) and root dry matter (RDM), while for number of nodules per plant (NN), nodule dry matter per plant (NDM), shoot nitrogen concentration (SNC), and total nitrogen content (TNC), differences were due to the inoculation treatments (Table 1).

Regarding the nodulation parameters, no nodules were found in the roots of non-inoculated plants, indicating that there was no contamination in the experiment. Inoculation also resulted in an increase in NDM in all plants. Regarding the variable NN, the plants inoculated with the bacteria ESA 69 and ESA 74 did not differ from the control treatments, according to statistical analysis. The other six bacteria increased the number of nodules in the roots, compared to the control treatments and the ESA 69 and ESA 74 inoculated plants. Plants inoculated with the reference strain BR 5609 showed a higher number of nodules per plant, compared to all the other treatments.



**Figure 2.** Results for PCR for amplification of *nodA* and *nodC* genes for the bacterial isolate ESA 71 (*Burkholderia* sp.) and BR 3407<sup>T</sup> (*Paraburkholderia* *sabiae*) using different primer sets. M - Molecular marker (GelPilot 1 kb Plus Ladder, Qiagen, Germany); 1 - Reaction of ESA 71 DNA with the primers NodCforB and NodCrevB; 2 - ESA 71 with NodAforB and NodArevB; 3 - ESA 71 with NodCF and NodCR(I); 4 - ESA 71 with NodA-1 and NodA-2; 5 - BR 3407<sup>T</sup> with NodCforB and NodCrevB; 6 - BR 3407<sup>T</sup> with NodAforB and NodArevB; 7 - BR 3407<sup>T</sup> with NodCF and NodCR(I); 8 - BR 3407<sup>T</sup> with NodA-1 and NodA-2; and 9-12 are negative controls.

**Table 1.** Shoot, root, and nodule dry matter (SDM, RDM, and NDM), number of nodules (NN), shoot nitrogen concentration (SNC), and total nitrogen content (TNC) in the shoots of *Erythrina velutina* inoculated with different root nodule bacteria in pots experiments with sterile sand and non-sterile soil as a substrate

Inoculation treatment	SDM	RDM	NDM	NN	SNC	TNC
	—— g per plant ——		mg per plant	nod per plant	mg N g <sup>-1</sup> plant	mg N per plant
Sterile sand <sup>(1)</sup>						
ESA 68	1.53 a	0.76 a	30 a	14 b	13.73 b	18.75 b
ESA 69	1.50 a	0.81 a	43 a	6 c	10.92 b	16.06 b
ESA 70	1.74 a	1.07 a	53 a	13 b	19.14 a	33.65 a
ESA 71	1.19 a	0.85 a	40 a	10 b	13.49 b	15.98 b
ESA 72	1.65 a	0.99 a	65 a	14 b	17.26 a	28.09 a
ESA 73	1.24 a	0.64 a	20 a	8 b	11.89 b	14.70 b
ESA 74	1.31 a	0.73 a	50 a	6 c	15.52 a	20.33 b
ESA 75	1.54 a	0.55 a	53 a	13 b	16.48 a	24.88 a
BR 5609	1.83 a	0.99 a	30 a	26 a	12.33 b	20.34 b
N control	1.67 a	0.81 a	0 b	0 c	17.69 a	30.38 a
Abs. control	0.98 a	0.43 a	0 b	0 c	6.60 c	5.29 c
CV (%)	9.9	10.2	39.1	32.7	10.3	10.5
Non-sterile soil <sup>(2)</sup>						
ESA 68	2.04 a	0.62 a	33 a	5 b	17.06 b	34.75 b
ESA 69	2.22 a	0.80 a	70 a	5 b	18.17 a	25.15 b
ESA 70	2.69 a	0.85 a	100 a	28 a	20.07 a	53.12 a
ESA 71	3.17 a	0.90 a	67 a	7 b	18.27 a	57.67 a
ESA 72	2.02 a	0.95 a	65 a	7 b	23.91 a	48.10 a
ESA 73	2.13 a	0.75 a	48 a	13 b	14.80 b	31.47 b
ESA 74	2.73 a	1.07 a	75 a	5 b	15.82 b	46.09 a
ESA 75	2.38 a	1.11 a	85 a	25 a	19.21 a	45.94 a
BR 5609	2.43 a	0.69 a	73 a	25 a	21.38 a	49.91 a
N control	1.98 a	1.04 a	48 a	3 b	22.43 a	43.56 a
Abs. control	2.09 a	0.42 a	40 a	3 b	12.45 b	25.83 b
CV (%)	7.6	8.9	30.4	35.3	8.7	11.5

<sup>(1)</sup> Data are means of three replications. <sup>(2)</sup> Data are means of four replications. Means with the same letter in the column, within in the same experiment, do not differ statistically by the Scott-Knott mean range test ( $p < 0.05$ ).

Evaluating the N nutritional aspects of the plants, it can be observed that all the inoculated treatments induced plants with higher SNC than that observed in the absolute control treatment. The bacteria ESA 70, ESA 72, ESA 74, and ESA 75 can be highlighted because they were statistically the same as the N-supplied control in this parameter. The isolates ESA 68, ESA 69, ESA 71, and ESA 74 also had good performance, inducing the same rate of N in the shoots as the reference strain did. The N accumulated in the shoots also showed statistical superiority in the inoculated treatments compared to the absolute control. *E. velutina* plants inoculated with ESA 70, ESA 72, and ESA 75 showed the same values for the variable TNC as observed in the plants that received the mineral N supply. The other bacteria, including the reference strain, had the same performance in regard to this parameter.

### Symbiotic efficiency in the non-sterilized soil substrate

The plants inoculated with bacterial isolates did not exhibit statistical differences in regard to the SDM and RDM plant development variables evaluated (Table 1). Regarding nodulation, no differences were observed comparing the NDM of all treatments, including those of the control. Nevertheless, the NN per plant of *E. velutina* inoculated with ESA 70, ESA 75, and BR 5609 were higher than in the other treatments.

Plants inoculated with the rhizobial isolates ESA 69, ESA 70, ESA 71, ESA 72, ESA 75, and BR 5609, as well as the N-supplied treatment, showed higher values for the variable SNC compared to the treatments ESA 68, ESA 73, ESA 74, and the absolute control. The TNC variable was also influenced by the inoculation treatment. Plants that received the bacteria ESA 70, ESA 71, ESA 72, ESA 74, ESA 75, and BR 5609 showed the same performance in this variable as the N-supplied treatment and were superior to the treatments inoculated with ESA 68, ESA 69, ESA 73, and the absolute control.

It is possible to verify that the better performance of bacterial isolates in the nitrogen nutrition of *Erythrina velutina* (Figure 3). The greener appearance of plants that received the native bacteria and the N supply can be clearly observed compared to the plants of the absolute control treatment, which has a smooth chlorotic appearance.



**Figure 3.** Plants of *Erythrina velutina* inoculated with different rhizobia at sowing, non-inoculated plants supplied with mineral nitrogen, or non-inoculated plants not supplied with nitrogen in an experiment using non-sterilized soil as a substrate, at 81 days after sowing. From left to right, the treatments are ESA 71, ESA 70, ESA 75, nitrogen supplied control, BR 5609, and an absolute control.



## DISCUSSION

Among the eight bacteria studied, seven were classified as *Bradyrhizobium* or *Rhizobium*. Both genera are often isolated from nodules of the Papilionoidae species (Peix et al., 2015). The results suggest that “mulungu” shows a preference for nodulation with  $\alpha$ -rhizobia rather than  $\beta$ -rhizobia, though few isolates were evaluated. Among the eight bacteria assessed, only one isolate was classified as a  $\beta$ -proteobacteria, which are isolates commonly obtained from Mimosoidae legumes (Bontemps et al., 2010; Gyaneshwar et al., 2011; Martins et al., 2015). These data are in agreement with our hypothesis that the bacterial isolates studied here belong to different taxonomical clusters. For different species of *Mimosa* sp., recent studies have already shown  $\beta$ -rhizobia isolated from the semi-arid region of Brazil (Reis Júnior et al., 2010; Martins et al., 2015), but there are not records in the scientific literature on the isolation of  $\beta$ -rhizobia from Papilionoidae legumes grown in soils from this region.

Among the Papilionoidae legumes, *Paraburkholderia* or *Burkholderia* was also isolated from nodules of herb species such as *Phaseolus vulgaris* (Dall’Agnol et al., 2016) and *Cyclopia* sp. (Elliott et al., 2007) and tree species of *Dalbergia* (Rasolomampianina et al., 2005; Lu et al., 2012), among other legumes in the tropics. The isolate ESA 71 was clustered with the *Burkholderia* and was not most closely related to traditional Mimosoideae nodulating species, such as *Paraburkholderia phymatum* or *Paraburkholderia sabiae*, for example, indicating the presence of new  $\beta$ -rhizobia nodulating the “mulungu” in the Brazilian semi-arid region. Low genetic similarity among ESA 71 and the nodulating strains of *Paraburkholderia sabiae* BR 3407<sup>T</sup> and BR 3405, by means of comparison of their Box-PCR profiles, has already been shown by Menezes et al. (2016). The results of the partial 16S rRNA gene sequences obtained corroborated the genetic diversity results previously found.

A recent proposal was to split the *Burkholderia* genus. In this new taxonomy, the bacteria from Bcc and other human pathogenic strains are placed within the same genus and the bacteria obtained from environmental sources are reclassified into a new genus called *Paraburkholderia* (Sawana et al., 2014). This division presumed that there are no nodulating Bcc bacteria. But the results found in the present study, together with those obtained by Rasolomampianina et al. (2005), who also obtained a Bcc isolate from nodules of *Dalbergia* sp. in Madagascar, indicates that there are other *Burkholderia* not reclassified as *Paraburkholderia* that are able to nodulate legume roots.

Amplification of an intragenic *nodA* gene fragment using a NodAforB and NodArevB primer set can exhibit positive results, with sizes ranging from 245 to 800 bp (Klonowska et al., 2012; Taulé et al., 2012). So, the amplification of different molecular size amplicons found in the present study can be considered positive for both bacteria, indicating that ESA 71 exhibits a *nodA* gene copy with some similarity to that found in the *Paraburkholderia*. The absence of a *nodC* gene amplification using the primers described by Elliott et al. (2007), widely used for *nodC* amplification of *Paraburkholderia*, suggests that ESA 71 has other symbiotic genes that probably evolved differently from those found in *Paraburkholderia*. The positive symbiotic performance of ESA 71 indicates that this bacterium has an effective symbiotic genetic apparatus that may be amplified with other primer sets. Absence of amplification of an intragenic fragment of *nodC* and *nodA* when degenerated and nonspecific primers designed to amplify the target genes from  $\alpha$ -rhizobial were applied also indicates that the bacterial isolate did not receive the symbiotic genes from other  $\alpha$ -rhizobia by horizontal gene transfer, and it also shows that the DNA of this sample was not contaminated with  $\alpha$ -rhizobia DNA. Rasolomampianina et al. (2005), who also isolated a Bcc bacterium from legume root nodules, failed to amplify any *nod* gene using several primer sets, indicating that the Bcc nodulating bacteria may have symbiotic genes different than those from *Paraburkholderia*.

The high similarity of ESA 71 to the Bcc bacteria indicates that, in spite of already being described as indigenous to the Caatinga (Reis Júnior et al., 2010; Martins et al., 2015),

the native  $\beta$ -rhizobia in the region should belong to a new species, different from those already described worldwide. Low genetic similarity of new  $\beta$ -rhizobia from the region and species already described was recently shown by Martins et al. (2015), who obtained *Paraburkholderia* isolates from *Mimosa caesalpinifolia* in the Brazilian semi-arid region, and analysis of 16S rRNA sequences showed that some isolates had low similarity with  $\beta$ -rhizobia described. Other taxonomical studies are needed to achieve better taxonomic positioning of ESA 71.

The symbiotic characteristics of the eight newly-isolated bacteria and the *Bradyrhizobium elkanii* BR 5609 strain showed that some isolates had the ability to fix large amounts of N, as we had hypothesized for the bacterial isolates tested in this study. In the experiment in gnotobiotic conditions, all bacteria were able to increase N concentration in their shoots and total N accumulation compared to the absolute control treatment, a strong indication of their symbiotic efficiency. In addition, in the experiment under non-sterile substrate conditions, the *Rhizobium* sp. ESA 69 and ESA 70, the *Burkholderia* sp. ESA 71, and the *Bradyrhizobium* sp. ESA 72, ESA 74, and ESA 75, together with BR 5609, also showed good performance because they induced an increase in N concentration in the shoots and/or in total N content of “mulungu” plants.

The statistical similarity among the inoculated and non-inoculated treatments regarding the mass of dried nodules indicate that, in the soil used for this experiment, there is a rhizobial community efficient in nodulating the “mulungu” roots. However, the higher number of nodules of plants inoculated with ESA 70, ESA 75, and BR 5609 and better performance of the seven bacteria cited above in the N nutrition variables (SNC and TNC) indicated that the bacteria tested are competitive and efficient in non-sterile conditions, with a desirable characteristic for tree seedling production under nursery conditions.

In spite of being nodulated both by  $\alpha$  and  $\beta$ -rhizobia, *Erythrina* seems to be a genus able to nodulate and fix N efficiently with some specific strains within the different rhizobia species. Bala and Giller (2001) evaluated the efficiency of tropical rhizobial isolates from different tree species like *Leucaena leucocephala*, *Gliricidia sepium*, and *Sesbania sesban* on *E. falcata*. The results indicated that among the ten isolates tested, the host nodulated only with three bacteria and, in spite of true nodules being formed, all of them were ineffective, with a white inner color and chlorotic plant aspect. In Brazil, the efficiency of BR 5609, originally isolated from *E. verna*, was also shown for other species within the genus, such as *E. fusca* (Faria and Uchôas, 2007) and *E. falcata* (Laste and Faria, 2009). The results found in the present study allow the potential range of *Erythrina* hosts to be increased for the recommended strain BR 5609.

Regarding the native isolates evaluated in the present study, Menezes et al. (2016) showed that the isolates ESA 70 and ESA 75 have high metabolic versatility are able to metabolize several carbon sources, and show tolerance to biotic stresses *in vitro*, growing in a medium with increased NaCl concentrations and under incubation temperatures up to 39 °C. In addition, they are indol acetic acid producing isolates, characteristics that can positively influence their competitive and diazotrophic behavior.

The results regarding the efficiency of all rhizobial bacteria allows selection of at least *Rhizobium* sp. ESA 70, *Burkholderia* sp. ESA 71, and *Bradyrhizobium* sp. ESA 72 and ESA 75, together with *Bradyrhizobium elkanii* BR 5609 for future experiments to validate these bacteria as candidates for official recommendation for *E. velutina*. It is important to keep in mind that the reference strain used is efficient and recommended for other *Erythrina* species, such as *E. verna* and *E. falcata* (Laste and Faria, 2009). The promising performance of the reference strain BR 5609 in *Erythrina velutina* is noteworthy and can, after further future experiments, increase the list of forest hosts officially recommended for this strain.

Several species found in the Brazilian “Caatinga” are able to efficiently associate with a wide range of native rhizobia (Souza et al., 2012). In spite of this knowledge and recent

advances regarding the diversity of bacterial communities isolated from soils in the Brazilian semi-arid region (Reis Júnior et al., 2010; Martins et al., 2015; Menezes et al., 2016), there are few experimental results seeking to evaluate bacterial efficiency for tree legumes in the Brazilian semi-arid region, specifically those from Papilionoidae legumes. To our knowledge, this is the first study reporting the selection of symbiotically efficient *Erythrina velutina* rhizobia. The data found in this study confirm our hypothesis that among the bacterial isolates evaluated there are some bacteria with symbiotic efficiency and that they belong to different taxonomical clusters.

## CONCLUSIONS

The Papilionoidae tree *Erythrina velutina* is able to establish symbiotic associations with  $\alpha$ -proteobacteria belonging to *Rhizobium* and *Bradyrhizobium* and with  $\beta$ -proteobacteria *Burkholderia* sp. The three genera harbor efficient bacterial isolates that can be suggested for further studies seeking their recommendation in inoculant production.

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