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Genetic diversity and inoculation of plant-growth promoting diazotrophic bacteria for production of *Eucalyptus urophylla* seedlings

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Abstract

The study of diversity and the established relationship between plants and endophytic bacteria contribute significantly to the plant development. This study aimed to isolate, identify plant-growth promoting diazotrophic bacteria from *Eucalyptus urophylla* plants and evaluate the plant seedling response to inoculation with selected strains. The study was conducted from July 2017 to August 2018 set up in a greenhouse in a completely randomized experimental design with 17 treatments and 16 replicates, with 12 strains isolated from *E. urophylla*, four standard bacteria and one control (not inoculated with bacteria). Twelve strains were isolated from the eucalyptus rhizosphere using the N-free semisolid media and the 16 rRNA sequencing identified species closely related to *Nitrospirillum amazonense*, *Stenotrophomonas maltophilia*, *Pantoea agglomerans*, *Herbaspirillum frisingense* and *Ideonella dechloratans*. All strains were able to produce indol compounds and the presence of the *nif*H gene (nitrogen fixation) was demonstrated by PCR analysis. There was a significant effect of the strain inoculation on the height, diameter, root dry matter, root length, root area surface and volume of the seedling plant. The strains L1E, L4E - *Azospirillum amazonense* and L7E - *Stenotrophomonas maltophilia* presented high values variables of height, diameter, root dry matter, root length, root area surface and volume. Many of the bacterial strains were able to promote plant growth either by root morphology changes or biomass accumulation in root and shoots. Therefore, there is a biotechnological potential of these plant-growth promoting diazotrophic bacteria strains to be applied as inoculants to improve growth of eucalyptus seedlings. This fact opens new opportunities to establish forests with economically viable and environmentally sustainable technologies.

Keywords: Auxin; rhizobacteria; *Eucalyptus*; nitrogen fixation; growth promotion.
Abbreviations: Sim_similarity; IFRO_Instituto Federal de Educação de Rondônia; L_LGI medium; JV_JMV medium; E_Eucalyptus urophylla; IAA_Indole acetic acid.

Introduction

Brazil has a vast area of forests composed of different biomes, with an estimated 493.5 million hectares of native forests (FRA, 2015). Also has an area of 7.84 million hectares of reforestation for production and exportation of pulp and paper, charcoal steelworks, wood panels and laminate flooring. These activities bring undeniable contributions to the trade balance and generate jobs and income in all regions of the country (IBA 2017). The planted forest areas of Brazil consist mainly of eucalyptus (*Eucalyptus* ssp.), which occupy 5.7 million hectares (IBA 2017). The wide variety of eucalyptus species and hybrids, with different climatic and soil adaptations, associated with easy seed propagation and cloning, allows the adaptation of plantations to several tropical and subtropical regions of Brazil (Gonçalves et al., 2013). Biotechnology, coupled with conventional breeding techniques, has emerged as a tool to overcome challenge in eucalyptus production. Technological innovation will lead to cultivation of trees with specific characteristics, such as higher productivity, better shape, more density, higher amount of fibers and resistance to pests and diseases, drought, cold or salinity, particularly relevant due to the effects of global climate change (IBA, 2015).

Despite the commercial and economic importance of eucalyptus, there are few studies on microbial communities in their ecosystems, and little is known about the interaction of soil microorganisms and nitrogen nutrition in eucalyptus (Silva et al., 2014; Puri et al., 2018). The great bacterial diversity present in the ecosystems can vary according to the type of soil, management and isolation methods applied (Brandão, 1992). Nitrogen can be obtained by plants via biological fixation process performed by free-living, associative and endophytic microorganisms (Reinhold-Hurek and Hurek, 2011; Puri et al., 2018).

Endophytic microorganisms may improve plant characteristics such as height, biomass and emergence of buds under nitrogen-limiting conditions (Puri et al., 2018), synthesis of cytokinins (Pirttilä, 2011), auxins (Taghavi et al.,

2005), auxin analogs and gibberellin analog, phytopathogen control, induction of germination, and increase of establishment of seedlings in the field after transplanting (Bottini et al., 2004; Waqas et al., 2012). Studies on the microbial community can increase knowledge about the interaction of plants with associative and endophytic microorganisms as well as their contributions to the nutrition of the forest.

Despite the existence of studies on bacterial diversity in *Eucalyptus* spp. (Ferreira et al., 2008; Procópio et al., 2009; Miguel et al., 2016) and their beneficial effects for the plant (Teixeira et al., 2007; Mafia et al., 2009; Castellanos et al., 2010; Raasch et al., 2013), only a small numbers have investigated the presence of genera identified in different plant species with emphasis on *Azospirillum* sp., *Herbaspirillum* sp., *Burkholderia* sp. among others outros (Döbereiner, 1990; Baldani et al., 1997; Moreira et al., 2010). Moreira et al. (2010) pointed out that, given the wide diversity of habitats, endophytic bacteria have been found in association with several crops, such as corn, rice, sugarcane, wheat, coffee, palm trees and grasses, which led to the first hypothesis that eucalyptus plants are associated with plant-growth promoting diazotrophic microorganisms isolated in both the root and shoot systems in semi-specific nitrogen-free culture media. The second hypothesis was that these microsymbionts exhibit characteristics of N-fixing organisms and 3-indoleacetic acid producers. The third hypothesis was that inoculation of seedlings of *Eucalyptus* spp. with microorganisms isolated from *Eucalyptus urophylla* promote an increase in phytomass. In this sense, the present study aimed to isolate, taxonomical identify diazotrophic bacteria isolated from plants of *Eucalyptus urophylla* and evaluate the plant-growth promoting effect on seedlings.

Results

Molecular and functional characterization of strains

The Most Probable Number (MPN) counting showed a low diazotrophic bacterial population associated with roots of *E. urophylla* with the nitrogen-free medium. No bacteria were detected in the semi-solid media NFb and JNFb using malic acid as a carbon source, while media using sucrose and mannitol were obtained strains of diazotrophic bacteria. The LGI allowed the isolation of many *Nitrospirillum amazonense* strains while the JMV increased the diversity and allowed the isolation of different bacterial species (Table 1). In total, 12 diazotrophic strains were isolated in both media, of which five strains were in the semisolid JMV culture medium and seven in the LGI medium. The diversity of endophytic bacteria was observed using the molecular and functional characterization of bacterial strains through the amplification of 16S rRNA primer sequences for comparison with similar sequences deposited in the NCBI (National Center for Biotechnology Information) database.

The amplified 16S rRNA fragment of the 12 strains that were compared with the sequences in the NCBI database and showed that the bacteria belonging to the classes Alphaproteobacteria (*N. amazonense – older Azospirillum amazonense*), Betaproteobacteria (*Herbaspirillum frisingesnse* and *Ideonella dechloratans*) according to (Garrity et al., 2005a) and Gammaproteobacteria (*Stenotrophomonas maltophilia* and *Pantoea agglomerans*), mentioned by Garrity et al. (2005b).

Phylogenetic analyses revealed the presence of strains closely related to *Pantoea agglomerans* (JV1E, JV4E and JV5E), *Stenotrophomonas maltophilia* (L7E), *Herbespirillum frisingense* (JV2E), *Ideonella dechloratans* (JV3E) and *Nitrospirillum amazonense* (L1E, L2E, L3E, L4E, L5E and L6E) colonizing roots of *Eucalyptus urophylla* plants (Figure 2).

Although these microorganisms were isolated in nitrogen-free media, amplification of the *nif*H gene was detected in all strains. An amplified fragment of approximately 350 bp (visible in the electrophoresis agarose gel) corresponding to the *nif*H gene was observed in all strains as well as in the reference strain (Bergmann et al., 2009) used as positive control. The phylogenetic tree generated with the sequenced *nif*H gene fragments, and alignment against sequences deposited in the genebank showed that some strains present high similarity among them, forming a group of diazotrophic bacteria (Figure 3).

The analysis of similarity of the *nif*H gene sequences of 8 strains corresponded to the same strains in the 16S rRNA phylogenetic tree. In this sense, the similarity of the 16S rDNA and *nif*H gene phylogentic tree, suggest the identification of the species.

Synthesis of indole compounds

The isolates showed statistical significant differences in the ability to synthesize indole compounds were observed among strains. Higher ability to produce indols was observed for strains IFROJV5E and JV4E-*Pantoea agglomerans* (70.1 and 44.96 µg indole compounds mg protein^{-1}) after 24H after growth and strains IFROJV4E, IFROJV5E - *Pantoea agglomerans*, IFROJV3E - *Ideonella dechloratans* and IFROL4E - *Nitrospirillum amazonense* (mean 48.86 µg indole compounds mg protein^{-1}) after 48 h period. The strains IFROL1E, L2E, L3E, L5E, L6E and L7E- *Nitrospirillum amazonense* showed lower IAA production when compared to the other strains. Even though they belong to the same species based on the 16S rRNA gene, the concentration of IAA varied indicating different potential of use of these strains (Figure 4).

Biomass production in Eucalyptus urophylla plants

There was a significant difference in the height and diameter of *Eucalyptus urophylla* plants inoculated with the bacterial strains along the growth time. The strains IFROL1E, L3E, L4E, L5E, L7E, JV3E, JV5E and the AC1 (reference strain) showed higher height development as compared to the other strains and uninoculated control (Figure 5). In relation to the diameter, the strains IFROL1E, L2E, L3E, L4E, L5E, L7E, JV3, JV5 and the reference strains AC1 and BR11366 performed better when compared among strains and the control along the evaluation time (Figure 6).

There was a statistical significant difference in the percentage of dry matter of roots. The strains IFROL1E, L4E, L7E, JV4E and the references AC1 and BR332 strains present higher biomass accumulation than the other strains and the uninoculated. For the percentage of dry matter of the shoot there was no significance (Table 2).

Concerning the root morphology, the results for root length, root surface area and volume showed a significant difference among the strains. The strains IFROL1E, L2E, L3E, L4E, L5E, L6E, L7E and the reference BR11366 stood out for the very fine root length (<0.5 mm) (Figure 7) while the surface area (> 3 mm) was higher for the strains IFROL1E, L3E, L4E, L5E, L7E, JV2, JV3, JV4, JV5 and the reference strains ACI, BR11366 and BR322 (Figure 9). The main root volume (> 3 mm) was higher for IFROL1E, L3E, L4E, L5E JV2E, JV3E, JV4E, JV5E and the strains ACI, BR11366 and BR322

While for total volume, the strains IFROL1E, L4E, L6E, L7E, JV2, AC1 and the reference BR322 strains performed much better than the others (Figure 9).

Discussion

The evaluation of the 16S rRNA gene allowed to efficiently identify the diversity of endophytic bacteria in *Eucalyptus urophylla* roots. The isolates are potentially diazotrophic, since they present the *nif*H gene, which is associated with N fixation (Weber et al., 2013). The sequence of the *nif*H gene is highly conserved among diazotrophs, and several primers for PCR are used to amplify regions of this gene in order to study microbial groups or populations (Auman et al., 2001; Mehta et al., 2003). It is observed the presence of *Nitrospirillum amazonense* (older *Azospirillum amazonense*) strains in large part due to the use of the nitrogen –free semi-specific semisolid LGI medium.

The *nif*H gene showed similarity of the strains JV1E, JV4E, JV5E with the bacterium *Pantoea agglomerans.* The genus *Pantoea* sp. was identified by Procópio et al. (2009) in studies with community of endophytic bacteria in six genotypes of *Eucalyptus* spp. Studies conducted by Miguel et al. (2016), working with the diversity and distribution of the endophytic bacteria community in different stages of growth in eucalyptus, observed that the genera *Stenotrophomonas* sp. and *Pantoea* sp. stood out among the isolates.

The identification of *Herbaspirillum frisingense* for both the 16S rDNA gene fragment and the *nif*H gene showed that this species can be considered host of eucalyptus plants. This species was described by Kirchhof et al. (2001), isolated from Poaceae such as *Pennisetum purpureum* in JNFb medium. However, in the present study this strain was isolated in JMV medium (Table 1), which presents mannitol as carbon source. It should be borne in mind that these media, although favoring certain species, allow the growth of other nitrogen-fixing species (Magalhães and Döbereiner, 1984; Nóbrega et al., 2004). The JV3E strain showed similarity with *Ideonella dechloratans*, not yet reported in studies with eucalyptus.

Our results confirm the high bacterial diversity found in association with *Eucalyptus* plants by other authors in (Ferreira et al. 2008; Prócopio et al, 2009; Castellanos et al. 2010; Miguel et al. 2016). Among them, the nitrogen-fixing genera are the majority of the species although other genera involved in other plant-bacteria interaction mechanisms were also present. Therefore, the selection of microorganisms with beneficial functions can contribute to nitrogen and other mechanisms that could improve the plant development and productivity.

In our case, all strains were able to produce indols that is part of an important mechanism involved in the plant growth promotion. The results showed that the amount of IAA produced varied among strains and also with growth period: 24 h (4.74 to 70.12) and 48 h (2.39 to 56.25), respectively. This variation in the amount of IAA is subjected to various regulatory mechanisms, such as regulatory sequences, pathways and location of biosynthesis genes and the presence of enzymes to convert active free IAA into conjugated forms (Duca et al. 2014).

The strain JV5 - *Pantoea agglomerans* was prominent in relation to the production of IAA e em contrapartida, the strains of *N. amazonense* with the exception of L4E and L2E strains showed low IAA production (mean 8.58 µg indole compounds mg protein^{-1}). Studies show that some species has potential for maximum production of IAA in a short period of time and others bacteria may present different pathways for IAA assimilation (Apine and Jadhav, 2011; Cecagno et al., 2015).

The positive effect of the bacterial inoculation on the phytomass production of *Eucalyptus urophylla* plants may be related to the ability of the strains to produce indols or other plant growth substances such as gibberellins and cytokinins (Yang et al. 1993). These phytohormones may induce a better root development of the seedlings therefore improving nutrient and water absorption.

Differences in the performance of growth promoting bacteria in IAA producing plants can be attributed to the individual inherent properties of each bacterium (Sarwar et al., 1992), presenting different behaviors in the interaction with the plant. The strains L1E, L4E - *Azospirillum amazonense* and L7E - *Stenotrophomonas maltophilia* presented high values for all variables except for the IAA level, which had benefits for biomass production of the plant. These results show that rhizobacteria can stimulate the growth of roots and shoots by an alternative mechanism that leads to increased absorption of nutrients. Brader et al. (2014) have reported on increasing evidence that endophytic bacteria have a high potential in the production of a wide range of metabolites that may influence plant development. The strains IFROL1E, L4E - *Nitrospirillum amazonense* and L7E - *Stenotrophomonas maltophilia* presented the highest values for all plant variables except for the indol production. The gains provided by inoculation to the root system are important for the production of seedlings in nurseries and field establishment.

The results of the present study show that there is a positive interaction between the isolates and the eucalyptus species used, evidencing a difference between the inoculated strains. Nevertheless, some factors must be taken into account, such as the abiotic factors (pH, nutrient availability, moisture, aeration, among others) and biotic factors (microbiota composition and others) that may favor colonization, survival and beneficial activity of plant growth promoting bacteria under the experimental conditions.

Materials and methods

Plant materials

The plant material used to isolate the bacteria was planted in July 2016 in the experimental field of the Federal Institute of Education, Science and Technology of Rondônia (IFRO), Colorado do Oeste at coordinates of latitude 13°06’ S and longitude 60°29’W and altitude of 407 m (Figure 1) . The soil is Eutrophic Red Argisol (Santos et al., 2018) and according to the Köppen classification, the climate is Am, tropical hot and humid, with two well defined seasons (dry and rainy), and average annual temperature of 24ºC (Alvares et al., 2013). The experiment was a completely randomized block design and the treatments consisted of four hybrids (VM1 - hybrid of *Eucalyptus urophylla* x *Eucalyptus camaldulensis*; H13 - hybrid of *Eucalyptus urophylla* x *Eucalyptus grandis*, GG100 - hybrid of *Eucalyptus urophylla* x *Eucalyptus grandis* and AEC144 - spontaneous hybrid of *Eucalyptus urophylla*) and four replications, each plot had 30 plants, totaling 480 plants. The distance between rows in the tree rows was 3 m, and the distance between the tree rows (number of trees arranged in the same row) of 10 m.

Table 1. Comparative analysis of similarity among 16 S rRNA sequences obtained from *Eucalyptus urophylla* roots and sequences deposited in the NCBI database.

Strain	Semisolidmedium	Dilution used for bacterial	16S rRNA (higher match%)	Number of accession	% Sim
IFROL1E	LGI	10^{-3}	*Nitrospirillum amazonense*	NR-104981.1	99
IFROL2E	LGI	10^{-3}	*Nitrospirillum amazonense*	NR-104981.1	99
IFROL3E	LGI	10^{-3}	*Nitrospirillum amazonense*	NR-104981.1	99
IFROL4E	LGI	10^{-2}	*Nitrospirillum amazonense*	NR-104981.1	99
IFROL5E	LGI	10^{-2}	*Nitrospirillum amazonense*	NR-104981.1	99
IFROL6E	LGI	10^{-2}	*Nitrospirillum amazonense*	NR-104981.1	99
IFROL7E	LGI	10^{-2}	*Stenotrophomonas maltophilia*	NR-041577.1	99
IFROJV1E	JMV	10^{-2}	*Pantoea agglomerans*	NR-111998.1	99
IFROJV2E	JMV	10^{-2}	*Herbaspirillum frisingense*	NR-114140.1	99
IFROJV3E	JMV	10^{-2}	*Ideonella dechloratans*	NR-026108.1	99
IFROJV4E	JMV	10^{-2}	*Pantoea agglomerans*	NR-111998.1	99
IFROJV5E	JMV	10^{-2}	*Pantoea agglomerans*	NR-111998.1042349.1	99

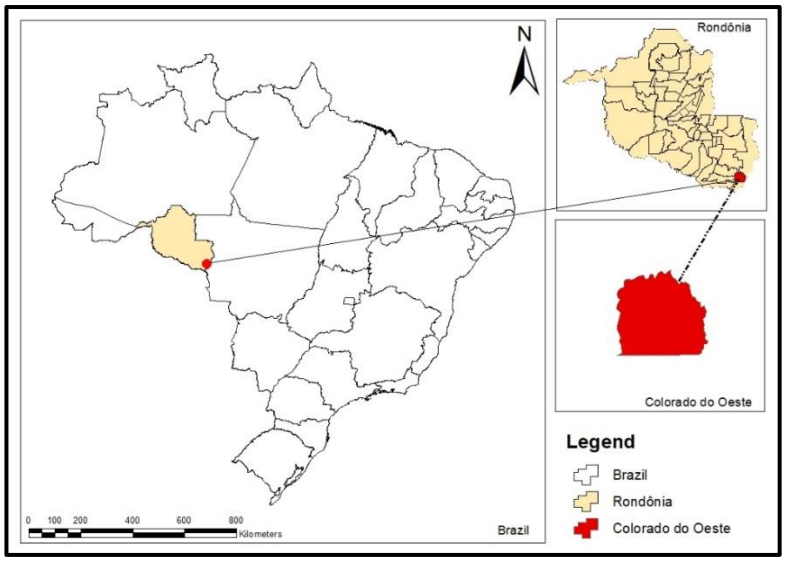

Fig 1. Location of the study area, Colorado do Oeste, State of Rondônia, Brazil.

Table 2. Dry biomatter accumulation in shoot and root of *Eucalyptus urophylla* plants inoculated with diazotrophic bacterial strains.

Dry matter (%)				
Strain	Bacterial species	Semisolid Medium	Shoot (mg plant^{-1})	Root (mg plant^{-1})
IFROL1E	*Nitrospirillum amazonense*	LGI	31.85a	82.53 a
IFROL2E	*Nitrospirillum amazonense*	LGI	42.62a	59.06 b
IFROL3E	*Nitrospirillum amazonense*	LGI	40.74a	55.54 b
IFROL4E	*Nitrospirillum amazonense*	LGI	37.28a	82.40 a
IFROL5E	*Nitrospirillum m amazonense*	LGI	33.98a	67.13 b
IFROL6E	*Nitrospirillum amazonense*	LGI	36.10a	58.01 b
IFROL7E	*Stenotrophomonas maltophilia*	LGI	37.57a	86.87 a
IFROJV1E	*Pantoea agglomerans*	JMV	37.36a	66.39 b
IFROJV2E	*Herbaspirillum frisingense*	JMV	30.45a	48.85 b
IFROJV3E	*Ideonella dechloratans*	JMV	37.46a	58.18 b
IFROJV4E	*Pantoea agglomerans*	JMV	34.64a	77.08 a
IFROJV5E	*Pantoea agglomerans*	JMV	26.68a	59.18 b
AC1	*Azotobacter chroococcum*	LG	34.05a	73.07 a
Aam82	*Azospirillum amazonense*	LGI	34.99a	61.44 b
BR11366	*Burkholderia tropica*	JMV	31.77a	61.11 b
BR322	*Rhizobium tropici*	TY	25.92a	73.98 a
Control			34.71a	57.38 b
CV[a] (%)			18.26	22.84

Means followed by different lowercase letters in the same column section are significantly different by the Scott-Knott test at 5% (P<0.05), [a]CV (Coefficient of variation).

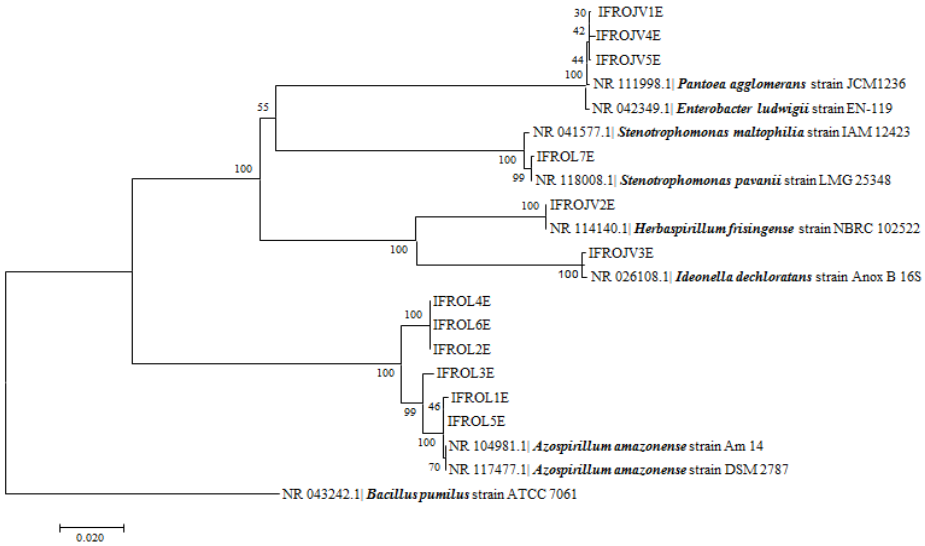

Fig 2. Phylogenetic tree based on 16S rRNA gene sequences (~ 1450 bp), including the isolates and reference diazotrophic bacterial species deposited in the NCBI database. Numbers located in the branches of the tree indicate the percentage of 1,000 samplings (bootstrap). The 16S rDNA gene sequence of *Bacillus pumilus* was used as an external group.

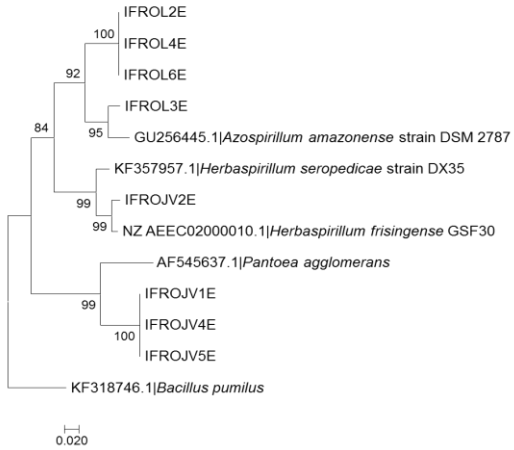

Fig 3. Phylogenetic tree based on sequences of the *nif*H gene (~350 bp), including the isolates and reference diazotrophic bacterial species deposited in the NCBI database. Numbers located on the branches of the tree indicate the percentage of 1,000 samplings (boots).

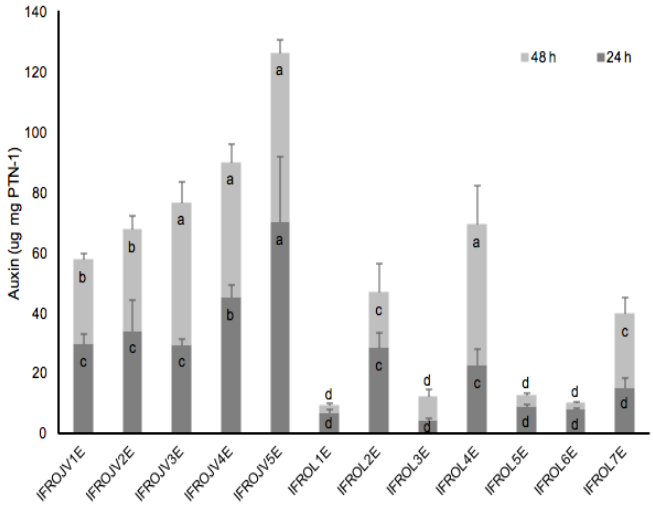

Fig 4. Indol production by diazotrophic bacterial strains isolated from roots of *Eucalyptus urophylla*. Mean values of three replicates and bars represent the standard deviation. Means followed by different lowercase letters in the same column section are significantly different by the Scott-Knott test at 5%.

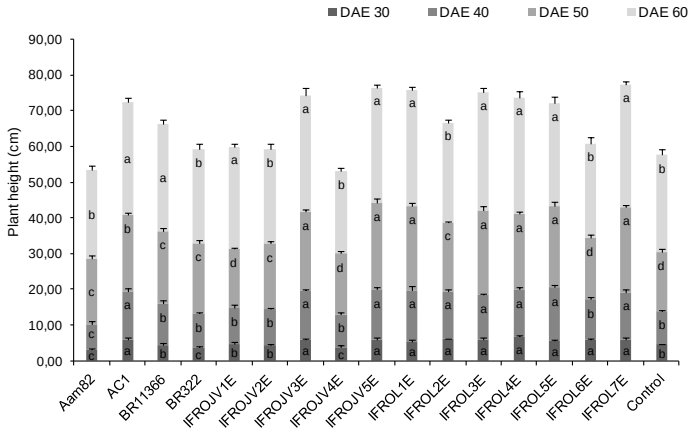

Fig 5. Effect of diazotrophic bacterial strains on the height of *Eucalyptus urophylla* plants grown in plastic tubes. Means followed by different lowercase letters in the same column section are significantly different by the Scott-Knott test at 5%.

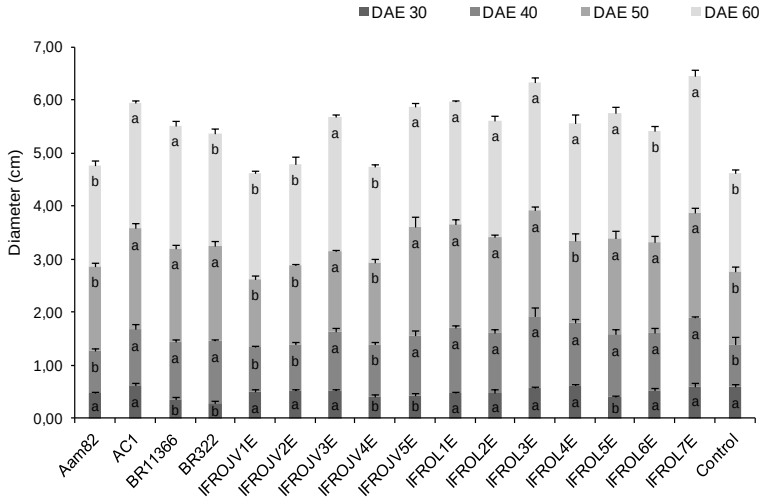

Fig 6. Diameter of *Eucalyptus urophylla* plants inoculated with diazotrophic bacterial strains. Means followed by different lowercase letters in the same column section are significantly different by the Scott-Knott test at 5%.

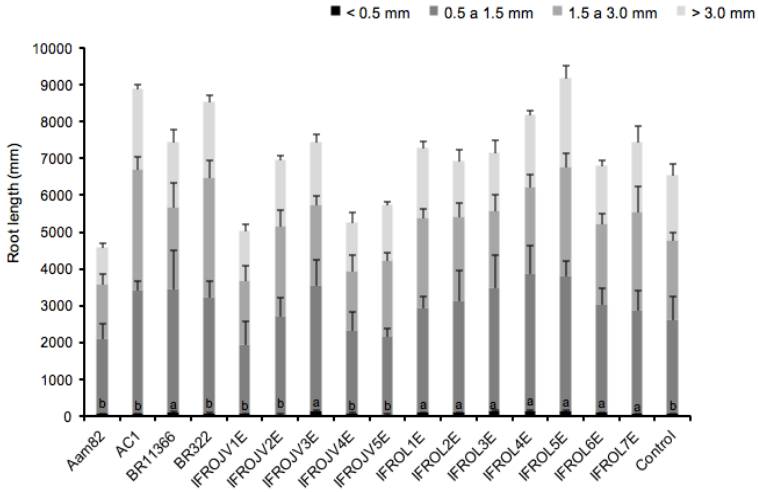

Fig 7. Effect of diazotrophic bacterial strains inoculation on root morphology of *Eucalyptus urophylla* : very fine root length CRMF1 <0.5 mm), very fine root length (CRMF2 0.5 to 1.5 mm), fine root length (CRF, 1.5 to 3.0 mm), main root length (CRP,> 3.0m). Means followed by different lowercase letters in the same column section are significantly different by the Scott-Knott test at 5%.

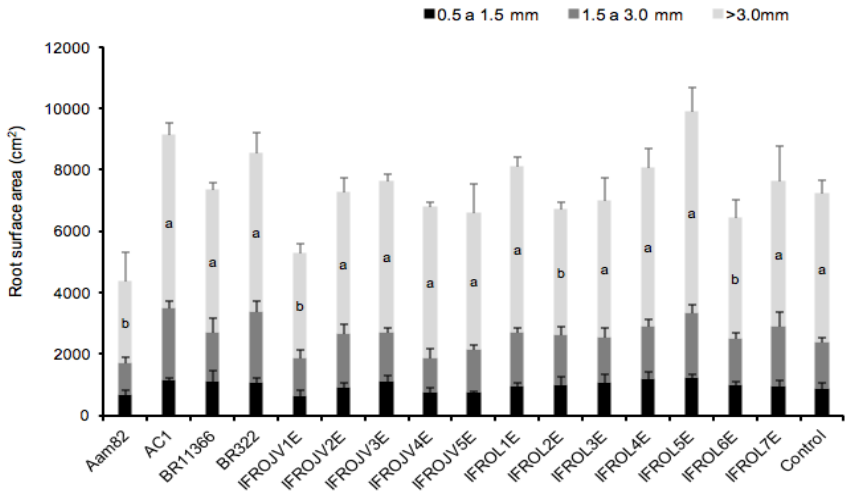

Fig 8. Effect of diazotrophic bacterial strains inoculation on root morphology of *Eucalyptus urophylla*: very fine root surface area (SAMF2 0.5 to 1.5 mm), fine root surface area (SAF 1.5 to 3.0 mm), main root surface area (SAP> 3 mm). Means followed by different lowercase letters in the same column section are significantly different by the Scott-Knott test at 5%.

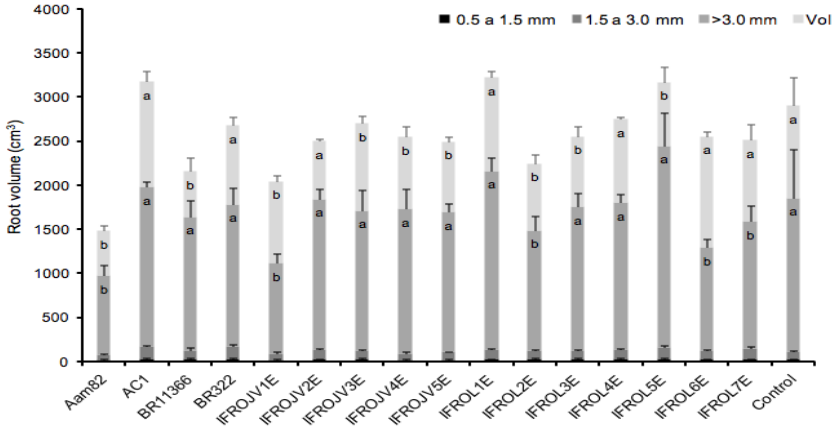

Fig 9. Effect of diazotrophic bacterial strains inoculation on root morphology of *Eucalyptus urophylla*: very fine root volume (VRMF2 0.5 to 1.5 mm), fine root volume (VRF 1.5 to 3 mm), main root volume (VRP> 3 mm) total volume (cm^3). Means followed by different lowercase letters in the same column section are significantly different by the Scott-Knott test at 5%.

After 18 months of cultivation, roots were collected from the clone AEC144 (*Eucalyptus urophylla*) for the isolation of bacteria.

Isolation of diazotrophic bacteria

The nitrogen-free semi-solid culture media used for counting and isolation of the diazotrophic bacteria were NFb (Baldani and Döbereiner 1980), JNFb (Baldani 1996), LGI (Magalhães et al. 1983) and JMV (Baldani 1996) and followed the methodology described by Döbereiner et al. (1995) and Baldani et al. (2014). For that, flasks inoculated with diluted root samples of eucalyptus were incubated at 30°C for seven days, and those with an aerotaxis typical pellicle near the surface of the medium were considered positive for estimation of the diazotrophic bacterial population. Flasks showing this characteristic pelliclewere used for bacterial isolation with a loop full containing bacteria transferred to a new semi-specific semisolid medium and let to grow until a new bacterial pellicle was formed. This procedure was repeated three times to make sure that the bacteria continued growth dependent on the nitrogen fixation process. The bacteria were then streaked on Petri dishes containing the specific solid media and incubated at 30°C for 5 days. Colonies showing diazotrophic characteristics were selected and transferred to flasks containing the same semi-solid media followed by purification on plates containing potato agar medium. The purified strains were stored in flasks containing specific slant medium under mineral oil.

Molecular characterization

The molecular and functional characterization of the bacterial strains was carried out at the Laboratories of Genetics and Biochemistry, Grasses and Molecular Biology of the Centro Nacional de Pesquisa Agrobiologia (EMBRAPA Agrobiologia) in Seropédica, State of Rio de Janeiro. The bacterial strains were grown in liquid DYGS medium (Rodrigues et al., 1986) at 30°C for 24 hours and then one milliliter of bacterial suspension was centrifuged at 16,000 g and used for total DNA extraction using the Wizard genomic DNA purification kit (Promega, USA) for Gram negative bacteria. The quality of the total genomic DNA was evaluated by electrophoresis gel agarose (1%) run for 1 hour and 90 V. The amount of total genomic DNA was quantified in a Nanodrop® 3300 spectrophotometer (Thermo Fisher Scientifica Inc., Waltham, USA).

Amplification and sequencing of 16S rRNA and nifH genes

The primers 27F (5´-AGA GTT TA TCC TG CTC AG-3´) (Furushita et al. 2003) and Amp2 (5´-AAG GAG GT ATC CAR CCG CA-3´) (Wang et al. 1996) as described by Videira et al. (2009) were used for amplification of the 16S rRNA gene fragment. In the case of *nif*H gene amplification fragment the primers PolF (5´-TC GAY CCS AAR GCB GAC TC-3´) e PolR (5´-ATS GCC ATC ATY TCR CCG GA-3´) were applied as described by Poly et al. (2001). The 16 S rRNA and *nif*H fragments were sequenced in the 3500 Genetic Analyzer (Applied Biosystem®) automatic sequencer at the Embrapa Agrobiologia.

Similarity analysis

The consensus among 16S rRNA sequences was performed using the Staden Package software. The sequences were submitted to the BLASTn program (Basic Local Alignment Search Tool) (Altschul et al. 1997), for comparison with similar sequences deposited in NCBI (National Center for Biotechnology Information) database. Phylogenetic trees were constructed based on fragments of the genes 16S rDNA (~1450 bp) and *nif*H (~350 bp). The sequences selected in FASTA format were processed in MEGA 7 program and the consensus tree constructed by the Neighbor-Joining method, considering a bootstrap of 1,000 replicates.

Indole production

The eucalyptus bacterial strains were analyzed for the indole compound production according to the method proposed by Sawar and Kremer (1995). Each bacterial strain was grown in three tubes containing 5 mL liquid DYGS supplemented with 100 µg mL^{-1} L-tryptophan and incubated at 30°C in the dark with shaking at 150 rpm. One mL aliquots of each tube were taken at 24 and 48 h after bacterial growth and centrifuged at 10,000 rpm for 15 minutes. Then, 150 µL the supernatant from each sample was mixed with 100 µL Salkowski reagent (1 mL 0.5 M FeCl 3 in 49 mL 35% perchloric acid). Samples were placed in the dark for 30 min at room temperature and then the absorbance was measured at a wavelength of 540 nm on Biochrom Anthos Zenyth 200 rt microplate reader. The concentration of the indole compounds was estimated using a standard curve previously prepared with amounts of 5 to 100 µg mL^{-1} of 3-indole acetic acid.

To normalize the values obtained from the synthesis of indole compounds, the protein content was determined as described by Bradford (1976). Centrifuged cells were then resuspended in 1 mL liquid DYGS medium and aliquots of 100 µL of that suspension were added to tubes containing 100 µL 1M NaOH and kept for 30 min at room temperature. Then, 25 µL of this solution was added to a microplate well and then added with 225 µL Bradford solution. The 96-well microplate was incubated at 37°C for 30 min. Absorbance readings were performed on Biochrom Anthos Zenyth 200 rt microplate reader at a wavelength of 595 nm. The protein concentration was determined using a standard curve previously prepared with amounts of 0.25 to 1.5 µg/mL of bovine serum albumin (BSA). Data were tested by analysis of variance and the Scott-Knott test at 5% significance.

Greenhouse experiment

A completely randomized experimental design with 17 treatments and 16 replicates was carried out with the genotype *Eucalyptus urophylla* cultivated by seeds at the IFRO, Colorado do Oeste Campus. The treatments consisted of 12 strains isolated from roots of *E. urophylla*, four reference bacterial species and one control (unnoculated). The reference strains were *Azotobacter chroococcum* AC1, *Nitrospirillum amazonense* Aam82 (Pereira et al., 1988), *Burkholderia tropica* (BR 11366) and *Rhizobium tropici* BR322.

The inoculum was prepared by growing the bacterial strains in Erlenmeyer containing 50 mL DYGS medium at 150 rpm for 24 h at 30 ° C.

Plastic tubes with of 90 cm^3 size containing the substrate Carolina Soil with the following composition: Sphagnum 70%, roasted rice straw 20%, perlite 10%, pH 5.65, presence of N-P-K (trace) and agricultural gypsum were used to carried out the experiment. Each plastic tube was sown with a portion of 50 mg seeds and inoculated with 1 mL liquid inoculant suspension (1 x 10^9 cell/mL). Fifteen days after emergence (DAE) only one plant per tube was maintained (Marques and Uesugi 2013).

The biometric evaluations of the seedlings were performed at 30, 40, 50 and 60 DAE. The height was measured using a millimeter ruler (from the substrate level to the apical bud of the plant) and while the diameter used a digital caliper (Estopa et al., 2007). After, The plants were harvested 60 DAE and the aerial part was separated from the root system that was washed in tap water. The fresh materials were packed in paper bags and dried in a incubator oven (65ºC) to determine the constant biomass.

Morphological characterization of roots

The root morphology analysis was performed using the WinRHIZO Arabidopsis program (Regent Instruments Inc., Canada), coupled to an EPSON XL 1000 professional scanner. The resolution of the images was 600 dpi. The roots were laid in a vat 2 cm deep, 40 cm long and 30 cm wide, and filled with destilled water up to 1 cm layer. The following root characteristics were determined: very fine root volume (0.5 to 1.5 mm); fine root volume (1.5 to 3.0 mm) and main root volume (> 3.0 mm); total volume (cm^3); very fine root length (<0.5 mm), very fine root length (0.5 to 1.5 mm), fine root length (1.5 to 3.0 mm); main root length (> 3.0 mm); very fine root surface (0.5 to 1.5 mm); fine root surface (1.5 to 3.0 mm); main root surface (> 3.0 mm). Data were tested by analysis of variance and compared by Scott-Knott at 5% significance.

Statistical analysis

The assumptions of the normal distribution of errors and homogeneity of variance were met, and the data were submitted to analysis of variance (ANOVA) by the F-test at 5%. When F was significant (p<0.05), means were compared by Scott-Knott's test (p<0.05). Statistical analyses were performed using Sisvar® software (Ferreira, 2011).

Conclusion

A high diversity of bacterial strains closely related to the species *Nitrospirillum amazonense*, *Stenotrophomonas maltophilia*, *Pantoea agglomerans*, *Ideonella dechloratans* and *Herbaspirillum frisingense* was detected associated to *Eucalyptus urophylla* roots. Besides to present the nifH genes, confirming the biological nitrogen fixation capacity of the species, the strains also have the functional ability to produce indole compounds, a hormone that is known to play a role during the association of plant-bacteria. Many of the bacterial strains were able to promote plant growth either by root morphology changes or biomass accumulation

in root and shoots. Therefore, there is a biotechnological potential of these plant-growth promoting diazotrophic bacteria strains to be applied as inoculants to improve growth of eucalyptus seedlings. This fact open new opportunities to establish forests with economically viable and environmentally sustainable technologies.

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