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ORIGINAL ARTICLE

Bradyrhizobium and *Pseudomonas* strains obtained from coal-mining areas nodulate and promote the growth of *Calopogonium muconoides* plants used in the reclamation of degraded areas

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Abstract

Aims: The objective of this work was to isolate and characterize indigenous rhizobia from coal-mining areas able to efficiently nodulate and fix nitrogen in association with *Calopogonium mucunoides* (calopo).
Methods and Results: Isolation, authentication and morphological, biochemical and molecular characterization of the autochthonous rhizobia were performed and their symbiotic efficiency (SE) evaluated. Efficient rhizobial isolates suitable for the inoculation of calopo in coal-mining regions were obtained. A total of 30 isolates were obtained after nodulation authentication, of which five presented high SE with plant-growth promoting traits such as indole-3-acetic acid production, phosphate solubilization and biofilm formation. These isolates were identified as belonging to *Bradyrhizobium*, *Pseudomonas* and *Rhizobium*.
Conclusions: *Bradyrhizobium* sp. A2-10 and *Pseudomonas* sp. A6-05 were able to promote calopo plant growth using soil obtained from coal-mining degraded areas, thus indicating their potential as inoculants aiming at land reclamation.
Significance and Impact of the Study: To our knowledge, this is the first report of *Pseudomonas* nodule formation in calopo. Furthermore, the results demonstrated that autochthonous rhizobia obtained from degraded soils presented high SE in calopo and possess a wide range of plant-growth promoting traits. Ultimately, they may all contribute to an increased leguminous plant growth under stress conditions. The selected rhizobia strains may be used as inoculants and present a valuable role in the development of strategies aiming to recover coal-mining degraded areas. Bacterial inoculants would greatly reduce the use of often harmful nitrogen fertilizers vastly employed in revegetation programmes of degraded areas.

Introduction

Coal-mining is one of the basic sectors for the Brazilian economy (Farias 2002). Yet, this practice has led to serious environmental problems associated mainly with the physical, chemical and biological changes resulting from the removal and deposition of soil, the mixing of other materials, and the removal of native vegetation from the impacted sites. An example of coal-mining pollution is seen in the Santa Catarina coal basin, located in southern Brazil, which has been classified as critical (Stahl *et al.* 2002). Soils from these areas are highly degraded, with

very low pH and low availability of macronutrients (Campos *et al.* 2010). An alternative to reverse this reality and recover the degraded soils consists on the revegetation of these areas with plants able to resist the harsh conditions found in those soils (e.g. low pH, low N levels, high levels of heavy metals). In this sense, herbaceous legumes such as *Calopogonium mucunoides* Desv. (calopo) present great adaptation to soils with low natural fertility, such as soils presenting low pH and containing high levels of Al. Therefore, the species is indicated for the revegetation of coal-mining degraded sites (Seiffert *et al.* 1985; Costa 2009). In addition, an important and vital characteristic of calopo plants is their ability to fix atmospheric N_2 in symbiosis with efficient nitrogen-fixing rhizobia. This potentially leads to an increased level of N (a common limiting nutrient in soils) (Camargos and Sodek 2010; Ferreira *et al.* 2016).

The legume-rhizobia symbiotic process begins with the selection of specific rhizobial strains able to induce the nodulation process. Upon the perception of plant flavonoids, rhizobia produce lipochitooligosaccharides, termed nodulation (Nod) factors that induce the plant symbiotic response and the development of nodules. Consequently, rhizobia enter the plant root hair cells, and reach the nodule via infection threads. Once in the nodule, rhizobia differentiate into a bacteroid, and start fixing providing nitrogen to the plant host (Gage 2004). Additionally, rhizobia can facilitate legume growth through other mechanisms, such as the modulation of phytohormone levels, production of siderophores and solubilization of inorganic phosphate (Brígido and Glick 2015). The rhizobia ability to form biofilms also improves the physicochemical properties of the soil, with consequent benefit to plant growth (Batool and Hasnain 2005; Qurashi and Sabri 2012).

Coal-mining degraded soils present very harsh conditions for the development of legume-associated rhizobia. Factors such as soil pH and the presence of heavy metals impair the nodulation process and the overall nitrogen fixation process. Moreover, low pH levels inhibit rhizobia growth, survival, abundance and competitiveness (Correa and Barneix 1997; Lin *et al.* 2012; Ferguson *et al.* 2013). Hence, in order to achieve an efficient symbiosis in these acidic soils it is extremely important not only to use stress-resistant plant species but also stress-resistant rhizobial strains presenting efficient nitrogen fixation abilities. Recent studies performed in our lab demonstrated that autochthonous microbial isolates (arbuscular mycorrhizal fungi and rhizobia) and not the common recommended rhizobial strains obtained from non-native soils, increased the nodulation and growth of *Mimosa* spp. (trees) and *Vicia sativa* (herbaceous) used in the revegetation of soils degraded by coal-mining (Moura *et al.* 2016; Stoffel *et al.* 2016; Hernández *et al.* 2017). Nevertheless, not much is understood about the nodulation of calopo and the rhizobial strains associating with this plant in natural or coal-mining areas. Therefore, the main objective of this work was to isolate, characterize and verify the symbiotic efficiency (SE) of indigenous rhizobia of coal-mining soils associated to calopo. Ultimately, obtaining autochthonous and efficient strains of rhizobia capable of surviving under adverse conditions is extremely important to the development of promising inoculants to be used in the revegetation of degraded soils.

Materials and methods

Soil sampling from coal-mining areas

Soil sampling was performed in June 2015, in areas previously submitted to revegetation procedures (Table S1). The areas were selected according to distinct recovering times: 2 years (A2), 4 years (A4), 6 years (A6) and 12 years (A12) under a revegetation regime, employing plant species recommended for the area (Table S1). The pH in water for the soils of those areas, measured according to EMBRAPA. Centro Nacional de Pesquisa de Solos (1997), were 4·66, 4·53, 3·80 and 4·91 respectively. The chemical characterization of the soils was performed according to the method described by Tedesco *et al.* (1995) and described in Table S1.

Soil samples were also collected in a reference area (RA), with no history of mining, with a typical vegetation cover of dense ombrophilous forest. In each site, five georeferenced points were selected, distanced 100 and 200 m from each other (depending on the size of the area). At each sampling point, a central point was chosen and in 4 m radius 4 soil subsamples (at each cardinal point) were collected, at a depth of 0–20 cm, totalling 400 g of soil per collection point. The collected soil was used as source of inoculum for the isolation of native rhizobia.

Isolation of rhizobia from calopo root nodules

Greenhouse experiment using soil inocula

An 60-day experiment using calopo as a trap plant was conducted in a greenhouse. Four calopo seeds, previously disinfected with 2% sodium hypochlorite for 2 min and washed six times in sterile distilled water, were placed in 280 cm^3 pots containing an autoclaved mixture of sand and vermiculite (1 : 2; v/v) and further received 50 g of each respective soil-inoculum. The experiment was conducted using a completely randomized design and consisted of five inoculation treatments corresponding to soil inoculum obtained from each of the recovery areas A2, A4, A6, A12 and RA (Table S1) and two control

treatments without inoculation: low (5·25 mg N) (C–N) and high concentration of mineral nitrogen (52·5 mg N) (C+N). A total of five replicates (pots) per treatment were used. Plants were watered daily with sterile distilled water and weekly with 50 ml of half strength modified nutrient solution (Hoagland and Arnon 1950).

At the end of the experiment, the shoots and roots of the plants were harvested and dried at 65°C until constant weight for the determination of shoot dry mass (SDM) and root dry mass (RDM). Nodules were detached, counted, and five nodules per pot selected for the isolation of rhizobia.

Nodule disinfection and bacterial isolation

Root nodules were disinfested with alcohol (95%) for 60 s, sodium hypochlorite (2·5%) for 2 min, and finally passed through six washes with sterile distilled water. Then, nodules were pinched and inoculated into Petri dishes containing Yeast Mannitol Agar (YMA) (Vincent 1970) and maintained at 28°C for 14 days. Different colonies were selected, maintained in YMA and the isolated bacteria tested for their nodulation ability. Each isolate was inoculated in calopo in sterile long-neck bottles containing Hoagland and Arnon (1950) nutrient solution (Araújo *et al.* 2017). Seeds were placed on a filter paper soaked in the nutrient solution. One ml of each bacterial inoculum, grown in YM medium for 72 h at 150 rev min^{-1} at 28°C, was then added per seed, and plants grown in a chamber for 90 days. After this period, the plants were removed and the presence of nodules determined. The bacterial isolates able to nodulate calopo were further conserved in YMA medium with glycerol (20%) at −80°C.

Morphological characterization and selection of representative calopo rhizobial isolates

To perform the phenotypic characterization, each authenticated isolate was transferred to YMA medium containing bromothymol blue for a period of 10 days at 28°C. Evaluated characteristics included: growth time, pH change, colour, shape, surface and border of the colony, absorption of the indicator and mucus production (Silva *et al.* 2006). Strains *Rhizobium leguminosarum* (SEMIA 384), *Rhizobium tropici* (CIAT 899), *Bradyrhizobium japonicum* (BR 1602) and *Bradyrhizobium* sp. (SEMIA6144) were used as references.

A BOX-PCR analysis was conducted in order to verify the similarity between the isolates obtained and discard potential replicate strains. For the molecular characterization by BOX-PCR, rhizobia DNA was extracted by the thermal lysis method. A colony of each bacterial isolate was suspended in 0·2 ml tubes containing 100 μl of ultrapure water and held at 100°C for 5 min, followed by a centrifugation at 13 000 ***g*** for 3 min. The supernatant was transferred to a fresh 0·2 ml tube and stored at −20°C (Hagen *et al.* 2002). BOX-PCR was run with the BOX A1R primer (5′CTACGGCAAGGCGACGCTGACG-3′) (Versalovic *et al.* 1994). The amplification reaction was performed using 1 μl of the extracted DNA, 50 pmol of the BOX A1R prime, 2·5 μl of 2 mmol l^{-1} dNTPs, 2·5 μl of 100% DMSO, 4·0 μl of BSA (1 mg ml^{-1}), 0·5 (1 U) of Taq DNA polymerase (Fermentas, São Paulo, Brazil), and 5 μl of 5X Gitschier Buffer (83 mmol l^{-1} of $(NH_4)_2SO_4$, 335 mmol l^{-1} Tris-HCl pH 8·8, 33·5 mmol l^{-1} $MgCl_2$, 33·5 μmol l^{-1} EDTA, 150 mmol l^{-1} β-mercaptoethanol) and ultrapure water to a final volume of 25 μl. Amplification conditions were 2 min at 95°C, 30 cycles of 3 s at 94°C, 30 s at 92°C and 1 min at 50°C, 8 min at 65°C and a final extension for 8 min at 65°C (Versalovic *et al.* 1994).

The amplified fragments were separated by 1·5% agarose gel electrophoresis in 1X TAE buffer (50 mmol l^{-1} Tris-HCl pH 8·8, 50 mmol l^{-1} glacial acetic acid, 25 mmol l^{-1} EDTA) at 55 V for 3 h. Image acquisition was performed in a ChemiDoc MP (Bio-Rad, Hercules, CA) and the amplicon profiles analysed in Gel Compar II ver. 6.5 (Biosystematica, Wales, UK) (Welsh *et al.* 2010) Representative isolates from each similarity group were randomly selected, totalling 17 samples.

SE of isolated rhizobia

Experiment #1

To perform an initial screening of the most efficient rhizobial isolates obtained, a greenhouse experiment using calopo was conducted (average temperatures in the greenhouse varying between 15·6 and 28·6°C). Initially, seeds were disinfected as previously described and pregerminated in a humid chamber. Then, four seeds per pot were placed on a mixture of sand and sterile vermiculite in the ratio of 1 : 2 (v/v). One ml of bacterial inoculum (optical density, OD_{535} = 0·5 corresponding to 10^7–10^8 cells per ml) was applied per seed. The inoculum was obtained following the growth of the selected isolates in YM medium for 72 h under stirring.

After the second cotyledon leaf was emitted, a thin layer of sterilized sand, chloroform and paraffin mixture (5 : 1 : 0·015 respectively) was applied to avoid contamination. Water was added daily and weekly fertilizations were performed using modified and sterilized half strength Hoagland and Arnon (1950) nutrient solution.

The assay was conducted in a completely randomized design with a number of treatments corresponding to the different rhizobia isolates (n = 17) and two control treatments without inoculation, one receiving mineral nitrogen

(C+N, 52·5 mg N) and the other without mineral nitrogen (C–N). A total of four replicates per treatment were used.

After 90 days, the plants were harvested and the number of nodules (NN), and the dry mass of nodules (NDM), shoots (SDM) and roots (RDM) determined. The nitrogen content in the shoots was determined by the Kjedahl semi-micro method, according to Tedesco *et al.* (1995). The accumulated nitrogen was calculated by multiplying the nitrogen content by the respective SDM. The SE of each rhizobia isolate and the recommended strains was calculated according to the formula SE = (total N fixed−total N without N)/(total N for C+N−total N for C−N) × 100 (Chagas-Junior *et al.* 2010), where SE = symbiotic efficiency; total N fixed = nitrogen accumulation of inoculated treatment; total N for C+N = nitrogen accumulation of the treatment with the application of N; total N for C–N = nitrogen accumulation of the treatment with no N application.

Experiment #2

In order to validate the results obtained in experiment #1, a second greenhouse experiment was conducted (average temperatures ranged between 21·5 and 35·9°C) following the same procedures described in experiment #1. In this second experiment, we evaluated the inoculation treatments that had high SE in experiment #1 ($n = 5$), low SE ($n = 1$), and the strain recommended by the Brazilian Ministry of Agriculture (MAPA) (*B. japonicum* BR 1602) with two noninoculated treatments with (52·535 mg N) and without mineral nitrogen. The assay was conducted following a completely randomized experimental design with seven replications. The evaluated parameters were the same as those described in experiment 1.

Promotion of calopo growth in a coal-mining recovery soil by selected rhizobial isolates

An experiment using coal-mining recovery soil was performed in order to test the selected strains ability to promote calopo growth under more realistic and stressful conditions. The soil used in this experiment was acid and had low fertility. Its characteristics were: organic matter – 1·6%; pH (H_2O) – 4·6; P and K (Mehlich-1) – 11.1 and 76·0 mg dm^{-3}, respectively; Ca, Mg, H^{+}Al, and Al – 5·7, 3·1, 7·54, and 0·7 cmol$_c$ dm^{-3}, respectively. Plant disinfection and germination were performed as previously described.

The experiment was conducted in the greenhouse with temperatures ranging from 17·2 and 35·4°C and followed a completely randomized design. The experiment consisted on four treatments: inoculation with strain A6-05; inoculation with strain A2-10; inoculation with strain *Bradyrhizobium* sp. BR1602 (recommended strain) and a negative control (noninoculated). A total of five replicates (four plants per pot) per treatment were performed.

The plants were watered daily and 50 days after inoculation harvested. Nodule number, nodule dry mass, shoot and RDM and N accumulation were measured as previously described.

Amplification and partial sequencing of the 16S rRNA gene and phylogenetic affiliation

Based on the SE experiments, the most efficient isolates were selected for amplification and sequencing of the 16S rRNA gene in the Genomic Division of Macrogen Inc., Korea. DNA from those isolates was extracted using the Ultra Clean Microbial DNA Isolation Kit (Mo Bio, Toronto, Canada) following the manufacturer's instructions. Universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-ACCTTGTTACGACTT-3′) (Lane 1991) were used for amplification. The sequences provided by Macrogen were processed and edited in FinchTV software ver. 1.4.0 (Geospiza, Denver, CO) and aligned using BLAST (Altschul *et al.* 1997). EzTaxon was used as a database to obtain the classification of the isolates up to gender level (Kim *et al.* 2012). Sequences obtained were submitted and deposited in GenBank and are available from accession number MF572134 through MF572140.

Strain biochemical characterization

Based on the SE experiments, the most efficient isolates were selected to evaluate the production of indole-3-acetic acid (IAA) and siderophores, phosphate solubilization capacity, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and biofilm formation.

The determination of IAA production was performed using the Salkowski's reagent prepared from ferric chloride in perchloric acid (50 ml of 35% $HClO_4$, 1 ml of 0·5 mmol l^{-1} FeCl) (Glickmann and Dessaux 1995). Bacteria were grown in 5 ml of YM medium containing 500 μg ml^{-1} tryptophan to induce the production of IAA. After 24 h of growth in the dark, under constant stirring (135 rev min^{-1}) and temperature of 28°C, the cultures were centrifuged (3248 ***g***, 15 min). The supernatant was then mixed with the Salkowski reagent in a 2 : 1 ratio and the absorbance read on a spectrophotometer at the wavelength of 535 nm. The reaction period for the quantification of EIA was 30 min and analyses performed in triplicates for each isolate. The concentration of IAA in each sample was calculated based on a standard curve ranging from 0 to 100 μg ml^{-1} IAA (Sigma, Shanghai, China).

To evaluate the ability to produce siderophores, the qualitative method described by Schwyn and Neilands (1987) was used. To this end, 10 μl of the bacterial cultures grown overnight in King B medium were inoculated onto a CAS agar plate (Alexander and Zuberer 1991). Triplicates were performed for each isolate and the autochthonous isolate UFCS-M8 was used as positive control (Hernández *et al.* 2017). After 72 h of incubation at 28°C the isolates were classified into producers or nonproducers of siderophores, due to the colour change in the reagent from blue to orange.

To determine the solubilizing capacity of calcium phosphate, 20 μl of bacterial inoculum were inoculated into Petri dishes containing NBRIP medium (Nautiyal 1999). The plates (in triplicate) for each isolate were incubated at 28°C for 14 days (Alikhani *et al.* 2007). The solubilizing capacity was determined by the existence of a transparent halo around the colony.

The formation of biofilm was measured as described in Timmusk *et al.* (2011), with some modifications. To this end, 2 ml of the bacterial suspensions of each isolate were diluted in YM medium until reaching an OD_{600} of 0·02. 150 μl aliquots were inoculated into 96-well plates and incubated for 72 h at 28°C. Subsequently, plates were carefully aspirated to remove unbound cells and washed with 150 μl of sterile distilled water. Then, biofilms were fixed with 200 μl of methanol for 15 min. After the methanol was completely withdrawn, 150 μl of crystal violet solution (1%) was added and incubated for 20 min. The plates were again washed three times with sterile distilled water and after drying, 200 μl of 33% acetic acid was added for the solubilization of the dye. It was then incubated for 20 min and the optical density measured on a plate reader at 590 nm wavelength. All samples were tested in five independent wells. Isolates that presented optical density values greater than one were considered as biofilm producers.

1-aminocyclopropane-1-carboxylate deaminase activity was tested qualitatively, by analysing the isolates ability to grow in DF minimal medium containing 3 mmol l^{-1} ACC as sole nitrogen source (Tavares *et al.* 2018).

Statistical analysis

The variables measured were submitted to normality (Shapiro–Wilk) and homogeneity of variances (Cochran) tests. The number of nodules was transformed by the log 10 function. The data were compared using analysis of variance and the means submitted to the SNK separation test ($P < 0·05$). These analyses were done in R (R Development Core Team, 2011). The phenotypic attributes of the rhizobia were submitted to a hierarchical grouping analysis using the program SYSTAT 11 (Systat Software Incorporated, 2004, San Jose, CA, USA). Obtained clusters from the BOX-PCR band profiles were analysed using the Jaccard's similarity coefficient and the UPGMA clustering model. Graphics containing bars with the standard error of the mean were generated using Sigma-Plot ver. 12 (Systat Corp., San Jose, CA).

Results

Effect of soil inoculum on the growth and nodulation of calopo

The SDM and the RDM of calopo was significantly influenced by the treatments. All plants inoculated with soils obtained from recovery and RAs presented root nodules and in some cases the inoculation resulted in increased plant growth (Fig. 1a,b).

The highest SDM was obtained in calopo inoculated with soils from areas A2 and A12, with average increases of 121 and 76% compared to the control (C+N) respectively. All the inoculation treatments led to the increase in RDM when compared with the controls without inoculation. Plants inoculated with soil from areas A2 and A6 presented the highest values of RDM in calopo, with mean increments of 328% in relation to control receiving nitrogen (C+N).

Isolation, characterization and selection of representative rhizobial isolates

A total of 50 bacterial isolates were obtained from the root nodules inoculated with soil from different recovery areas, of which 30 were able to nodulate calopo (data not shown). Among the phenotypic characteristics evaluated, it was observed that most of the isolates presented fast to intermediate growth and mucus production. Hierarchical grouping analysis, based on morphological and cultural characteristics, showed separation of those 30 isolates in three groups (Figure S1). One group, composed of 11 isolates (AR-18, A12-2, A4-04, A12-05, A12-08, A2-06, A4-03, AR-12, AR-14, A6-04, AR-15) presented morphological similarity with the reference strains *Bradyrhizobium* sp. SEMIA 6144 and BR1602. Another group composed of five isolates (A6-11, A6-14, AR-04, A12-11, AR-02) presented morphological similarity with strains of *R. tropici* CIAT 899 and *R. leguminosarum* SEMIA 384. A group of 14 isolates (A6-05, A2-11, A6-05, A6-06, A4-02, A2-10, A2-08, A2-01, A4-12, A4-07, A2-07, A6-08, A6-09, A2-09) showed no similarity to any of the reference strains evaluated.

Of the 30 authenticated isolates, only two did not have an acceptable amplification of the BOX region. The hierarchical cluster analysis based on the BOX-PCR showed

that most of the isolates presented different profiles (Figure S2). Based on these results, 17 of the 30 isolates were randomly selected and tested for their SE.

SE of calopo-associated rhizobia

Experiment 1

Results obtained from the SE experiment 1 showed that several rhizobial isolates were efficient and promoted calopo growth in great extent when compared with the noninoculated controls (Table 1). Amongst the 17 isolates tested, A6-05, AR-12, A2-10, A6-11, AR18 presented an increased and significant ability to form nodules and, consequently, increased calopo SDM and nitrogen content. Three of these isolates (A6-05, AR-12, A2-10) presented an outstanding ability to form nodules (221, 122 and 91 nodules respectively), with nodules presenting an increased dry mass when compared with the other rhizobial treatments (Table 1). Consequently, the highest SDM values were obtained in plants inoculated with isolate

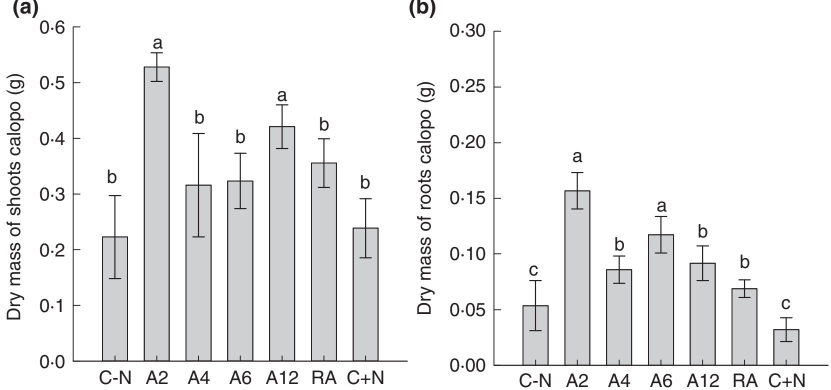

Figure 1 Effect of soil inoculation from coal mining areas under different recovery times, on attributes related to the growth of calopo: Dry mass of shoots (SDM) (a) and roots (RDM) (b) after 60 days of growth. R = reference soil without mining tailings. 2, 4, 6, 12 = years of recovery; C+N = control not inoculated and with N; C-N = control not inoculated and with no N. Means followed by the same letter do not differ by the SNK test ($P < 0·05$). Vertical bars represent the standard error of the mean (n = 5).

Table 1 Effect of the inoculation with autochthonous rhizobia on the number of nodules, dry mass of nodules, dry mass of shoots, accumulation of nitrogen and Symbiotic efficiency of bacterial strains inoculated to *Calopogonium mucunoides* (calopo) after 90 days of growth

Treatments	Nodules (# pots^{-1})	Dry mass of nodules (mg per pots)	Dry mass of shoots (mg per pots)	Nitrogen content in the shoots (g kg^{-1})	Nitrogen accumulation in the shoots (mg per pots)	Symbiotic efficiency (%)
C-N	0 e	0·0 e	97·0 d	29·00 b	2·75 c	–
A2-06	40 e	11·0 d	136·8 d	39·36 a	5·09 c	49·30 c
A2-10	91 c	22·3 c	241·5 c	33·57 b	7·92 b	102·08 b
A4-07	50 d	9·5 d	136·0 d	45·44 a	6·18 b	67·69 c
A6-04	31 e	10·5 d	133·0 d	36·41 b	7·73 c	39·12 c
A6-05	221 a	48·5 a	613·3 a	32·72 b	19·96 a	339·97 a
A6-06	55 d	11·0 d	95·5 d	28·70 b	2·74 c	1·93 c
A6-08	31 e	6·3 d	108·0 d	34·85 b	3·65 c	17·93 c
A6-11	81 c	32·5 b	250·0 c	30·55 b	7·64 b	96·53b
A6-15	18 e	9·0 d	125·3 d	34·70 b	4·23 c	41·42 c
A12-02	21 e	4·0 e	120·0 d	27·08 b	3·39 c	0·00 c
A12-05	7 e	0·8 e	57·5 d	31·90 b	1·80 c	0·00 c
A12-11	38 e	10·0 d	180·5 d	33·01 b	5·31 c	71·17 b
AR-02	22 e	8·5 d	152·3 d	32·53 b	4·40 c	36·65 c
AR-04	22 e	4·3 e	108·3 d	34·75 b	3·76 c	20·26 c
AR-12	122 b	42·5 a	331·0 b	25·95 b	8·59 b	115·21 b
AR-15	23 e	8·0 d	128·0 d	34·90 b	4·56 c	35·63 c
AR-18	76 c	18·0 c	215·5 c	39·66 a	8·29 b	109·51 b
C+N	0 e	0·0 e	157·0 d	50·55 a	7·80 b	–

Values followed by different letters in the same column for each consortium are statistically different according to the SNK test ($P < 0·05$).

A6-05 (382 and 290% increment in relation to the controls, C-N and C+N respectively), followed by isolates AR-12 (241 and 110% increment in relation to the controls, C-N and C+N respectively) and A2-10 (149 and 53% increment in relation to the controls, C-N and C+N respectively).

The plant nitrogen accumulation was significantly increased in plants inoculated with isolate A6-05, resulting in a 155% increase over the C+N control. For the other treatments, gains in nitrogen accumulation were observed but these were not statistically different from the values presented by the C+N treatment.

The SE was calculated and these values indicated that the most efficient isolate was A6-05, with efficiencies higher than 300%. Isolates AR-12, A2-10, A6-11and AR-18 also showed high SE (98% average) (Table 1).

Experiment 2

Based on the data obtained in experiment 1, five isolates presenting high SE (A6-05, AR-12, A2-10, A6-11, AR18) and one isolate (AR-02) presenting low SE were selected and their SE compared to that of the recommended strain *Bradyrhizobium* sp. BR1602. Sixty days after inoculation it was observed that isolates A6-05 and BR1602 presented the highest plant growth promotion abilities and no significant statistical differences were found between those treatments. Both A6-05 and BR1602 presented on average, 328, 58 and 87% increments for the variables NDM, SDM and Nitrogen accumulation respectively (Figure 2).

Genetic and biochemical characterization of selected rhizobia

The six isolates selected in experiment 1 were identified by means of partial sequencing of the 16S rRNA gene (Table 2). Interestingly, isolates A6-05, A6-11 and AR18 were identified as *Pseudomonas* spp. Moreover, BlastN analysis against sequences from type strains indicated that strain A6-05 presented high identity to *Pseudomonas koreensis,* whereas strains A6-11 and AR-18 presented high identity to *Pseudomonas azotoformans.* The isolates A2-10 and AR-12 were identified as *Bradyrhizobium* sp., and presented high identity to *B. embrapense.* Isolate AR-

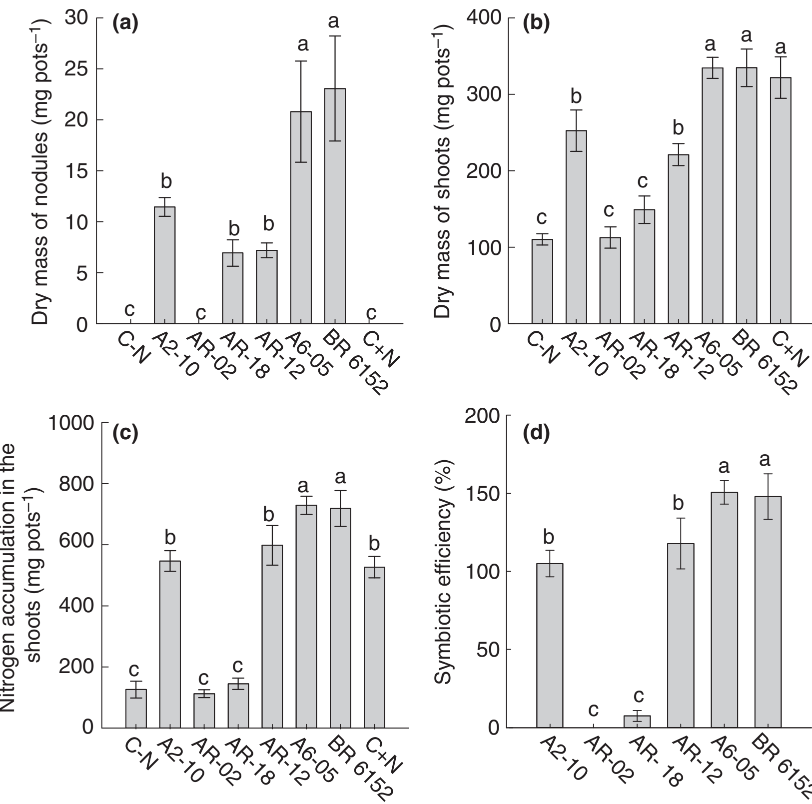

Figure 2 Nodule dry mass (a), shoot dry mass (b), Nitrogen accumulation (c) and symbiotic efficiency (d) of *Calopogonium mucunoides* (calopo) inoculated with rhizobia, after 60 days of growth. Means followed by the same letter did not differ significantly from each other by the SNK test ($P < 0{\cdot}05$). Vertical bars represent the standard error of the mean (n = 7).

02 was identified as *Rhizobium* sp. and presented high identity to *R. tropici*.

Pseudomonas strains presented phosphate solubilization capacity, produced siderophores and low levels of IAA but were unable to form biofilms or present ACC deaminase activity under the tested conditions (Table 2). *Bradyrhizobium* strains were unable to form biofilms and did not present ACC deaminase activity, siderophore or IAA production. Yet, strain A2-10 presented phosphate solubilization capacity. The strain *Rhizobium* sp. AR-02 produced IAA and biofilms, however, did not present ACC deaminase, siderophore production and phosphate solubilization activities.

Table 2 Taxonomic identification by partial sequencing of the 16S rRNA gene and levels of IAA, PSI, ACCD, and siderophore by plant growth promoting rhizobia isolated from coal mining areas

Isolate/Identification	Accession number	IAA (μg ml^{-1})	PS	SID	BIO
A2-10 *Bradyrhizobium* sp.	MF572134	0·0 ± 0·0	+	–	–
A6-05 *Pseudomonas* sp.	MF572140	11·79 ± 0·2	+	+	–
A6-11 *Pseudomonas* sp.	MF572137	9·80 ± 0·3	+	+	–
A12-11 *Rhizobium* sp.	MF572139	17·52 ± 0·2	–	–	+
AR-02 *Rhizobium* sp.	MF572135	22·27 ± 0·2	–	–	+
AR-12 *Bradyrhizobium* sp.	MF572136	0·0 ± 0·0	–	–	–
AR-18 *Pseudomonas* sp.	MF572138	ne	+	+	–

IAA, Indole-3-acetic acid; PS, phosphate solubilization; SID, siderophore production; BIO, biofilm formation; ne, not evaluated.

Promotion of calopo growth in coal-mining recovery soil by *Bradyrhizobium* sp. A2-10 and *Pseudomonas* sp. A6-05

The inoculation of calopo plants with autochthonous *Bradyrhizobium* sp. A2-10 and *Pseudomonas* sp. A6-05 resulted in a significant increase in plant growth and stress resistance when compared with the control and *Bradyrhizobium* sp. BR1602 inoculation treatments (Fig. 3a–d). On the other hand, the inoculation with the recommended strain *Bradyrhizobium* sp. BR1602 did not positively impact calopo growth and development.

The inoculation of calopo with *Bradyrhizobium* sp. A2-10 resulted in 1000, 479, 186 and 162% increments in NN, NDM, SDM and accumulation of N, respectively, when compared with the inoculation of the recommended strain BR1602. Similarly, when compared with the recommended strain BR1602 inoculation treatment, calopo inoculation with *Pseudomonas* sp. A6-05 resulted in increases of 300, 517, 173 and 166% in NN, NDM, SDM and accumulation of N respectively.

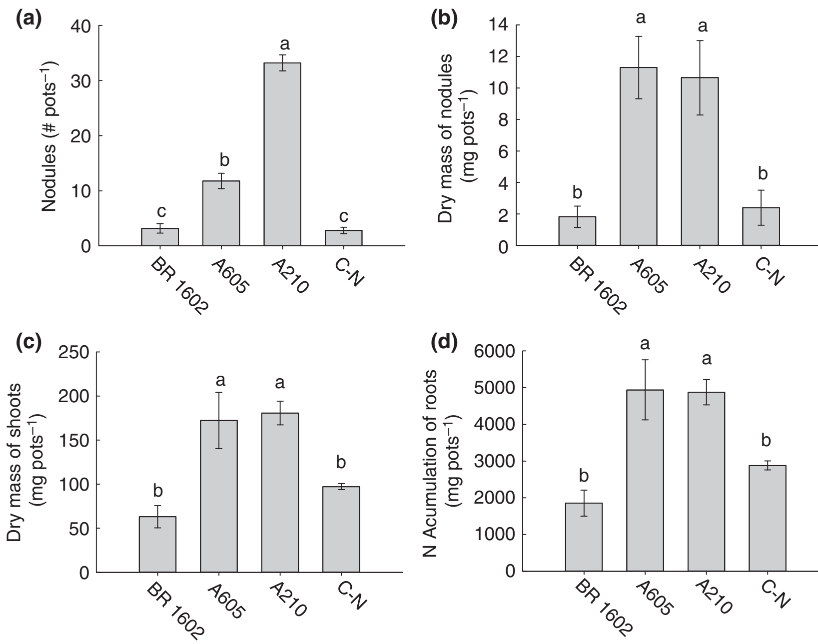

Figure 3 Nodule dry mass (a), dry mass of nodules (b), shoot dry mass (c) and Nitrogen accumulation (d) of *Calopogonium mucunoides* (calopo) inoculated with rhizobia, after 50 days of growth in low fertility soil. Means followed by the same letter did not differ significantly from each other by the SNK test ($P < 0{\cdot}05$). Vertical bars represent the standard error of the mean ($n = 5$).

Discussion

In this work, we isolated, characterized and selected efficient rhizobial strains to be applied in the revegetation of coal-mining degraded areas using calopo plants. The obtained results demonstrated that selected autocthtonous rhizobia adapted to coal-mining degraded soils significantly promoted calopo growth under normal and stressful conditions. Moreover, these autocthtonous rhizobia promoted calopo growth in a coal-mining recovery soil, whereas the recommended strain *Bradyrhizobium* sp. BR1602 was not able to promote calopo growth under these conditions. This data further confirms the results obtained in previous studies, demonstrating that selected autochthonous microorganisms present an increased ability to promote plant growth under stressful conditions (Yang *et al.* 2009; Timmusk *et al.* 2011, 2014), and, therefore, present an alternative to the use of non-native rhizobial inoculants that are mainly used in general agricultural processes but are inefficient in the recovery of degraded areas (Moura *et al.* 2016; Stoffel *et al.* 2016; Hernández *et al.* 2017; Meyer *et al.* 2017). One of the reasons behind the increased nodulation and plant growth promotion abilities of autochthonous rhizobia may be their natural adaptation to soil characteristics, resistance to pH and heavy metal stress, which are factors known to greatly inhibit the nodulation process (Ferguson *et al.* 2013).

Bacteria belonging to the *Bradyrhizobium*, *Rhizobium* and *Pseudomonas* genera were able to form nodules in calopo. Still, these bacteria presented different nodulation and symbiotic efficiencies. *Bradyrhizobium* and *Pseudomonas* strains were highly efficient, however, *Rhizobium* presented a decreased nodulation and SE, thus suggesting, an unspecific symbiotic relationship. Moreover, their nodulation and plant growth promotion abilities seem to be independent from their biochemical properties.

It is known as rhizobia those bacteria that in symbiosis with legumes have the capacity to produce nodules and fix atmospheric nitrogen. Such association has been studied during the last century or more (Brígido and Glick 2015). In the last two decades, the number of known nodulating species has increased considerably due to the increase in the number of taxonomic studies and the advances of molecular techniques used for species characterization (Shiraishi *et al.* 2010). Weir (2016) described that the rhizobia group was composed by α and β-proteobacteria, comprising 13 genera and 98 species. However, the finding of nodulation by rare genera has been frequently described such as *Acinetobacter*, *Bacillus*, *Klebsiella*, *Enterobacter* and *Curtobacterium* spp. (Soares *et al.* 2014; Hossain and Lundquist 2016; Hernández *et al.* 2017). Benhizia *et al.* (2004) reported, for the first time, members of γ-proteobacteria nodulating legumes. However, in that work, essential aspects such as the authentication to verify the Koch postulates and/or analyses of the symbiotic genes of the isolates were not considered. These same limitations can also be found in the work of Sbabou *et al.* (2016), who described species of the three subclasses (α, β and γ-proteobacteria) present in *Hedysarum* nodules. From that data it can be inferred only that the bacteria are endophytic to the nodules, but not necessarily that they have the capacity to form the nodules. The first report describing the subclass γ-proteobacteria nodulating legumes dates back to the last decade, in which nodulation of *Pseudomonas* sp. was described for *Robinia pseudoacacia*, an arboreal legume species (Shiraishi *et al.* 2010). The authors suggested that *Pseudomonas* sp. Ch10048 isolated from *R. pseudoacacia* acquired symbiotic genes from other rhizobia trough horizontal gene transfer events.

Interestingly enough, one of the most efficient strains obtained in this study (A6-05) belongs to the genus *Pseudomonas*. Other *Pseudomonas* strains (A6-11, AR-18) were also recovered from calopo root nodules and presented increased nodulation abilities. These strains were isolated from calopo plants inoculated with soils obtained from different and distant areas (e.g. A6 and AR) (Table S1), further suggesting their presence and distribution in soils of South Brazil. To our knowledge, this is the first report of *Pseudomonas* with high SE nodulating herbaceous legumes, such as *C. mucunoides*. Some authors have suggested that endophytic bacteria may acquire genes related to nodulation via horizontal gene transfer, which may be the case for the presently sampled strains (Moulin *et al.* 2001; Minamisawa *et al.* 2002).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1. Hierarchical clustering based on morpho-logical-cultural characteristics of rhizobia isolated from areas with different stages of recovery after coal mining in the Santa Catarina coal basin.

Figure S2. Hierarchical clustering analysis of the BOX-PCR data of rhizobia isolated from areas under different stages of recovery after coal mining in the Santa Catarina coal basin.

Table S1. Location and characteristics of the areas in different stages of recovery in the Carboniferous region of Santa Catarina. Trait elements according to USEPA 3051A (1998).