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Selection and characterization of coal mine autochthonous rhizobia for the inoculation of herbaceous legumes

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Abstract Coal open pit mining in the South of Santa Catarina state (Brazil) was inappropriately developed, affecting approximately 6.700 ha. Re-vegetation is an alternative for the recovery of these areas. Furthermore, the use of herbaceous legumes inoculated with nitrogen fixing bacteria is motivated due to the difficulty implementing a vegetation cover in these areas, mainly due to low nutrient availability. Therefore, the aim of this work was to evaluate, among 16 autochthonous rhizobia isolated from the coal mining areas, those with the greatest potential to increase growth of the herbaceous legumes *Vicia sativa* and *Calopogonium mucunoides*. Tests were conducted in greenhouse containing 17 inoculation treatments (16 autochthonous rhizobia + Brazilian recommended strain for each plant species), plus two treatments without inoculation (with and without mineral nitrogen). After 60 days, nodulation, growth, N uptake, and symbiotic efficiency were evaluated. Isolates characterization was assessed by the production of indole acetic acid, ACC deaminase, siderophores, and inorganic phosphate solubilization. The classification of the isolates was performed by 16 S rDNA gene sequencing. Only isolates UFSC-M4 and UFSC-M8 were able to nodulate *C. mucunoides*. Among rhizobia capable of nodulating *V. sativa*, only UFSC-M8 was considered efficient. It was found the presence of more than one growth-promoting attributes in the same organism, and isolate UFSC-M8 presented all of them. Isolates were classified as belonging to *Rhizobium, Burkholderia* and *Curtobacterium*. The results suggest the inoculation of *Vicia sativa* with strain UFSC-M8, classified as *Rhizobium* sp., as a promising alternative for the revegetation of coal mining degraded areas.

Keywords Biological nitrogen fixation · *Rhizobium* · Degraded areas · Symbiotic efficiency

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Introduction

Coal is considered one of the most abundant fossil fuels on the planet, and represents an extremely important source of energy worldwide (WEC 2013). In Brazil, coal is mostly located in the states of Rio Grande do Sul (RS) and Santa Catarina (SC), with reserves of approximately 32.6 billion tones. In those regions, coal-mining activities were originally performed in open, improperly developed pits, therefore leading to large environmental impacts (Rocha-Nicoleite et al. 2013). In the South of Santa Catarina, 6.700 ha and 2/3 of the water streams suffer from heavy pollution due to acid mine drainage (AMD) or aquifer residue deposition (Alba 2007). As a result, soil fertility is severely impacted, thus leading to vegetation loss, landscape modifications, and overall decrease in biodiversity (Soares et al. 2008).

Conventional physical and chemical treatments used for soil recovery present several limitations, such as high

costs and the possible irreversible changes in soil properties (Delgadillo et al. 2011). One potential low cost alternative for soil and landscape recovery lies on re-vegetation using leguminous plants (Nascimento and Biondi 2008; Siqueira et al. 2008). Some studies have been developed with leguminous species for the recovery of degraded soils in Brazil (Melloni et al. 2004; Carneiro et al. 2008; Nunes et al. 2015; Moura et al. 2016).

Vicia sativa (vetch) and *Calopogonium mucunoides* (calopo) have shown great potential for soil recovery (Rocha-Nicoleite et al. 2013). These herbaceous plants (Fabaceae) are able to grow in low fertility soils presenting high acidity and high aluminum concentrations (Seiffert et al. 1985; Calegari et al. 1993). Nevertheless, its growth is severely affected by low nitrogen, which limits its efficiency in the recovery of degraded areas (Ferrari and Wall 2004).

The use of plant-growth-promoting bacteria (PGPB) might be a useful strategy to increase plant growth and resistance to multiple stresses (Glick 2014). In this sense, rhizobia play a significant role promoting plant growth through its ability to form a symbiotic relationship and provide nitrogen to the host plant (Glick 2012). Moreover, many rhizobia also possess other plant-growth promotion abilities such as phosphate solubilization, siderophore, ACC (1-aminocyclopropane-1-carboxylate) deaminase, and IAA (indole acetic acid) production that play important roles towards plant growth and development (Glick 2014). *V. sativa* and *C. mucunoides* usually form a symbiotic relationship with rhizobia belonging to α-Proteobacteria. In Brazil, the recommended rhizobia strains for *V. sativa* and *C. mucunoides* are *Rhizobium etli* SEMIA 384 and *Bradyrhizobium japonicum* BR 1602, respectively (Brasil 2011). However, not much is known about the symbiotic efficiency of these rhizobial strains under limiting conditions, such as those found in coal-mining degraded soils. Hence, obtaining autochthonous and compatible rhizobial strains able to survive under these limiting conditions is of extreme importance to obtain potential inoculants that can be used under field conditions.

Therefore, the aim of this study was to characterize and assess the symbiotic compatibility and plant-growth promotion abilities of rhizobial strains previously obtained from coal-mining degraded areas in the state of Santa Catarina, using *V. sativa* and *C. mucunoides* as host plants.

Materials and methods

Rhizobial strains and leguminous plant species

This study tested 16 strains of rhizobia from the Soil Microbiology Laboratory Collection at the Federal University of Santa Catarina, isolated in October 2010 in an area degraded from coal extraction near the non-operational Indústria Carboquímica Catarinense—ICC, in Criciúma, SC, southern Brazil (28° 44′ 18.40″S, 49° 24′ 42.62″W). A soil sample, representative of the mining area, was chemically analyzed, exhibiting pH (H_2O) 3.8 and levels of trace elements (mg kg^{-1}) of 8.60, 17.3, 125 and 422 for As, Cd, Pb and Zn, respectively. These are all higher than standard levels for Brazil. Table 1 shows the tested strains previously assessed as able to establish symbiosis with leguminous trees (Moura et al. 2016).

In addition, strains SEMIA 384 (*R. etli*) and BR 1602 (*B. japonicum*) (Menna et al. 2006a, b) were also tested. *Pseudomonas* sp. (UW4) and *P. fluorescens* (YsS6) (Li et al. 2000; Ali et al. 2012) were used as positive controls for siderophore and ACC deaminase production, respectively.

Leguminous species investigated included *Vicia sativa* L. and *Calopogonium mucunoides* Desv. Each species was tested in independent experiments.

Table 1 Bacterial strains/isolates used in the study

Host species	Isolate	Trap species	Origin
Mimosa scabrella Benth (bracatinga)	UFSC-B1, UFSC-B2, UFSC-B3, UFSC-B4, UFSC-B5, UFSC-B6 UFSC-B8, UFSC-B9, UFSC-B12 UFSC-B16, UFSC-B17	*Vigna unguiculata* (Cowpea)	Criciúma (SC)
Mimosa bimucronata (DC) Kuntze (maricá)	UFSC-M1, UFSC-M2 UFSC-M4, UFSC-M8 UFSC-M9	*Vigna unguiculata* (Cowpea)	Criciúma (SC)
–	SEMIA 384 (*Rhizobium etli*)		Fepagro (RS)
–	BR 1602 (*Bradyrizhobium japonicum*)		Embrapa Agrobiologia (RJ)
–	YsS6 (*Pseudomonas fluorescens*), UW4 (*Pseudomonas* sp.)		Bernard Glick personal Collection, University of Waterloo, Canada

Symbiotic efficiency

The experiment was conducted in Leonard jars (Vincent 1970) in a greenhouse under natural lighting. *V. sativa* was grown from June to Aug 2013 (average temperarure 16.8 °C) while *C. mucunoides* from March to May 2014 (average temperature 22.3 °C) (Labclimagri, Metereological Station from the Centro de Ciências Agrárias-UFSC). The experimental design was completely randomized, consisting of three replicates and 19 treatments. Treatments were inoculated with each of the 17 strains tested (16 strains UFSC+ one recommended strain for each plant) and two non-inoculated controls (with and without mineral nitrogen) (52.535 mg N).

Seeds were disinfected with 70% alcohol for 30 s, followed by 2% sodium hypochlorite for 2 min. Seeds were then rinsed ten times with sterile distilled water to remove residues, and germinated on Petri dishes with sterile paper film, maintained in a growth chamber at 28 °C for 24 h. Two pre-germinated seeds were placed in each Leonard jar containing 500 cm³ of a mixture of sterile sand and vermiculite in a 1:1 (v/v) ratio. The down part of the jars were filled with fourfold diluted sterile Hoagland and Arnon (1950) nutrient solution. Seeds were then inoculated with 1 mL of the tested bacterial strain taken during the log growth phase (10^8 cells mL^{-1}). The bacteria strains were cultured in YM medium (Vincent 1970). For the non-inoculated controls, 1 mL of sterile YM medium was added. After seeding, 2.0 cm layer of a sterile mixture of sand, chloroform, and paraffin (5:1:0.015) was placed in the jar to avoid contamination.

Solution levels were periodically refilled with sterile Hoagland and Arnon (1950) nutrient solution. After 60 days, plants were harvested to assess number of nodules (NN), dry weight of nodules (DWN), shoot dry matter (SDM), root dry matter (RDM), *N* content (Tedesco et al. 1995), and *N* accumulation in the shoots (*N* content times SDM). The symbiotic efficiency (SE) was calculated following Chagas-Junior et al. (2010).

$$SE = (N_{\text{total fixed}} - N_{\text{total without } N}/N_{\text{total with } N} - N_{\text{total without } N}) \times 100.$$

In vitro evaluation of plant growth-promoting characteristics

IAA (Indole-3-acetic acid) production

The bacterial IAA production ability was measured following the method described by Glickmann and Dessaux (1995). An aliquot of 1 mL of grown bacterial culture, at 28 °C for 48 h at 135 rpm, previously adjusted to an optical density of 0.5 was used to inoculate 5 mL YM medium containing tryptophan (500 mg mL^{-1}). After 24 h of incubation, 1 mL of each bacterial suspension was centrifuged

at 3248×*g* for 15 min. Subsequently, 2 mL supernatant was mixed with 4 mL Salkowski's reagent (Gordon and Weber 1951), incubated in the dark for 25 min and the absorbance read (OD$_{535}$). The concentration of IAA in each sample was calculated based on a standard curve ranging from 0 to 100 µg mL^{-1} IAA (Sigma, China).

Phosphate solubilization

The bacterial phosphate solubilization activity was screened according to Son et al. [2014] in NBRIP medium. Three plates of NBRIP-CaP were inoculated with 20 µL of each isolate grown for 48 h in YM medium, and incubated at 28 °C for 14 days (Alikhani et al. 2006). The phosphate solubilization activity was determined by measuring the clearance zone (solubilization area) developed around the colony, compared to the colony diameter. The phosphate solubilization index (SI) was = (colony + clearance zone diameter)/colony diameter.

Siderophore production

Siderophore production was measured as described by Schwyn and Neilands (1987). This is a qualitative method where a positive reaction is indicated by a color change of the Chrome Azurol S (CAS) reagent from blue to orange. Around 20 µL of 48 h bacterial culture grown in King's B medium (King et al. 1954) were spotted onto CAS agar plate (Alexander and Zuberer 1991) and incubated at 28 °C for 72 h.

ACC (1-aminocyclopropane-1-carboxylate) deaminase activity

Bacterial cultures were grown for 48 h in YM medium, centrifuged at 10,000×*g* and washed twice with DF minimal medium (Dworkin and Foster 1958), prior to a resuspension in DF minimal medium, with ACC final concentration of 5 mM as the sole source of nitrogen. Cells were then incubated for 48 h at 28 °C and 135 rpm. After induction, ACC deaminase activity was measured based on the determination of α-ketobutyrate resulting from ACC cleavage by ACC deaminase, as described by Penrose and Glick (2003). The concentration of α-ketobutyrate in each sample was calculated based on a standard curve ranging from 0 to 0.5 mmol L^{-1} α-ketobutyrate (Sigma, China) (Duan et al. 2009).

16 S rDNA gene amplification and sequencing

Genomic DNA from three isolates forming nodules (UFSC-M4, UFSC-M8 and UFSC M9) was extracted using the UltraClean Microbial DNA Isolation Kit (Mo Bio, Canada)

according to the manufacturer's instructions. Isolates were identified by amplifying and partial sequencing the 16 S rDNA, using the universal primers 27F (5′-AGAGTTTGA TCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTT ACGACTT-3′) (Lane 1991) at the Genomic Division, Macrogen Inc., Korea. The results of the 16 S rDNA sequence analysis were compared with registered sequences deposited at GenBank using the NCBI Blast server (http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were submitted to GenBank and are available under the accession numbers KY488191, KY488192 and KY488193.

Statistical analysis

Data were submitted to analysis of variance (ANOVA), and means compared by the Scott-Knott test at $p < 0.05$ using R-project (Team R 2008). Values for N content and N accumulation in the shoots were previously transformed into $x^{1/2}$, and values for symbiotic efficiency into $Log10(x + 1)$.

Results

Isolates nodulation abilities

From the 16 rhizobia isolates tested (Table 1), only UFSC-M8 and UFSC-M9 were able to form effective nodules with *V. sativa*. Nevertheless, those isolates were not able to form as many nodules as the reference strain *R. etli* SEMIA 384 (Fig. 1a). Even though UFSC-M8 formed fewer nodules, the NDW was 22% higher those of *R. etli* SEMIA 384 (Fig. 1b). Moreover, UFSC-M8 average weight per nodule was higher than *R. etli* SEMIA 384, showing a greater nodule development (data not shown). On the other hand, isolate UFSC-M9 formed less nodules with less weight when compared to UFSC-M8 and SEMIA 384 (Fig. 1b).

Only two of the 16 rhizobia isolates tested were able to form an effective symbiotic relationship with *C. mucunoides*. Isolates UFSC-M4 and UFSC-M8 formed nodules, however, in lower extent when compared to the reference strain *B. japonicum* BR 1602 (Fig. 1c). Similar results were obtained in total NDW, with the reference strain presenting a better performance (Fig. 1d). UFSC-M4 and UFSC-M8 formed the same number of nodules and had similar NDW. The NDW for BR 1602 was significantly higher than the average of UFSC-M4 and UFSC-M8.

Isolates growth promoting abilities

Isolates UFSC-M8, UFSC-M9 and SEMIA 384 significantly enhanced the growth of *V. sativa* (Fig. 2a). The

SDW was similar between treatments UFSC-M8 and SEMIA 384, and statistically higher than the treatment without N. UFSC-M9 showed lower SDW than UFSC-M8 and SEMIA 384. However, it showed statistically higher SDW than all other inoculated treatments.

The RDW was significantly higher in plants inoculated with isolate UFSC-M8 in comparison to all other inoculated treatments (Fig. 2b), suggesting a significant impact of this isolate in root development. When compared to SEMIA 384, isolate UFSC-M8 was able to increase RDM in about 33%. Isolate UFSC-M9 and SEMIA 384 were not able to increase RDW, presenting no differences with the treatment without nitrogen.

For *C. mucunoides*, reference strain BR 1602 increased biomass production when compared to all UFSC isolates tested. The average SDW value for the UFSC isolates was 18 times lower than the treatment with nitrogen. Higher RDW and SDW were obtained for the treatment with *N*, followed by BR 1602. Increments in RDW were in the order of 788% for the treatment with nitrogen and 157% for strain BR 1602. Besides that, there were not significant differences on RDW between the treatments inoculated with the UFSC isolates and the treatment without nitrogen (Fig. 2d).

Strain characterization

Phosphate solubilization abilities were detected in 14 isolates. Only isolates UFSC-B4 and UFSC-M4 were unable to solubilize phosphate, together with the reference strains *R. etli* SEMIA 384 and *Bradyrhizobium* sp. BR 1602. Isolates UFSC-B12, UFSC-M9 and UFSC-B17 presented the highest ability to solubilize phosphate with SI of 7.07, 6.67 and 4.47, respectively (Table 2). Still, the majority of the isolates presented medium to low phosphate solubilization ability.

Siderophore production was detected in three out of the 16 isolates tested. Only isolates UFSC-M1, UFSC-M4 and UFSC-M8 were able to produce siderophores. Reference strains *R. etli* SEMIA 384 and *Bradyrhizobium* sp. BR 1602 did not produced siderophores under the tested conditions.

IAA production was detected in all isolates and reference strains, ranging from 2.6 to 62.7 μg mL^{-1}. Isolates UFSC-B12 and UFSC-M9 produced high levels of IAA followed by *R. etli* SEMIA 384. The majority of the tested strains (72%, $n = 13$) produced IAA concentrations in the range of 10 to 40 μg mL^{-1} (Table 2).

Only two isolates, UFSC-M4 and UFSC-M8, were able to use ACC as sole nitrogen source. An ACC deaminase activity of 2.43 and 1.64 μmol mg^{-1} protein h^{-1} was detected for UFSC-M4 and USFC-M8, respectively.
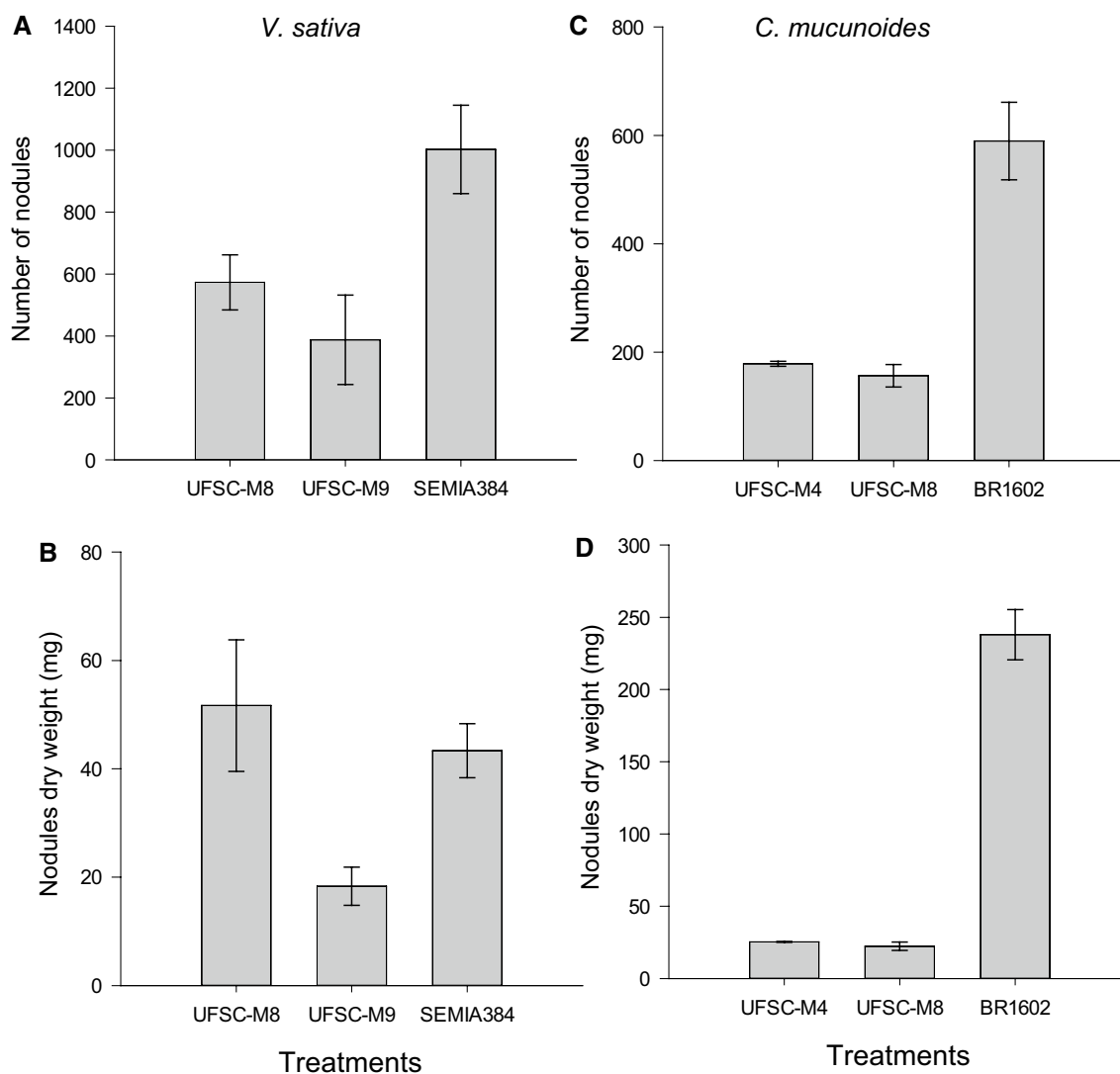
Fig. 1 Number of nodules (**a, c**) and nodule dry weight (**b, d**) in *Vicia sativa* and *Calopogonium mucunoides* inoculated with the rhizobial strains UFSC-M8, UFSC-M9 and the recommended strain SEMIA384, after 60 days of growth. *Vertical bars* represent the standard error of the mean ($n = 3$)

Symbiotic efficiency

From the growth and N uptake (not shown) data, it was calculated the symbiotic efficiency for UFSC-M8, SEMIA 384 and UFSC-M9 to *V. sativa*, with values of 147, 90 and 31%, respectively. These values show an increased ability to promote growth and nutrition of *V. sativa* by the UFSC-M8 and SEMIA 384 strains, whereas UFSC-M9 presented a less significant impact and efficiency. Only BR 1602 strain presented symbiotic efficiency with *C. mucunoides* (61.77%).

Based on 16 S rDNA gene sequences, three isolates were able to nodulate legumes could be classified as belonging to two distinct orders, α-Proteobacteria and β-Proteobacteria. The α-Proteobacteria representatives belonged to *Rhizobium* and *Curtobacterium*, whereas β-Proteobacteria belonged to *Burkholderia* (Table 2). Isolates of *Rhizobium* sp. (UFSC-M8) and *Curtobacterium* sp. (UFSC-M9) were able to nodulate *V. sativa*, while isolates of *Burkholderia* sp. (UFSC-M4) and *Rhizobium* sp. (UFSC-M8) were able to nodulate *C. mucunoides*.

Discussion

To our knowledge, no other report on the isolation, characterization and symbiotic performance of autochthonous rhizobia from coal mining areas is available in the current literature. The majority of works related to coal mining areas only report the bacterial diversity in the impacted
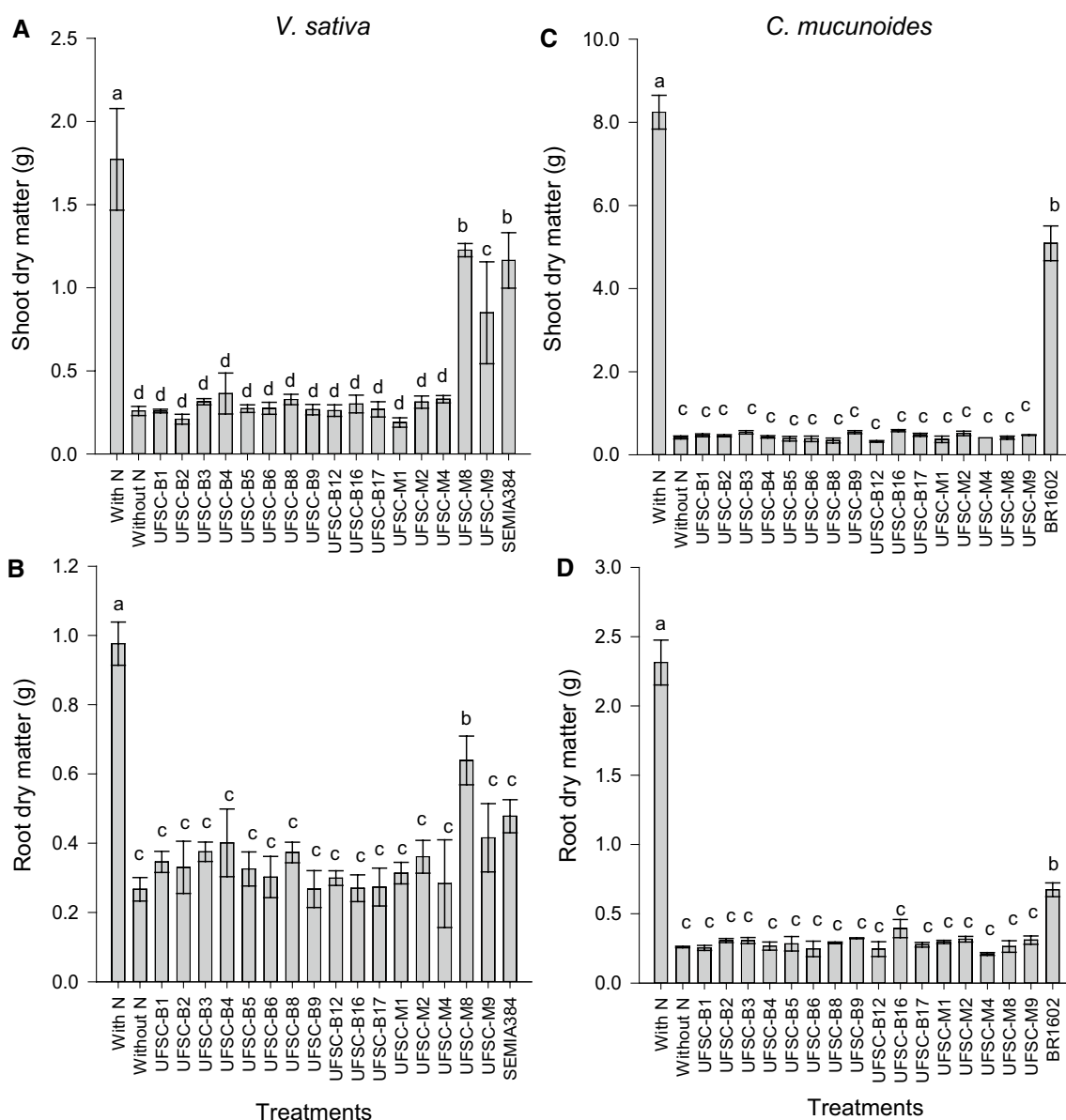
Fig. 2 Shoot (**a, c**) and root dry matter (**b, d**) of *Vicia sativa* and *Calopogonium mucunoides* inoculated with rhizobia isolates, after 60 days of growth. Means followed by the same letter do not differ statistically by Scott Knott test ($p < 0.05$). *Vertical bars* represent the standard error of the mean ($n = 3$)

soils (Quadros et al. 2016; Zhan and Su 2011). In the present study, 16 rhizobial isolates were characterized and their symbiotic efficiency evaluated in *V. sativa* and *C. mucunoides* plants. Previous works performed in our laboratory demonstrated that these isolates nodulated *V. unguiculata* and some nodulated and promoted the growth of leguminous trees (*Mimosa* spp.) (Moura et al. 2016). However, not much is known about their ability to promote the growth of herbaceous leguminous plants like *V. sativa* and *C. mucunoides*, which present an important role in the re-vegetation of degraded areas, especially due to its high biomass production, tolerance to high levels of acidity, and

nitrogen concentrations (Seiffert et al. 1985; Calegari et al. 1993). Results obtained in this work showed that only two isolates from our collection (UFSC-M8 and UFSC-M9) were able to efficiently nodulate *V. sativa*. In addition, two autochthonous rhizobial strains (UFSC-M4 and UFSC-M8) nodulated *C. mucunoides*, albeit, inefficiently. Curiously, *V. sativa* symbionts are often described as belonging to the *Rhizobium etli* group, while *C. mucunoides* symbionts mostly belong to the genus *Bradyrhizobium* (Menna et al. 2006a, b; Brasil 2011). None of the isolates in our collection belongs to *Bradyrhizobium*, which might help explain the inability to efficiently nodulate and promote the growth

Table 2 Levels of IAA, PSI, ACCD, and siderophore by plant growth promoting rhizobia isolated from coal mining areas and strains recommended by Brazilian Ministry of Agriculture

Isolate code (accession numbers)	IAA (µg mL^{-1})	PSI	ACCD	SID
UFSC-B1	25.7 ± 6.5d[*]	2.43 ± 0.2	–	–
UFSC-B2	15.7 ± 0.1f	1.97 ± 0.4	–	–
UFSC-B3	22.0 ± 1.0e	1.88 ± 0.1	–	–
UFSC-B4	26.9 ± 0.1d	0.0 ± 0.0	–	–
UFSC-B5	14.9 ± 0.1f	1.63 ± 0.3	–	–
UFSC-B6	21.9 ± 0.3e	1.87 ± 0.2	–	–
UFSC-B8	20.3 ± 0.1d	1.62 ± 0.2	–	–
UFSC-B9	19.4 ± 1.2f	2.08 ± 0.1	–	–
UFSC-B12	62.7 ± 3.5a	7.07 ± 0.7	–	–
UFSC-B16	14.4 ± 1.5f	1.93 ± 0.1	–	–
UFSC-B17	39.9 ± 6.4c	4.47 ± 0.5	–	–
UFSC-M1	15.5 ± 3.8f	1.48 ± 0.4	–	+
UFSC-M2	12.4 ± 0.0f	1.35 ± 0.4	–	–
UFSC-M4 (KY488191)	4.7 ± 3.4g	0.0 ± 0.0	+	+
UFSC-M8 (KY488192)	33.4 ± 17.4e	1.68 ± 0.1	+	+
UFSC-M9 (KY488193)	60.1 ± 2.6a	6.67 ± 0.3	–	–
SEMIA 384	46.9 ± 0.0b	0.0 ± 0.0	–	–
BR 1602	2.6 ± 0.7g	0.0 ± 0.0	–	–
YsS6	nd	nd	+	+

IAA indole-3-acetic acid

PSI phosphate solubilization index

ACCD ACC deaminase

SID Siderophore

*Values followed by different letters in the same column for each consortium are statistically different according to the Scott Knott test ($p < 0.05$)

of *C. mucunoides*. In fact, Bala and Giller (2001) showed that multiple bacteria belonging to *Rhizobium, Mesorhizobium* and *Sinorhizobium* were unable to nodulate *C. mucunoides*, suggesting, therefore, that *C. mucunoides* has a very restricted symbiotic host range.

Interestingly, the bacteria that nodulated belong to *Burkholderia* (UFSC-M4) and *Curtobacterium* (UFSC-M9). While species of *Burkholderia* have been described as efficient symbionts of leguminous trees (Moulin et al. 2001; Chen 2005; Taulé et al. 2012), not much is understood about its nodulation abilities in different leguminous species. Results obtained in this work show that *Burkholderia* strains that nodulate leguminous trees (*Mimosa* spp.) and *V. unguiculata* (Moura et al. 2016) can also nodulate *C. mucunoides*. The bacterium species *B. phymatum* (STM 815), isolated from the root nodules of *Machaerium lunatum* (Moulin et al. 2001; Elliott et al. 2007), is able to form nodules with a wide range of leguminous trees and *Phaseolus vulgaris* (Gyaneshwar et al. 2011; Liu et al. 2011). In addition, other *B. phymatum* strains have been isolated from the nodules of common bean in Morocco (Talbi et al. 2010). *Burkholderia tuberum* is also able to form nodules with a wide variety of hosts (Barrett and Parker 2005; Gyaneshwar et al. 2011). Overall, these results suggest that at least

some *Burkholderia* strains possess the ability to form nodules in a wide range of hosts, which might be extremely important for agricultural applications and for studies on the molecular mechanisms of nodule formation.

Remarkably, we found that isolate UFSC-M9, able to form nodules with *V. sativa*, belongs to *Curtobacterium*, a genus that has recently being known for promoting plant growth endophytically (Azevedo et al. 2016; Sturz et al. 1998). This is the first report on the nodulation abilities of a *Curtobacterium* species. Other non-symbiotic bacteria have also proven to form nodules with legume species. Ampomah and Huss-Danell (2011) suggest that the ability of *Paenibacillus* and *Stenotrophomonas*, two non-symbiotic bacteria, to form nodules on legumes was acquired by horizontal gene transfer. This event has been described earlier for other bacteria, as presented by Wood et al. (2001) and Welch et al. (2002). It is possible that the dynamic behavior of the symbiotic rhizobia genome allows them to adapt to a changing environment, such as the presence of a new legume at a given site (Barcellos et al. 2007). Nodulation abilities have been described in at least fifteen Proteobacteria genera. Recent examples include *Devosia, Ochrobactrum, Methylobacterium* and *Phyllobacterium* (Gyaneshwar et al. 2011; Oliveira-Longatti et al. 2013). Further studies

are necessary to understand the molecular basis of the nodulation abilities of *Curtobacterium*.

Despite forming fewer nodules than *R. etli* SEMIA 384, UFSC-M8 (*Rhizobium* sp.) presented the highest symbiotic efficiency upon inoculation with *V. sativa*. This isolate formed more developed nodules (with increased weight) when compared to SEMIA 384, suggesting that the high symbiotic efficiency of this isolate might be related to nodule development and not nodule number. Positive correlations between nodule weight and total amount of fixed nitrogen have been previously described (Pereyra et al. 2015). Ferreira et al. (2012) showed that higher nodule dry weight was directly related to the ability to promote growth of five different leguminous plants. Curiously, UFSC-M8 was able to induce root development in both *V. sativa* and *C. mucunoides* when compared to all other treatments. It is possible that UFSC-M8 not only promotes plant growth through its symbiotic features (nitrogen fixation), but also through other mechanisms. Even though the mechanisms for plant growth promoting capacity are not completely elucidated according to Ahemad and Kibret (2014), plant growth promoting rhizobacteria mediate plant growth by altering the microbial community in the rhizospheric niche through the production of various substances. By the traditional route, bacteria can provide necessary nutrients (N, P, essential minerals, enzymes) and/or modulate plant hormonal levels. Alternatively, mechanisms such as the production of IAA, ACC deaminase, siderophores, and the capacity to solubilize phosphate mediate plant growth (Brígido and Glick 2015). These mechanisms have been primarily investigated in agricultural environments, while in other ecosystems, such as degraded environments, they are minimally addressed (Timmusk et al. 2011).

In this respect, UFSC-M8 was able to synthesize IAA, produce ACC deaminase, siderophores, and solubilize phosphate. All these traits are known to play a significant role in plant-growth promoting capabilities (Glick 2014). For instance, IAA increases the rate of xylem and root development and initiates lateral and adventitious root formation (Glick 2014). In addition, IAA plays an important role in the nodulation abilities of many rhizobial strains (Glick 2012). Nevertheless, IAA is known to induce plant ACC synthase, and consequently the levels of ethylene, the ACC plant precursor (Ma et al. 2002). Ethylene regulates many plant developmental processes including root and shoot elongation and root nodule development (Stearns and Glick 2003). In addition, plants produce high levels of ethylene when facing stress conditions (stress ethylene), like those present in coal-mining areas, therefore leading to growth inhibition (Mayak et al. 2004; Cheng et al. 2007). Bacteria that produce ACC deaminase are able to efficiently decrease plant ACC and, consequently, deleterious ethylene levels responsible for plant growth inhibition

(Honma and Shimomura 1978). In general rhizobia producing ACC deaminase present increased nodulation abilities and plant-growth promoting abilities (Ma et al. 2003, 2004; Conforte et al. 2010; Nascimento et al. 2012a, b; Brígido et al. 2013). By producing both IAA and ACC deaminase, it is possible that UFSC-M8 is able to form more developed nodules. Nascimento et al. (2012c) showed that *Mesorhizobium ciceri* LMS-1 formed more developed nodules in *Cicer arietinum* when expressing an exogenous ACC deaminase gene.

Bacterial siderophore production is another important trait for impacted environments. This trait plays an important role in facilitating iron uptake in the host plant, increasing growth (Neilands 1995; Hider and Kong 2010). This mechanism is of extreme importance in contaminated soils (heavy metals, high acidity), where iron is not available for plant absorption due to the presence of other competing metal ions (Burd et al. 2000; Belimov et al. 2005; Braud et al. 2006). In fact, it has been described that iron deficiency induces chlorosis and leaf abscission in plants growing in coal-mining areas (Imsande 1998; Ferreira et al. 2012). Hence, it is conceivable that siderophore and ACC deaminase-producing bacteria, such as *Rhizobium* sp. UFSC-M8, can increase plant iron uptake and reduce the negative effects of ethylene-induced stress.

Generally, soils possess high levels of phosphate. However, it its insoluble and not available for plant assimilation (Khan et al. 2007). Therefore, bacteria that solubilize phosphate have the ability to promote plant growth in great extent by making phosphate available for plant uptake (Pradhan and Sukla 2005; Tao et al. 2008; Marra et al. 2011).

Conclusions

Our results suggest that UFSC-M8 presents the potential to be used as an effective inoculant in coal-contaminated areas. By being naturally present in the local soil, we can postulate that this bacterium can be able to resist the stresses and increase the growth of leguminous plants like *V. sativa*, thus potentiating the re-vegetation of those areas with leguminous species. By presenting traits such as activity of ACC deaminase, siderophore production, and phosphate solubilization, this bacterium might have potential for plant-growth promoting abilities when compared to strains like the recommended *R. etli* SEMIA 384 that only produces IAA.

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