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Rhizobia Isolated from Coal Mining Areas in the Nodulation and Growth of Leguminous Trees

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ABSTRACT: An alternative for recovery of areas degraded by coal mining is revegetation with rapidly growing leguminous trees, which often do not establish in low fertility soils. The objective of this study was to evaluate the efficiency of native rhizobia isolated from coal mining areas in the nodulation and growth of leguminous trees. We isolated 19 strains of rhizobia from a degraded soil near Criciúma, SC, Brazil, and evaluated the nodulation and growth-promoting capacity of the inoculated isolates for *bracatinga* (*Mimosa scabrella*), *maricá* (*M. bimucronata*) and *angico-vermelho* (*Parapiptadenia rigida*). Isolates UFSC-B2, B6, B8, B9, B11 and B16 were able to nodulate *bracatinga*, providing average increases of 165 % in shoot dry matter, with a significant contribution to N accumulation. Isolates UFSC-B5, B12, and M8 favored nodulation and growth of *maricá*, especially isolate UFSC-B12, which promoted increases of 370 % in N accumulation compared to treatment with N fertilizer. All strains were inefficient in promoting growth and N uptake by *angico-vermelho*. In conclusion, isolation and use of selected rhizobia for *bracatinga* and *maricá* plant inoculation can contribute to the growth and accumulation of N, with prospects for use in programs for revegetation of degraded soils in coal mining areas.

Keywords: environmental recovery, revegetation, diazotrophs, bioremediation.

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INTRODUCTION

Coal is important for the Brazilian economy, contributing to generation of power and economic returns. In Brazil, about two thirds of non-renewable energy comes from coal, with reserves 20 times greater than oil and 65 times greater than natural gas. Main coal reserves in Brazil are in the states of Rio Grande do Sul and Santa Catarina, with approximately 28 and 3 billion Mg, respectively (SEMC, 2011). However, mining causes negative effects on the physical, chemical and biological properties of the soil, water and air, endangering natural resources and ecosystems (Silvas et al., 2011).

Improper waste disposal and inversion of soil horizons in open-pit mining negatively affect vegetation, due to loss of soil fertility, causing problems of water availability and impairment of soil biota, immediately affecting the supply of nutrients important for plants. In these areas, degradation is aggravated by constant soil disturbances, resulting in a highly unstructured substrate, therefore susceptible to erosion, with low pH and high levels of trace elements (such as Cd and As) (Siqueira et al., 2008). This scenario impairs spontaneous recovery of biodiversity and soil properties, requiring human intervention with reclamation strategies (Nascimento and Biondi, 2008).

An alternative for recovery of degraded areas is revegetation using fast-growing species of legumes, which promote nutrient cycling, increase incorporation of C in the soil and minimize erosion, therefore functioning as a nursery for newly introduced species (Rocha-Nicoleite et al., 2013). Legume species associate with symbiotic N-fixing bacteria (generally known as rhizobia), enhancing their potential for use in revegetation programs (Ferreira et al., 2012). The association promotes increased deposition of N, reducing the soil C/N ratio, which favors mineralization and cycling of nutrients, in addition to increasing soil organic matter (humus), a fundamental condition for recovery of degraded soils (Siqueira et al., 2007). In addition, some legume species are adapted to low fertility soils (Melloni et al., 2004), which are commonly found in areas degraded by coal mining (Rocha-Nicoleite et al., 2013).

The ability of legumes to establish symbiosis with rhizobia is variable, with different degrees of efficiency (Carvalho et al., 2008). Furthermore, symbiosis can lose effectiveness after transplanting of seedlings due to adaptation to local conditions and competition with the indigenous rhizobium population (Geetha and Joshi, 2013). Nevertheless, studies have shown the potential of N-fixing bacteria for the recovery of degraded areas (Melloni et al., 2004). However, there are no studies with the purpose of selecting native rhizobia from coal mining areas to be used in reclamation programs.

The hypothesis of this work is that rhizobia from coal mining degraded areas will increase symbiotic efficiency and, hence, the establishment of plant species in these contaminated environments. Therefore, the objective of this study was to evaluate the effectiveness of indigenous rhizobia isolated from coal mining areas in nodulation and capacity to promote the growth of leguminous trees of rapid growth, and their potential for use in revegetation of areas degraded by coal mining.

MATERIALS AND METHODS

Soil sampling and rhizobium isolation

Samples of an anthropogenic soil, characterized by the presence of mine tailings and inversion of soil horizons, were collected 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). Soils from the 0.0-0.10 m layer were collected near the roots of three leguminous tree species from an area revegetated in 2008. Plant species included black *acacia* (*Acacia mearnsii* De Wild), *maricá* (*Mimosa bimucronata* (DC) Kuntze) and *bracatinga* (*Mimosa scabrella* Benth).

Five plants of each species were randomly selected, with a minimum distance of 200 m between individuals. Samples were composed of 12 sub-samples taken under the projection of the canopy of the selected species. Soil samples were packed in a Styrofoam box with ice and transported to the laboratory, and rhizobia isolated.

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 (Cetesb, 2014).

A mixture composed of 50 g of soil from the mining area with the addition of sand and vermiculite (1:1, v/v) was used as a rhizobium-trapping assay in 300 cm^3 pots. Three seeds of cowpea (*Vigna unguiculata*) were placed in each pot. Pots had been previously sterilized in 2 % sodium hypochlorite for 2 min and rinsed six times with sterile distilled water. Plants were grown in a greenhouse for 60 days and irrigated daily with sterile distilled water. Every five days, plants received a Hoagland and Arnon nutrient solution (Hoagland and Arnon, 1950) with the original concentration (final concentration of 5.25 mg L^{-1}).

At the end of the experiment, three nodules were randomly collected per pot, which were detached from the root and then surface disinfected by immersion in 95 % ethanol for 20 s, 2 % sodium hypochlorite for 2 min and 5 % formalin for 2 min, followed by six rinses in sterile distilled water. Nodules were then macerated in YMA culture medium (Vincent, 1970) and incubated at 28 °C for 14 days.

After bacterial growth, pure cultures were obtained and stored in YMA medium with 20 % glycerol at -80 °C in an Ultrafreezer. Forty rhizobium isolates were obtained: 20 from *bracatinga* (B) and 10 each from black *acacia* (A) and *maricá* (M).

To confirm nodulation ability, we performed authentication of the isolates. Each isolate was inoculated in cowpea in pots as described above. The substrate was composed of sand and vermiculite in a 2:1 (v/v) ratio. Cell multiplication was obtained by growing each isolate in YM medium (Vincent, 1970) for 24 h under stirring (150 rpm) at a constant temperature of 28 °C. Two mL of inoculum were then added per pot, and plants were grown in a greenhouse for 60 days, at which time the presence or absence of nodules was determined.

Treatments without inoculation were used as negative controls. Strain INPA 03-11B (*Bradyrhizobium* sp.), recommended for cowpea, was used as a positive control.

Autentication yielded 19 rhizobium isolates, including 1 for black *acacia* (UFSC-A8), six for *maricá* (UFSC-M1, M4, M5, M7 and M9) and 12 for *bracatinga* (UFSC-B1, B2, B3, B4, B5, B6, B8, B9, B11, B12, B16 and B17). These isolates were used to evaluate symbiotic efficiency with leguminous trees, as described below.

Symbiotic efficiency evaluation

Independent assays were conducted in a greenhouse to determine the symbiotic efficiency of the isolates. To that end, *maricá*, *bracatinga* and *angico-vermelho* (*Parapiptadenia rigida* (Benth.) Brenan) were used as symbionts. Seeds of the three plants species were surface sterilized by immersion in 2 % sodium hypochlorite and rinsed in sterile distilled water. Pots of 300 cm^3 capacity were autoclaved and used for growing the leguminous plants. The substrate was composed of sand and vermiculite in a 2:1 (v/v) ratio. At the time of seeding, 2 mL of the bacterial inoculum was added to the seeds. The inoculum was obtained by incubation of the isolates in YM medium (Vincent, 1970) until the OD_{560} = 0.5, which corresponded to approximately 10^8 cells mL^{-1}. A saline solution (0.85 %) was used to adjust the optical density when it exceeded 0.5.

The experimental design was completely randomized with four replications. Evaluations included the 19 indigenous rhizobium isolates from the mining area and seven reference strains provided by Embrapa Agrobiology, including BR3437 (*Burkholderia nodosa*),

BR3461, BR3454, BR9002 and BR3470 (*Burkholderia*), BR2811 (*Bradyrhizobium elkanii*), and BR827 (*Sinorhizobium fredii*). Two non-inoculated control treatments, one with high (52.5 mg L^{-1}) and another with low (5.25 mg L^{-1}) N concentration were also tested. Plants were grown for a period of seven months and irrigated every three days with autoclaved distilled water and every five days with an autoclaved nutrient solution.

At the end of the experiment, we determined plant height and number and dry weight of nodules. Dry weight was obtained by drying the nodules in an oven at 60 °C to constant weight. The roots and shoots were placed in paper bags and dried as described for determination of nodule biomass. Shoots were processed in a Willey mill mounted on a 1.0 mm stainless steel sieve. For determination of N, samples were digested with sulfuric acid and then distilled using the Kjeldahl method as described by Tedesco et al. (1995). The cumulative amount of N in the shoots was calculated by multiplying the concentration of the element by the respective amount of dry matter.

Symbiotic efficiency was calculated based on the accumulated amount of N in the shoots, determined by: $SE = [(N_{fixed} - N_{total\ low\ N}) / (N_{total\ high\ N} - N_{total\ low\ N}) \times 100]$, where N_{fixed} = accumulated amount of N in the inoculated treatments; $N_{total\ low\ N}$ = accumulated amount of N in the control with low N; $N_{total\ high\ N}$ = accumulated amount of N in the control with high N (Chagas-Júnior et al., 2009).

Data were subjected to analysis of variance using the SISVAR Software 4.2 (Ferreira, 2008), where number of nodules were transformed using $(x + 1)^{0.5}$. For the other variables, no data transformations were performed. In each treatment we calculated the standard error of the mean (n = 4), and means were separated by the Scott Knott test at 5 % probability.

RESULTS AND DISCUSSION

Plant shoot dry matter (SDM) and plant height were significantly affected by inoculation with rhizobia ($p<0.01$; Figure 1). Among the reference strains, only BR3437 for *bracatinga* and BR3454 and BR3437 for *maricá* benefited plant growth in comparison with treatments with N fertilization. In contrast, for *angico-vermelho*, all strains showed lower growth when compared to the treatment with high N.

Isolates UFSC-B2, B6, B8, B9, B11 and B16 promoted SDM of bracatinga similar to the BR3437 reference strain, with an average increase of 165 % when compared to the high N treatment. Isolates UFSC-B2, B6 and B16 were most beneficial for plant height of *bracatinga*. For *maricá*, isolates UFSC-B5, B12 and M8 showed SDM yields similar to the BR3454 reference strain. These strains promoted average increases of 550 % when compared to the high N treatment. UFSC-B5 and B12 were also beneficial towards plant height. Inoculation did not affect *angico-vermelho*, since SDM and plant height were similar to the low N treatment. These results show that the specificity is a characteristic of the relationship between N-fixing bacteria and legumes, which means that N fixation is strongly influenced by the genotypic characteristics of the plant and the bacteria. These characteristics regulate a system that exchanges molecular signals responsible for the specificity of the interaction and, consequently, symbiotic efficiency (Xavier et al., 2006).

Only indigenous rhizobia isolated from *bracatinga* favored the growth of that species. Meanwhile, rhizobia isolated from both *bracatinga* and *maricá* were effective in promoting plant growth of *maricá*. In general, isolates obtained from *Mimosa* sp. were genus specific but not species specific, and were able to nodulate and efficiently fix N with many species within a specific genus, which explains and confirms the results observed in the present study (Chaer et al., 2011).

Finding species of rhizobia from the soil degraded by mining that are promising for inoculation of arboreal leguminous species is a possibility, except for *angico-vermelho*. Rhizobia inoculation initiatives are of great interest for the revegetation and restoration of areas

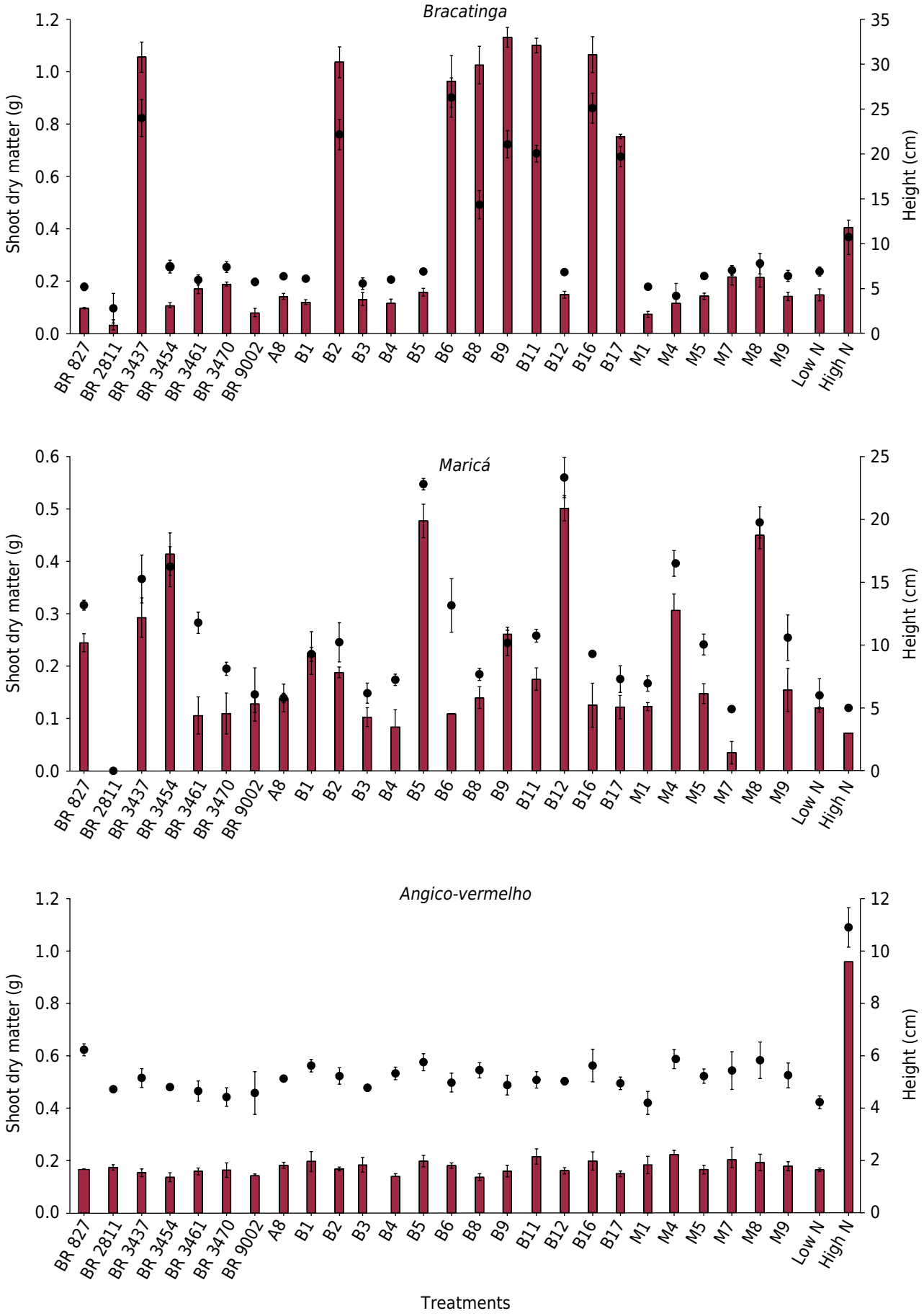

Figure 1. Shoot dry matter (magenta bars) and plant height (black circles) of *bracatinga*, *maricá* and *angico-vermelho* inoculated with rhizobia indigenous to coal mining areas or with the recommended strains of reference. Vertical bars represent the standard error of the mean (n = 4).

impacted by mining since rhizobium strains adapt to the soil and climatic conditions of a region, effectively contributing to plant growth, a characteristic that may not be present in the reference strains recommended. The importance of selecting microorganisms indigenous to degraded areas for use as inocula to favor plant development and, consequently, environmental recovery were observed by Melloni et al. (2006). This biotechnology may effectively contribute to soil N and organic matter enrichment, and would be a factor to stimulate acceleration of plant succession in these environments (Siqueira et al., 2007).

The number and dry matter of nodules (DMN) were significantly influenced by the inoculation treatments ($p<0.01$; Figure 2). Nitrogen-added treatments were nodule-free, showing that there was no external contamination throughout the experiments. Out of 26 inoculation treatments for *bracatinga*, only nine showed absence of nodules. The highest number of nodules in this species was obtained through inoculation with the BR3437 reference strain, followed by the isolates UFSC-B2, B8 and M4. The remaining isolates promoted fewer nodules. However, in some treatments, DMN was an average of 17 % higher than for the reference strain. This condition was seen for isolates UFSC-B6, B8, B9, B11, B16, B17 and M7.

For *maricá*, the largest number of nodules was obtained for strains BR3461 and BR3454, followed by UFSC-M4. Inoculation with UFSC-M8 increased the dry matter of nodules an average of 35 % compared to the reference strains (BR3461and BR3437). For *angico-vermelho*, nodulation was seen for most inoculation treatments, especially for isolate UFSC-M8, which promoted the largest number of nodules, and UFSC-B6, B16, B17, M4, M9 and BR3470, which provided higher dry matter of nodules.

The weight and number of nodules are considered important indicators of effective nodulation (Araujo et al., 2008). However, studies have shown that this variable would be most useful for evaluation of the symbiotic efficiency of rhizobium strains since it is strongly correlated with plant growth and N accumulation (Dobereiner et al., 1966).

The number and dry weight of nodules corroborate results found in the literature, even for *angico-vermelho*, where nodulation resulted in low growth promotion in plants (Dias et al., 2012). Working with the model plant *Medicago truncatula*, Laguerre et al. (2012) observed that even when inoculating mutant Nod+ Fix- strains (capable of stimulating nodulation, but incapable of fixing N), the ability to nodulate the legume species was not affected. Nevertheless, plants showed what could be called “compensation”, which led to an increase in the size of nodules for strains capable of fixing N (Fix+). Moreover, in that particular case, the number of bacteria per nodule was affected when plants were inoculated with the mutant strains. These data may indicate that size of nodules, as well as number of bacteria per nodule, can be good indicators of symbiotic efficiency, especially for setting up trials aiming at the selection of indigenous N-fixing bacteria from degraded areas.

Considering that nodules are the main site for biological N fixation in legumes, larger nodulation percentages can effectively contribute to plant N supply. This significantly increases the levels of this nutrient in the soil by the decomposition of deposited plant material, as well as by the breakdown of nodules following senescence (Geetha and Joshi, 2013).

For *bracatinga*, several rhizobium isolates showed levels of fixed N similar to the treatment with high N, particularly those that benefited the growth of this species, as presented earlier (Table 1). In addition, isolate UFSC-M9 yielded high levels of N in the plant, but this may be due to a concentration effect, since there was little SDM yield for this treatment. The largest amounts of N accumulated in *bracatinga* was obtained when the plants were inoculated with isolates UFSC-B2, UB8, B9, B11, B16, and BR3437, which were, on average, 128 % higher than the treatment with high N.

For *maricá*, isolates UFSC-B1, M4 and the reference strains BR827 and BR3437 promoted N levels in SDM similar to the treatment with high N (Table 1). In general, the amount of N accumulated for *maricá* was lower than for *bracatinga*. However, for *maricá*, isolates UFSC-B12 and BR3437 promoted average increases in N of 340 % compared to the treatment with high N.

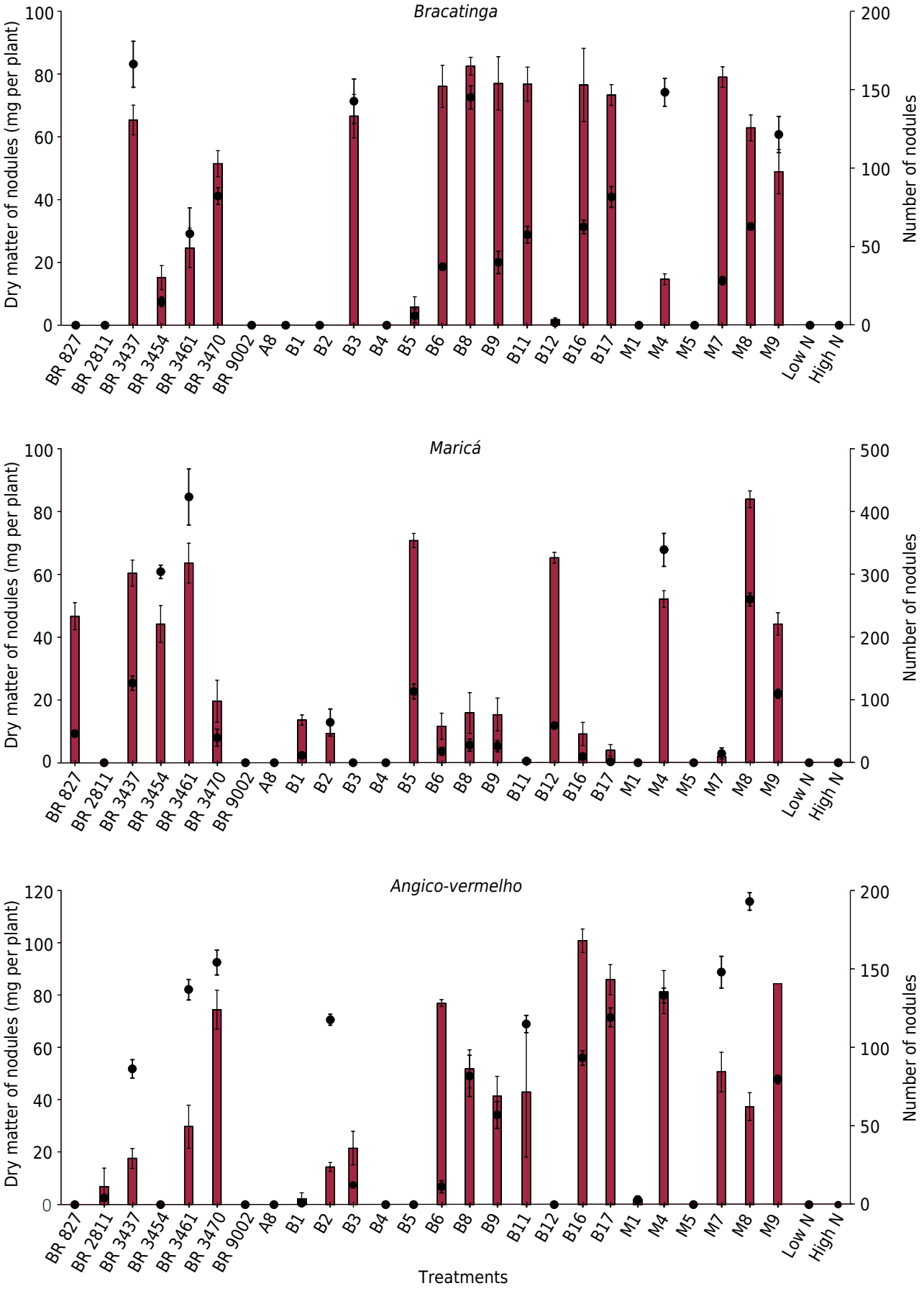

Figure 2. Dry matter (magenta bars) and number of nodules (black circles) from seedlings of *bracatinga*, *maricá* and *angico-vermelho* inoculated with rhizobia indigenous to coal mining areas or with the recommended strains of reference. Vertical bars represent the standard error of the mean (n = 4).

For *angico-vermelho*, most treatments had lower N contents than the treatment with N fertilization and, therefore, little N accumulated in the shoots (Table 1). These results show that inoculation with selected rhizobia yields higher plant biomass, especially for *bracatinga* and *maricá*. This enriched biomass will eventually increase N levels in the degraded soils, which are, for the most part, nutritionally poor (Santos et al., 2001).

Considering the results of the symbiotic efficiency tests shown in table 1, it appears that the most promising isolates tested in inoculation of *bracatinga* for reforestation of areas degraded by coal mining are UFSC-B2, B6, B8, B9, B11, B16 and B17. All seven isolates were able to nodulate *bracatinga*. For *maricá*, several rhizobium isolates exhibited symbiotic efficiency above 600 %, such as UFSC-B1, B12 and M4. Symbiotic efficiency for *angico-vermelho* was less than 20 %, indicating the need for more studies to select efficient indigenous rhizobia for this legume species. It is also important to mention that some rhizobium strains exhibited negative values for symbiotic efficiency, such as UFSC-M4 for *bracatinga*, UFSC-B6 and

Table 1. Symbiotic efficiency (SE) of isolates and contents and accumulation of N in the shoots of leguminous tree species inoculated with rhizobia isolated from coal mining areas, in comparison to recommended strains of reference

Treatment	*Bracatinga*			*Maricá*			*Angico-vermelho*		
	Level N	Accumulation N	SE	Level N	Accumulation N	SE	Level N	Accumulation N	SE
	g kg^{-1}	mg per plant	%	g kg^{-1}	mg per plant	%	g kg^{-1}	mg per plant	%
BR 827	8.8 c	0.9 d	n/n	26.3 a	6.3 b	650	10.5 d	1.7 c	n/n
BR 2811	4.4 c	0.3 d	n/n	0.0 d	0.0 d	n/n	12.3 c	2.1 c	5.8
BR 3437	25.4 a	27.4 a	235	26.7 a	7.8 a	838	12.3 c	1.9 c	4.5
BR 3454	9.2 c	1.0 d	-0.9	6.1 d	2.5 c	175	11.4 c	1.6 c	n/n
BR 3461	8.8 c	1.5 d	3.6	9.2 c	1.3 d	25	10.5 d	1.7 c	3.2
BR 3470	10.5 c	2.0 d	8.0	17.1 b	2.4 c	163	10.5 d	1.7 c	3.2
BR 9002	12.3 b	1.0 d	n/n	8.8 c	1.1 d	n/n	12.3 c	1.7 c	n/n
A8	12.7 b	1.8 d	n/n	10.5 c	1.5 d	n/n	10.1 d	1.8 c	n/n
B1	17.9 b	2.2 d	n/n	26.3 a	5.9 b	601	10.1 d	2.1 c	5.8
B2	26.7 a	27.6 a	237	14.0 c	2.6 c	188	23.2 a	3.8 b	16.7
B3	14.0 b	1.8 d	n/n	5.7 d	0.6 d	n/n	18.8 b	3.4 b	14.1
B4	9.6 c	1.1 d	n/n	4.4 d	0.5 d	n/n	15.3 b	2.0 c	n/n
B5	16.2 b	2.6 d	13	7.0 d	3.3 c	275	9.6 d	1.9 c	n/n
B6	23.2 a	22.3 b	189	7.0 d	0.8 d	-38	17.1 b	3.1 b	12.2
B8	28.4 a	29.8 a	256	20.1 b	2.7 c	200	17.5 b	2.4 c	7.7
B9	24.5 a	27.7 a	238	15.8 b	4.1 c	375	12.3 c	2.0 c	5.1
B11	25.8 a	28.5 a	245	12.7 c	2.2 c	138	7.9 d	1.7 c	3.2
B12	14.0 b	2.1 d	9	17.5 b	9.0 a	988	10.5 d	1.7 c	n/n
B16	25.8 a	27.4 a	235	10.1 c	1.7 d	75	10.5 d	2.1 c	5.8
B17	28.4 a	21.4 b	181	14.0 c	1.7 d	75	7.0 d	1.1 c	-0.6
M1	12.3 b	0.9 d	n/n	15.8 b	1.9 d	n/n	7.4 d	1.3 c	0.6
M4	7.9 c	0.9 d	-1.8	22.8 a	7.0 b	738	13.1 c	3.0 b	11.5
M5	9.2 c	1.3 d	n/n	13.1 c	2.0 d	n/n	17.5 b	2.9 b	n/n
M7	10.5 c	2.3 d	10.7	8.8 d	0.6 d	-63	14.0 c	2.9 b	10.9
M8	14.0 b	3.0 d	17	5.7 d	2.5 c	175	14.0 c	2.7 b	9.6
M9	24.9 a	3.5 d	21	20.1 b	3.7 c	325	8.8 d	1.5 c	1.9
Low N	7.9 c	1.1 d	-	8.8 c	1.1 d	-	7.0 d	1.2 c	-
High N	30.6 a	12.3 c	-	26.3 a	1.9 d	-	17.5 b	16.8 a	-

Means followed by the same letter in each column are not statistically different (Scott-Knott test, 5 %). n/n = no nodules.

M7 for *maricá*, and UFSC-B17 for *angico-vermelho*. This indicates that those symbioses may have a parasitic nature, in which there is the formation of nodules, but the nodules are ineffective (Dias et al., 2012).

In addition to the contribution of rhizobia to biological N fixation, it is known that these microorganisms act as plant growth promoters and are involved in actions such as phosphate solubilization (Marra et al., 2012), production of siderophores (Jin et al., 2006), and production of phytohormones (Schlindwein et al., 2008), among other effects, all mechanisms that facilitate establishment of plants in degraded environments. This may be the explanation for the growth stimulation promoted by isolates UFSC-B5 and M8 in *maricá*, where even with low nodulation percentages there was considerable plant growth. Therefore, these mechanisms for plant growth promoting capacity mediated by the rhizobia tested in this study need to be further investigated.

CONCLUSIONS

Isolates of rhizobia indigenous to coal mining areas were able to promote nodulation and efficiently fix N in symbiosis with *bracatinga* and *maricá*. However, the inoculation of indigenous rhizobia did not stimulate plant growth and N accumulation in *angico-vermelho*.

The most promising rhizobium isolates recommended for programs of revegetation of areas degraded by coal mining are UFSC-B2, B6, B8, B9, B11, B16 and B17 for *bracatinga*, and UFSC-B1, B12 and M4 for *maricá*.

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