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Root symbioses in two legume-grass consortia inoculated with soils obtained from degraded coal mining areas in reclamation

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The intense exploitation of coal deposits in Southern Brazil has caused severe degradation problems in extensive areas. In order to recover such areas, the use of revegetation with species adapted to disturbed environments and with the capacity to establish mutual relationships with microorganisms have been of great value. The objective of this study is to characterize plant-root symbioses in two grass-legume (*Calopogonium mucunoides* with *Brachiaria decumbens* and *Vicia sativa* with *Brachiaria decumbens*) consortia inoculated with soil of the Carboniferous Basin in the state of Santa Catarina. Areas evaluated were at different stages of land reclamation and the influence of those consortia on the occurrence of rhizobia, arbuscular mycorrhizal fungi, and in the community of endophytic bacteria were evaluated. The study was done in two independent experiments in a completely randomized design, with five replications; done under greenhouse condition. There were seven treatments - five inoculated (with soil obtained from areas of 2, 4, 6 and 12 years of recovery, and a reference area) and two control treatments without inoculation (with low and high concentrations of mineral nitrogen). After 50 days of implantation, soil and plant material were collected to characterize root symbioses by nodule counting, nitrogen fixing bacteria isolation, mycorrhizal occurrence (%), and characterization of root endophytic bacterial communities. Only the calopogonium-brachiaria consortium was able to nodulate with rhizobia from the recovering coal mining areas. Arbuscular mycorrhizal fungi (AMF) and endophytic bacteria occur in the vetch-brachiaria and calopogonium-brachiaria consortia regardless of the time of recovery. The microbial communities present in soils with different stages of recovery are more efficient in promoting plant growth in the calopogonium-brachiaria consortium and this behavior may be associated with the calopogonium's ability to associate with autochthonous rhizobia.

Key words: Environmental recovery, revegetation, plant-growth-promoting, rhizobia, *arbuscular mycorrhiza.*

INTRODUCTION

The Carboniferous Basin in the state of Santa Catarina (CBSC) is the second most important in Brazil, and for a long time the deposition of tailings, following coal extraction, was done with no control or soil preservation (Lopes et al., 2009). In the last decades, many areas were abandoned after open-pit mining, which entailed the removal of large strips of vegetation, erosive processes, the release of toxic gases into the atmosphere, and the loss of soil organic matter. The sites lost or significantly decreased their self-healing capacity, requiring active intervention to reestablish a non-degraded condition (Rocha-Nicoleite et al., 2013). Faced with such facts, a civil action was proposed, which obliges the coal industry, the State and the Federal Government to carry out projects aimed at recovering those areas. Revegetation is a rehabilitation alternative that can aid in this process, since it promotes the control of erosive processes, resulting in the recovery of soil properties (Siqueira et al., 2008).

Plant species used in the revegetation of such areas must have adaptive capacity to degraded environments. Works involving revegetation with tree legumes have been developed in the region in the last decade. However, the use of these plants presents limitations due the deep root systems that may affect the structure of soil built which is preformed in tailings areas with the purpose of being a barrier for confinement the residues. Hence, the use of other legumes, mainly herbaceous species, may pose an alternative. Leguminous plants such as *Calopogonium mucunoides* Desv. (calopo) and *Vicia sativa* L. (vetch) consorted with grasses such as *Brachiaria decumbens* Stapf. (brachiaria) are used for the revegetation programs in the region, as they increase the organic material deposited in the soil surface, favoring biological activity, and accelerating the recovery process (Rocha-Nicoleite et al., 2013). Other important characteristics of these plants are good adaptation to acid soils of low natural fertility containing high levels of aluminum. These plants are able to establish mutualistic relationships with symbiotic microorganisms such as rhizobia (in the case of leguminous plants) and arbuscular mycorrhizal fungi (AMF) (leguminous plants and brachiaria), which provide nutrients (such as phosphorous) to the plant (Ampomah and Huss-Danell, 2016; Ferreira et al., 2016; González et al., 2018). Besides rhizobial and mycorrhizal symbionts there is a great diversity of other microorganisms occupying the interior of the plant tissues, also known as endophytes. Those may present the capacity to promote plant growth through several mechanisms, including production of phytohormones and siderophores, solubilization of phosphates, among others (Timmusk et al., 2011; Brígido and Glick, 2015; Santoyo et al., 2016). Altogether, these microorganisms interacting with plants can play a crucial role for the establishment and development of plants in degraded environments, such as those commonly found in coal mining areas.

Despite calopo and vetch being able to form a symbiotic relationship with rhizobia, not much is understood about the development of the symbiosis under stressful conditions, which greatly affect the outcome of the symbiotic process. Similarly, little is known about the impact of the stressful conditions imposed by the coal-mining degraded soil in the mycorrhizal and root endophytic communities of brachiaria, calopo and vetch. Moreover, in order to maximize the revegetation procedure, future inoculants adapted to the harsh environmental conditions present in the coal-mining recovery areas need to be developed. Therefore, the objective of the present work is to characterize plant-root symbioses in calopo+brachiaria and vetch+brachiaria consortia inoculated with soils from mining areas at different stages of land reclamation, and determine the influence of those consortia on the occurrence of rhizobia, AMF, and in the community of endophytic bacteria.

MATERIALS AND METHODS

Data collection

The collection of data was done in June 2015. Different areas were chosen according to the recovery time after mining, therein designated: two years (A2), four years (A4), six years (A6) and 12 years (A12) under a revegetation regime. The pH for the soils of those areas was 4.66, 4.53, 3.80 and 4.91, respectively. A2 and A4 are located in the municipality of Lauro Muller and have the following coordinates: 28º19'08.97"S 49º26'20.93"W and 28º33'26.62"S 49º27'56.19"W respectively. A6 is located in Treviso 28º26'10.78"S 49º23'36.04"W and A12 in Siderópolis 28º35'09.30"S 49º25'25.93"W. Soil samples were also collected in a reference area (RA) with no mining history in Lauro Muller-SC (28°22'32.1"S 49°20'31.9"W), and with a typical vegetation cover of dense ombrophilous forest. The chemical characterization of the soils and the plant species present in these areas was summarized in Silva (2016). For the collection, five random sites were chosen within each area, located 100 to 200 m apart from each other (depending on the size of the area). At each sampling site a central point was chosen and at a 4 m radius 4 soil samples were collected (at each cardinal point), at a depth of 0-20 cm, forming together 400 g of soil per sample, which was used as source of inoculum.

Plant growth assay

The experiment was conducted under greenhouse conditions using

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two consortia: one of *Calopogonium mucunoides* and *Brachiaria decumbens* Stapf. (calopo+brachiaria), and another with *Vicia sativa* and brachiaria (vetch+brachiaria). Experiments were done in 280 cm³ pots containing an autoclaved mixture of sand and vermiculite (1:1; v/v) inoculated with 50 g of soil-inoculum. For this purpose, 15 mL of brachiaria seeds and two legume seeds, previously disinfected with 2% sodium hypochlorite for two minutes and washed six times in sterile distilled water, were sown. For each consortium, a completely randomized design (five replications) was used, consisting of five treatments with the inoculum source (corresponding to soils obtained from areas A2, A4, A6, A12 and RA), and two control treatments without inoculation: low (5.25 mg N) (C-N) and high concentration of mineral nitrogen (52.535 mg N) (C+N), totaling 35 experimental units for each consortium. Weekly, 50 ml of a half-strength Hoagland and Arnon (1950) nutrient solution were added to the pots. The experiment was conducted for 50 days, when the soil and plant material were collected for evaluation. The aerial part of the plants was placed in a drier with air circulation at 65°C until constant weight, to determine the shoot dry biomass (SDB). The nitrogen content in the shoots was determined by the Kjedahl semi-micro method according to Tedesco et al. (1995). Accumulated nitrogen was calculated by multiplying the nitrogen content with the respective SDB content.

Rizhobia and mycorrhizal occurrence

Nodules were detached and counted from the roots of the leguminous plants. Then, ten nodules were selected per pot to isolate the rhizobia. For this, nodules were disinfected with alcohol (95.5%) for 60 seconds, sodium hypochlorite (2.5%) for 2 minutes, and washed six times in sterile distilled water. Nodules were then macerated and inoculated into Petri dishes containing yeast mannitol agar medium- YMA extract (Vincent, 1970). Subsequently, plates were incubated for a period of 14 days at 28 °C. Then, isolates were characterized morphologically after 10 days of incubation via bromothymol blue in YMA medium and congo red in YMA medium. The following morphological characteristics were evaluated: growth time, pH change, color, shape, surface and border of the colony, absorption of the indicator, and mucus production. Strains of *Rhizobium leguminosarum* (SEMIA 384), *R. tropici* (CIAT899), *Bradyrhizobium japonicum* (BR 1602), and *Bradyrhizobium* sp. (SEMIA 6144) were used as reference.

Root samples from each treatment were separated, washed and stained according to Koske and Gemma (1989), and the percentage of colonization estimated (Giovannetti and Mosse, 1980). The soil spore density was obtained from samples of 50 g of soil collected in each treatment. Spores were obtained by wet sieving (Gerdemann and Nicolson, 1963), followed by sucrose gradient centrifugation. After extraction, spore counting was performed using a stereomicroscope (16X).

Root endophytic bacteria community determination

Samples of 0.5 g of consortium roots were disinfected following Da Silva et al. (2016). To verify the effectiveness of the disinfestation process, an aliquot of the last wash water was used for DNA amplification. Root samples were macerated in liquid nitrogen for DNA extraction using the 2% CTAB method (Doyle and Doyle, 1990). Amplification was done for the V3 region of the bacterial 16S rDNA gene using primers BAC338FGC and UN518R (Ovreås et al., 1997). Amplification was performed using 10 μmol L^{-1} of the primers and the PCR products analyzed by denaturing gradient gel electrophoresis (DGGE) following Da Silva et al. (2016). Acquisition of gel images was done on a Gel Logic 2200 Pro Photo Documentator (Carestream Health, New York, USA). The fragment (band) patterns were analysed with the program BIONUMERICS 7.10 (BioSystematica, Wales, UK).

Statistical analysis

Normality test (Shapiro-Wilk) and the homogeneity of variances (Cochran) were performed for the variables measured. The number of spores was transformed with the $\log_{10}$ function. The data were compared using analysis of variance and the means submitted to the SNK test ($p<0.05$) (ASSISTAT 7.7). The phenotypic attributes of the rhizobia were evaluated by a hierarchical clustering analysis using the software SYSTAT 11. The clusters obtained from the band profiles of the PCR-DGGE were analyzed using the Jaccard index and the UPGMA clustering model.

RESULTS

Occurrence of autochthonous rhizobia and mycorrhiza

The visual inspection of roots revealed the presence of nodules in the calopo+brachiaria consortium in all inoculated treatments (Table 1). No nodules were observed in the vetch+brachiaria consortium.

In the calopo+brachiaria consortium plants inoculated with soil from Areas A2, A4 and A12 exhibited on average 55 nodules per pot, 243% higher than those for area A6 (16 per pot). Fifty rhizobia isolates were obtained and their morphological and cultural features characterized. Two thirds of the isolates evaluated did not alter the pH of the culture medium. Most isolates of rhizobia (76%) exhibited intermediate or slow growth, and 60% of them had scant or low mucus production. These characteristics pointed to the low representativeness of the genus *Rhizobium*, which is fast growing, reduces the pH of the medium, and presents abundant mucus production.

Unlike the rhizobia, AMF were verified in both consortia. The percentage of mycorrhizal colonization ranged from 14 to 51% in the calopo+brachiaria consortium, and from 19 to 47% in the vetch+brachiaria consortium (Table 1). Plants inoculated with soil from Areas A2 and A6 showed the highest mycorrhizal colonization in both consortia (on average 47%). For the calopo+brachiaria consortium, the number of spores in areas in the initial stages of recovery (A2 and A4) was a range of 5- 6 times greater than in RA. On the other hand, in the vetch+brachiaria consortium AMF spores were detected in all treatments with the lowest values in areas A12 and RA. In area A6, about four times more spores were found in the vetch+brachiaria than in the corresponding calopo+brachiaria consortium.

Characterization of the community of endophytic bacteria

The results of the hierarchical cluster analysis for

Table 1. Mycorrhizal colonization (%) and number of AMF spores present in the calopo-brachiaria and vetch-brachiaria consortia inoculated with coal mined soils at different stages of recovery in the state of Santa Catarina.

Consortia	Treatments	Nodule number	Mycorrhizal colonization (%)	#spores 50 mL soil^{-1}
Calopo+brachiaria	A2*	62^a	46^a**	377^b
	A4	51^b	21^b	722^a
	A6	16^c	51^a	188^d
	A12	52^b	21^b	282^c
	RA	66^a	14^b	76^e
Vetch+brachiaria	A2	0	46^a	626^a
	A4	0	19^b	464^a
	A6	0	47^a	707^a
	A12	0	24^b	271^b
	RA	0	27^b	118^c

*A2, 2 years of recovery, A4: 4 years of recovery; A6, 6 years of recovery; A12, 12 years of recovery; RA, reference area. **Values followed by different letters in the same column for each consortium are statistically different according to the Scott-Knott test (p<0.05).

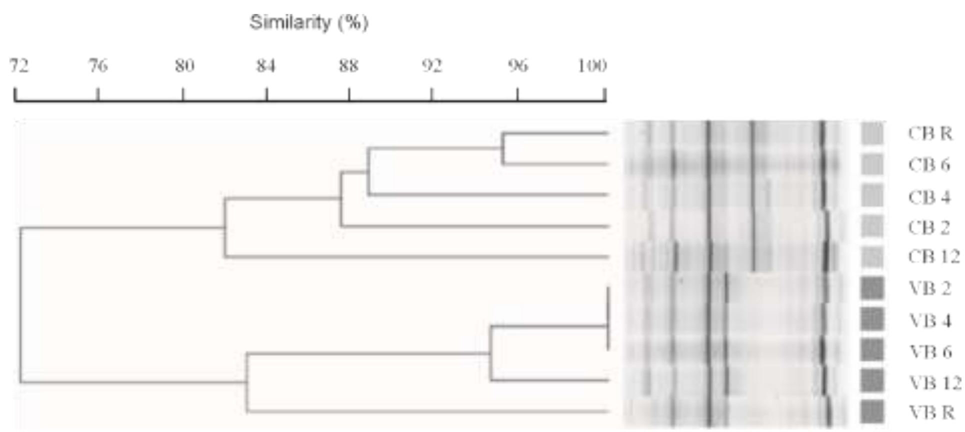

Figure 1. Hierarchical grouping of the community structure of endophytic bacteria. CB = calopo+brachiaria, VB= vetch+brachiaria, R = reference soil without mining tailings. 2, 4, 6, 12 = different recovery times for soils with mining tailings.

bacteria communities in the consortia can be observed in Figure 1. It can be seen that the structure of the endophytic community presents 72% similarity between consortia. There is also a formation of two groups with 82 and 83% similarity, made by samples from the vetch+calopo consortium, respectively. Thus, the determining factor of clustering was the type of legume present in the consortium and not the area used as inoculum source.

In the calopo+brachiaria consortium, the grouping presented some heterogeneity: AR was closer to areas with the lowest recovery times, whereas A12 had communities of more differentiated endophytic bacteria. In the grouping obtained for the vetch+brachiaria consortium, the grouping presented lower heterogeneity. Moreover, there was a separation between the RA and the areas under recovery. There were no marked differences between the microbial groups evaluated in soils with different recovery times in this consortium.

Effect of soil inoculation on plant growth and nitrogen accumulation

Shoot dry matter (SDM) and nitrogen accumulation of the calopo+brachiaria consortium were significantly influenced by the treatments. The highest SDM was accumulated by plants of the C+N treatment, followed by those inoculated with soil-inoculum of areas A2, A4, and A12 (Figure 2A). Treatments A6 and RA presented a biomass 33% lower than the C-N. Nitrogen accumulation was highlighted for the treatments with the soil-inoculum from areas A2, A4 and A12, which did not differ from C+N (Figure 2C). For those treatments, the average increase was 57% higher in relation to the C-N treatment. In the RA, the accumulation of nitrogen presented an intermediate value, and the A6 area again presented the lowest levels. On the other hand, in the vetch+brachiaria consortium there were increases in plant growth only in the C+N treatment. The inoculation did not influence

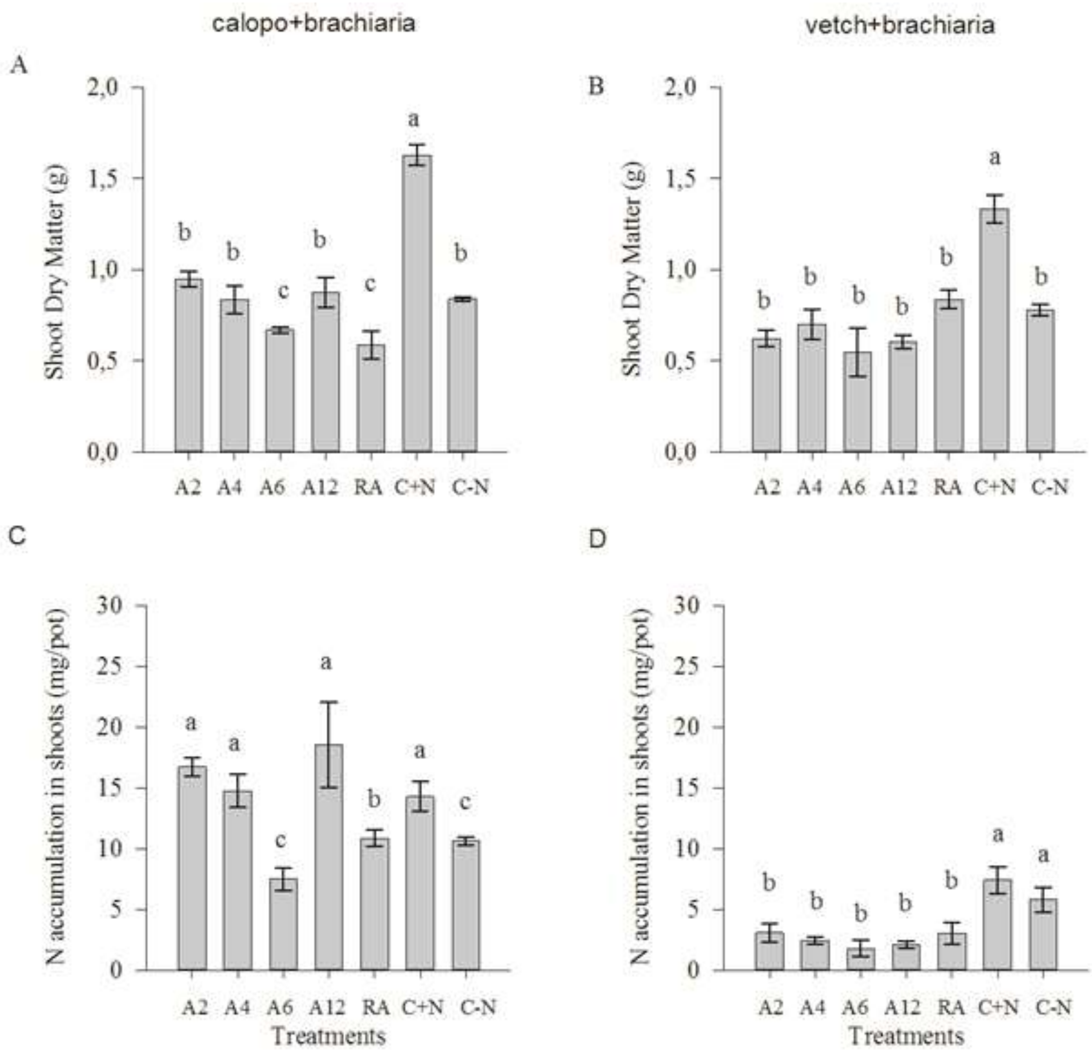

Figure 2. Effect of soil inoculation from coal mining areas under different times of recovery, on attributes related to growth and nutrition of plants grown in consortia: shoot dry matter for the calopo+brachiaria (A) and vetch+brachiaria (B) consortia, nitrogen accumulation in calopo+brachiaria (C) and vetch+brachiaria (D) consortia; RA = reference area without mining tailings. 2, 4, 6, 12 = years of recovery; C+N = non-inoculated control with high N after 50 days of growth; C-N = non-inoculated control with low N after 50 days of growth. Means followed by the same letter do not differ statistically by the Scott-Knott test ($p<0.05$). Vertical bars represent the standard error of the mean ($n = 5$).

accumulation of nitrogen since the inoculated treatments were lower when compared to the controls without inoculation (Figure 2B and 2D).

DISCUSSION

Symbiotic potential of calopo vs. vetch in different soils

Rhizobia

After water, nitrogen is the second limiting factor for plant growth. The Biological Nitrogen Fixation (BNF) process for nitrogen addition appears as a very environmentally friendly alternative since the nitrogenous industrial fertilizer inputs are reduced. Currently, it is accepted as a consensus that both the production and the use of nitrogen fertilizers result in a serious threat to the environment, since in both situations considerable amounts of nitrous oxide (N_2O) are generated, being one of the more powerful greenhouse gases there is (Crews and Peoples, 2004; Jensen et al., 2012). In this sense, the use of nitrogen fixing bacteria as an important way to make this nutrient available after the establishment of symbiosis with legumes has been intensively studied. Numerous studies have shown that symbiosis increases N accumulation for many legumes (Calheiros et al., 2013;

Moura et al., 2016).

In the present study, calapo was found to have a better response to inoculation than vetch, with mean increases in N accumulation four times higher in most inoculated treatments. In that respect, the selection of strains with high efficiency has become a constant search. Nevertheless, the choice should be for autochthonous strains, which have much higher survival rates in relation to non-autochtonous strains (Geetha and Joshi, 2013). In the present work, 40 indigenous rhizobia from recovered mining areas were obtained. They all share a common trait, which is the fact that they are all adapted to the local conditions and have competitive capacity with the microbial community for their establishment and permanence in coal mining degraded areas. These isolates were compatible with calopo but not with vetch.

The rhizobium-legume symbiosis is a highly specific interaction (Lopes et al., 2016). A previous study developed by our group described that autochthonous rhizobia from coal mining areas show low symbiotic compatibility with vetch, since nodulation was confirmed in only 12.5% of the isolates evaluated (Hernández et al., 2017). Furthermore, Spaink et al. (1991) showed that vetch is a very restricted plant that establishes interaction with only the genus *Rhizobium* that has receptors for a single type of nodulation factor. Recently, Ampomah and Huss-Danell (2016) studied the genetic diversity and phylogeny of bacteria isolated from nodules of six species of *Vicia* in Norway. In that study, 25 isolates were obtained, all of are which classified as *Rhizobium leguminosarum* sv. *viciae*. These descriptions suggest that isolates compatible with vetch are uncommon, a fact that matches the results presented in the current study.

AMF

AMFs are highly tolerant to abiotic stresses; plants colonized with AMF can mobilize more nutrients, tolerate water shortages, and reduce the impact of trace elements, among other characteristics that increase plant survivability in the initial stages of establishment and development (Siqueira et al., 2008). In this study, mycorrhizal colonization was similar in both consortia. With the exception of area A6, it was verified that in the vertch+brachiaria consortium there was a reduction in the number of AMF spores with the increase of recovery time. Several authors have described that in areas in the early stages of succession, mycorrhizal colonization is greater than for areas in more advanced stages (Zangaro et al., 2012; Sousa et al., 2014). In addition, plants inoculated with the A12 and RA soils present the lowest values of mycorrhizal colonization and number of spores, probably because the mycorrhizal symbiosis, as explained previously, presents a smaller competitive advantage in stable ecosystems. There are, however, variations in these patterns, as observed in area A6, which may be related to the inoculum potential of AMF present in the soils of the loan areas. In the process of revegetation, soils of different places, called loan areas, are added and provide topographic remodeling aside from supporting the vegetation to be introduced. The quality of this soil determines the number of propagules and the initial microbial diversity in each area to be recovered, so the starting point for the different areas in recovery is not necessarily the same.

Endophytic bacterial communities

In relation to the endophytic bacterial community structure, a high similarity between the consortia was expected, due to the presence of brachiaria in both treatments. However, the formation of two distinct groups was verified, and can be attributed to the genotype of the legume present in the consortium. These results are in agreement with those described in other studies in which it was shown that the genotype of the host, its stage of development, and the type of organism studied determine the community of endophytic microorganisms (Ottesen et al., 2013; Bodenhausen et al., 2013; Hardoim et al., 2015).

Moreover, in the vetch+brachiaria consortium, in the inoculated treatments, regardless of time, a very specific bacterial community structure can be observed, with similarity varying between 95-100% (Figure 1). In this work, restricted associations can be observed not only in the bacterial endophyte community structure, but also with rhizobia. This symbiosis was absent in vetch and may justify the different community structure of the consortium, being these microorganisms absent in the banding pattern or even influencing the bacterial community associated to the species.

Effect of soil inoculation on plant growth and nitrogen accumulation

The increases in N accumulation verified for treatments A2, A4 and A12 showed that in the calopo+brachiaria consortium the inoculation may substitute completely the addition of nitrogen fertilizers since the values were not significantly different between those treatments and the C+N treatment. The contribution of N in the calopo+brachiaria consortium is positively related to the presence of rhizobia ($r = 0.5^{**}$). On the other hand, in the vetch+brachiaria consortium the inoculation may contribute negatively for the accumulation of N, as shown in Figure 2.

In general, calopo was more responsive than vetch when inoculated with soil from coal mining areas, presenting increased ability to form symbiotic relationships. Calopo seems to be a better alternative to be used in future recovery programs since 80% of the evaluated soils presented a microbiota that in symbiosis has the capacity to promote the growth of this legume. This fact is very positive considering that this is a legume native to South America adapted to low fertility soils and low pH, meanwhile vetch is a native legume from the temperate regions of the world (Ferreira et al., 2016; Camargos and Sodek, 2010). In the present study, there was no symbiotic association between vetch and any native rhizobia. However, the vetch+brachiaria consortium could be used as a prior crop once it promotes abundant AMF propagules that can establish symbioses later with the plants inserted by the recovery programs. Rhizobia isolates and AMF spores obtained in this study will be tested in symbiotic efficiency assays in calopo in order to obtain microbial inoculants appropriate to areas degraded by mining.

Conclusion

The calopogonio-brachiaria and vetch-brachiaria consortia are similar in the presence of root symbioses involving arbuscular mycorrhizal fungi and endophytic bacteria, regardless of the recovery time of the coal mining areas. The microbial communities present in soils with different stages of recovery are more efficient in promoting plant growth in the calopogonium-brachiaria consortium, and this behavior may be associated with the calopogonium's ability to associate with autochthonous rhizobia.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Ampomah OY, Huss-Danell K (2016). Genetic diversity of rhizobia nodulating native *Vicia* spp. in Sweden. Systematic and Applied Microbiology 39:203-210.

Bodenhausen N, Horton MW, Bergelson J (2013). Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. PloS One 8:e56329.

Brígido C, Glick BR (2015). Phytoremediation using Rhizobia. In: Ansari AA, Gill SS, Gill R, Lanza GR, Newman L (eds) Phytoremediation: management of environmental contaminants, Springer Science 2:95-114

Calheiros AS, Junior L, de Andrade M, Soares DM, Figueiredo MDVB (2013). Symbiotic capability of calopo rhizobia from an agrisoil with different crops in Pernambuco. Revista Brasileira de Ciência do Solo 37:869-876.

Camargos LS, Sodek L (2010). Nodule growth and nitrogen fixation of *Calopogonium mucunoides* L. show low sensitivity to nitrate. Symbiosis 5:167-174

Crews TE, Peoples MB (2004). Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agriculture, Ecosystems and Environment 102:279-297.

Da Silva KJ, De Armas RD, Soares, CRF, Ogliari JB (2016). Communities of endophytic microorganisms in different developmental stages from a local variety as well as transgenic and conventional isogenic hybrids of maize. World Journal of Microbiology and Biotechnology 32:189.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Ferreira TC, Aguilar JV, Souza LA, Justino GC, Aguiar LF, Camargos LS (2016). pH effects on nodulation and biological nitrogen fixation in *Calopogonium mucunoides*. Brazilian Journal of Biology 39:1015-1020.

Geetha SJ, Joshi SJ (2013). Engineering rhizobial bioinoculants: a strategy to improve iron nutrition. The Scientific World Journal 2013:1-15.

Gerdemann, JW, Nicolson TH (1963). Spores of mycorrhizal endogone species extracted from soil by wet sieving and decanting. Transactions of the British Mycological Society 46(2):235-244.

Giovannetti M, Mosse B (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytologist 84:489-500.

González AH, Londoño DM, Pille da Silva E, Nascimento FXI, de Souza LF, Garcez B, et al (2018). *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. Journal of Applied Microbiology (online).

Hardoim PR, Van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A et al., (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiology and Molecular Biology Reviews 79:293-320.

Hernández AG, de Moura GD, Binati RL, Nascimento FXI, Londoño DM, Mamede ACP, da Silva EP, de Armas RD, Giachini AJ, Rossi MJ, Soares CRFS (2017). Selection and characterization of coal mine autochthonous rhizobia for the inoculation of herbaceous legumes. Archives of Microbiology 199:991-1001.

Hoagland D, Arnon DI (1950). The water culture method for growing plants without soil. Riverside: California Agriculture Experimental Station/Davis: College of Agriculture, University of California.

Jensen ES, Peoples MB, Boddey RM, Gresshoff PM, Hauggaard-Nielsen H, Alves BJ, Morrison MJ (2012). Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. Agronomy for Sustainable Development 32:329-364.

Koske RE, Gemma JN (1989). A modified procedure for staining roots to detect VA mycorrhizas. Mycological Research 92: 486-488.

Lopes ÉCP, de Moraes A, Lang CR (2016). Estudo do fracionamento isotópico de nitrogênio aplicado à gramíneas e leguminosas forrageiras. Brazilian Journal of Applied Technology for Agricultural Science 9(1):121-130.

Lopes RP, Lopes R, Santo E, Galatto S (2009). Mineração de carvão em Santa Catarina: geologia, geoquímica e impactos ambientais. In: Milioli G et al., (eds) Mineração de carvão, meio ambiente e desenvolvimento sustentável no sul de Santa Catarina: uma abordagem interdisciplinar. Curitiba: Juruá pp. 51-70

Moura GGDD, Armas RDD, Meyer E, Giachini AJ, Rossi MJ, Soares CRFS (2016). Rhizobia isolated from coal mining areas in the nodulation and growth of leguminous trees. Revista Brasileira de Ciência do Solo 40:1-10.

Ottesen AR, Peña AG, White JR, Pettengill JB, Li C, Allard S et al., (2013). Baseline survey of the anatomical microbial ecology of an important food plant: *Solanum lycopersicum* (tomato). BMC Microbiology 13:1.

Ovreås L, Forney L, Daae, FL, Torsvik V (1997). Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Applied and Environmental

Microbiology 63:3367-3373.
Rocha-Nicoleite ER, Campos ML, Citadini-Zanette V, Santos R, Martins R, Soares CRFS (2013). Mata Ciliar: implicações técnicas sobre a restauração após a mineração de carvão. Criciúma, SATC.
Santoyo G, Moreno-Hagelsieb G, del Carmen Orozco-Mosqueda M, Glick BR (2016). Plant growth-promoting bacterial endophytes. Research in Microbiology 183:92-99.
Silva EPD (2016). Atributos do solo e comunidades microbianas associadas à bracatinga (*Mimosa scabrella* Benth.) em áreas de mineração de carvão em recuperação. Dissertação. Universidade de Santa Catarina.
Siqueira JO, Soares CRFS, Silva C (2008). Matéria orgânica em solos de áreas degradadas. In: Santos GA et al., Fundamentos da matéria orgânica do solo ecossitemas tropicais e subtropicais. 2ed. Porto Alegre: Metropole 654 p.
Sousa CDS, Menezes RSC, Sampaio EVDSB, Lima FDS, Maia LC, Oehl F (2014). Arbuscular mycorrhizal fungi in successional stages of Caatinga in the semi-arid region of Brazil. Ciência Florestal 24:137-148.
Spaink HP, Sheeley DM, van Brussel AA, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJJ (1991). A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:125-130.
Tedesco MJ, Gianello C, Issani CA, Bohnen H, Volkweiss SJ (1995). Análise de solo, plantas e outros materiais. 2nd ed. Universidade Federal do Rio Grande do Sul, Porto Alegre.
Timmusk S, Paalme V, Pavlicek T, Bergquist J, Vangala A, Danilas T, Nevo E (2011). Bacterial distribution in the rhizosphere of wild barley under contrasting microclimates. PLoS One 6:e17968.
Vincent JMA (1970). Manual for the pratical study of root-nodule bacteria. Blackwell Scientific, Oxford.
Zangaro W, Alves RA, Lescano LE, Ansanelo AP, Nogueira MA (2012). Investment in fine roots and arbuscular mycorrhizal fungi decrease during succession in three Brazilian ecosystems. Biotropica 44:141-150.