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RIZÓBIOS AUTÓCTONES DE ÁREAS DE MINERAÇÃO DE CARVÃO NO CRESCIMENTO E NA MICROBIOTA DE LEGUMINOSAS HERBÁCEAS EMPREGADAS NA REVEGETAÇÃO DE ÁREAS DEGRADADAS

Florianópolis
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Rizóbios autóctones de áreas de mineração de carvão no crescimento e na microbiota de leguminosas herbáceas empregadas na revegetação de áreas degradadas

por

Anabel González Hernández

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Aos meus pais

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“Man has always to be busy with his thoughts if anything is to be accomplished” **Antonie van Leeuwenhoek**

RESUMO

Historicamente, as atividades relacionadas com a extração e beneficiamento do carvão mineral na região carbonífera catarinense ocasionaram mudanças físicas, químicas e biológicas no solo. Estas modificações resultaram, principalmente, do processo de inversão das camadas do solo tornando difícil o estabelecimento de uma cobertura vegetal nesses locais. As bactérias promotoras do crescimento vegetal têm se mostrado como alternativa eficiente para auxiliar no processo de revegetação. Desse modo, o objetivo do presente trabalho foi caracterizar rizóbios autóctones de áreas de mineração de carvão e avaliar a influência da inoculação destes sobre o crescimento e a microbiota de espécies herbáceas empregadas na recuperação de áreas degradadas. No primeiro capítulo descreve-se sobre a coleta de solo em quatro locais com processos de recuperação em andamento e que variam apenas no tempo de execução (2, 4, 6 e 12 anos), e um área referência onde não existem registros de atividades de mineração. Os solos foram utilizados para o cultivo de dois consórcios *Calopogonium mucunoides+Brachiaria decumbens* e *Vicia sativa+Brachiaria decumbens* no intuito de caracterizar as simbioses radiculares por meio da contagem de nódulos, do isolamento de bactérias fixadoras de nitrogênio, da ocorrência micorrízica e da caracterização da estrutura das comunidades bacterianas endofíticas de raízes. Foi observado que as comunidades microbianas presentes em solos com diferentes estágios de recuperação são mais eficientes na promoção do crescimento de plantas no consórcio *C. mucunoides + B. decumbens*, e esse resultado pode estar associado à capacidade de *C. mucunoides* de se associar com rizóbios autóctones. No segundo capítulo foi descrito o isolamento, a autenticação e a caracterização morfológica, bioquímica e molecular, assim como a eficiência simbiótica destes rizóbios. O isolado autóctone UFSC-A605, classificado como *Pseudomonas* sp., apresentou elevada eficiência simbiótica com *C. mucunoides* em condições controladas utilizando tanto substrato estéril como solo não autoclavado. No terceiro capítulo está contida a avaliação do efeito associado à inoculação de isolados autóctones sobre a microbiota bacteriana endofítica das leguminosas *C. mucunoide* e *V. sativa* utilizando técnicas de sequenciamento de nova geração, especificamente, a plataforma MiSeq. Em *V. sativa* inoculadas houve aumento de representantes do gênero *Sideroxydans* nas raízes e não detecção de *Nocardiopsis* nas folhas. Já nas raízes inoculadas de *C. mucunoides* verificou-se um aumento de representantes do gênero *Bradyrhizobium*. Estas mudanças não geraram

diferenças significativas nos índices de riqueza em ambas as espécies. No entanto, estudos envolvendo as capacidades funcionais das comunidades microbianas no ecossistema se fazem necessários para introduzir a inoculação como uma prática sustentável para a recuperação de áreas degradadas pela mineração de carvão.

Palavras-chave: *Calopogonium mucunoides*, *Vicia sativa*, revegetação, promoção do crescimento vegetal, microbiota, sequenciamento de nova geração.

ABSTRACT

Historically, the activities related to the extraction and beneficiation of mineral coal in the Santa Catarina coal region caused physical, chemical and biological changes in the soil. These changes resulted mainly from the inversion process of the soil layers making it difficult to establish a vegetation cover at these sites. Plant growth promoting bacteria have been shown to be an efficient alternative to assist in the revegetation process of those areas. Thus, the objective of the present work was to characterize indigenous rhizobia of coal mining areas and to evaluate the influence of their inoculation on the growth and microbiota of herbaceous species used in the recovery of degraded areas. The first chapter describes the soil collection in four sites under recovery and varying only in execution time (2, 4, 6 and 12 years). The soils were used for the cultivation of two consortiua *Calopogonium mucunoides* + *Brachiaria decumbens* and *Vicia sativa* + *Brachiaria decumbens* in order to characterize the root symbioses by means of nodule counting, isolation of nitrogen-fixing bacteria, mycorrhizal occurrence and characterization of structures belonging to endophytic bacteria in the roots. It was observed that the microbial communities present in soils with different stages of recovery are more efficient in promoting the growth of plants in the *C. mucunoides* + *B. decumbens* consortium, and this result may be associated to the fact that *C. mucunoides* associates with autochthonous rhizobia in the soil. The second chapter describes the isolation, authentication and morphological, biochemical and molecular characterization, as well as the symbiotic efficiency determination of those isolated rhizobia. The autochthonous isolate UFSC-A605, classified as *Pseudomonas* sp., showed high symbiotic efficiency with *C. mucunoides* under controlled conditions using both sterile and non-autoclaved soil. In the third chapter the evaluation of the effect associated with the inoculation of native isolates on the endophytic bacterial microbiota of the legumes *C. mucunoides*

and *V. sativa* using the new generation sequencing techniques, specifically the MiSeq platform, is contained. In *V. sativa* inoculated there was increase of representatives from the genus *Sideroxydans* in the roots and no detection of *Nocardiopsis* in the leaves. In the inoculated roots of *C. mucunoides* there was an increase of representatives from the genus *Bradyrhizobium*. These changes did not generate significant differences in richness for both plant species. However, studies involving the functional capabilities of microbial communities in the ecosystem are needed to introduce inoculation as a sustainable practice for the recovery of areas degraded by coal mining.

Key words: *Calopogonium mucunoides, Vicia sativa*, revegetation, plant growth promoting capacity, microbiota, new generation sequencing.

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1 JUSTIFICATIVA

A mineração de carvão possui importante papel para a economia do sul do Estado de Santa Catarina, porém o impacto ambiental causado pela sua exploração é também expressivo, sendo considerada área crítica nacional para fins de controle de poluição (BRASIL, 1980). Calcula-se que existam cerca de 5.655 hectares impactados pela mineração em Santa Catarina (SIECESC, 2017). Jica (1997) relatou que 2/3 dos cursos d'água nessa região encontravam-se comprometidos pela drenagem ácida de mina (DAM) e pela deposição de rejeitos nos aquíferos. Os locais onde foram realizados a extração e o beneficiamento de carvão de forma inadequada perderam ou diminuíram sua capacidade de autorrecuperação. Nesses locais, os tratamentos físico-químicos convencionais podem apresentar eficácia limitada (DELGADILLO et al., 2011). Assim, minimizar os danos ambientais ocasionados pela mineração torna-se um desafio.

A revegetação é uma estratégia econômica com ampla aceitação pública que pode auxiliar na reversão da degradação e redução dos impactos sobre a qualidade e funcionalidade dos ecossistemas (LAMB et al., 2005; DOMINGUEZ et al., 2010). Para isto, é realizada a implantação de uma cobertura vegetal com objetivo de controlar os processos erosivos e recuperar as propriedades do solo (NASCIMENTO e BIONDI, 2008; SIQUEIRA et al., 2008). Na atualidade, em cerca de 2/3 das áreas degradadas na região carbonífera catarinense os impactos estão sendo mitigados pelo uso de processos de revegetação visando a recuperação destes locais (SIECESC, 2017). No entanto, áreas em processo de recuperação após a mineração apresentam restrições físicas, químicas e biológicas que podem dificultar o estabelecimento das plantas, como por exemplo acidez, baixa fertilidade, níveis tóxicos de Al e Mn, baixa atividade microbiana, compactação, entre outros. Nesse contexto, é necessário o uso de plantas capazes de se adaptar a essas condições desfavoráveis (SIQUEIRA et al., 2008). Espécies vegetais com capacidade de fixação de N_2 atmosférico são consideradas ideais para programas de recuperação devido a escassez deste elemento em solos degradados. Destaca-se assim a importância das espécies pertencentes a família Leguminosae (BRÍGIDO e GLICK, 2015).

Vicia sativa L. (ervilhaca) é uma leguminosa herbácea exótica que vem sendo utilizada em programas de revegetação de ambientes degradados na região carbonífera de Santa Catarina, uma vez que apresenta adaptação a regiões de clima temperado e capacidade de se estabelecer em solos de baixa fertilidade, elevada acidez e toxicidade de

Al no solo. Mesmo nessas condições a espécie em questão consegue fixar cerca de 120 a 180 kg de N ha^{-1} (ENNEKING, 1994). Por sua vez, *Calopogonium mucunoides* Desv. (calopogônio) é uma leguminosa herbácea nativa da América do Sul que também tem sido recomendada para uso em programas de revegetação na região, pois também possui grande adaptação a solos ácidos e de baixa fertilidade (SEIFFERT et al, 1995; FERREIRA et al., 2016) e capacidade de fixar N_2 atmosférico na ordem de 370 a 450 kg ha^{-1} N (CALEGARI e MONDARDO, 1993). SOUZA et al., (2012) descreveram o calopogônio como micotrófico e com alta produção de biomassa, atingindo 4 a 5 Mg ha^{-1} ano^{-1} de massa seca, tornando-a interessante na revegetação de áreas degradadas. Por outro lado, a mistura de gramíneas com estas leguminosas tem sido uma metodologia sugerida a fim de produzir uma cobertura espessa em um curto período de tempo, possibilitando uma diminuição da erosão e facilitando o processo de recuperação (MAITI e PRASAD, 2016).

Sabe-se que os microrganismos são fundamentais para o bom funcionamento de processos importantes como a ciclagem de nutrientes e a mobilização e mineralização de C e de N, essenciais para a revegetação de ambientes degradados (BARGET e SHINE, 1999; ZAK et al., 2003). Como dito anteriormente, solos de áreas degradadas apresentam baixos teores de matéria orgânica com limitada atividade microbiológica do solo. Para garantir o sucesso da implantação da vegetação nesses ambientes são necessários mecanismos que promovam o crescimento vegetal e auxiliem a planta a superar as adversidades destas áreas, sendo uma das opções a inoculação com microrganismos promotores do crescimento vegetal, tais como rizóbios, microrganismos endofíticos e fungos micorrízicos arbusculares (FMA) (KLOEPPER et al., 1989; GLICK, 2012).

Os microrganismos podem promover o crescimento das plantas por uma série de mecanismos diretos, indiretos ou a combinação de ambos que resultam na alteração de toda a comunidade microbiana na rizosfera (KLOEPPER e SCHROTH, 1981; GUPTA et al., 2000; BULGARELLI et al., 2015). Os mecanismos diretos envolvem principalmente a produção de variadas substâncias que facilitam a absorção de nutrientes pelas plantas (GLICK et al., 1999; GAMALERO e GLICK, 2011). Dentre esses mecanismos se destaca a fixação biológica de nitrogênio, a qual é sem dúvidas o mecanismo de promoção de crescimento mais bem estudado. Também a produção de sideróforos, metabólitos que sequestram o ferro do solo e o disponibilizam para as plantas. Além disso, um mecanismo considerado como um dos principais para a promoção de crescimento vegetal é a produção de

fitohormônios como as auxinas, citocininas e giberelinas (BELIMOV et al., 1995; BARAZANI e FRIENDMAN, 1999; GUTIERREZ-MANERO et al., 2001). As bactérias também podem promover o crescimento vegetal pelo fornecimento de fósforo por meio da solubilização de fosfatos, assim como pela produção de enzimas, por exemplo, ACC deaminase, que modula os níveis de etileno na planta (GLICK, 2014).

Alguns estudos realizados no Brasil têm demonstrado o potencial da simbiose rizóbio-leguminosa na recuperação de áreas degradadas (FRANCO e FARIA, 1997; FARIA et al., 1998; LASTE et al., 2008). Vários trabalhos, ao redor do mundo, têm demonstrado o potencial de microrganismos promotores do crescimento vegetal isolados de áreas de mineração de cobre, zinco, cádmio, chumbo e arsênio (TRANNIN et al., 2001; MATSUDA et al., 2002; CARRASCO et al., 2005; MATIAS et al., 2009; GLICK et al., 2010; FERREIRA et al., 2012). Entretanto, ainda são poucas as pesquisas sobre microrganismos promotores do crescimento como inoculantes em plantas utilizadas em programas de revegetação de áreas degradadas pela mineração de carvão (ZHANG e SUN, 2011; NAVARRO-NOYA et al., 2012, DAHMER, 2014; QUADROS et al., 2016). Na última década, o grupo de pesquisa do Laboratório de Microbiologia do Solo da Universidade Federal de Santa Catarina tem trabalhado explorando esta temática. Como resultado, possui uma coleção de isolados autóctones de áreas de mineração (MOURA et al., 2016; HERNÁNDEZ et al., 2017; MEYER et al., 2017; STOFFEL et al., 2017; DOS SANTOS et al., 2017). Esses isolados apresentam potencial de emprego como inoculantes na revegetação de áreas degradadas, uma vez que podem possibilitar que as plantas simbiontes utilizadas sejam total ou parcialmente independentes da aplicação de fertilizantes químicos. Desse modo, tornam-se interessantes estudos que avaliem a eficiência destes isolados nas espécies vegetais recomendadas nos programas de revegetação.

Sabe-se que os microrganismos podem colonizar todos os tecidos vegetais, podendo atingir densidade de células maior do que as células da própria planta. Essa comunidade recebe o nome de microbiota endofítica e apresenta aplicações biotecnológicas para as mais diversas áreas (AZEVEDO, 2000; VAN DER LELIE et al., 2009; HARDOIM et al., 2015). A estrutura e composição da microbiota podem ser influenciadas por diversos fatores (LUNDBERG et al., 2012; BULGARELLI et al., 2015). MITTER et al. (2016) descreveram que os perfis bacterianos endofíticos variam dentro das mesmas espécies de

plantas em diferentes locais de amostragem, sendo essa variação impulsionada por fatores ainda não bem esclarecidos. No entanto, a influência da inoculação de rizóbios autóctones de áreas impactadas pela mineração de carvão sobre a microbiota dessas plantas ainda constitui uma incógnita. Em um primeiro estudo, apresentado no primeiro capítulo do presente trabalho, verificou-se que a presença de rizóbios foram determinantes na promoção do crescimento vegetal de consórcios gramíneas-leguminosas (HERNÁNDEZ et al., 2018). Deste modo, se fazem necessários trabalhos para a caracterização de rizóbios autóctones de áreas de mineração de carvão e a influência da inoculação de isolados selecionados no crescimento vegetal e na estrutura e composição da microbiota endofítica de espécies herbáceas empregadas na recuperação destas áreas.

2 HIPÓTESE

Rizóbios autóctones de áreas de mineração de carvão apresentam diferentes mecanismos de promoção do crescimento vegetal, favorecendo o estabelecimento das plantas em solos de áreas de mineração de carvão.

A inoculação de rizóbios autóctones selecionados não altera a estrutura e a composição da microbiota endofítica de espécies herbáceas empregadas na recuperação de áreas degradadas.

3 OBJETIVOS

3.1.1 Objetivo geral

Caracterizar rizóbios autóctones de áreas de mineração de carvão e avaliar a influência da inoculação destes sobre o crescimento e a microbiota de espécies herbáceas empregadas na recuperação de áreas degradadas.

3.1.2 Objetivos específicos

Caracterizar os grupos microbianos que estabelecem interações simbióticas em consórcios gramíneas-leguminosas utilizadas em programas de revegetação;

Caracterizar rizóbios autóctones de áreas de mineração de carvão e a sua eficiência simbiótica para *Calopogonium mucunoides*;

Avaliar a influência da inoculação de rizóbios autóctones eficientes na microbiota de duas leguminosas herbáceas empregadas na revegetação.

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5 CAPÍTULO I

Manuscript 1: Original article

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

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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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5.1 ABSTRACT

The intense exploitation of coal deposits in Southern Brazil, resulted in extensive areas with severe degradation problems. 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 was 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 carried in two independent experiments in a completely randomized design, with five replications under the greenhouse condition, in 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

5.2 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 Governments 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 performed 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 was 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.

5.3 MATERIAL AND METHODS

5.3.1 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 were 4.66, 4.53, 3.80 and 4.91, respectively. A2 and A4 are located in the municipality of Lauro Muller and have the 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.

5.3.2 Plant growth assay

The experiment was conducted under greenhouse conditions using 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 substrate 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.

5.3.3 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 (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 substrate 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).

5.3.4 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).

5.3.5 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.

5.4 RESULTS

5.4.1 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 1.1). 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.

Table 1.1 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 and RA: reference area
**Values followed by different letters in the same column for each consortium are statistically different according to the Scott-Knott test ($p<0.05$)

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 1.1). 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.

5.4.2 Characterization of the community of endophytic bacteria

The results of the hierarchical cluster analysis for endophytic bacteria communities in the consortia can be observed in Figure 1.1. 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.

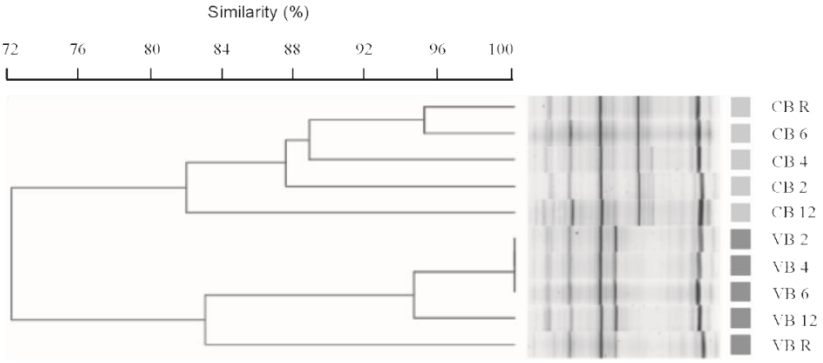

Figure 1.1 Hierarchical grouping of the community structure of endophytic bacteria. CB = calopo+brachiaria, VB= vetch+brachiaria, R = reference soil without mining tailings. 2, 4, 6, 12 = different recovery times for soils with mining tailings.

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.

5.4.3 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 (Fig 1.2A). 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 (Fig 1.2C). 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 accumulation of nitrogen since the inoculated treatments were lower when compared to the controls without inoculation (Fig 1.2B and D).

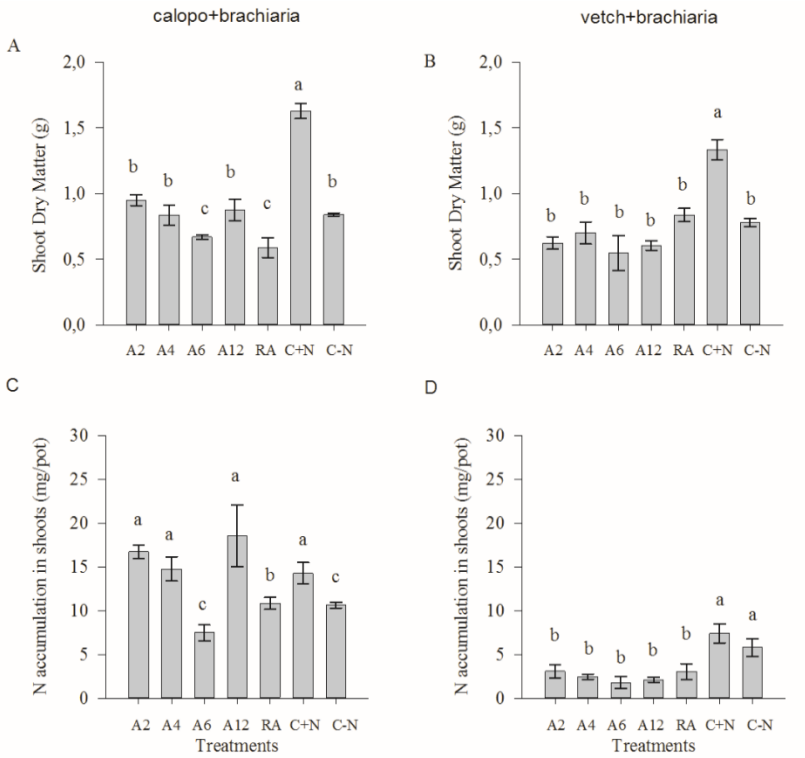

Figure 1.2 Effect of soil inoculation from coal mining areas under different times of recovery, on attributes related to growth and nutrition of plants grown in consortia. Shoot dry matter for the calopo+brachiaria (A) and vetch+brachiaria (B) consortia, nitrogen accumulation in calopo+brachiaria (C) and vetch+brachiaria (D) consortia; R = reference soil 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 from each other by the Scott-Knott test ($P<0.05$). Vertical bars represent the standard error of the mean (n = 5).

5.5 DISCUSSION

5.5.1 Symbiotic potential of calopo vs. vetch in different soils

5.5.1.1 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 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.

5.5.1.2 AMF

AMF are highly tolerant to abiotic stresses, so 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.

5.5.1.3 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% (Fig 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.

5.5.1.4 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 fig 1.2.

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.

5.6 CONCLUSIONS

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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6 CAPÍTULO II

Manuscript 2: 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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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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Autochthonous rhizobia for recovery

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Keywords: *Calopogonium mucunoides*, degraded areas, plant growth promotion, *Pseudomonas*, reclamation.

6.1 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 evaluated. Efficient rhizobial isolates suitable for the inoculation of calopo in coal-mining regions were obtained. A total of thirty isolates were obtained after nodulation authentication, of which five presented high symbiotic efficiency 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 symbiotic efficiency 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 programs of degraded areas.

6.2 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. 1995, 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) (Camargo 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 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.

6.3 Material and Methods

6.3.1 Soil sampling from coal-mining areas

Soil sampling was performed in June/2015, in areas previously submitted to revegetation procedures (**Table S2.1**). The areas were selected according to distinct recovering times: two years (A2), four years (A4), six years (A6) and 12 years (A12) under a revegetation regime, employing plant species recommended for the area (Table S2.1). The pH in water for the soils of those areas, measured according to Embrapa (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 S2.1.

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, totaling 400 g of soil per collection point. The collected soil was used as source of inoculum for the isolation of native rhizobia.

6.3.2 Isolation of rhizobia from calopo root nodules

6.3.2.1 Greenhouse experiment using soil inocula

A 60-day experiment using calopo as a trap plant was conducted in a greenhouse. Four calopo seeds, previously disinfected with 2% sodium hypochlorite for two minutes 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 S2.1**) 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.

6.3.2.2 Nodule disinfection and bacterial isolation

Root nodules were disinfested with alcohol (95%) for 60 s, sodium hypochlorite (2.5%) for two minutes, 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 d. 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 d. 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.

6.3.3 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 d at 28 °C. Evaluated characteristics included: growth time, pH change, color, 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 (1U) 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, California, USA) and the amplicon profiles analyzed in Gel Compar II version 6.5 (Biosystematica, Wales, UK) (Welsh *et al.* 2010) Representative isolates from each similarity group were randomly selected, totaling 17 samples.

6.3.4 Symbiotic efficiency of isolated rhizobia

6.3.4.1 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 ºC). Initially, seeds were disinfected as previously described and pre-germinated 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 to 10^8 cells ml^{-1}) 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 symbiotic efficiency (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) x 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.

6.3.4.2 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 ºC) 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) (*Bradyrhizobium japonicum* BR 1602) with two

non-inoculated 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.

6.3.4.3 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 – 16%; pH (H_2O) – 4.6; P and K (Mehlich-1) – 11.1 and 760 mg dm^3, respectively; Ca, Mg, H^+Al, and Al – 57, 31,754, and 07 cmolc 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 ºC 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 (non-inoculated). A total of five replicates: four plants per pot (280 cm^3) per treatment were performed.

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

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

Based on the symbiotic efficiency 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 V1.4.0 (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.

6.3.6 Strain biochemical characterization

Based on the symbiotic efficiency 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 x 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 IIA was 30 min and analyzes 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 non-producers of siderophores, due to the color change of 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 d (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.

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

6.3.7 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 log10 function. The data were compared using analysis of variance and the means submitted to the SNK separation test ($P<0.05$). These analyzes 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 analyzed 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 v. 12 (Systat Corp., San Jose, USA).

6.4 Results

6.4.1 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 reference areas presented root nodules and in some cases the inoculation resulted in increased plant growth **(Figure 2.1A, 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 of 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).

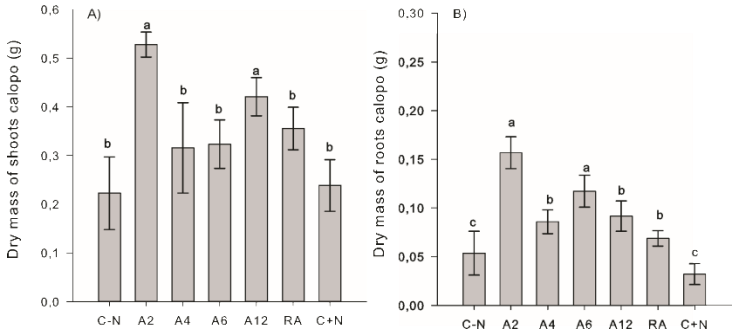

Figure 2.1 Effect of soil inoculation from coal mining areas under different recovery times, on attributes related to the growth of calopo: Dry mass of shoots (SDM) (A) and roots (RDM) (B) after 60 days of growth. R = reference soil without mining tailings. 2, 4, 6, 12 = years of recovery; C+N = control 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).

6.4.2 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 *Rhizobium 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 symbiotic efficiency.

6.4.3 Symbiotic efficiency of calopo-associated rhizobia

6.4.3.1 Experiment 1

Results obtained from the symbiotic efficiency experiment 1 showed that several rhizobial isolates were efficient and promoted calopo growth in great extent when compared to the non-inoculated controls (**Table 2.1**). 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 to the other rhizobial treatments (**Table 2.1**). Consequently, the highest SDM values were obtained in plants inoculated with isolate 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).

Table 2.1. 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 pots^{-1})	Dry mass of shoots (mg pots^{-1})	Nitrogen content in the shoots (g kg $^{-1}$)	Nitrogen accumulation in the shoots (mg pots^{-1})	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	-

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 symbiotic efficiency was calculated and these values indicated that the most efficient isolate was A6-05, with efficiencies higher than 300%. Isolates AR-12, A2-10, A6-11 and AR-18 also showed high symbiotic efficiency (98% average) (**Table 2.1**).

6.4.3.2 Experiment 2

Based on the data obtained in experiment 1, five isolates presenting high symbiotic efficiency (A6-05, AR-12, A2-10, A6-11, AR18) and one isolate (AR-02) presenting low symbiotic efficiency were selected and their symbiotic efficiency 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 2.2**).

6.4.4 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 2.2**). 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 *P. koreensis* while strains A6-11 and AR-18 presented high identity to *P. azotoformans.* The isolates A2-10 and AR-12 were identified as *Bradyrhizobium* sp., and presented high identity to *B. embrapense.* Isolate AR-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 2.2**). *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.

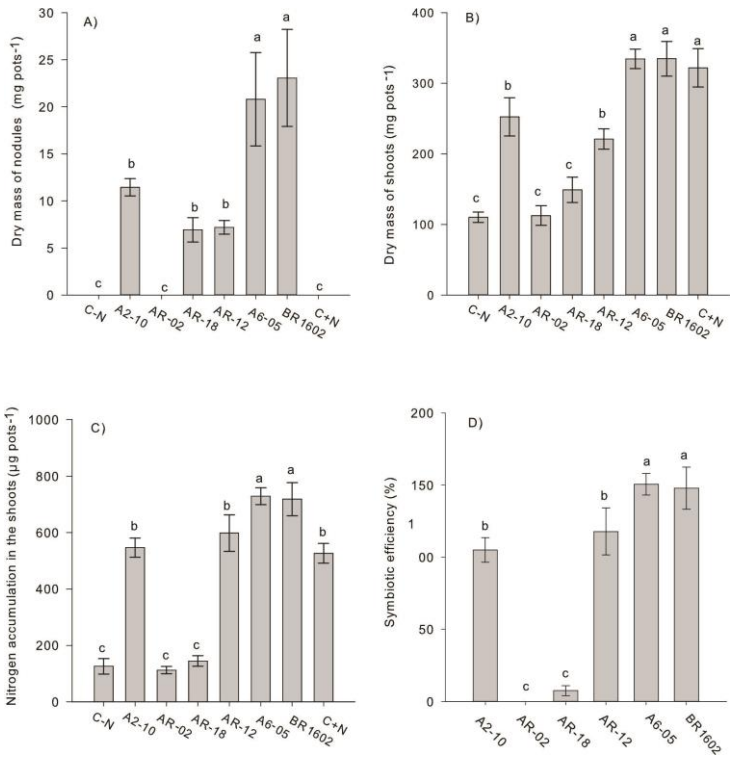

Figure 2.2. Nodule dry mass (A), shoot dry mass (B), Nitrogen accumulation (C) and symbiotic efficiency (D) of *Calopogonium mucunoides* (calopo) inoculated with rhizobia, after 60 days of growth. Means followed by the same letter did not differ significantly from each other by the SNK test (P<0.05). Vertical bars represent the standard error of the mean (n=7).

Table 2.2. 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.

6.4.5 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 to the control and *Bradyrhizobium* sp. BR1602 inoculation treatments (**Figure 2.3, A-D**). On the other hand, the inoculation with the recommended strain *Bradyrhizobium* sp. BR1602 did not positively impact calopo growth and development.

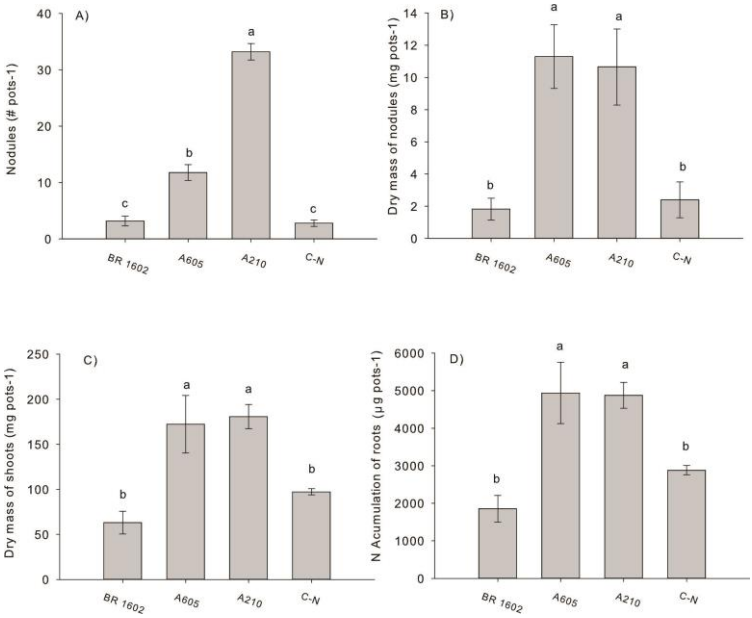

Figure 2.3. Nodule dry mass (A), dry mass of nodules (B), shoot dry mass (C) and Nitrogen accumulation (D) of *Calopogonium mucunoides* (calopo) inoculated with rhizobia, after 50 days of growth in low fertility soil. Means followed by the same letter did not differ significantly from each other by the SNK test ($P<0.05$). Vertical bars represent the standard error of the mean (n=5).

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 to the inoculation of the recommended strain BR1602. Similarly, when

compared to 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.

6.5 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, while 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 symbiotic efficiency, 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 analyzes 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 S2.1**), further suggesting their presence and distribution in soils of South Brazil. To our knowledge, this is the first report of *Pseudomonas* with high symbiotic efficiency nodulating herbaceous legumes, such as *Calopogonium 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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6.7 Suplementary Material

Table S2.1. Location and characteristics of the areas in different stages of recovery in the Carboniferous region of Santa Catarina. Trait elements according to USEPA 3051A.

(Site location/ Time of recovery)	Contaminant/ Added material	Arboreal species / Herbaceous species	Zn/ As/ Cd/ Pb /Mn (mg kg^{-1})
28º19'08.97''S 49º26'20.93"O elevation 351 m **2 years (A2)**	Opencast mining/ Clay and organic material (poultry litter)	*Ceiba speciosa* A. St.-Hil., *Croton celtidifolius* Baill, *Ingasessilis* (Vell.) Mart., *Mimosa scabrella* Benth., *Myrsine coriácea* (Sw.) R. Br. ex Roem. & Schult., *Pitadenia gonoacantha* (Mart.) J.F. Macbr, *Psidium cattleyanum* Sabine, *Schinus terebinthifolius* Raddi, *Senna multijuga* (Rich.) H.S. Irwin & Barneby, *Trema micrantha* (L.) Blume, and *Vitex megapotamica* (Spreng.) Moldenke / From the seed bank of the substrate used	29.8/0.04/ 0.09/ 5.24/ 418
28º33'26.62"S 49º27'56.19"O elevation 150 m **4 years (A4)**	Presence of contaminating materials (tailings from coal washing)/ Clay and organic material (poultry litter and peat)	*Alchornea triplinervia* (Spreng.) Müll. Arg., *Ceiba speciosa* A. St.-Hil., *Citharexylum myrianthum* Cham., *Garcinia gardneriana* (Planch. & Triana) Zappi, *Mimosa scabrella* Benth., *Phytolacca diooica* L., *Psidium cattleyianum* Sabine., *Pseudobombax grandiflorum* (Cav.) A. Robyns, *Schinus terebinthifolius* Raddi, and *Senna multijuga* (Rich.) H.S. Irwin & Barneby / *Avena sativa* L. and	43.6/0.09/ 0.01/ 5.65/1215

		Lolium multiflorum Lam.	
28º26'10.78"S 49º23'36.04"O elevation 259 m **6 years (A6)**	Presence of contaminating materials (tailings from coal washing)/ Clay and organic material (poultry litter and peat)	*Alchornea triplinervia* (Spreng.) Müll. Arg., *Allophylus edulis* (A. St.-Hil., Cambess. & A. Juss.) Radlk., *Cabraleacanjerana* (Vell.) Mart., *Caesalpinia peltophoroides* Benth., *Campomanesia xanthocarpa* O. Berg, *Cedrelafissilis* Vell., *Ceiba speciosa* A. St.-Hil., *Cupania vernalis* Cambess., *Eugenia brasiliensis* Lam., *Eugenia involucrata* DC., *Eugenia pyriformis* Cambess., *Eugenia uniflora* L., *Inga sessilis* (Vell.) Mart., *Guapira opposita* (Vell.) Reitz, *Ingastriata* sp., *Mimosa scabrella* Benth., *Myrcia* sp., *Myrcia splendens* (Sw.) DC., *Myrsine coriacea* (Sw.) R. Br. ex Roem. & Schult., *Occotea odorifera* Rohwer, *Phytolacca dioica* L., *Pseudobombax grandiflorum* (Cav.) A. Robyns, *Psidium cattleyianum* Sabine, *Schinus terebinthifolius* Raddi, *Senna macranthera* (DC. ex Collad.) H.S. Irwin & Barneby, *Tabebuia chrysotricha* (Mart. ex A. DC.) Standl., *Tabebuia umbellata* (Sond.) Sandwith, *Tabebuia heptaphylla* (Vell.) Toledo, *Tabernaemontana catharinensis* A. DC., *Trema micrantha* (L.) Blume, and *Vitex megapotamica* (Spreng.) Moldenke *Avena sativa* L. and *Lolium multiflorum* Lam.	56.1/0.13/ 0.10/ 4.81/ 610

28º35'09.30"S 49º25'25.93"O elevation 154 m **12 years**	Opencast mining, presence of sterile/ Clay and organic material (poultry litter and peat)	*Mimosa scabrella* Benth. *Melinis minutiflora* and *Urochloa humidicola*	34.9/0.04/ 0.07/ 5.11/ 976
28°22'32.1"S 49°20'31.9"W reference area (RA)		typical species of dense ombrophilous forest	not determined

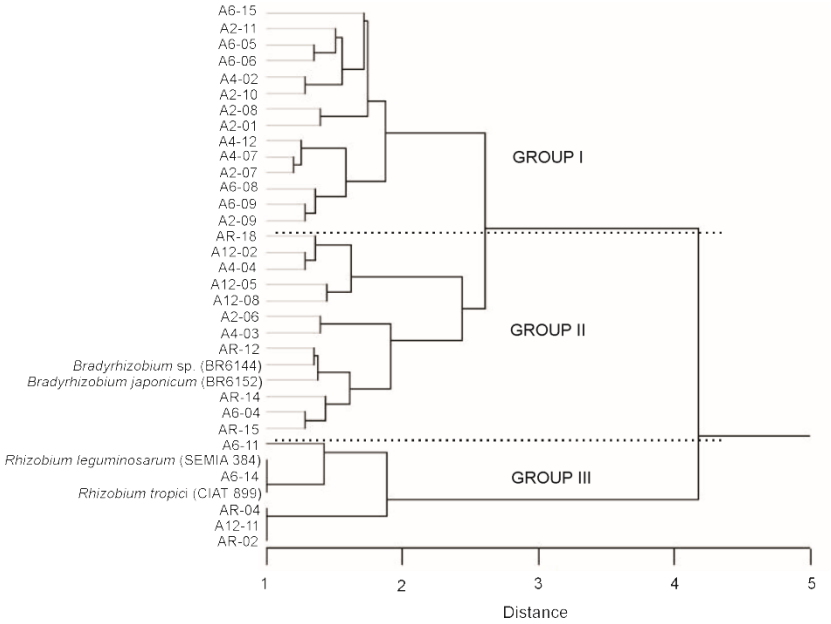

Figure S2.1 Hierarchical clustering based on morphological-cultural characteristics of rhizobia isolated from areas with different stages of recovery after coal mining in the Santa Catarina coal basin

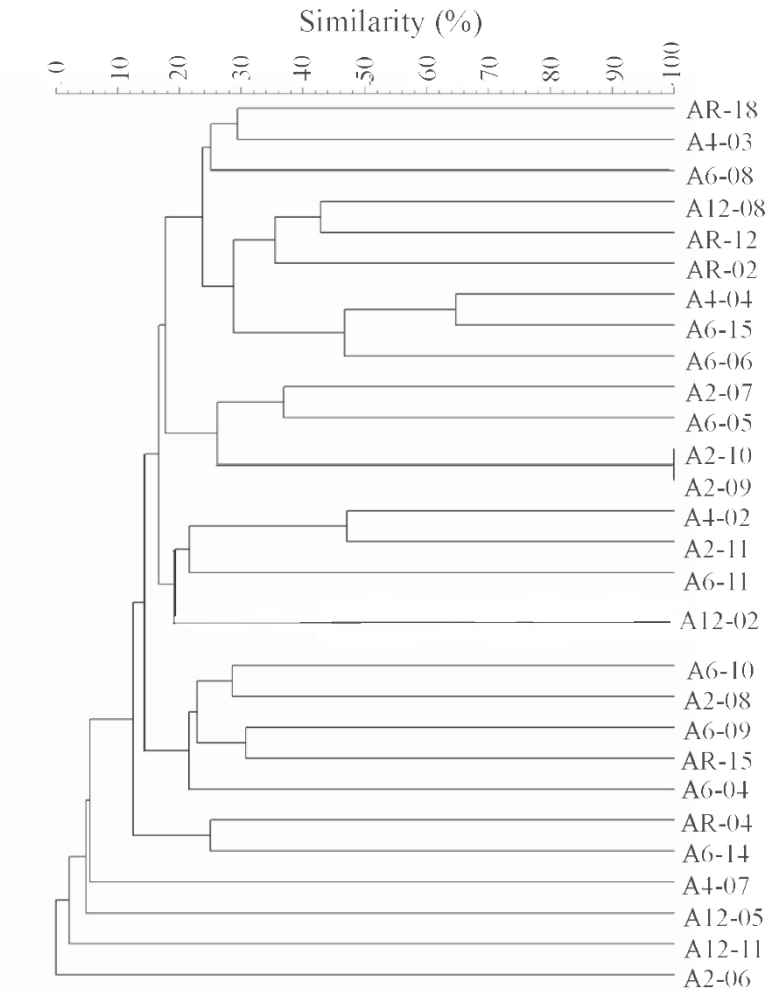

Figure S2.2 Hierarchical clustering analysis of the BOX-PCR data of rhizobia isolated from areas under different stages of recovery after coal mining in the Santa Catarina coal basin.

7 CAPÍTULO III

Manuscript 3: Original article

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Rizóbios autóctones de áreas de mineração e sua influência sobre a microbiota endofítica de leguminosas herbáceas

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Inoculation and legume microbiota

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7.1 Resumo

Atualmente, a recuperação de áreas degradadas constitui uma preocupação para a comunidade científica. Diversos esforços vêm sendo realizados no intuito de adotar práticas sustentáveis que permitam minimizar danos ao meio ambiente decorrentes do uso de fertilizantes industriais. Neste sentido, o emprego de bactérias promotoras do crescimento vegetal tem sido uma alternativa de sucesso. Entretanto, o impacto destes inoculantes na comunidade bacteriana endofitica das espécies vegetais utilizadas é ainda desconhecido. Desse modo, o objetivo do presente trabalho foi avaliar a influência da inoculação de rizóbios na microbiota endofítica de duas leguminosas herbáceas

empregadas na recuperação de áreas degradadas após a mineração do carvão por meio de dois experimentos independentes em casa de vegetação. Foram utilizados como inoculantes as estirpes UFSC-M8 e UFSC-A605 isoladas e selecionadas previamente como altamente eficientes para *Vicia sativa* e *Calopogonium mucunoides*, respectivamente. O delineamento experimental foi inteiramente casualisado, constituído em dois tratamentos, com e sem inoculação, e quatro repetições. Após 60 dias, amostras de raízes e folhas foram desinfectadas e foi realizada a extração de DNA para posterior amplificação e sequenciamento parcial do gene 16S rDNa bacteriano na plataforma MiSeq. A nível de gênero houve aumento significativo de *Sideroxydans* nas raízes e ausência de *Nocardiopsis* nas folhas para *V. sativa* nos tratamentos inoculados. Já para *C. mucunoides* houve aumento significativo de *Bradyrhizobium* nas raízes, não sendo obervadas diferenças entre os gêneros microbianos recuperados das folhas. Estes resultados proveem de forma inédita demonstrações sobre o grau de alteração que a inoculação pode ocasionar sobre a estrutura da microbiota endofítica de leguminosas em solos de áreas de mineração de carvão em recuperação.

Palavras-chave: microbiota, *Vicia sativa*, *Calopogonium mucunoides*, inoculantes, bactérias endofíticas.

7.2 Introdução

As bactérias promotoras do crescimento vegetal têm sido bastante estudadas sobretudo pela comprovada contribuição dos inoculantes microbianos na agricultura sustentável (Berg, 2009; Schlaeppi and Bulgarelli, 2015; Santoyo et al., 2016). Nas últimas décadas vários estudos têm descrito as potencialidades desse grupo também em outros ambientes como aqueles que apresentam baixa disponibilidade de nutrientes, temperaturas extremas, salinidade, acidez e contaminação com elementos traço (Glick, 2004; 2012; Nascimento et al., 2016; Young et al., 2018).

A mineração representa um dos setores básicos da economia brasileira (Farias, 2002), mas tal prática acarreta graves problemas ambientais devido, principalmente, a mudanças físicas, químicas e biológicas resultantes da remoção e deposição do solo, mistura de outros materiais e a retirada da vegetação autóctone desses locais (Gastauer et al., 2018; Silva et al., 2018).

Pesquisas envolvendo o isolamento e a seleção de rizóbios autóctones de áreas impactadas pela mineração de carvão na região Sul do Brasil têm sido realizadas pelo Laboratório de Microrganismos e Processos Biotecnológicos da UFSC. Esses estudos demonstraram que os isolados UFSC-M8, classificado como *Rhizobium* sp. e UFSC-A605, classificado como *Pseudomonas* sp. possuem elevada eficiência simbiótica para as leguminosas herbáceas *Vicia sativa* (ervilhaca) e *Calopogonium mucunoides* (calopogônio) (Hernández et al. 2017; González et al. 2018), respectivamente. Dessa forma, estes isolados bacterianos apresentam potencial de emprego como inoculantes na revegetação de áreas degradadas pela mineração de modo a reduzir total ou parcialmente a necessidade de aplicação de fertilizantes nitrogenados, os quais incidem consideravelmente no aumento dos custos do processo (Naylor, 1996), bem como podem impactar negativamente os balanços energéticos e ambientais de sistemas em recuperação.

A totalidade de microrganismos em um ambiente particular denomina-se microbiota (Schlaeppi and Bulgarelli, 2015). Nas plantas, os microrganismos podem colonizar todos os tecidos, podendo atingir densidade de células maior do que as células da própria planta (Azevedo, 2000). As bactérias endofíticas que compõem esta microbiota têm sido estudadas pelas suas diversas aplicações biotecnológicas (Van Der Lelie et al., 2009). A estrutura e composição desta microbiota podem ser influenciadas por diversos fatores, como a densidade do inóculo (Pillay and Nowak, 1997), variações sazonais (Mocali et al., 2003), tipo de solo (Fromin et al., 2001), dinâmica do habitat, entre outros.

Estudos envolvendo os efeitos da microbiota endofitica de plantas em solos degradados após o emprego de inoculantes microbianos são escassos. Alguns trabalhos descrevem o efeito que a inoculação pode ter na comunidade microbiana. Trabelsi et al. (2011) descreveram que a inoculação com rizóbios aumentou a riqueza da família Rhizobiaceae nas raízes de feijão-comum diretamente pela presença de rizóbios e indiretamente pelo efeito da rizodeposição. Em estudos envolvendo a inoculação com *Azospirillum* não foram observados efeitos significativos na estrutura das comunidades bacterianas das raízes de plantas de milho e arroz (Lerner et al., 2006). O ácido indol acético produzido por *Azospirillum* aumenta a área superficial do sistema radicular o que melhora a absorção de nutrientes como o nitrogênio, aumentando também os rendimentos. Assim, a diversidade geral da rizosfera parece ser mais influenciada pelo incremento no nitrogênio

residual do que pelo efeito direto da inoculação (García de Salamone et al., 2012). Desse modo, o presente trabalho teve como objetivo avaliar a influência da inoculação de rizóbios autóctones na microbiota endofítica de duas leguminosas herbáceas empregadas na recuperação de áreas degradadas após a mineração do carvão.

7.3 Material e Métodos

Para a realização dos experimentos foram obtidos solos de áreas impactadas pela mineração de carvão na região carbonífera de Santa Catarina. A coleta foi realizada no município de Siderópolis para o ensaio com calopogônio e no município de Treviso para o ensaio com ervilhaca. Os solos foram peneirados em malha de 2 mm e acondicionados em sacos plásticos para o transporte. A caracterização química dos mesmos foi realizada na Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (EPAGRI) (Tabela 3.1), sendo o pH do solo corrigido para 5,7 após aplicação de calcário dolomitico com PRNT de 70%.

Foram produzidos inoculantes com os isoladados UFSC-M8 e UFSC-A605 pertencentes à Coleção do Laboratório de Microrganismos e Processos Biotecnológicos da UFSC. Estes rizóbios foram obtidos utilizando plantas iscas crescidas em solos rizósfericos de áreas degradadas após mineração de carvão em Santa Catarina. As estirpes foram caracterizadas como *Rhizobium* sp. e *Pseudomona*s sp., e selecionadas por sua elevada eficiência simbiótica nas leguminosas ervilhaca e calopogônio, respectivamente (Moura et al., 2016; González et al., 2018).

Tabela 3.1 Caracterização química dos solos de áreas de mineração de carvão em recuperação utilizados para o crescimento de leguminosas herbáceas.

Local de coleta do solo	P	K	MO	pH_{H2O}	Índice SMP	Al	Ca	Mg	H^+Al
	mg kg^{-1}		% (m/v)			$cmol_c\ dm^{-3}$			
Siderópolis 28º35'49.77"S 49º26'09.16"O	11.1	76.0	1.6	4.6	5.5	0.7	5.7	3.1	7.54
Treviso 28º28'32.4"S 49º27'11.1"O	2.5	96.0	1.6	4.1	3.8	8.7	0.83	1.24	47.0

Ensaios independentes foram conduzidos para cada espécie vegetal com delineamento experimental inteiramente casualizado, sendo testados para cada planta dois tratamentos (com e sem inoculação) em que se avaliou os efeitos sobre a comunidade de bactérias endofíticas em amostra de raízes e folhas. As amostras foram assim designadas: IL (folhas de plantas inoculadas), IR (raízes de plantas inoculadas), NIL (folhas de plantas não-inoculadas) e NIR (raízes de plantas não-inoculadas). Para compor o tratamento inoculado os isolados foram preparados em meio YM (Vincent, 1970) e crescidos a 28 ^{o}C, 150 rpm durante 48 horas. Na sequência foi realizada a padronização do inóculo (10^8-10^9 UFC mL^{-1}) e a desinfecção das sementes utilizando álcool (30s), hipoclorito de sódio (2 min) e sucessivas lavagens com água destilada estéril (Hernández et al. 2018). Posteriormente, 200 sementes de cada planta foram adicionadas em 250 mL do respectivo inóculo e colocadas em agitação por 30 min antes da semeadura. Para compor o tratamento não inoculado, 200 sementes de cada planta foram submetidas ao mesmo procedimento, porém empregando meio YM estéril. Após estes procedimentos as sementes foram dispostas em vasos de 1,5 dm^3 (10 sementes/vaso), totalizando sete repetições de cada tratamento para cada planta.

Após 60 dias da implantação foram selecionados aleatoriamente quatro vasos e realizada a coleta de amostras (~1g) de folha e raízes sadias e, posteriormente, acondicionadas em sacos plásticos estéreis e armazenadas a -80 ^{o}C até seu processamento. Realizou-se a desinfecção dos tecidos vegetais para eliminação dos microrganismos epifíticos (Hernández et al. 2018). Em seguida o material foi macerado em almofariz com nitrogênio líquido e 100 mg de cada amostra foi adicionada em microtubos e realizada a extração de DNA utilizando o kit DNeasy Plant Mini Kit (Qiagen, Germany), seguindo as instruções do fabricante. A concentração de DNA foi determinada utilizando Nanodrop e cada amostra foi ajustada para uma concentração final de 100 ng μL^{-1}. Foi amplificado um fragmento de 250 bases da região hipervariável V4 do gene ribossomal 16S rRNA utilizando-se os iniciadores universais 515F e 806R e as seguintes condições de PCR: 94 °C por 3 min; 18 ciclos de 94 °C por 45 seg, 50 °C por 30 seg e 68 °C por 60 seg; seguido de 72 °C por 10 min. A partir destes amplificados foi construída a biblioteca metagenômica utilizando-se o kit comercial "Nextera DNA Library Preparation Kit" da Illumina®. Os amplificados foram reunidos em pools e posteriormente sequenciados no sequenciador "MiSeq" da plataforma Illumina® (Degnan and Ochman, 2012).

As sequências obtidas foram analisadas na plataforma QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010; 2011), seguindo-se um fluxo de trabalho desde a remoção de sequências de baixa qualidade, filtração, remoção de quimeras e classificação taxonômica. As sequências foram classificadas em gêneros bacterianos pelo reconhecimento de unidades taxonômicas operacionais (OTUs) com identidade ≥97%. Para comparar as sequências foi utilizada a atualização Silva 128 do ano 2017 do banco de dados de sequências ribossomais SILVA database (Yilmaz et al., 2013).

Para gerar a classificação das comunidades bacterianas por identificação de OTUs foram utilizadas 18.500 leituras por amostra para ervilhaca e 10.500 leituras para calopogônio, isso com a finalidade de normalizar os dados e não comparar amostras com diferente número de leituras, evitando dessa forma um erro metodológico. Posteriormente, OTUs identificados como cloroplastos e mitocôndria foram eliminados da tabela com a classificação taxonômica gerada no QIIME. Dessa forma foi refeita a análise de abundância utilizando-se 107 e 67 leituras por amostra para ervilhaca e calopogônio, respectivamente.

Para as análises estatísticas foram utilizados os resultados de sequenciamento de quatro réplicas por tratamento (com exceção do grupo IL do qual foram eliminadas duas amostras de ervilhaca por apresentarem um número muito baixo de leituras no sequenciamento). O programa estatístico utilizado foi o STAMP: Statistical Analysis of Metagenomic Profiles (Parks et al., 2014). Para determinar se as médias dos filos e gêneros bacterianos apresentavam a mesma abundância entre os tratamentos foram comparados pelo teste de ANOVA com posterior teste *post hoc* Tukey-Kramer, com nível de confiança de 95%. Por sua vez o teste de Welch foi utilizado para comparar a média entre os pares de tratamentos ($P<0.05$). As médias referentes à biodiversidade foram comparadas usando o pacote stats e ggplot2 do R pelo número de unidades taxonômicas operacionais observadas (OTUs) e ao índice Chao1 pelo teste de Kruskal Wallis ($P<0.05$), por apresentarem distribuição não paramétrica.

7.4 Resultados

Em ervilhaca foram identificados um total de 21 filos do Domínio Bacteria e um filo do Domínio Archaea (Tabela S3.1). Os filos mais abundantes foram *Proteobacteria*, que representou 43,32% do total das sequências obtidas, seguido de *Firmicutes* com 27,24% e *Actinobacteria* com 19,95%. Foram verificadas diferenças significativas na abundância

relativa apenas nos filos *Proteobacteria* e *Firmicutes* (Figura 3.1A). *Proteobacteria* foi o filo mais abundante nas raízes (Figura 3.1B), enquanto Firmicutes foi o mais abundante nas folhas (Figura 3.1C).

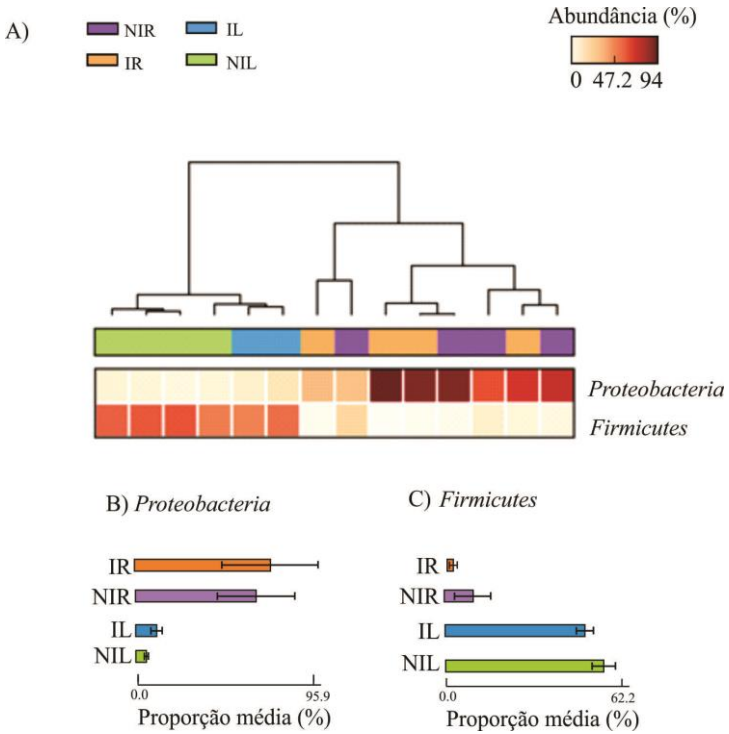

Figura 3.1 Dendograma e *heatmap* baseado na abundância relativa dos filos significativamente diferentes da comunidade endofítica bacteriana associada a raízes e folhas de *Vicia sativa* (A). Média e desvio padrão da abundância de *Proteobacterias* (B) e *Firmicutes* (C) em folhas inoculadas (IL), raízes inoculadas (IR), folhas não inoculadas (NIL) e raízes não inoculadas (NIR).

Por sua vez, em calopogônio foram identificados 20 filos do Domínio Bacteria e dois filos do Domínio Archaea (Tabela S3.2). Das bactérias, os filos mais abundantes foram *Proteobacteria* que representou 82,76%, *Firmicutes* com 2,94%, e *Actinobacteria* com 2,83%. Foram observadas, a nível de filo, diferenças significativas na abundância relativa apenas no filo *Firmicutes*. Constatou-se que *Firmicutes* foi o filo mais abundante nas folhas (Figura 3.2).

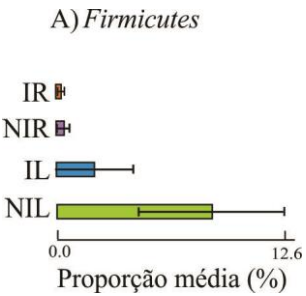

Figura 3.2 Média e desvio padrão da abundância de *Firmicutes* na comunidade endofítica bacteriana associada a raízes e folhas de *Calopogonium mucunoides* (A) em folhas inoculadas (IL), raízes inoculadas (IR), folhas não inoculadas (NIL) e raízes não inoculadas (NIR).

Em ervilhaca, na análise de componentes principais (PCA) o eixo PC1 explica 87.5% das diferenças nas comunidades bacterianas na planta a nível de filo (Figura 3.3A). Nota-se um agrupamento definido das amostras por órgãos (folha ou raiz), destacando-se a pouca dispersão das amostras das folhas. Já para o calopogônio observou-se que o eixo PC1 explica 77.6% das diferenças nas comunidades bacterianas a nível de filo (Figura 3.3B). Nesse caso, as amostras dos grupos IR e NIR apresentaram agrupamento evidente, ao contrário das amostras de folhas que apresentaram maior dispersão.

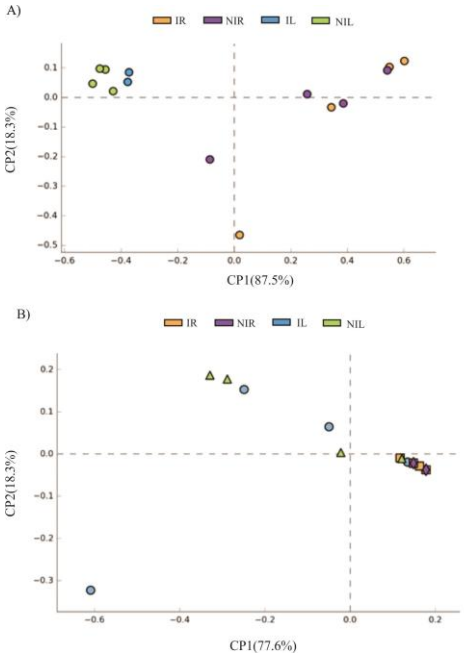

Figura 3.3 Análise de componentes principais (ACP) baseada no método de distâncias Bray-Curtis entre amostras de *Vicia sativa* (A) e *Calopogonium mucunoides* (B), em folhas inoculadas (IL), raízes inoculadas (IR), folhas não inoculadas (NIL) e raízes não inoculadas (NIR).

Em ervilhaca foram identificados 318 gêneros bacterianos (Tabela S3.3), sendo os mais abundantes *Burkholderia-Paraburkholderia* que representou 11,27% de todas as sequências, seguido por *Sideroxydans* (6,98%), *Salinicoccus* (5,11%) e por *Staphylococcus* (4,94%). No total foram identificados 15 gêneros com abundância significativamente diferente entre todos os tratamentos (Figura 3.4A). Entretanto, a comparação entre os pares de tratamentos com e sem inoculação mostrou que houve aumento significativo do gênero *Sideroxydans* nas raízes inoculadas (Figura 3.4B), enquanto que a inoculação aparentemente levou a não detecção do gênero *Nocardiopsis* nas folhas da ervilhaca (Figura 3.4C).

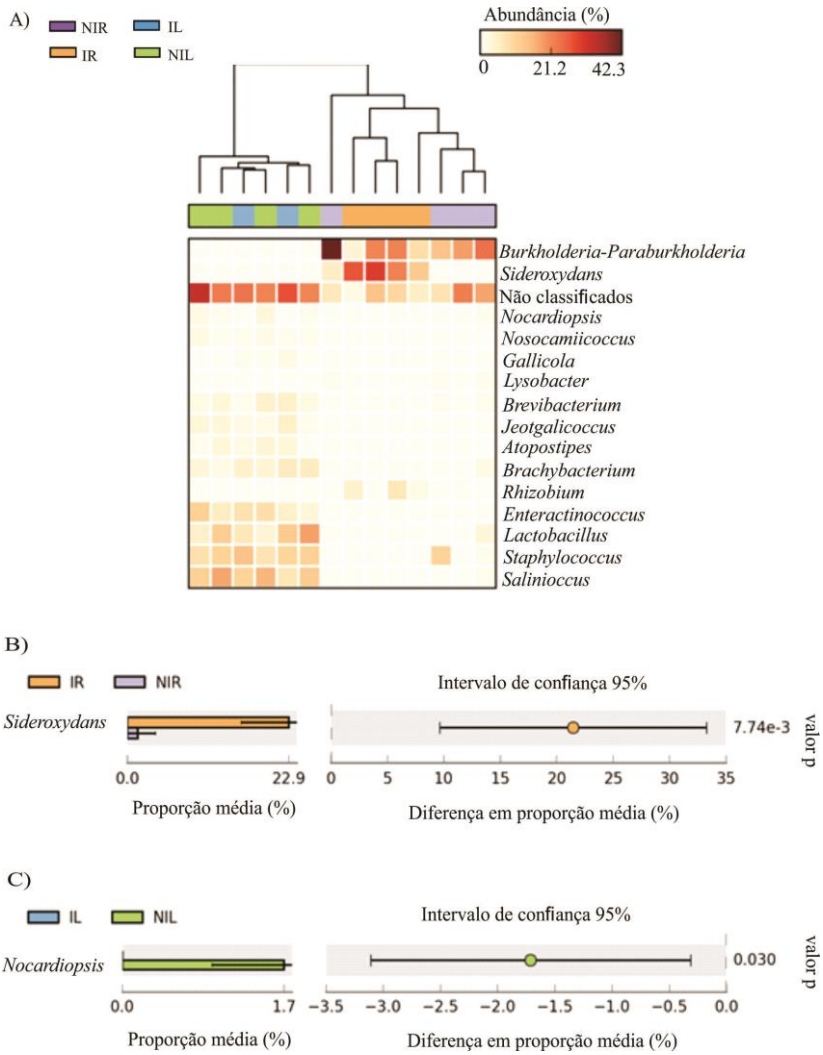

Figura 3.42 Dendograma e *heatmap* baseado na abundância relativa aos gêneros significativamente diferentes da comunidade endofítica bacteriana associada a raízes e folhas de *Vicia sativa* (A). Média, desvio padrão da abundância e representação gráfica do teste post hoc Tukey-Kramer ($P<0.05$) de *Sideroxydans* (B) e *Nocardiopsis* (C) em folhas inoculadas (IL), raízes inoculadas (IR), folhas não inoculadas (NIL) e raízes não inoculadas (NIR).

Já em calopogônio foram identificados 273 gêneros do Domínio Bacteria e cinco gêneros do Domímio Archeae (Tabela S3.4). Os gêneros bacterianos mais abundantes foram *Bradyrhizobium*, que representou 27,12% de todas as sequências, seguido por um gênero não identificado da família *Bradyrhizobiaceae* (26,08%), *Rickettsia* (6,94%), e por "*Candidatus* Portiera" (5,61%). Além disso, foram identificados quatro gêneros com abundância significativamente diferente entre todos os tratamentos, sendo eles *Acinetobacter*, "*Candidatus* Portiera*"*, *Rickettsia* e *Bradyrhizobium* (Figura 3.5A). A comparação entre pares de tratamentos mostrou aumento significativo do gênero *Bradyrhizobium* nas raízes do tratamento IR quando comparado ao grupo NIR (Figura 3.5B). Também foi observado uma diminuição significativa de "Não Classificados" (soma de vários táxons identificados como não classificados (Figura 3.5C) nas raízes analisadas. Em relação as folhas não foram verificadas diferenças significativas entre os tratamentos IL e NIL nas condições avaliadas.

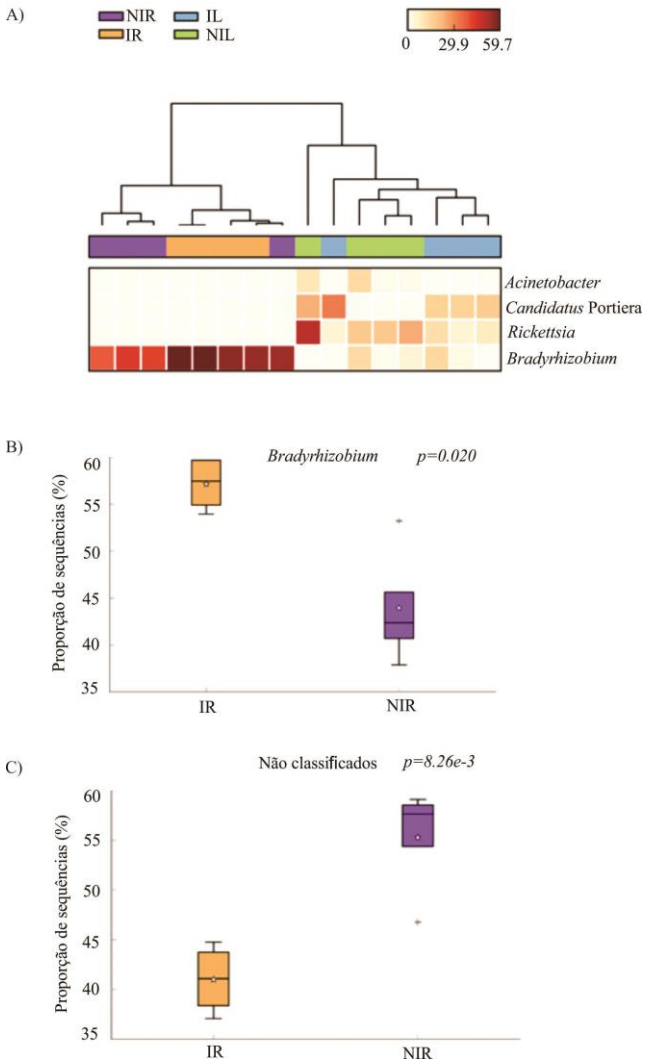

Figura 3.5 Dendograma e *heatmap* baseado na abundância relativa aos gêneros significativamente diferentes da comunidade endofítica bacteriana associada a raízes e folhas de *Calopogonium mucunoides* (A). Média e desvio padrão da abundância de *Bradyrhizobium* (B) e Não classificados (C) após ANOVA e posterior teste post hoc Tukey-Kramer ($P<0.05$), em folhas inoculadas (IL), raízes inoculadas (IR), folhas não inoculadas (NIL) e raízes não inoculadas (NIR).

Referente à biodiversidade das comunidades bacterianas, tanto em ervilhaca (Figura 3.6) quanto em calopogônio (Figura 3.7) não foram observadas diferenças significativas entre os tratamentos IL vs. NIL e IR vs. NIR para nenhum dos dois índices de riqueza avaliados (OTU e Chao1).

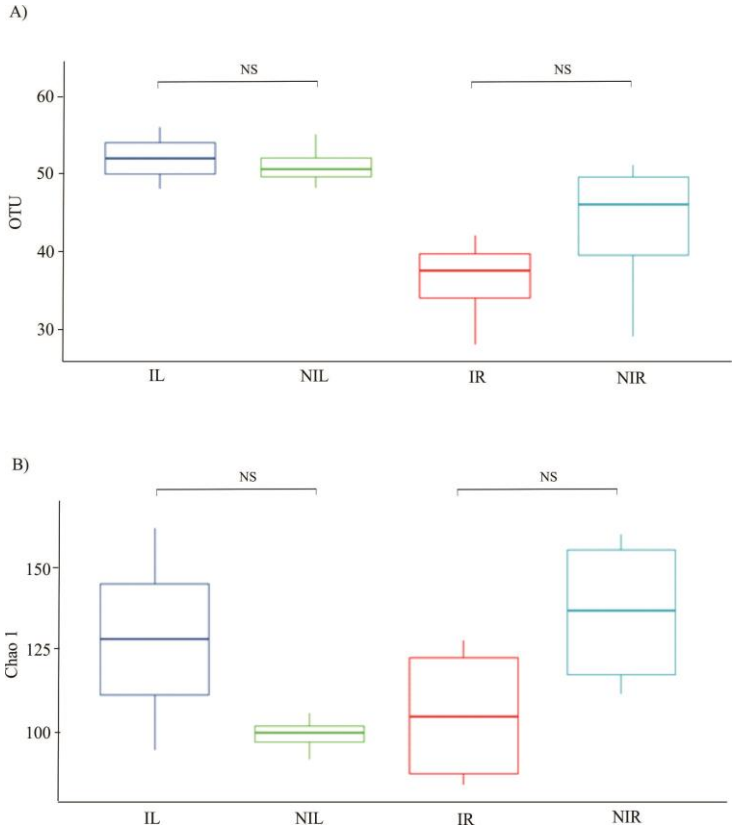

Figura 3.6 Índices de riqueza OTUs (A) e Chao1 das comunidades endofíticas bacterianas associadas a raízes e folhas de *Vicia sativa*. Mediana e quartis são apresentadas. NS = Não houve diferença significativa entre tratamentos pelo teste de Kruskal Wallis ($P < 0.05$).

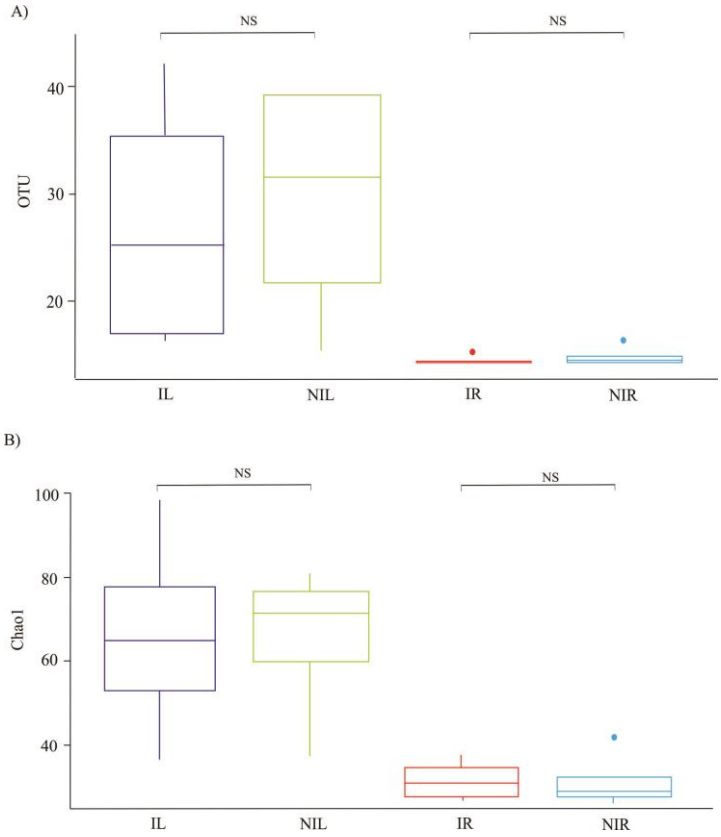

Figura 3.7 Índices de riqueza OTUs (A) e Chao1 das comunidades endofíticas bacterianas associadas a raízes e folhas de *Calopogonium mucunoides*. Mediana e quartis são apresentadas. NS = Não houve diferença significativa entre tratamentos pelo teste de Kruskal Wallis ($P < 0.05$).

7.5 Discussão

A inoculação com bactérias bacterias fixadoras de N é uma técnica amplamente estudada e com evidências de que pode contribuir em até dez vezes com a diminuição dos custos decorrentes da adubação (ANPI, 2018). Além disso, é uma prática mais amigável com o meio, pois diminui o grande impacto ambiental ocasionado pelo uso dos fertilizantes industriais (Brígido and Glick 2015). Alguns estudos têm

descrito que em ambientes estressantes, como em solos com elevados teores de elementos traços, ocorre uma redução significativa da fixação biológica de nitrogênio de várias leguminosas (Broos et al., 2004; Wani et al., 2007; 2008) devido fundamentalmente à diminuição na população de bactérias fixadoras de nitrogênio (Alexander et al., 1999; Younis, 2007). Desta forma, encontrar isolados eficientes em ambientes estressantes torna-se um desafio. Estudos prévios no nosso grupo de pesquisa permitiram o isolamento de duas estirpes altamente eficientes para a inoculação de calopogônio e ervilhaca (González et al., 2018; Hernández et al., 2017). Esses resultados sugerem a utilização dessas estirpes como potenciais inoculantes já que ambas leguminosas são recomendadas para revegetação de áreas impactadas pela mineração de carvão.

Historicamente, os impactos relacionados à inoculação têm sido negligenciados. Sabe-se que o aumento da concentração microbiana após inoculação pode produzir uma perturbação ao menos transiente do equilíbrio das comunidades do solo (Trabelsi and Mhamdi 2013). Informações sobre a influência da inoculação de rizóbios autóctones de áreas de mineração na microbiota das plantas utilizadas em programas de revegetação são necessárias para o desenvolvimento de novos inoculantes microbianos a serem empregados na promoção do crescimento de plantas. Sendo assim, se fazem necessários estudos que abordem esta temática e que esclareçam se a inoculação é uma prática justificada.

No presente trabalho observou-se que o filo *Proteobacteria* foi o mais abundante para as duas espécies vegetais testadas. Tal fato era esperado, pois este é o maior filo em diversidade metabólica, constituído por bactérias com importância agrícola, industrial e médica, as quais podem ser encontradas nos mais diversos ambientes. As bactérias deste grupo são conhecidas por ter estratégia tipo r, responder prontamente às diferentes fontes de carbono, e ser de rápido crescimento (Pieffer et al., 2013). Vários trabalhos descrevem a presença deste filo em inúmeras espécies de plantas (Philippot et al., 2013; Edwards et al., 2015; Hong et al., 2015). Bulgarelli et al. (2013) relatam que esta abundância é uma resposta quimiostatica à rizodeposição.

Firmicutes apresentou a segunda maior abundância, sendo majoritário nos grupos IL e NIL, sugerindo um domínio deste filo na microbiota das folhas. Os *Firmicutes* compõem um grupo de bactérias de alta diversidade fenotípica e metabólica, que inclui espécies aeróbias, anaeróbias, termofílicas, fototróficas, entre outras (Madsen, 2008). Estes resultados coincidem com Mitter et al. (2017) que encontraram o filo

Firmicutes com elevada abundância em folhas de trevo e cevada crescidas em uma área de recuperação de areias petrolíferas. Além disso, esses autores relataram uma marcada dispersão nas amostras que representam a comunidade da endosfera da raiz de trevo, que coincide com o presente trabalho para a ervilhaca. Os resultados deste estudo sugerem que para cada planta existe diferenças entre as microbiotas de folhas e de raízes.

Os gêneros mais abundantes identificados no presente estudo foram *Bradyrhizobium* e *Burkholderia-Paraburkholderia* para calopogônio e ervilhaca, respectivamente. O gênero *Bradyrhizobium* tem sido amplamente estudado para fins agrícolas, uma vez que compreende as espécies que nodulam soja (Peix et al., 2015). Madigan et al. (2012) descreveram o gênero *Burkholderia* como muito heterogêneo, abrangendo desde espécies patogênicas ao homem, plantas e animais, até espécies muito eficientes no controle biológico (Beneduzi et al., 2013).

As diferenças significativas na abundância relativa a nível de filos foram em *Proteobacteria* para ervilhaca e em *Firmicutes* para ambas a espécies vegetais. Em relação à comparação entre os pares de tratamento após inoculação, em ervilhaca houve aumento significativo de representantes do gênero *Sideroxydans* e não detecção de representantes do gênero *Nocardiopsis* nas folhas. Deste modo, é preciso avaliar quais são as implicações dessa seletividade de gêneros para a atividade metabólica da ervilhaca. Estudos envolvendo o microbioma poderão ajudar a esclarecer neste sentido, uma vez que técnicas metagenômicas permitem identificar genes e as rotas metabólicas em diferentes condições.

As bactérias que compõem o gênero *Sideroxydans* são vibrios gram negativos, microaerófilos, com duas espécies descritas atualmente (Weiss et al., 2015). As mesmas têm sido associadas aos ambientes com toxicidade, como áreas de mineração, por possuírem a capacidade de derivar a energia para seu crescimento a partir da oxidação do Fe. Estas bactérias oxidam o Fe de forma extracelular, evitando o acúmulo de óxidos de ferro dentro de suas células (Liu et al., 2012). A drenagem ácida de mina (DAM) é formada pela oxidação contínua de ferro ferroso em férrico, essas bactérias podem contribuir para a redução do ferro e de parte da sua carga via oxidação do ferro ferroso com subsequente precipitação destes minerais a hidroxisulfato de ferro amorfo (Janneck et al., 2010). Sendo assim, poderia ser esperada a presença destas bacterias em solos de areas de mineração.

O gênero *Nocardiopsis* inclui actinobactérias aeróbias, gram positivas, com colônias rugosas, bem desenvolvidas que formam esporos, sendo catalase-positivos (Bennur et al., 2015). Até o momento foram descritas 14 espécies deste gênero, sendo o solo apontado como seu habitat natural (Kroppenstedt and Evtushenko, 2006). Entretanto, também têm sido encontrado como endofítico de folhas com atividade antagônica contra vários microrganismos patogênicos por meio da produção de compostos antimicrobianos (Bouras et al., 2015). Desta forma não seria inesperada a presença deste gênero nas folhas das espécies avaliadas. No presente trabalho também foi verificado aumento significativo de representantes do gênero *Bradyrhizobium* em calopogônio. Estes resultados vão ao encontro daqueles de Zhang et al. (2011) que descreveram que após a inoculação com rizóbios houve modificação na composição dos grupos taxonômicos da comunidade microbiana do solo aumentando o gênero *Bradyrhizobium.*

Andreote (2007) relatou que a microbiota endofítica é influenciada apenas nos estádios iniciais do desenvolvimento da planta, havendo um efeito transitório que parece ser neutralizado em estádios posteriores. Isto poderia explicar porque após os 60 dias não foram constatadas diferenças significativas em relação aos gêneros *Rhizobium* e *Pseudomonas*, as quais foram utilizadas para a inoculação de ervilhaca e calopogônio, respectivamente. Kennedy (1999) menciona que a modificação na estrutura da comunidade microbiana pode ser diminuída pela resiliência do ecossistema. A perda ou ganho de algumas bactérias pode não alterar o funcionamento do sistema devido à redundância funcional bacteriana (Trabelsi and Mhamdi 2013).

A diversidade microbiana recuperada a partir de técnicas de sequenciamento apresenta limitações permitindo que mudanças no número e na composição possam ser superou subestimadas devido fundamentalmente à etapa de amplificação (Lundberg et al., 2012). No presente trabalho foram obtidas poucas leituras após a retirada das OTUs correspondentes aos cloroplastos. Esta limitação poderia ser contornada em próximos trabalhos com a utilização do iniciador 799F, o qual foi desenhado para evitar o amplificação de células de cloroplasto ao invés de *Cyanobacterias* (Chelius and Triplett, 2001). Os dados aqui apresentados proveem de forma inédita esclarecimentos sobre alterações que a inoculação pode ocasionar na estrutura da microbiota endofítica de leguminosas em solos de áreas de mineração de carvão em recuperação.

7.6 Conclusões

Em *Vicia sativa* inoculadas com o isolado UFSC-M8 (*Rhizobium* sp.) houve aumento de representantes do gênero *Sideroxydans* nas raízes e não detecção de *Nocardiopsis* nas folhas. Já nas raízes de *Calopogonium mucunoides* inoculadas com UFSC-A6-05 (*Pseudomonas* sp.) verificou-se um aumento de representantes do gênero *Bradyrhizobium.*

Estudos futuros são necessários para compreender como essa mudança influencia as capacidades funcionais das comunidades microbianas destas leguminosas em ambientes degradados pela mineração de carvão e quais são as implicações para o ambiente em recuperação.

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7.8 Material Suplementar

Tabela S3.1 Filos e abundância relativa da comunidade endofítica associada a raízes e folhas de *Vicia sativa*

Phylum	NIR1	NIR2	NIR3	NIR4	NIL5	NIL6	NIL7	NIL8	IR9	IR10	IR11	IR12	IL14	IL15	Total	%Freq
Proteobacteria	0,74	0,36	0,92	0,63	0,08	0,06	0,07	0,05	0,35	0,86	0,72	0,96	0,11	0,16	6,06	43,32
Firmicutes	0,05	0,20	0,05	0,10	0,57	0,59	0,50	0,61	0,01	0,02	0,07	0,00	0,49	0,55	3,81	27,24
Actinobacteria	0,14	0,38	0,02	0,11	0,19	0,23	0,22	0,25	0,59	0,06	0,17	0,01	0,20	0,20	2,79	19,95
Acidobacteria	0,02	0,01	0,00	0,07	0,03	0,04	0,07	0,01	0,01	0,02	0,02	0,00	0,08	0,05	0,44	3,14
Bacteroidetes	0,03	0,04	0,00	0,04	0,02	0,01	0,04	0,01	0,03	0,02	0,01	0,02	0,00	0,00	0,26	1,84
Verrucomicrobia	0,00	0,01	0,00	0,01	0,03	0,01	0,02	0,01	0,00	0,00	0,00	0,00	0,05	0,01	0,16	1,12
Gemmatimonadetes	0,00	0,00	0,00	0,01	0,02	0,00	0,03	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,08	0,55
Nitrospirae	0,00	0,00	0,00	0,01	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,02	0,02	0,07	0,47
Chloroflexi	0,00	0,00	0,00	0,00	0,01	0,02	0,00	0,01	0,00	0,00	0,01	0,00	0,01	0,00	0,06	0,43
Tectomicrobia	0,00	0,00	0,00	0,00	0,03	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,05	0,33
Planctomycetes	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,04	0,28
Latescibacteria	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,13
Spirochaetae	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,09
GAL15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,08
Cyanobacteria	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,07
Armatimonadetes	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,03
Deinococcus-Thermus	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,03
Chlamydiae	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02
Tenericutes	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Elusimicrobia	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Saccharibacteria	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01

Tabela S3.2 Filos e abundância relativa da comunidade endofítica associada a raízes e folhas de *Calopogonium mucunoides*.

Phylum	NIL3	NIR4	NIL5	NIR6	NIL7	NIR8	NIL9	NIR10	IL11	IR12	IL13	IR16	IL17	IR18	IL19	IR20	Total	%Freq
Proteobacteria	0,56	0,99	0,89	1,00	0,79	1,00	0,48	0,99	0,37	0,98	0,95	1,00	0,54	0,99	0,72	1,00	13,24	82,76
Firmicutes	0,04	0,00	0,08	0,00	0,13	0,00	0,09	0,00	0,01	0,00	0,01	0,00	0,06	0,00	0,04	0,00	0,47	2,94
Actinobacteria	0,12	0,00	0,01	0,00	0,01	0,00	0,07	0,00	0,01	0,01	0,02	0,00	0,16	0,00	0,03	0,00	0,45	2,83
Acidobacteria	0,09	0,00	0,00	0,00	0,00	0,00	0,12	0,00	0,04	0,01	0,00	0,00	0,05	0,00	0,03	0,00	0,34	2,15
Bacteroidetes	0,03	0,00	0,00	0,00	0,01	0,00	0,07	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,06	0,00	0,20	1,26
Gemmatimonadetes	0,03	0,00	0,00	0,00	0,00	0,00	0,07	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,13	0,78
Chloroflexi	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,05	0,00	0,02	0,00	0,12	0,72
Thaumarchaeota	0,03	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,07	0,00	0,11	0,69
Verrucomicrobia	0,03	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,01	0,00	0,00	0,00	0,03	0,00	0,02	0,00	0,10	0,65
Planctomycetes	0,03	0,00	0,00	0,00	0,00	0,00	0,06	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,09	0,56
Nitrospirae	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,03	0,19
BRC1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,00	0,00	0,00	0,02	0,15
Tectomicrobia	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,00	0,00	0,02	0,15
Other	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,00	0,01	0,05
Fusobacteria	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,03
Latescibacteria	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Tenericutes	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01
Candidate TM6	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01

Tabela S3.3 Resumo dos táxons bacterianos identificados em *Vicia sativa* com frequência superior a 1%.

Táxon	%Freq
Burkholderia-Paraburkholderia	11,27
Sideroxydans	6,98
Enterobacteriaceae	6,43
Salinicoccus	5,11
Staphylococcus	4,94
Lactobacillus	3,95
Corynebacterium	3,45
Ralstonia	3,31
Enteractinococcus	2,97
Bacillaceae	2,81
Amycolatopsis	2,48
Streptomyces	2,17
Brachybacterium	1,91
Bacillaceae uncultured	1,84
Pseudomonas	1,58
Brevibacterium	1,50
Methylobacterium	1,40
Actinomadura	1,23
Rhizobium	1,10
Bradyrhizobiaceae	1,07
Outros	32,50

Tabela S.4 Resumo dos táxons bacterianos identificados em *Calapogonium mucunoides* com frequência superior a 1%

Táxon	**%Freq**
Bradyrhizobium	27,12
Bradyrhizobiaceae	26,08
Rickettsia	6,94
Candidatus Portiera	5,61
Sphingomonas	2,34
Pseudomonas	2,18
Rickettsiaceae	1,74
Candidatus Hamiltonella	1,40
Acinetobacter	1,20
Methylobacterium	1,03
Outros	24,34