UNIVERSIDADE FEDERAL DE VIÇOSA

MOMOKO KAYASHIMA

SEEDLINGS PRODUCTION OF BRAZILIAN NATIVE SPECIES BY INOCULATION OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT GROWTH-PROMOTING RHIZOBACTERIA

VIÇOSA – MINAS GERAIS
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Dissertação apresentada à Universidade Federal de Viçosa como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

Orientadora: Maria Catarina Megumi Kasuya
Coorientadores: Paulo Prates Júnior
Marliane de Cássia Soares da Silva
Cynthia Canedo da Silva

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Maria Catarina Megumi Kasuya
Orientadora

I dedicate

To God

To my family

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ABSTRACT

KAYASHIMA, Momoko, M.Sc., Universidade Federal de Viçosa, June 2021. **Seedling production of Brazilian native species by inoculation of arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria.** Adviser: Maria Cataria Megumi Kasuya. Co-advisers: Paulo Prates Júnior, Marliane de Cássia Soares da Silva and Cynthia Canêdo da Silva.

The success of reforestation contributes to biodiversity, and the use of seedlings of native species with good quality in these areas is essential. In this study, three native Brazilian plants were focused: macauba (*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.), canafistula (*Peltophorum dubium* (Spreng.) Taub.) and pau-viola (*Citharexylum myrianthum* Cham.). These plants are considered pioneer species, and biological inputs such as microbial inoculants in the soil are significant to the seedling establishment that can be applied to develop high-quality seedling production. Thus, the objective of this work was to evaluate the seedling's growth inoculated with beneficial microorganisms, arbuscular mycorrhizal fungi (AMF), produced by on-farm method, and Plant Growth Promoter Rhizobacteria (PGPR), isolated from native Brazilian tree species. We assessed the seedlings growth and microbial community in the soil by PCR-DGGE technique. We detected no effect of AMF inoculation in the biomass accumulation of macauba, but higher AMF colonization was confirmed when we inoculated in the early stage. Additionally, it was revealed that 90 % of similarity on the AMF community in the soil between inoculated seedlings and non-inoculated ones by the PCR-DGGE technique, which probably explain why the plant biomass was not accumulated by AMF inoculation. Conversely, AMF inoculation increased canafistula and pau-viola seedling's growth. Canafistula seedling also responded positively to PGPR inoculation as well as the combination of AMF and PGPR inoculation. Consequently, the AMF and PGPR inoculation contribute to the seedling's production with sustainability and soil quality. Therefore, we assessed that the different responses to inoculation with FMA and PGPR among native forest species. Additional studies are required to investigate the effect of bioinoculant on the seedling's growth and the tolerate biotic and abiotic conditions in the field.

Keywords: Beneficial microorganism. Symbioses. On-farm.

RESUMO

KAYASHIMA, Momoko, M.Sc., Universidade Federal de Viçosa, junho de 2021. **Seedling production of Brazilian native species by inoculation of arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria.** Orientadora: Maria Cataria Megumi Kasuya. Coorientadores: Paulo Prates Júnior, Marliane de Cássia Soares da Silva e Cynthia Canêdo da Silva.

O sucesso do reflorestamento contribui para a biodiversidade, e o uso de mudas de espécies nativas com boa qualidade nessas áreas é fundamental. Neste estudo, três plantas nativas brasileiras foram enfocadas: macaúba (*Acrocomia aculeata* (Jacq.) Lodd. Ex Mart.), canafístula (*Peltophorum dubium* (Spreng.) Taub.) e pau-viola (*Citharexylum myrianthum* Cham.). Essas plantas são consideradas espécies pioneiras e os insumos biológicos, como inoculantes microbianos no solo, são significativos para o estabelecimento de mudas, podendo ser aplicados no desenvolvimento de uma produção de mudas de alta qualidade. Assim, o objetivo deste trabalho foi avaliar o crescimento de mudas inoculadas com microrganismos benéficos, fungos micorrízicos arbusculares (FMA), produzidos pelo método *on-farm*, e rhizobactérias promotoras do crescimento de plantas (PGPR), isolados de espécies arbóreas nativas brasileiras. Avaliamos o crescimento das mudas e a comunidade microbiana no solo pela técnica de PCR-DGGE. Não detectamos efeito da inoculação de FMA no acúmulo de biomassa de macaúba, mas alta colonização de FMA foi confirmada quando inoculamos no estágio inicial. Adicionalmente, foi revelado que 90% de similaridade na comunidade de FMA no solo entre mudas inoculadas e não inoculadas pela técnica de PCR-DGGE, o que provavelmente explica porque a biomassa vegetal não foi acumulada pela inoculação de FMA. Por outro lado, a inoculação de FMA aumentou o crescimento de mudas de canafístula e pau-viola. Mudas de canafístula também responderam positivamente à inoculação de PGPR, bem como à combinação de inoculação de FMA e PGPR. Consequentemente, a inoculação de AMF e PGPR contribuem para a produção de mudas com sustentabilidade e qualidade do solo. Portanto, avaliamos as diferentes respostas à inoculação com FMA e PGPR entre as espécies florestais nativas. Estudos adicionais são necessários para investigar o efeito do bioinoculante no crescimento da muda e as condições bióticas e abióticas toleradas no campo.

Palavras-chave: Microrganismo benéfico. Simbioses. O*n-farm*.

SUMMARY

INTRODUCTION

The global human population will reach 9 billion by 2050 (Prasad et al., 2019). As human activity has increased, we face more environmental problems such as climate change and deforestation, which make a range of impacts in ecosystems from a local to world-scale (Potapov et al., 2017). Large areas of the original forests have been cleared for fuel and buildings and make way for cultivation (Ryan et al., 2012). Deforestation results in damage to the ecosystems and decreases the biological diversity of these areas (Gavassi et al., 2018), representing one of the environmental challenges of our time.

Conversely, reforestation, which is reestablishing trees where they once dominated (Novick & Katul, 2020), has been proposed as a forest establishment, climate mitigation strategy, and protection of the ecosystem (Zhang et al., 2020). The success of reforestation contributes to biodiversity. As exotic tree species used to be fast growth (Günter et al., 2009), seedlings with good quality of native species in these areas are essential. In this study, three native Brazilian plants were focused: macauba (*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.), canafistula (*Peltophorum dubium* (Spreng.) Taub.) and pau-viola (*Citharexylum myrianthum* Cham.). These plants are some of the notable species for reforestation of degraded areas due to their acclimation capacity in a wide range of environmental conditions (Bazzo et al., 2018; Tenfen et al., 2018; Marques et al., 2019), and they can be used for reforestation as pioneer species (Bazzanella et al., 2019).

The quality and rapid establishment of seedlings are prerequisites for reforestation (Riikonen & Luoranen, 2018). Biological inputs such as microbial inoculants in the soil are significant to the seedling establishment contributing to developing high-quality seedling production (Tiepo et al., 2020). Microorganisms, such as arbuscular mycorrhizal fungi (AMF) (Prates Júnior et al., 2019, Prates Júnior et al., 2021a) and Plant Growth Promoter Rhizobacteria (PGPR) (Li et al., 2020), used to promote seedlings growth and also represent a replacement

for the chemical fertilizers and pesticides, which in turn contribute to the soil biodiversity, playing an essential role in soil health (Karličić et al., 2016).

The importance of the association of trees with mycorrhizal fungi is highlighted (Averill et al., 2014) to understand the complexity of symbioses that drives physiological alterations (Venkateshwaran et al., 2013). These fungi can have the most significant effect on plant performance, functioning as an extension of the root system and increasing the absorption area. Plants that grow in soils deficient in phosphorus (P) develop strategies to acquire this element, such as symbiotic associations with AMF. AMF increase the absorption of water and nutrients by the plant and the plant's tolerance to extreme environments such as those affected by tailings and mining waste (Goetten et al., 2016).

AMF contribute to the stability of soil aggregates, primarily due to the physical effect, due to the formation of a mycelial network around soil particles, which increases the resistance to soil erosion (Magurno et al., 2019). It has been suggested that the glomalin content of the soil is related to changes associated with land use and the rehabilitation of degraded soils (Lopes Leal et al., 2016).

The combination of AMF and beneficial bacteria, such as PGPR favors agricultural and forestry cultivations and areas undergoing environmental restoration (Prates Júnior et al., 2021b). PGPR can directly promote plant growth by either facilitating nutrient acquisition of certain plant nutrients from the environment, such as nitrogen, phosphorus, potassium, and essential minerals (Ortega et al., 2017), or modulating plant hormone levels (Khan et al., 2020; Yasmin et al., 2020). They have indirect effects by decreasing the damage from various pathogens on plant growth and development due to the antagonistic substances production or inducing resistance to pathogens (Khan et al., 2020), which works in the forms of biocontrol agents. Some PGPR can exert stimulation of mycorrhizae development (Bhattacharyya and Jha, 2012). PGPR applications can modulate plant-soil chemistry altogether, which in turn pave the way for an

alternative biocontrol for plant production that is environmentally friendly and sustainable (Ortega et al., 2017, Li et al., 2020).

AMF and PGPR allow better responses of inoculated seedlings (Nunes, 2019). Here we hypothesized that AMF and/or PGPR increase the growth of native seedlings and the nutrients contents, producing seedlings with high quality. Thus, this study aimed to explore the potential of AMF produced by the on-farm method and PGPR isolated from native Brazilian tree species, for producing seedlings of macauba, canafistula and pau-viola by evaluating the higher growth capacity and the richer microbial community of AMF, total bacteria and nitrogen-fixing bacteria (NFB) in the soil.

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CHAPTER 1: Can the inoculation with arbuscular mycorrhizal fungi improve the production of macauba (*Acrocomia aculeata*) seedlings?

Can the inoculation with arbuscular mycorrhizal fungi improve the production of macauba (*Acrocomia aculeata*) seedlings?

Abstract

Macauba is a native palm tree in Brazil whose main economic primary interest is oil production for biodiesel. The objective of this work was to evaluate the effect of arbuscular mycorrhizal fungi (AMF) inoculation by examining the plant biomass accumulation, root colonization, spore's number, and community of AMF and total bacteria in the soil by PCR-DGGE of macauba seedlings inoculated AMF at the stage of semi-germinated seed (EXP1) and the time of transplantation with the 3-month-old seedling (EXP2). At 11 months of the cultivation, the biomass accumulation showed no difference ($p>0.05$) between seedlings inoculated (INOC) and not inoculated (NON) with AMF. The colonization of AMF and the spore's number were higher on INOC than NON in EXP1. However, they were not influenced by the inoculation in EXP2. Furthermore, molecular community analysis by PCR-DGGE revealed that inoculation with AMF made the AMF diversity in soil change and increase; however, there was no difference in these diversity indices for the bacterial community. We concluded that inoculation of AMF does not influence the biomass accumulation of macauba in substrate seedlings $P > 66$ mg dm^{-3} but favors the diversity of AMF and does not alter the bacterial community in the soil. Further work is needed to reveal whether macauba seedlings inoculated with AMF can promote plant growth in the field, including tolerate biotic and abiotic stress.

Keyword: Sustainability. Symbiosis. AMF. Bacteria. PCR-DGGE.

Introduction

Macauba (*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.) is an oil palm, which is distributed throughout the American tropic (Motta et al., 2002) from the Arecaceae family, which has a great potential for oil production (Moreira et al. 2018). The primary economic interest in this species lies in the oil production for biodiesel as a profitable and renewable energy source (de Sousa et al., 2016). Additionally, residues generated by the extraction of oil can be used as sources for different biofuels. (Evaristo et al. 2018) and also in the mushroom production (Kayashima, 2018).

This palm tree can be used to recover degraded areas with agroforestry systems because it adapts to different ecosystems with remarkable drought tolerance (Rosa et al., 2019, Bazzo et al., 2018). For instance, macauba agroforestry system in the degraded pasture in the Brazilian Cerrado contributes to the positive environmental impacts, including increased carbon sequestration (Feliciano et al. 2018, Moreira et al. 2020). Furthermore the agroforestry system of coffee intercropped with macauba can mitigate climate effect (Moreira et al. 2018, Prates Júnior et al., 2021a).

Biological inputs such as microbial inoculants in the soil play an essential role in the seedling establishment, maintaining ecosystem services, and promoting plant growth. AMF are soil microorganisms, making better use of the nutrients present in the soil through their extra-radical mycelium, which helps plants absorb and carry soil elements of low mobility, especially phosphorus (Clark & Zeto, 2000). The mycelial network by AMF allows the soil microbial communities to coexist (Svenningsen et al., 2018). AMF can establish the tripartite symbiosis (fungus-plant-bacteria) (Hao et al., 2019), which can assist in decreasing chemical fertilizers and contributes to the soil biodiversity, playing an essential role in soil health (Karličić et al. 2016).

The macauba palm represents the high mycorrhizal colonization of the root system without depending on the soil's fertility (Souza et al., 2018). Still, little is known about the benefit of the mycorrhizal association to macauba. Hence, there are studies on mycorrhizal associations with other palm trees, such as oil palm (*Elaeis guineenses* Jacq.) (Phosri et al., 2010) and *Phoenix dactylifera* L. (Diatta et al., 2014). Oil palm is very responsive to mycorrhizae, which means that there is potential to explore this symbiosis (Phosri et al., 2010).

We hypothesized that inoculation of AMF can increase the biomass accumulation and improve the AMF and bacterial community in the soil. Thus, the objective of this work was to evaluate the bioaccumulation of macauba seedlings in symbiosis with arbuscular mycorrhizal fungi (AMF) and the impact on the community AMF and total bacteria in the soil, to decrease the economic costs of seedling production and the levels of fertilizers used.

Material and Methods

This experiment was conducted at the Laboratory of Mycorrhizal Associations, Institute of Biotechnology Applied to Agriculture (Bioagro) and in the greenhouse belonging to the Department of Microbiology at the Universidade Federal de Viçosa (UFV), in Viçosa, located in the Atlantic Rainforest Brazilian biome, in Minas Gerais State, Brazil (19° 52′ 20″ S and 44° 25′ 12″ W). The entire period of the experiment was from March in 2019 through February in 2020. Annual ambient temperature in this region is 25 ºC and the humidity is 50 % (Melo et al., 2018).

Macauba seeds and seedlings were provided by Acrotech Biotecnologia (MG, Brazil), an agricultural technological company that works, especially the macauba production chain. The germination was performed by Acrotech, which has a dormancy-breaking protocol for macauba seeds in advance.

The substrates were prepared by a mixture of Oxisol (Latossolo Vermelho-Amarelo Distrófico), collected in the region of Viçosa – Minas Gerais (Table 1) and goat manure (2:1, v:v).

The AMF inoculation was multiplied by on-farm method (Moreira *et al.*, 2019), whose substrate was composed of soil (horizon B), sugarcane bagasse, and vermiculite (1: 1: 1, v:v:v) added with 10 % organic compost, using sorghum plants. The species identification was based on analyzing their spore wall structure and taxonomic information described by (Stürmer et al., 2018) in Botany Laboratory of Regional University of Blumenau Foundation, in Blumenau, Santa Catarina, Brazil. The principal species were detected as follow: *Acaulospora scrobiculata, Claroideoglomus etunicatum, Paraglomus albidum* and *Paraglomus brasilianum*. Each inoculated treatment received 15 Ml of AMF inoculant, containing about three spores Ml^{-1}.

Table 1: Result of the analysis of a soil mixture, collected in the region of Viçosa – Minas Gerais for macauba seedlings cultivation.

Ph	P	K	Zn	Fe	Mn	Cu	B	Ca^{2+}	Mg^{2+}	Al^{3+}	H+Al	SB	CEC(t)	V	OM	P-rem
H_2O	mg dm^{-3}							cmolc dm^{-3}						%		mg L^{-1}
6.3	66.7	230	2.9	85.8	16.4	0.5	0.1	2.5	0.9	0	2.8	4	4	59	2.25	27.3

Extractors used: P, K, Zn, Cu = extractor Mehlich1; Al^{3+}, Ca^{2+} and Mg^{2+} = extractor KCl 1 mol L^{-1}; H + Al = extractor Ca acetate 0.5 mol L^{-1}; SB = sum of exchangeable bases; CTC (t) = effective cation exchange capacity; V = base saturation index; OM = organic matter; P-rem = remaining phosphorus.

The experiment was conducted as a completely randomized design, with two treatments in each experiment and five replications for each treatment. The treatments used in the experiment 1 were: i) seedlings inoculated with AMF in the germinated seed stage (EXP1.INOC), ii) seedlings without inoculation (EXP1.NON). In experiment 2 were i) seedlings inoculated with AMF in the three-month-old stage (EXP2.INOC), and ii) seedlings without inoculation (EXP2.NON). The seedlings were irrigated daily to maintain the substrate's adequate moisture for plant development during the experiment for 11 months.

Experiment 1: The germinated seeds (with and without inoculation of AMF) remained in 150 Ml "*Ellepots*" tubes for four months, and after that, they were transplanted into the substrate in plastic bags of 6 L, which stayed for another seven months in a greenhouse.

Experiment 2: The three-month-old seedlings were transplanted to substrate in plastic bags of 6 L. Inoculation with AMF were performed during transplantation. The seedlings were kept for another eleven months in the greenhouse.

After having kept in greenhouse for the period described above shoots were cut out, and root systems were removed from the soil and washed carefully to remove the adhering soils. Samples of fine roots (< 2 mm) were separated for evaluating mycorrhizal colonization. Shoot dry mass (SDM) and root dry mass (RDM) were determined after drying at 70 ºC in an oven with forced ventilation, until constant mass.

Samples for determination of mycorrhizal colonization were stored in FAA (formaldehyde: alcohol: acetic acid; 5:90:5; v: v: v), for the conservation of the fungal structures and the root. Then, roots were cleared by submerging in 10 % KOH (w:v) and/or sodium hypochlorite 2 % for 10 min, followed by three successive washes in tap water. Afterward, the roots were immersed in HCl 2 % (v:v) for 10 min. Finally, roots were dyed with trypan blue in lactoglycerol 0.05 % (w:v) for a week and then stored in a solution of lactoglycerol (Phillips & Hayman 1970). To evaluate mycorrhizal colonization, the sample of roots were randomly chosen, and 10 root fragments (approximately 3 cm each) were placed on three microscopic glasses, arranged parallel to one another, evaluating the top, median, and bottom of each root fragment (Trouvelot et al. 1986). The percentage of mycorrhizal colonization was estimated according to the presence of fungal structures in the roots (arbuscules, hyphae, vesicles, and spores) observed under an optical microscope at 400 times magnification. The percentage of the AMF colonization was calculated as the following formula: AMF colonization (%) = (the number of AMF structure counted / total count amount) *100 (Moreira et al. 2021).

AMF spores were extracted from 100 g of soil by sieving and decanting method (Gerdemann & Nicolson, 1963) with water centrifugation and subsequent centrifugation in a 50 % sucrose solution for separation by osmotic pressure (Jenkins, 1964). The enumeration of spores was performed under a microscope.

Molecular analysis was included in experiment 2 to evaluate the soil microbial community using PCR-DGGE technique. The total DNA was extracted from 0.3 g of the soil, using the NucleoSpinSoil® (Machereye-Nagel, GmbH & Co. KG, Germany), according to the manufacturer's guidelines. The total DNA were submitted to electrophoresis on agarose gel (0.8 %, w:v) stained with ethidium bromide and visualization under UV light in the photo documentation imaging system (Loccus Biotecnologic L-Pix Chemi), to check bands indicating the presence and quality of genomic DNA in the soil.

The extracted DNA was amplified by PCR and Nested-PCR reaction to amplify specific regions of the 18S Rdna fraction from AMF and 16S Rdna fraction corresponding to total bacteria. Each PCR reaction mixture consisted of 1 µL of DNA, 2 µL of Dntp (200 µM), 4µL of $MgCl_2$ (2 Mm), 2 µL of each primer (0.2 µM), 10 µL of Buffer (20 Mm), 0.25 µL of Cellco SuperTaq Pol DNA polymerase (1.25 u), 28.75 µL of nuclease-free water for a final volume reaction 50 µL and all reaction was carried out in a thermocycler (Mastercycle Epgradient Eppendorf).

To amplify specific regions of the 18S Rdna fraction from AMF, PCR and Nested-PCR reaction was realized, using primer pairs, AML1 (5′-ATCAACTTTCGATGGTAGGATAGA-3′) and AML2 (5′- GAACCCAAACACTTTGGTTTCC-3 ′) (Lee et al. 2008). The first PCR was taken place as follow: an initial denaturation of 3 min at 94 °C and 30 cycles of denaturation of 1min at 94 °C, annealing of 1min at 55 °C, and extension of 1 min at 72 °C, followed by a final extension step of 10 min at 72 °C (Prado et al., 2019). After that, Nested-PCR was carried with another set of primer, NS31-GC (5′-

CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGTTGGAGGGCAGTCTG GTGCC-3′) (Kowalchuk et al. 2002) and Glo1 (5′-GCCTGCTTTAAACACTCTA-3′) (Cornejo et al. 2004), and with following thermocycling conditions: an initial denaturation of 5 min at 94 °C and 35 cycles of denaturation of 1min at 94 °C, annealing of 45 s at 52 °C, and extension of 1 min at 72 °C, followed by a final extension step of 30 min at 72 °C (Prado et al. 2019).

The 16S Rdna gene was amplified using the primer set, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1494R (5′- GGTTACCTTGTTACGACTT -3′) (Weisburg et al., 1991) in the first PCR. The amplification was carried out as follow: an initial denaturation at 94 °C for 4 min and 35 cycles of denaturation of 1min at 94 °C, annealing of 1min at 55 °C, and extension of 2 min at 72 °C, followed by a final extension step of 10 min at 72 °C (Cardoso et al. 2020, dos Santos et al. 2020). The Nested-PCR reaction for the amplification of the 16S Rdna fraction was performed with a set of primers, 984FGC (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAAC -3′) (Nübel et al., 1996) and 1378R (5′- CGGTGTGTACAAGGCCCGGGAACG -3′) (Heuer et al., 1997), and was carried with the following thermocycling conditions: PCR was taken place as follow: an initial denaturation of 5 min at 94 °C and 30 cycles of denaturation of 45 s at 94 °C, annealing of 45 s at 52 °C, and extension of 1 min at 72 °C, followed by a final extension step of 30 min at 72 °C (Pimentel et al. 2017).

Aliquots of 5 μL of each PCR and Nested-PCR products were analyzed by electrophoresis on 1.5 % (w/v) agarose gel, stained with ethidium bromide (0.5 μg Ml−1), using TAE 1 × buffer at a constant temperature of 60 ˚C to 100 V for 40 min. Then confirmed the presence and quality of the DNA fragments amplified under UV light and UV light photo documentation imaging system (Loccus Biotecnologic L-Pix Chemi. Then, the PCR products were stored at −20 °C until the gel electrophoresis denaturing gradient (DGGE) analyses.

Nested-PCR amplicons from the sample were used in the first DGGE fingerprint. The fragments obtained by the nested-PCR technique were analyzed with the DGGE to compare bacterial and AMF communities present in the sample. A 20 Ml aliquot of the nested-PCR reaction ranging from 150 to 200 ng of DNA was loaded on an polyacrylamide gel (37.1:1 acrylamide: bisacrylamide) 8 % (w:v) in Tris-acetate-EDTA buffer (TAE) 1X (Tris/acetic acid/EDTA, Ph 8.0) (da Silva et al., 2021). The gel was prepared on a denaturation gradient ranging from 35 to 55 %, the 100 % denaturation condition consisting of 7 mol/L urea (Sigma, Cat # U5378) and formamide 40 % (v/v) (Sigma, Cat # F9037). The gel was subjected to vertical electrophoresis at 120V for 10 min at 60 °C, followed by 100 V for 12 h at 60 °C. The gel was stained for 40 min in a 1X SYBR GOLD solution® (Sigma Aldrich), According to the manufacturer's recommendations. The images were observed under UV light, captured and digitized by means of Molecular Imaging System (Loccus Biotecnologic L-Pix Chemi). The DGGE profiles were analyzed using the Bionumerics software program (Version 6.0, Applied Maths NV). Dendrogram were constructed by Cosine Coefficient similarity index, followed by cluster analysis using the minimum variance (Ward) method. The samples were compared based on the presence/absence, and intensity of the bands and the index differences of diversity among treatments were detected using the software PAST ® (version 3.20) (Hammer et al., 2001).

The results were submitted to Kolmogorov-Smirnov normality verification and analysis of variance (ANOVA). The statistical differences were considered with 10 % probability significance for data of diversity indices and with 5 % probability significance for other data obtained. The analysis was performed in the statistical package R Studio v1.4.1103 and software system MINITAB 19 free version.

Results and discussion

Mycorrhizal colonization was present and high in all treatments. The soil's phosphorus level in this study was 66.7 mg dm^{-3} (Table 1), which is classified as high phosphorus levels in the soil for mycorrhizal development (around 50 mg dm^{-3}) (Fakhech et al. 2020). The presence of AMF in non-inoculated treatments confirmed the presence of a natural AMF community because the soil was not sterilized in this experiment.

Given the morphology of the root systems, macauba seems to be strongly mycorrhizal dependent (Phosri et al. 2010), reaching values as high as 75 % even in high natural fertility soils (Souza et al. 2018) and in this study we observed high mycorrhizal colonization in macauba.

EXP1.INOC had higher colonization of AMF than EXP1.NON, which obtained an increase of 31 % of the colonization of AMF (Table 2). Thus possibly, our AMF inoculation made symbioses stronger and favored in EXP1.INOC. This result could be an advantage that the AMF inoculation used in this experiment was produced by on-farm method that multiplies local species in a handmade way and is low cost to farmers (Prates Júnior et al., 2021b).

On the other hand, the EXP2.INOC and EXP2.NON did not show any difference (Table 2) for both the percentage of AMF colonization and spore's number. It indicates that macauba showed mycorrhizal responsiveness with indigenous fungi and inoculated ones, establishing mycorrhizal symbioses because the roots pick up natural symbioses with indigenous populations of AMF when palm trees are grown in unsterilized soils (Phosri et al. 2010).

The evaluation of AMF spore's number showed a difference in experiment 1 (Table 1). EXP1.INOC obtained a higher spore's number ($p < 0.05$) than EXP1.NON. In experiment 2, the spore's number did not show a difference ($p > 0.05$) by the inoculation with AMF. Thus, the colonization and spore's number (Table 1) indicate that inoculating AMF in the early stage promotes the increase of the AMF colonization and spore's number to macauba seedlings. The study of another palm tree (*Elaeis guineesis* Jacq.) has demonstrated that the formation of AMF

began from the second month and the seedlings developed AMF network until the third month; therein lies the reason that the host plant may have encouraged the AMF growth after depending on the nutrients by seed (Galindo-Castañeda & Romero 2013), making the interactions between AMF and host plant stronger.

Table 2: Percentage of AMF colonization and AMF spores, shoot (SDM) and root dry mass (RDM) of macauba, after 11 months of growth in the greenhouse, comparing plants inoculated and non-inoculated with AMF, in experiments 1 and 2 separately.

Parameter		Percentage of AMF colonization (%)	Number of AMF spores $100g^{-1}$	SDM (g)	RDM (g)
EXP.I	NON	66.86 b	29.6 b	9.74 a	4.912 a
	INOC	87.34 a	86.0 a	12.79 a	4.832 a
EXP. II	NON	61.3 A	65.0 A	31.21 A	15.87 A
	INOC	74.6 A	118.4 A	33.34 A	15.22 A

Means followed by the same letter on the same line are not significantly different according to the t-test at $p < 0.05$. Lowercase letters were used for experiment 1, and uppercase letters were used for experiment 2.

Inoculation of AMF did not increase biomass accumulation in the initial growth of macauba seedlings, neither experiment 1 nor experiment 2 (Table1). Notably, the inoculated plant has a high cost to maintain mycorrhizal symbiosis by supplying photosynthates to the associated AMF (Prates Júnior et al., 2021c). Therefore, it is worth mentioning that they have not reduced the growth even as in this condition with high availability of P. Furthermore, when the mycorrhized plant was transferred to the field, it might have better conditions to tolerate stress conditions, improving plant defenses (Tian et al., 2019), which should be included in a further evaluation.

There is evidence that inoculation with AMF promoted the accumulation of the dry matter in other palm trees such as oil palm (*Elaeis guineesis* Jacq.) (Dumar & Fernando 2005) and juçara (*Euterpe edulis* Martiuss) seedlings (Moreira et al. 2016). Conversely, other study also demonstrated growth depression on dry weight of oil palm (*Elaeis guineesis* Jacq.) by inoculation with AMF during the nursery stage, having higher root colonization (Sundram

2010). Differently, our results showed that AMF inoculation does not influence biomass accumulation in macauba seedling.

No evidence that altering the AMF colonization percentage brought a difference in the biomass quantity in the seedling's initial development, showing that native AMF are promising to promote seedling's growth (Galindo-Castañeda & Romero 2013), whereby AMF play an essential role in acquiring nutrients by plants, water absorption, and tolerance of biotic and abiotic environmental stress (Gumiere et al. 2019).

Although the present study did not go into the analysis of the tolerance to biotic and abiotic stress, macauba may have received some benefit through the AMF capacity, for example, phosphorous absorption, increases in water and nutrient uptake surfaces, and a great hyphae network of mycorrhizal symbiosis (Frank, 2005). Further studies are needed to evaluate biomass accumulation and plant health for the subsequent stage in the field. We suggest that the more the seedlings associate with AMF, the more helpful the growth promotion, considering macauba's high mycorrhizal dependency (Souza et al. 2018).

Microbial profile of inoculant by DGGE

The structure of AMF communities was analyzed through PCR-DGGE technique. Cluster analysis of the soil AMF community revealed two distinct, and both groups showed more than 70 % similarity (Figure 1). The first group included the treatment without AMF inoculant, having more similarity to each other. The second group comprises INOC R1 and INOC R2, and INOC R3 was classified as a more distant group in terms of similarity to the others.

Figure.1 Cluster analysis, Cosine-ward, obtained by the DGGE bands profile of the 18S rDNA gene from Glomeromycota communities of AMF inoculants from 2 sources of experiment 2. R1, R2 and R3 are repetitions.

Although there is a difference in the composition of AMF community, according to the cluster analysis (Figure.1) the treatments EXP2.INOC and EXP2.NON (excluding INOC R3) represents 90 % of similarity, which probably explain why the biomass accumulation did not show the difference between EXP2.INOC and EXP2.NON by the inoculation of AMF.

Cluster analysis of the soil bacterial community also showed two groups. However, there was no difference between bacterial profile treatments, showing the high similarity between these two groups. Two repetitions of INOC and the other one of NON formed the first group. The second group had two repetitions of NON. The addition of inoculation with AMF had no effects on the profile of the bacterial community (Figure 2). Bacterial community structure depends more on AMF than on host plant identity (Bonfante & Anca, 2009). AMF inoculant used in this study did not favor enhancing the bacterial community, as the previous research about Glomus-cucumber symbiosis showed little influence on the bacterial profile (Mansfeld-Giese et al. 2002).

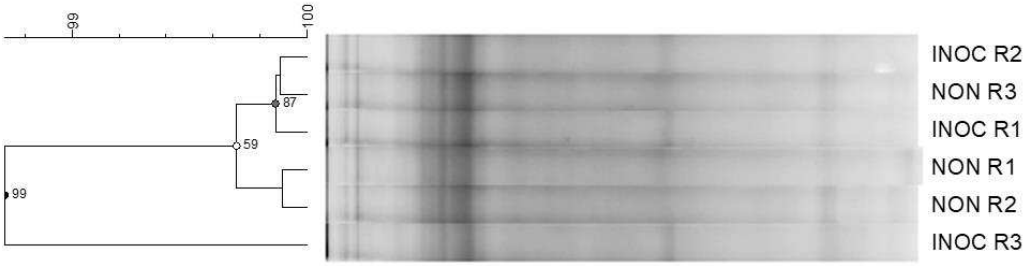

Figure.2 Cluster analysis, Cosine-ward, obtained by the DGGE bands profile of the 16S rDNA gene from bacterial communities of AMF inoculants from 2 sources of experiment 2. R1, R2 and R3 are repetitions.

As evidenced by the diversity indices, AMF communities in EXP2.INOC has more richness of species and diversity than EXP2.NON (Table 2). This result suggests that AMF inoculation is essential to manage the diverse AMF community, which maybe favor macauba seedlings to adapt to the field condition in the next stage. Rich AMF profiles contribute to the recruitment of other beneficial microorganisms, which can consequently happen to the soil fertile and promote plant growth (El Kinany et al. 2019).

We considered that AMF inoculation did not make a difference to the bacterial community since the AMF community in the soil showed 90 % similarity between EXP2.INOC and EXP2.NON (Figure 1), Additionally, the combination of bacterial and AMF strains in this study did not positively or negatively alter the symbiotic efficiency (Júnior et al. 2017).

Table 3: Diversity indices of arbuscular mycorrhizal fungi (AMF) and bacteria obtained by the PCR-DGGE technique in experiment 2.

		Richness		Dominance		Simpson		Shannon_H		Chao	
AMF	NON	10.33	b	0.10	a	0.90	b	2.33	b	58.67	b
	INOC	12.67	a	0.08	b	0.92	a	2.53	a	87.33	a
Bacteria	NON	9.33	A	0.11	A	0.89	A	2.22	A	49.67	A
	INOC	9.67	A	0.10	A	0.90	A	2.26	A	52.00	A

The averages followed by the same letter do not differ by Tukey test at 10 % probability. Means followed by the same letters in a column are not significantly different according to an analysis of the variance test ($p < 0.1$). Lowercase letters were used for AMF, and uppercase letters were used for bacteria.

Conclusion

Our result shows that inoculation of AMF does not influence the biomass accumulation in macauba seedlings when the value of P is high but increases the diversity of AMF and does not alter the bacterial community in the soil. We detected that early inoculation can favor mycorrhizal colonization by macauba. Further work is needed to reveal whether macauba

seedlings inoculated with AMF can promote plant growth, including tolerate biotic and abiotic stress in the field.

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CHAPTER 2: Inoculation of arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria improve *Peltophorum dubium* seedlings growth

Inoculation of arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria improve *Peltophorum dubium* seedlings growth

Abstract

Bioinoculants such as arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) can improve plant growth and represent a replacement for chemical fertilizers and pesticides. Canafistula (*Peltophorum dubium* (Spreng.) Taub.) is a native Brazilian tree and widely used for reforestation in degraded and contaminated areas. The objective of this work was to evaluate the growth of canafistula seedling with inoculation of AMF and PGPR to improve seedling development. Canafistula seeds were germinated in sterile sand for seven days, and the seedlings were transplanted into tubes of 150 cm^3 with a substrate composed of fine charcoal, sugar cane bagasse and broiler litter in a 1: 1: 1 (v:v:v) ratio added with 20 % organic compost from soil (horizon B). The experiment was performed by a completely randomized 5 × 2 factorial design. Five inoculation levels of PGPR consisted of non-inoculation of PGPR or inoculation of four isolated of PGPR, identified as *Klebsiella (B1)*, *Burkholderia (B2)*, *Lysinibacillus (B3)* and *Lysinibacillus (B4)*. Inoculation level of AMF consisted of non-inoculation or inoculation of AMF. The seedlings growth was evaluated after five months by determining diameter (mm), height (cm) and shoot dry matter (SDM) (g). Besides, the determination of AMF colonization percentage was performed. Both AMF and PGPR inoculation (*Klebsiella* and *Burkholderia*) significantly affect canafistula seedlings growth, showing the high potential of these isolates to be used as biotechnological products.

Keyword: Native tree species. Symbiosis. Beneficial microorganisms. Canafistula.

Introduction

The species *Peltophorum dubium* (Spreng.) Taub. Is a member of the Fabaceae family, popularly known as canafistula and this species is native to South America (Jesus et al. 2020) and has widely distributed in Brazil (Mantovani et al. 2017). Canafistula can have a low incidence of AMF (Jesus et al. 2005) and is a leguminous (Fabaceae) nitrogen-fixing tree (Coelho et al. 2007). Due to their tolerance and resistance to a wide range of environmental conditions (Marques et al. 2019) and their rapid growth (Silva et al. 2017), canafistula is widely used in reforestation strategies (Van-Lume & Souza 2018) in degraded (Lorenzi 2002) and contaminated areas (Cordeiro et al. 2018, Cruz et al., 2020) because the quality and rapid establishment of seedlings are prerequisites for successful reforestation (Riikonen & Luoranen, 2018).

Microorganisms, such as arbuscular mycorrhizal fungi (AMF) (Prates Júnior et al., 2021) and plant growth-promoting rhizobacteria (PGPR) (Li et al. 2020), are known as microbial inoculants in the soil. They play an essential role in developing seedling growth with quality (Tiepo et al. 2020), allowing better responses of inoculated seedlings (Nunes 2019). Furthermore, these bioinoculants represent a replacement for chemical fertilizers and pesticides (Saranraj 2014), contributing to soil biodiversity and health (Karličić et al. 2016).

AMF associate with plants, establish fungal structures that are efficient to absorbing water and nutrients (especially phosphorus) from the soil transferred to plants (Bernardo et al. 2017) and increasing the tolerance to biotic and abiotic stresses (Jesus et al. 2020). PGPR improve plant health and enhance plant growth by plant growth-promoting mechanisms directly and indirectly. Some PGPR affect the balance of plant phytostimulators such as Indole 3-acetic acid (IAA) (Prasad et al. 2019). Others induce systemic resistance to plant pathogens (Pathak et al. 2020) and protect against unhealthy environmental conditions (Glick 2014), thereby having the potential as a bioinoculant (Jha & Saraf 2015).

The improvement of the plant growth by these bioinoculants largely depends on the association's specificity between microorganisms and host plants (Candido et al. 2015). Thus, the objective of this work was to evaluate the effects of inoculation with AMF and PGPR in the growth of canafistula seedlings. We hypothesized that AMF and PGPR inoculation improve the growth of canafistula seedlings in the nursery stage.

Material and methods

The soil for the production of the AMF inoculant was obtained in Paracatu, MG, Brazil by on-farm method (Moreira *et al.*, 2019), where the spores present in the soil of a region of native forest were multiplied in pots (Prates Júnior et al. 2020). The AMF inoculation was prepared by Cogumê biotecnologia, and an substrate was composed of soil (horizon B), sugarcane bagasse, and vermiculite (1: 1: 1, v:v:v) added with 10 % organic compost, using sorghum plants. After three months of cultivation, the plants underwent stress due to the absence of water (one month). Then the soil was sieved for homogenization and was used as an inoculum. Each inoculated treatment received 15 mL of AMF inoculant, containing about 3 spores mL^{-1}. The AMF species present in the inoculation was identified as described by (Stürmer et al., 2018) in Botany Laboratory of Regional University of Blumenau Foundation, in Blumenau, Santa Catarina, Brazil, and the principal species were detected as follow: *Acaulospora scrobiculata, Claroideoglomus etunicatum, Paraglomus albidum* and *Paraglomus brasilianum*.

Canafistula seeds were disinfected superficially using 70 % alcohol for 30 s and 2 % sodium hypochlorite (v:v) for 5 min, and then, rinsed in abundant sterilized water. Then, they were placed in sterilized sand and, after seven days, uniform plantlets were transferred into tubes of 150 cm^3 containing unsterilized substrate composed of fine charcoal, sugar cane

bagasse and broiler litter (1: 1: 1, v:v:v) (Prates Júnior et al., 2020) added with 20 % organic compost from soil (horizon B). The characteristics of substrate used are shown in Table 1.

Table. 1: Chemical characteristics of substrates for cultivation of canafistula.

pH	N	P	K	Ca	Mg	S	Zn	Fe	Mn	Cu	B
(H_2O)	g dm^{-3}						mg dm^{-3}				
5.56	7.8	6.2	10.4	11.5	6.9	3.8	51.9	11122	427.2	16.7	18.1

Extractors used: N = Kjeldhal; P, K, Ca, Mg, S, Zn, Fe, Mn, Cu, B = nitric acid + perchloric acid mixture.

Canafistula seedlings were grown under greenhouse conditions in an experimental period, from September in 2019 to February in 2020. A completely randomized 5 × 2 factorial design (five levels of inoculation of PGPR and two levels of inoculation of AMF) with 5 repetitions of 10 treatments was used. Five inoculation levels of PGPR consisted of non-inoculation of PGPR or inoculation of four isolated PGPR, which were identified as Klebsiella (B1), Burkholderia (B2), Lysinibacillus (B3) and Lysinibacillus (B4) by degerming 16S rDNA sequence. The PGPR were isolated from two native Brazilian tree species, being braúna (Melanoxylon brauna Schott), and vinhático (Plathymenia reticulata Bentham) (Table 2). The DNA extraction was performed, and subsequently, the gene was amplificated by the polymerase chain reaction (PCR) method, using primers 27F and 1429R (Weisburg et al., 1991). Afterward, the products of PCR were sequenced by Macrogen, Inc. (Korea). All sequences obtained in the present study were analyzed by comparing the obtained sequences with those deposited in the GenBank database using the BLASTx search tool (NCBI).

Inoculation level of AMF consisted of non-inoculation or inoculation of AMF. Substrates in treatments NON.CONTROL, NON.B1, NON.B2, NON.B3 and NON.B4 were not inoculated with AMF, whereas substrates in treatments INOC.CONTROL, INOC.B1, INOC.B2, INOC.B3 and INOC.B4 were inoculated with AMF (Table 2).

Table 2: Origen of plant species and *in vitro* assays characteristics of isolated plant growth-promoting rhizobacteria culture.

Plant species' origins	Rhizo-Bacterial strain	Identification	Biochemical test			
			[a]P solubilization (CP)	[b]GA production	[c]BNF	[d]IAA production
Braúna	B1	*Klebsiella*	−	(+)	−	+
Vinhático	B2	*Burkholderia*	+	+	−	(+)
Braúna	B3	*Lysinibacillus*	−	+	−	(+)
Vinhático	B4	*Lysinibacillus*	+	+	−	(+)

'+'= positive; '–'= negative; '(+)'= slow positive; CP= calcium phosphate; GA= gibberellins; BNF= biological nitrogen fixation; IAA= Indole 3-acetic acid;

[a] P solubilization was evaluated by (Berraquero et al., 1976)

[b] GA production was evaluated by (Graham & Henderson, 1961).

[c] BNF was evaluated in N-free semi-solid media with pellicle formation indicating the ability to fix atmospheric nitrogen (Boddey et al., 1995)

[d] IAA production was indicated by the cultivation in tryptic soy agar (TSA) medium 10% containing L-tryptophan (5 mm), pH 7.0 (Glickmann & Dessaux, 1995).

After 150 days, measures of the stem diameter (D) at 1.5 cm from the soil, plant height (H), and shoot dry mass (SDM) were evaluated. SDM were determined after drying at 70 ºC in an oven with forced ventilation until constant mass.

About 2 g of thin roots (<2 mm in diameter) were selected and washed with tap water and stored in FAA (formaldehyde: alcohol: acetic acid; 5:90:5; v: v: v) to conserve the root. Root staining method was performed to determine the percentage of mycorrhizal colonization, according to the previous study of Phillips & Hayman (1970) with some adjustment. After washing roots with tap water to remove excess FAA, they were immersed in sodium hypochlorite 2 % for 10 min, followed by washing roots in tap water. Afterward, the roots were immersed in HCl 2 % (v:v) for 10. Finally, roots were dyed with trypan blue in lactoglycerol 0.05 % (w:v) for 3 days, and then the sample roots were stored with lactoglycerol.

The microscopic glass slides were prepared by placing ten root fragments chosen randomly and arranged parallel to one another, evaluating the top, median, and bottom of each root fragment (Ruscitti et al. 2011, Trouvelot et al. 1986). A total of three slides were prepared per plant. The presence of fungal structures in the roots (arbuscules, hyphae, vesicles and spores) were observed under an optical microscope at 400 times magnification to estimate the percentage of mycorrhizal colonization. The percentage of the AMF colonization was

calculated as the following formula: AMF colonization (%) = (the number of AMF structure counted / total count amount) *100 (Moreira et al. 2021).

The data were submitted to Shapiro-Wilk test for the normality verification and analysis of two-way ANOVA, and the averages were compared by the Tukey test ($P \leq 0.05$). Data were transformed using log(X), where it did not meet the requirements for normality and homoscedasticity. Nonetheless, the results are presented in their original numeral scale. The analysis was performed in the statistical package R.

Results and discussion

There was a significant ($p < 0.05$) effect of AMF and PGPR inoculation in the seedling's growth measure (Table 3). ANOVA showed a significant interaction (AMF × PGPR) for the results of the seedling growth (Table 3). The result of seedling growth in diameter on NON.B1, INOC.B1 and INOC.B2 were higher than CONTROL.NON. For plant height, the higher values were observed in NON.B1, INOC.B1 and INOC.B2 as well as CONTROL.INOC. An increase in SDM in plants of on INOC.B1 and INOC.B2 was observed, with a 160 % and 147 % SDM increase, respectively, in comparison to CONTROL.NON (Table 3), which were the better values of SDM among all treatments. Subsequently, NON.B1, INOC.CONTROL, INOC.B3 showed better results on SDM significantly, compared to NON.CONTROL.

Overall, the interaction between AMF and PGPR induced seedling growth. Notably, INOC.B1 showed the best performances of seedling growth among all treatments (Table 3).

According to the analysis (*in vitro*) to characterize isolated PGPR, all four strains could produce Indole-3-acetic acid (IAA) hormone, and especially B1 has a better capacity to produce IAA hormone than B2, B3 and B4; therein lies the probable reason to explain the positive influence on the seedling's development by PGPR-related effect (Table 2). Consequently, inoculation of B1 is expected to change in the hormonal levels and promoted the increase of

seedlings biomass (Table 1) during the nursery phase, and thereby the accumulation of IAA induced the root growth and biomass (Agarwal et al. 2019; Valente Lima et al. 2020). Additionally, we attributed the increase of the seedling growth by the inoculation of B1 isolate to the fact that canafistula and braúna are classified as subfamily of Caesalpinioideae. Furthermore, the alteration of rhizospheric microbial community may have induced better results of canafistula seedlings growth (Verma et al. 2019), which indicated the importance of inoculation with PGPR to promote the seedling's development.

Table. 3: Diameter, height, and shoot dry mass (SDM) of canafistula after 150 days of greenhouse cultivation, comparing plants inoculated and non-inoculated with Arbuscular Mycorrhizal Fungi (AMF) (NON= non-AMF, INOC= with AMF) and/or Plant Growth-Promoting Rhizobacteria (CONTROL= non-PGPR, B1= Klebsiella, B2= Burkholderia, B3= Lysinibacillus and B4= Lysinibacillus).

AMF	CONTROL	B1	B2	B3	B4
			Diameter (mm)		
NON.	2.144 b	2.838 a	2.414 ab	2.280 ab	2.726 ab
INOC.	2.492 ab	2.738a	2.760 a	2.644 ab	2.338 ab
			Height (cm)		
NON.	10.33 b	14.0 a	10.0 b	10.33 b	12.0 ab
INOC.	14.9 a	14.8 a	13.5 a	12.3 ab	12.6 ab
			SDM (g)		
NON.	0.43 d	0.94 ab	0.56 cd	0.53 cd	0.67 bcd
INOC.	0.84 abc	1.12 a	1.06 a	0.82 abc	0.69 bcd

Means followed by the same letters in a column and a line within a measure type are not significantly different at $p<0.05$ (ANOVA).

For the results of the percentage of AMF colonization, ANOVA detected interaction between AMF and PGPR (Table 4), showing the importance of both groups of microorganisms for better growth of canafistula.

Except for NON.B3 and NON.B4, the percentage of AMF colonization on other treatments was observed (Table 4). The AMF colonization on non-AMF treatment indicated

that not only AMF inoculated but also native AMF favored canafistula seedlings. The treatments NON.B1 and INOC.B1 showed no difference on the growth (Table 3) as well as the percentage of AMF colonization (Table 4)

Table 4: Percentage of AMF colonization in canafistula, comparing plants inoculated and non-inoculated with Arbuscular Mycorrhizal Fungi (AMF) (NON= non-AMF, INOC= with AMF) and/or Plant Growth-Promoting Rhizobacteria (CONTROL= non-PGPR, B1= Klebsiella, B2= Burkholderia, B3= Lysinibacillus and B4= Lysinibacillus).

AMF	CONTROL	B1	B2	B3	B4
	Percentage of AMF colonization (%)				
NON.	31 bc	74 a	7 bc	0 c	0 c
INOC.	38 bc	70 a	8 bc	42 ab	31 bc

Means followed by the same letter on a column and line are not significantly different according to the t-test at $p < 0.05$.

Considering higher height and SDM INOC.B2 than NON.B2, fungal-bacterial symbioses function between B2 and AMF was implied. The previous studies reported *Burkholderia*-related endobacteria in AMF by the identification of bacterium related to the genus free-living *Burkholderia* in AMF isolates (Hildebrandt et al. 2006; Lastovetsky et al. 2018; Pawlowska et al. 2018), provided evidence that B2 establishes the interactions between AMF and plants.

There was no difference in growth between NON.B3 and INOC.B3 (Table 3); however, INOC.B3 on the SDM was better than NON.CONTROL, which indicated the beneficial interaction between *Lysinibacillus* and AMF inoculation to canafistula seedlings growth. Moreover, the previous study about the potential of *Lysinibacillus* as PGPR mentioned about the IAA production (Nauren et al. 2017), which was also detected in our strain (Table 2).

Altering microbial and biochemical composition in the soils by the inoculation of the microorganisms might have occurred (Dos Santos et al. 2020), being one of the contributing factors to promote seedling's development. The optimization of chemical fertilizer by

microorganism's inoculation for canafistula seedlings can happen because of the association between plants and microorganisms.

Conclusions

Inoculation of AMF and PGPR can enhance the canafistula seedling growth. The two PGPR isolate, Klebsiella and the combination of AMF and Burkholderia considerably increased the plant growth, which may be used as sustainable biofertilizers. Further studies are needed to explore the fungal-bacterial symbioses function.

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CHAPTER 3: Seedlings production of *Citharexylum myrianthum* Cham. with arbuscular mycorrhizal fungi for the restoration of iron-ore mining impacted areas

Seedlings production of *Citharexylum myrianthum* Cham. with arbuscular mycorrhizal fungi for the restoration of iron-ore mining impacted areas

Abstract

Microorganisms play an important role in maintaining ecosystem services, as well as promoting the growth and health of plants, such as *Citharexylum myrianthum* Cham. (pau-viola). Thus, we aimed to evaluate the growth of pau-viola seedlings when inoculated with arbuscular mycorrhizal fungi (AMF) and/or plant growth-promoting rhizobacteria (PGPR) by determining the seedling's growth and microbial community of AMF, total bacteria and nitrogen-fixing bacteria (BFN) in the soil. The AMF inoculation was multiplied by on-farm method, which included *Acaulospora scrobiculata, Claroideoglomus etunicatum, Paraglomus albidum* and *Paraglomus brasilianum,* as a principal species. PGPR isolates B1, B2, B3 and B4, identified as *Klebsiella, Burkholderia, Lysinibacillus* and *Lysinibacillus*, respectively, were used combined or not with AMF inoculation. We used substrate composed of fine charcoal, sugar cane bagasse and broiler litter during the first six months (EXP I). Afterwards, the seedlings were transplanted to an iron-ore mining waste (IOMW) (EXP II). The inoculation with AMF improved the seedling's growth in EXP I, but no effect was observed by the inoculation with PGPR in this study. There was no negative influence by IOMW in the microbial community evaluated in non-AMF treatments, which indicated that some AMF and bacteria species may have adapted IOMW conditions. We concluded that inoculation with AMF helps in better growth of pau-viola in the substrate used. Consequently, the AMF inoculation contributes to the seedling's production with sustainability and soil quality.

Keyword: Pau-viola. Arbuscular mycorrhizal fungi. Bacteria. Sustainability.

Introduction

Pau-viola (*Citharexylum myrianthum* Cham. (Verbenaceae)) is a Brazilian native tree species (Gavassi et al. 2018), and it widely occurs in in the northeast, southeast and south of Brazil, in Cerrado, Caatinga and Atlantic Forest formations (Amaral et al., 2013). Pau-viola is a critical species for reforestation of degraded areas (Tenfen et al. 2018) due to its rapid growth and adaption to a very humid and swampy area and is classified as a pioneer (Bazzanella et al. 2019). Thus, it is widely used in rehabilitation programs in riparian areas of Brazilian regions (Gavassi et al. 2018). It is a deciduous tree that blooms white flowers (Tenfen et al. 2018) and reaches 8 to 15 m in height and 20 to 40 cm in breast height diameter (Marcati et al. 2014). The fruits are reddish, ripen in January to March, and are eaten by various bird species (Bazzanella et al. 2019). Its fruits are appreciated by local fauna, becoming an essential component in the regeneration of degraded areas (Gavassi et al. 2018). Pau-viola can be used in an agroforestry system (SAF), having some benefits. For instance, it is possible to increase nutrient cycling, biodiversity, carbon storage and provide a moderate microclimate for other plants and fauna (Viégas et al. 2019; Gomes et al. 2020).

Taking advantage of some beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) (Prates Júnior et al., 2019) and plant growth-promoting rhizobacteria (PGPR) (Jha and Saraf 2015), is a key to encourage the seedlings development. Arbuscular mycorrhizal fungi (AMF) increase the absorption of water and nutrients of the host plant and the plant's tolerance to extreme environments such as those affected by tailings and mining waste (Goetten et al. 2016). For instance, AMF can be favorable to the success of reforestation programs, given the possibility of reducing the use of chemical fertilizers, less time for seedling formation in the nursery stage, and a higher chance of survival in the field (Goetten et al. 2016). Additionally, PGPR can be a nitrogen-fixing bacteria and/or solubilize phosphate. Furthermore, they can

produce indole 3-acetic acid, which stimulates plant cell proliferation and/or plant cell elongation.

We hypothesized that inoculation of AMF and PGPR can increase the seedlings growth and improve the AMF and bacterial community in the soil. Thus, this study aimed to evaluate the pau-viola seedling's growth with inoculation of AMF and, or PGPR, and analyze microbial community in the soil.

Material and methods

Study area and soil preparation

The experiment was carried out at the Laboratory of Mycorrhizal Associations, Institute of Biotechnology Applied to Agriculture (Bioagro).

Surface disinfection of the pau-viola's seeds was performed by 70 % alcohol for 30 s and 2 % sodium hypochlorite (v:v) for 5 min. The seeds were germinated using sterilized sand. After germination, uniform seedlings were selected and transferred into tubes of 150 cm^3 with a substrate composed of fine charcoal, sugar cane bagasse, and broiler litter in a 1: 1: 1 (v:v:v) (Prates Júnior et al., 2020). After 180 days of seedlings cultivation in tubes, half of these seedlings were used for some measurements. Another part was transplanted into a plastic bag of 3000 cm^3 with 2.2 L of iron ore mining waste (IOMW), collected in Mariana, Minas Gerais, where a rupture of the Fundão tailing dam occurred on November 5th, 2015. The characteristics of the substrate and the IOMW are shown in Table 1.

Table 1: Chemical characteristics of substrate used in experiment I and Iron-ore mining waste (IOMW) used in experiment II to cultivate pau-viola seedling.

Experiment I -substrate											
pH	N	P	K	Ca	Mg	S	Zn	Fe	Mn	Cu	B
(H_2O)	g dm^{-3}						mg dm^{-3}				

5.56	7.8	6.2	10.4	11.5	6.9	3.8	51.9	11122	427.2	16.7	18.1

Experiment II - IOMW										
pH	P	K	Ca^{2+}	Mg^{2+}	Al^{3+}	H+Al	SB	CEC(t)	V	OM
(H_2O)	g dm^{-3}		cmolc dm^{-3}						%	dag kg^{-1}
6.9	0.009	0.016	0.8	0	0	0.33	0.8	0.8	7.2	1.15

CO = organic carbon; C/N = carbon-nitrogen ratio; SB = sum of exchangeable bases; CTC (t) = effective cation exchange capacity; V = base saturation index; OM = organic matter.
Extractors used in experiment I: N = Kjeldhal; P, K, Ca, Mg, S, Zn, Fe, Mn, Cu, B = nitric acid + perchloric acid mixture. Extractors used in experiment II: P, K, Zn, Cu = extractor Mehlich1; Al^{3+}, Ca^{2+} and Mg^{2+} = extractor KCl 1 mol L^{-1}; H + Al = extractor Ca acetate 0.5 mol L^{-1}.

Greenhouse experiment and inoculation of PGPR and AMF

The experiment performed was conducted with 5 × 2 factorial scheme with five inoculation levels of PGPR and two inoculation levels of AMF in each treatment, and 5 repetitions in each treatment, totaling 30 experimental units. Five inoculation levels of PGPR consisted of non-inoculation of PGPR or inoculation of four isolated PGPR, which were identified as Klebsiella (B1), Burkholderia (B2), Lysinibacillus (B3) and Lysinibacillus (B4) (Table 2) by degerming 16S rDNA sequence. The PGPR were isolated from two native Brazilian tree species, being braúna (Melanoxylon brauna Schott) and vinhático (Plathymenia reticulata Bentham) (Table 2). The DNA extraction was performed, and subsequently, the gene was amplificated by the polymerase chain reaction (PCR) method, using primers 27F and 1429R (Weisburg et al., 1991). Afterward, the products of PCR were sequenced by Macrogen, Inc. (Korea). All sequences obtained in the present study were analyzed by comparing the obtained sequences with those deposited in the GenBank database using the BLASTx search tool (NCBI).

Table 2: Origen of plant species and *in vitro* assays characteristics of isolated plant growth-promoting rhizobacteria culture.

Plant species' origins	Rhizo-Bacterial strain	Identification	Biochemical test			
			[a]P solubilization (CP)	[b]GA production	[c]BNF	[d]IAA production
Braúna	B1	*Klebsiella*	−	(+)	−	+
Vinhático	B2	*Burkholderia*	+	+	−	(+)

Braúna	B3	*Lysinibacillus*	−	+	−	(+)
Vinhático	B4	*Lysinibacillus*	+	+	−	(+)

'+'= positive; '–'= negative; '(+)'= slow positive; CP= calcium phosphate; GA= gibberellins; BNF= biological nitrogen fixation; IAA= Indole 3-acetic acid;

[a] P solubilization was evaluated by (Berraquero et al., 1976)

[b] GA production was evaluated by (Graham & Henderson, 1961).

[c] BNF was evaluated in N-free semi-solid media with pellicle formation indicating the ability to fix atmospheric nitrogen (Boddey et al., 1995)

[d] IAA production was indicated by the cultivation in tryptic soy agar (TSA) medium 10% containing L-tryptophan (5 mm), pH 7.0 (Glickmann & Dessaux, 1995).

AMF spores were multiplied by on-farm method (Moreira *et al.*, 2019), using a substrate composed of soil (horizon B), sugarcane bagasse, and vermiculite (1: 1: 1; v:v:v) with the addition of 10 % organic compost, using sorghum plants. After three months of cultivation, the plants underwent stress due to the absence of water (one month). Then the soil was used as an inoculum after the homogenization. The inoculation of AMF was principally consisted of *Acaulospora scrobiculata, Claroideoglomus etunicatum, Paraglomus albidum* and *Paraglomus brasilianum*, which were identified as described by (Stürmer et al., 2018) in Regional University of Blumenau Foundation, in Blumenau, Santa Catarina, Brazil. Each inoculated treatment received 15 mL of AMF inoculant, about 45 spores per pot.

Inoculation level of AMF consisted of non-inoculation or inoculation of AMF. Substrates in treatments NON.CONTROL, NON.B1, NON.B2, NON.B3 and NON.B4 were not inoculated with AMF, whereas substrates in treatments INOC.CONTROL, INOC.B1, INOC.B2, INOC.B3 and INOC.B4 were inoculated with AMF. The study was divided into two parts, the first sampling after 180 days of seedling cultivation as Experiment I and the second sampling after 360 days of seedling cultivation as Experiment II (Table 1). The experiment was carried out in the greenhouse condition during the season between October 2019 and October 2020.

Evaluation of growth parameters

The stem diameter (D) at 1.5 cm from the soil, plant height (H) and dry mass (SDM) of the aerial part were measured. SDM was determined after drying to constant mass at 70 ºC in an oven under forced ventilation. The routine chemical analyses of soil and foliar samples were performed according to the methods of Defilipo and Ribeiro (1997) in the Laboratory of Analysis of Soil Viçosa (Table 2) in Viçosa, MG, Brazil.

PCR-DGGE analyses

Evaluation of molecular biology was performed by DNA extraction and PCR-DGGE technique to analyze the microbial community of Glomeromycota (AMF), total bacteria and nitrogen-fixing bacteria (NFB) and in the soil using a small DNA fragment. The total DNA extraction of the soil sample was performed with the NucleoSpinSoil® (Machereye-Nagel, GmbH & Co. KG, Germany) following the manufacture's protocol.

After the extraction, the DNA solutions were heated to 60 ºC for 10 min, vortexed for 5 seconds and centrifuged for 5 seconds, aiming at better homogenization. Then, a composite sample (45 µL) was obtained by mixing 15 µL of the DNA solution obtained for each of the three sample points in each repetition.

The 18S and 16S rDNA genes were amplified using a nested polymerase chain reaction (PCR) approach to obtain specifically the sequences of Glomeromycota, total bacteria and the *nif*H gene for nitrogen-fixing bacteria (NFB). Primer sets AML1/AML2 (Lee et al. 2008) and NS31GC/Glo1 (Kowalchuk et al. 2002, Cornejo et al. 2004), 27F/1494R (Weisburg et al., 1991) and 984FGC/1378R (Nübel et al., 1996, Heuer et al., 1997) and 19F/407R and 19F-C/278R (Ueda et al. 1995) were used for the first and second PCR, respectively for the 18S rDNA fraction from Glomeromycotan, 16S rDNA fraction from total bacteria, and *nif*H gene.

Aliquots of 5 µL of each PCR product were analyzed by agarose gel electrophoresis (1.5 %, w/v), stained with ethidium bromide (0.5 µg mL^{-1}). Subsequently, the presence and

quality of the DNA fragments were confirmed under UV light and UV light photo documentation imaging system (Loccus Biotecnologic L-Pix Chemi). The fragments obtained by the nested-PCR were separated by the denaturing gradient gel electrophoresis (DGGE) technique whereby AMF, total bacteria (Prado et al. 2019), and *nif*H (Silva et al. 2014) profiles present in the soil samples were analyzed.

Data analyses

The data were subjected to analysis of two-way ANOVA followed by a Tukey test ($p \leq 0.05$) and the results were submitted to Kolmogorov-Smirnov normality verification. The DGGE profiles, aligned according to external markers, were analyzed using the Bionumerics software system (Version 6.0, Applied Maths NV).

Dendrograms were constructed by Cosine Coefficient similarity index, followed by cluster analysis using the minimum variance (Ward) method. The samples were compared based on the bands' presence/absence and intensity and differences of the index of diversity among treatments were detected using the software PAST ® (Hammer et al., 2001). The analysis was performed in the statistical program Minitab.

Results

Bioaccumulation

Experiment I

On the whole, pau-viola seedlings did not respond to inoculation of PGPR but positively to AMF (Table 3), which were beneficial ($p<0.05$) for the plant growth. The statistic results showed a significant interaction (AMF × PGPR) for H and SDM of the seedling growth.

For the plant diameter, the treatment INOC (inoculated with AMF) had higher performance than that of NON (non-AMF), whereas inoculation of PGPR did not influence

these growth measures (Table 3). For the plant height, NON.B1 and INOC.CONTRO were higher ($p<0.05$) than the others four treatments, NON.B2, INOC.B2, NON.B3 and NON.B4. Any inoculation of PGPR did not respond ($p>0.05$) in relation to the NON.CONTROL (Table 3). For the SDM, better results were obtained on all INOC treatments than in NON-inoculated treatments.

Table 3: Stem diameter (D), plant height (H) and shoot dry mass (SDM) of pau-viola after 180 days (experiment I) and 360 days (experiment II) of greenhouse cultivation, comparing plants inoculated and non-inoculated with AMF and/or PGPR. (NON= non-AMF, INOC= with AMF) and/or Plant Growth-Promoting Rhizobacteria (CONTROL= non-PGPR, B1= Klebsiella, B2= Burkholderia, B3= Lysinibacillus and B4= Lysinibacillus)

Experiment I					
D (mm)					
AMF		NON. 3.22 b		INOC. 3.82 a	
H (cm)					
AMF	CONTROL	B1	B2	B3	B4
NON.	23.92 abc	25.62 a	20.14 bc	20.70 bc	15.87 c
INOC.	31.20 a	25.56 ab	18.58 bc	24.10 abc	23.8 abc
SDM (g)					
AMF	CONTROL	B1	B2	B3	B4
NON.	0.91 c	1.14 c	1.20 c	1.33 bc	1.07 c
INOC.	2.18 a	2.18 a	1.87 ab	1.99 a	2.02 a
Experiment II					
D (mm)					
AMF	CONTROL	B1	B2	B3	B4
NON.	6.94 ab	7.88 a	6.48 ab	7.20 ab	7.83 a
INOC.	7.00 ab	5.60 b	7.38 ab	6.90 ab	7.16 ab
H (cm)					
AMF	CONTROL	B1	B2	B3	B4
NON.	32.66 ab	34.98 a	30.72 abc	29.25 abc	22.10 c
INOC.	29.56 abc	33.55 ab	23.68 bc	27. 20 abc	35.65 a
SDM (g)					
AMF	CONTROL	B1	B2	B3	B4
NON.	5.45 ab	6.86 a	4.54 ab	5.54 ab	5.97 ab
INOC.	5.10 ab	3.36 b	5.20 ab	5.51 ab	6.83 ab

Means followed by the same letters in a column and a line within a measure type are not significantly different at $p<0.05$ (ANOVA).

Shoot chemical characteristics showed a difference between NON and INOC ($p< 0.05$), resulting in higher nutrients content in nitrogen, phosphorus, potassium, calcium, magnesium in treatments of INOC than that of NON (Table 4).

Table 4: Nutrient content (mg/plant) in the shoot of pau-viola, inoculated and non-inoculated with AMF, after 180 days of growing in the greenhouse.

	N	P	K	Ca	Mg	S	Zn	Fe	Mn	Cu	B
NON	12.79 b	2.84 b	36.86 b	7.12 b	1.91 b	3.55 a	0.15 a	0.53 a	0.02 a	0.01 a	0.11 b
INOC	16.75 a	3.89 a	48.34 a	11.85 a	3.25 a	4.01 a	0.11 a	0.55 a	0.03 a	0.02 a	0.16 a

N = nitrogen; P = phosphorus; K = potassium; CA = calcium; MG = magnesium; S = sulfur; Zn = zinc; Fe = iron; Mn = manganese; Cu = Copper; B = boron; Different letters in the column indicate a significant difference at $p<0.05$ (ANOVA).

Experiment II

All plants survived in IOMW conditions. Although the statistic results showed interaction (AMF × PGPR) on seedling growth ($p<0.05$), the microbial inoculation showed little difference among the ten treatments. When we compare the result with CONTROL and other treatments, there was no difference ($p>0.05$).

However, for the stem diameter (D), NON.B1 and NON.B4 presented higher values comparing to INOC.B1 (Table 3). For the plant heigh, NON.B1 and INOC.B4 were higher than INOC.B2 and NON.B4 (Table 3). For the SDM, NON.B1 showed better results than INOC.B1 (Table 3).

Overall, seedling growth in all treatments was not influenced by inoculation of either AMF or PGPR, although in one case (NON.B4 in height), there was an exception that had a negative effect by PGPR inoculation.

Analysis of PCR-DGGE

PCR-DGGE technique evaluated three microbial communities in the soil, AMF, total bacteria and nitrogen-fixing bacteria. Cluster analysis of the soil AMF community formed two distinct groups (Fig. 1). The first group presented approximately 70 % similarity, including the treatments NON from experiment I and all the NON and INOC from experiment II. One subgroup was composed of treatments INOC from experiment II with 94 % similarity. Additionally, there are three subgroups of treatments NON from the experiment I and II with more than 90 % similarity in the first group. The second group had 98 % similarity, formed by treatments INOC from experiment 1 (Fig. 1), showing the different AMF community composition from the first group.

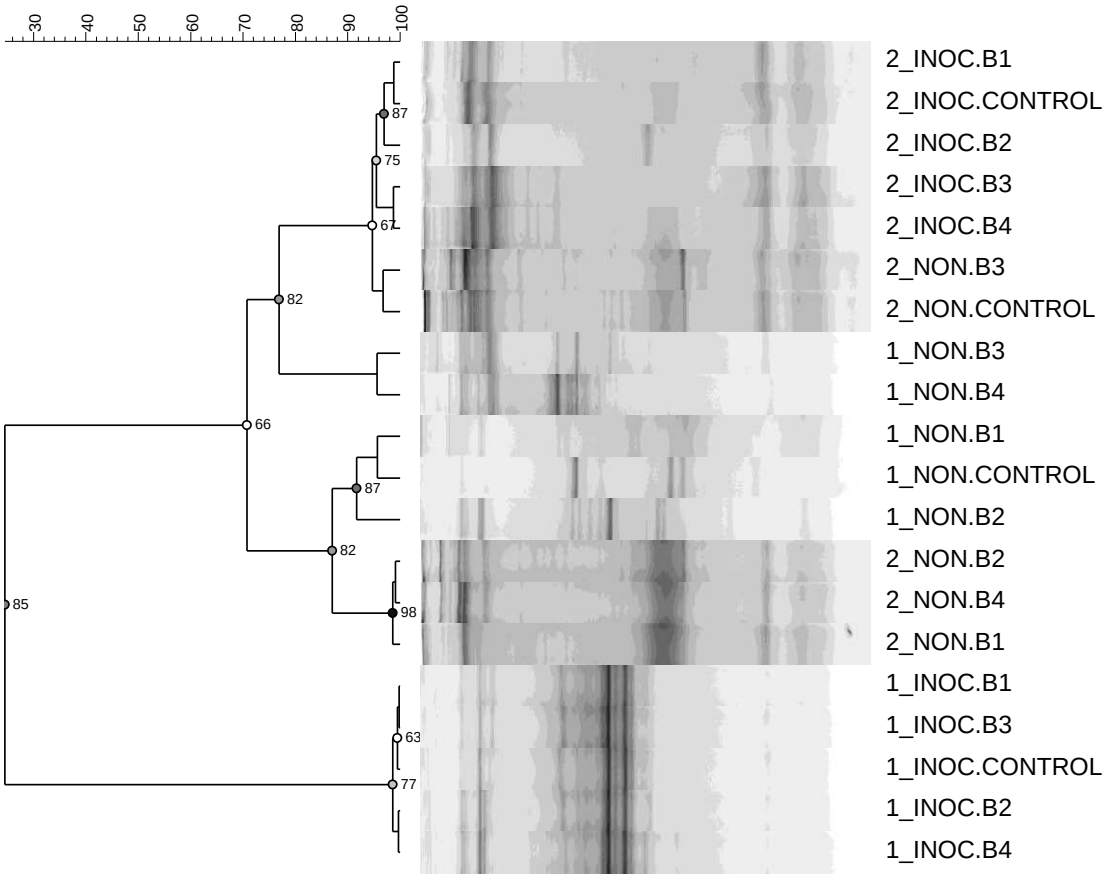

Fig.1: Cluster analysis, Cosine-ward, obtained by the DGGE bands profile of the 18S gene from Glomeromycota communities of AMF inoculants from 20 sources. The soil samples were collected after 180 days (1) and 360 days (2) of greenhouse cultivation of pau-viola.

Cluster analysis of the soil total bacterial community was separated only by experiment I and experiment II. Two distinct groups obtained 50 % similarity, and each group showed 97 % similarity, which indicated that neither AMF nor PGPR influenced the classification (Fig.2).

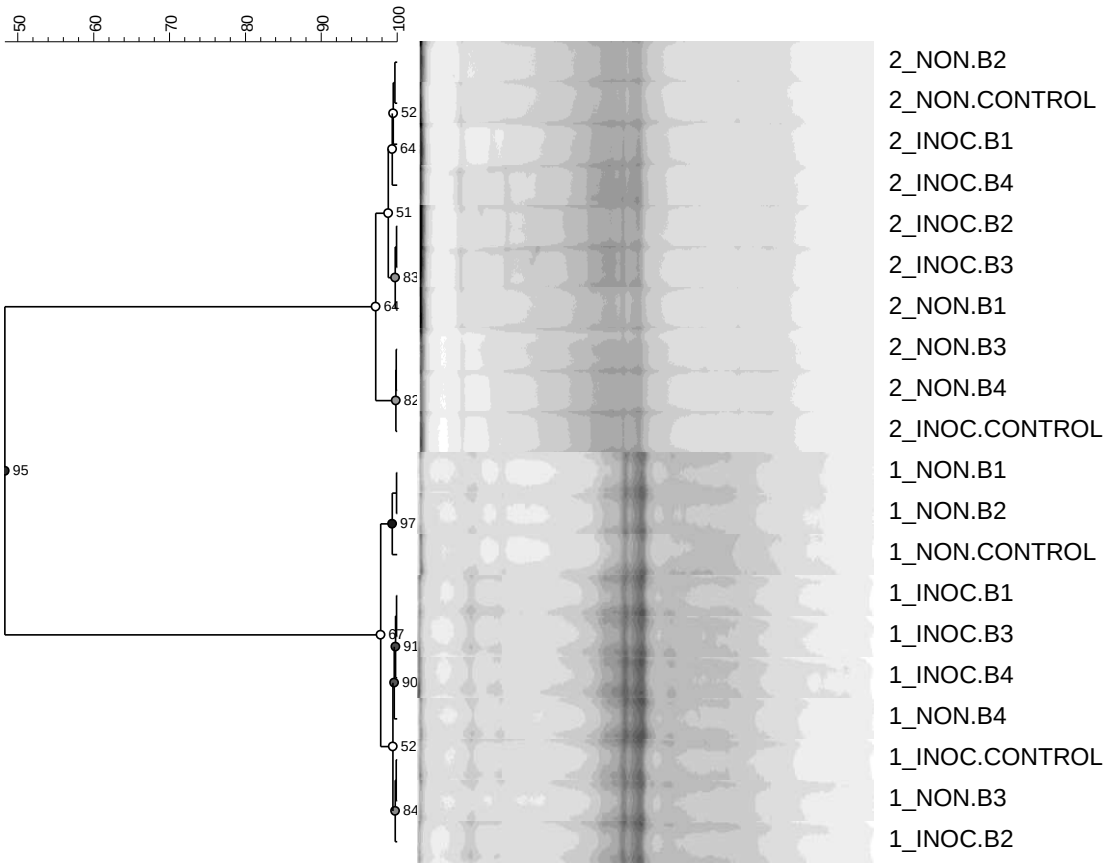

Fig.2: Cluster analysis, Cosine-ward, obtained by the DGGE bands profile of the 16S gene from Glomeromycota communities of AMF inoculants from 10 sources. The soil samples were collected after 180 days (1) and 360 days (2) of the greenhouse cultivation of pau-viola.

Cluster analysis of the soil NFB community also formed two distinct groups by experiment I and experiment II with 86 % similarity, having exceptions of tree treatment, 2_NON.B1, 2_NON.CONTROL, and 2_INOC.B3, which were classified with other treatments from the experiment I. The first and second groups obtained 97 % and 98% similarity,

respectively (Fig.3). Thus, inoculation of AMF and PGPR was not the factor for separating the nitrogen-fixing bacterial community in the soil.

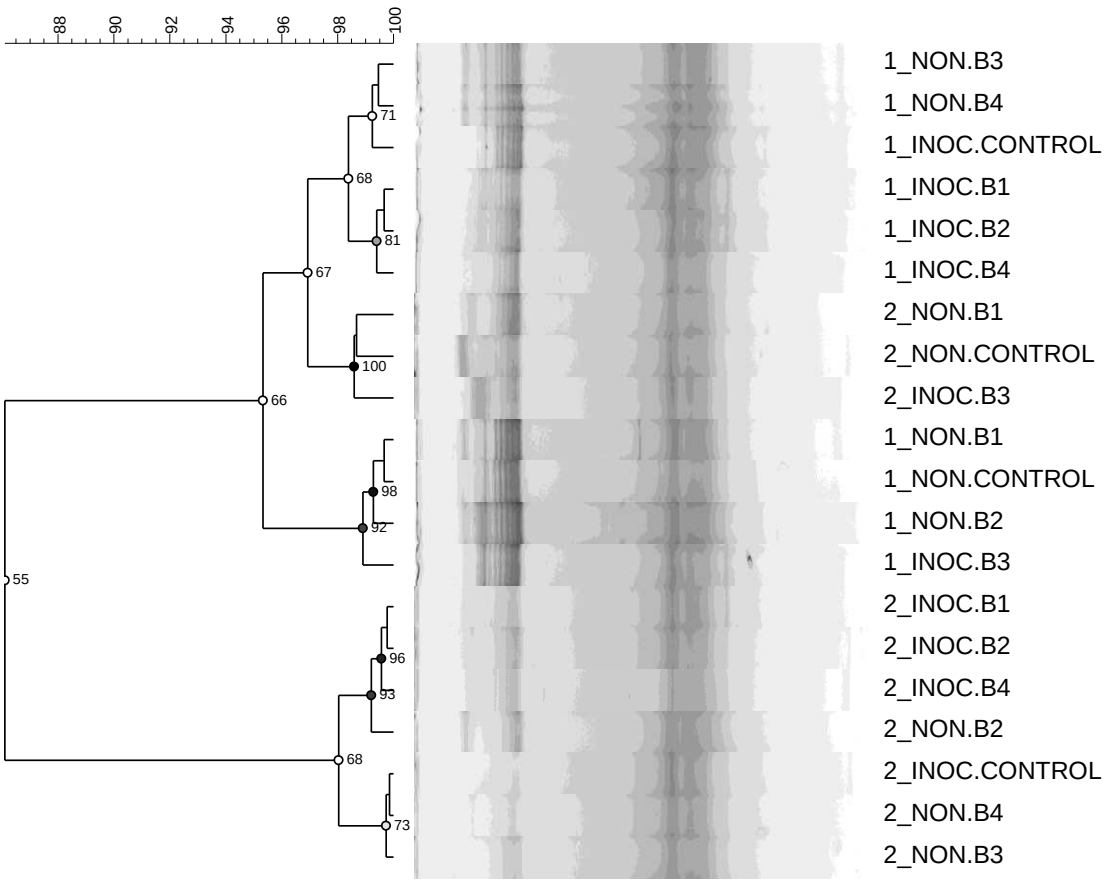

Fig.3: Cluster analysis, Cosine-ward, obtained by the DGGE bands profile of the 16S gene from NIF communities of AMF inoculants from 10 sources. The soil samples were collected after 180 days (1: experiment I) and 360 days (2: experiment II) of the greenhouse cultivation of pau-viola.

The statistic results did not show the difference ($p>0.05$) on indices of diversity for AMF; however, higher indices of diversity ($p<0.05$) on treatments of INOC from experiment I (1_INOC) were obtained for total bacteria as well as nitrogen-fixing bacteria community in the soil, compared to treatments of NON from experiment II.

Table 5: Indices of diversity for arbuscular mycorrhizal fungi (AMF), total bacteria, and nitrogen-fixing bacteria (NFB) obtained by the PCR-DGGE technique in the substrate (1_NON and 1_INOC from experiment I) and contaminated soil (2_NON and 2_INOC from experiment II) with and without inoculation of AMF.

AMF				
Treatments	Richness	Simpson	Shannon (H)	Chao
1_NON	16.8 A	0.93430 A	2.7764 A	160.0 A
1_INOC	16.8 A	0.93636 A	2.7574 A	133.4 A
2_NON	15.8 A	0.93950 A	2.8132 A	151.8 A
2_INOC	14.6 A	0.93016 A	2.6710 A	116.0 A
Total bacteria				
Treatments	Richness	Simpson	Shannon (H)	Chao
1_NON	8.2 AB	0.87562 AB	2.0942 AB	38.4 AB
1_INOC	10.0 A	0.89864 A	2.2962 A	55.6 A
2_NON	6.0 B	0.80920 B	1.7286 B	26.0 B
2_INOC	6.6 B	0.84142 AB	1.8644 AB	23.0 B
NFB				
Treatments	Richness	Simpson	Shannon (H)	Chao
1_NON	17.6 AB	0.9425 AB	2.8618 AB	165.6 AB
1_INOC	18.8 A	0.9453 A	2.9208 A	190.4 A
2_NON	13.8 B	0.9270 B	2.6214 B	102.8 B
2_INOC	14.2 AB	0.9280 B	2.6424 B	110.0 B

Means followed by the same letters in a column, within an index type and a microbial community are not significantly different at $p<0.05$ (ANOVA)

Discussion

Biomass accumulation

Experiment I

The result suggest that symbiosis between AMF and pau-viola was established, having a significant effect on water and nutrient uptake (Prates Júnior et al. 2021a). This positive influence assists in the better overall performance of plants associated with AMF, which indicates the potential as a bioinoculant. On the other hand, PGPR used as inoculation did not positively influence the seedling growth compared to the control in the condition of the substrate used. An effective symbiosis depends on the specificity of association in both plants and bacteria (Mus et al., 2016). Therefore, the symbiosis may not have been compatible between the host plant and bacteria inoculated. We suggest that inoculation of PGPR strains

used in this study is not necessary to increase the seedling's development because what improved plant growth was AMF inoculation but further study is needed to evaluate the potential of other PGPR inoculation.

Experiment II

Since the seedlings grew well in the IOMW condition independent of the microbial inoculation, we considered that pau-viola could tolerate the stress conditions. In other words, both AMF and PGPR inoculation did not influence the seedlings growth in this experiment II. Nonetheless, AMF and bacteria, including NFB, were present in the soil (Table 5). AMF multiplication by the on-farm method requires the use of non-sterile soil and local materials. Given that AMF inoculation contains taxonomically diverse microorganisms adapted to local conditions besides AMF (Moreira *et al.*, 2019), the inoculated seedlings could help recover the local microbial community in the soil, especially in degraded regions (Prates Júnior et al., 2021b). Therefore, further work is needed to study the inoculation effect on inoculated seedlings in the field.

Analysis of PCR-DGGE

Although the alteration of richness and diversity in AMF community did not reach a significant level ($p>0.05$) (Table 5), the dendrogram of AMF community (Fig.1) separated 1_NON from 1_INOC by the application of AMF inoculation, having different band pattern. AMF community composition has shifted considerably in 1_INOC, which probably resulted in the seedling growth promotion (Table 3). According to the indices of AMF diversity (Table 5), negative influence by IOMW in AMF, total bacteria and NFB community in NON was not observed, which indicated that some fungus and bacteria species may have adapted new environmental conditions (Angelard et al. 2014), by changing chemical properties in the soil in experiment II (Dong et al. 2017).

As shown in Table 5, in general, indices of diversity for AMF, bacteria total and NFB had less richness and diversity in experiment II than in experiment I (Table 5). In this context, the relatively high pH value of IOMW could have made a considerable impact on the microbial community in the soil because pH is the critical environmental factor determining fungal communities (Veresoglou et al. 2013) and influence AMF spore germination (Siqueira et al. 1982). Since *Glomus* and *Acaulospora* occur in soil with pH below 6.2 (Stürmer and Bellei 1994; Stürmer et al. 2006), we considered that inoculated AMF species did reduce the community in the critical condition of IOMW. Thus, overall, the smaller microbial community in experiment II may have attributed to abiotic stress from the inadequate soil condition, mainly by higher pH value than neutral Brazilian soil.

Neither total bacteria nor NFB community were influenced by AMF inoculation (Fig.2 and Fig.3), which was also reported in the previous study about Glomus-cucumber symbiosis with no influence on bacterial diversity (Mansfeld-Giese et al. 2002). When comparing experiment I and experiment II, 1_INOC showed higher species richness and diversity in the indices than 2_NON (Table 5). This effect over AMF inoculation on total bacteria and NFB community probably ascribed the AMF community from inoculation that has favored fungal-bacterial symbiosis development. Mutualisms like fungal-bacterial symbiosis are stabilized over evolutionary time. Thus the associations are highly coevolved (Pawlowska et al. 2018). Further work is needed to study phylogenic placement to know the evolutionary history.

Conclusion

We concluded that inoculation with AMF helps in better growth of pau-viola during the nursery and indicates that AMF inoculation application adds a new AMF community that favors the host. Consequently, the AMF inoculation contributes to the seedling's production with sustainability and soil quality. Since the drastic changes in soil condition, including alkaline pH

value of IOMW, alter microbial community composition and AMF inoculation does not increase the seedling's growth in the IOMW in this condition of the study. Additional experiments will be required to study the effect of the inoculation in the field condition.

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FINAL CONSIDERATION

Selected beneficial microorganisms can be used for producing seedlings of native forest plants, since they are capable of promoting the growth and increase the tolerance to stress, resulting in seedlings with high quality. We expected that the seedlings using this technology is able to develop more vigorously over the years that will remain in the field, and guarantee the reforestation using native trees species.