**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Arbuscular mycorrhizal fungi and *Eucalyptus* species with different
levels of water stress tolerance**

Bruna Andréia de Bacco Lopes

Dissertation presented to obtain the degree of
Master in Science. Area: Soil and Plant Nutrition

**Piracicaba
2019**

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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*To my friend, Karen Cristina de Medeiros (in memoriam),
I dedicate*

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If I have seen further than others, it is by standing upon the shoulders of giants.

Isaac Newton

SUMMARY

RESUMO.....	8
ABSTRACT	9
ABBREVIATION LIST.....	11
1. INTRODUCTION AND JUSTIFICATIVE	103
2. LITERATURE REVIEW	15
2.1 THE GENUS <i>EUCALYPTUS</i> : ORIGIN AND ECONOMIC IMPORTANCE	15
2.2 THE GENUS <i>EUCALYPTUS</i> AND THE WATER DEFICIT IN SOIL	16
2.3 CHARACTERIZATION OF <i>EUCALYPTUS</i> SPECIES.....	16
2.4 RELATIONS AMONG MYCORRHIZAL FUNGI, <i>EUCALYPTUS</i> AND WATER DEFICIT IN SOIL	17
3. HYPOTHESIS AND OBJECTIVES	19
3.1 HYPOTHESIS	19
3.2 OBJECTIVES.....	19
4. MATERIAL AND METHODS	21
4.1 FIELD EXPERIMENT.....	21
4.2 GREENHOUSE EXPERIMENT	28
5 RESULTS AND DISCUSSION	31
5.1 FIELD EXPERIMENT	31
5.1.1 Spores number and roots colonization	31
5.1.2 Glomalin-related soil protein.....	34
5.1.3 Enzymatic activity.....	36
5.1.4 Microbial biomass carbon and soil basal respiration	38
5.1.5 Abundance of ITS genes.....	40
5.1.6 Fungal community structure	41
5.1.7 Redundancy analysis	43
5.2 GREENHOUSE EXPERIMENT	44
5.2.1 Spores number and root colonization.....	44
5.2.2 P leaves and shoots dry matter	45
6. CONCLUSION	47
REFERENCES.....	49
ADDITIONAL CONTENT.....	55

RESUMO

Fungos micorrízicos arbusculares e espécies de *Eucalyptus* com diferentes níveis de tolerância ao estresse hídrico

As espécies de eucalipto respondem por quase 80% das florestas plantadas no Brasil, o que faz da silvicultura uma atividade de grande importância no cenário nacional e internacional. Apesar de sua adaptabilidade, o eucalipto está sujeito a danos que as alterações climáticas podem causar, especialmente o déficit hídrico. A maioria das plantas possui estratégias de sobrevivência em situações de déficit hídrico. A associação com fungos micorrízicos arbusculares (FMA) é uma delas, pois aumenta a absorção de água e nutrientes pelas raízes. Assim, o objetivo principal deste trabalho foi avaliar a colonização de fungos micorrízicos em seis espécies de *Eucalyptus* com diferentes níveis de tolerância à seca: *E. brassiana*, *E. camaldulensis*, *E. citriodora*, *E. cloeziana*, *E. grandis* e *E. urophylla*. Dois ensaios foram realizados: um no campo e outro em casa de vegetação. Em casa de vegetação, os vasos foram submetidos a diferentes níveis de água: 50, 75 e 100% da capacidade de campo, por três meses. No campo, as amostras foram coletadas em dois períodos diferentes: após o período seco e após a estação chuvosa. As amostras de solo e raízes finas coletadas nos dois experimentos foram submetidas à análise microbiológica e molecular. A colonização da raiz por FMA foi avaliada. Os genes ITS foram analisados por PCR quantitativo (qPCR) e a estrutura da comunidade fúngica por polimorfismo de fragmentos de restrição (T-RFLP). Dentre as espécies estudadas, *E. urophylla* apresentou maior colonização em ambos os ensaios. A atividade microbiana do solo foi fortemente afetada pela umidade do solo. A estrutura da comunidade fúngica não se diferenciou claramente entre os tratamentos. A idade das plantas e a umidade do solo parecem ter maior efeito no desempenho das espécies de eucalipto do que seu nível de tolerância ao estresse hídrico.

Palavras-chave: Microbiologia do solo; Eucalipto; Micorrizas; Tolerância ao estresse hídrico

ABSTRACT

Arbuscular mycorrhizal fungi and *Eucalyptus* species with different levels of water stress tolerance

Eucalyptus species account for almost 80% of the forests planted in Brazil, which makes silviculture an activity of great importance in the national and international scenario. Despite its adaptability, *Eucalyptus* is subject to damages caused by climate change, especially the water deficit. Most plants have survival strategies in water deficit situations. Association with mycorrhizal fungi (FM) is one of them, since they increase water and nutrients uptake by the roots. Thus, the main objective of this study was to evaluate the colonization of mycorrhizal fungi in six *Eucalyptus* species with different levels of drought tolerance: *E. brassiana*, *E. camaldulensis*, *E. citriodora*, *E. cloeziana*, *E. grandis* and *E. urophylla*. Two trials were carried out, one in the field and another in a greenhouse. In the greenhouse, the substrate of the pots were submitted to different levels of water retention, 50, 75 and 100% of the field capacity, for three months. In the field, soil and roots were sampled at two different periods: after the dry period and after the rainy season. The soil samples and fine roots collected from the two experiments were submitted to microbiological and molecular analyses. AMF root colonization was evaluated. The ITS genes were analyzed by quantitative PCR (qPCR) and the fungal community structure by restriction fragment polymorphism (T-RFLP). Among the eucalypt species studied, *E. urophylla* presented higher colonization in both trials. Soil microbial activity was strongly affected by soil moisture. The fungal community structure did not differ clearly among the treatments. Age of plants and soil moisture seemed to affect the plant species more than levels of water stress tolerance.

Keywords: Soil microbiology; *Eucalyptus*; Mycorrhiza; Water stress tolerance

ABBREVIATION LIST

Al – Aluminum

AMF – Arbuscular mycorrhizal fungi

B – Boron

Bra – *Eucalyptus brassiana*

BSA – Bovine serum albumin

C – Carbon

Ca – Calcium

Cam – *Eucalyptus camaldulensis*

CEC – Cation exchange capacity

Cit – *Eucalyptus citriodora*

Clo – *Eucalyptus cloeziana*

Cmic – Biomass microbial carbon

Cu – Copper

ECM – Ectomycorrhiza

Fe – Iron

Gra – *Eucalyptus grandis*

GRSP_{EE} – Easily extractable glomalin-related soil protein

H₂O₂ – Hydrogen peroxide

HCl – Hydrochloric acid

IAC – Instituto Agronômico de Campinas (The Agronomy Institute of Campinas)

IBGE – Instituto Brasileiro de Geografia e Estatística (The Brazilian Institute of Geography and Statistics)

INVAM - International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi

K – Potassium

KOH – Potassium hydroxide

Mg – Magnesium

Mn – Manganese

MUB – Modified universal buffer

NaOH – Sodium hydroxide

NH_4^+ - Ammonium

NO_3^- - Nitrate

P – Phosphorus

PNF – *p*-nitrophenyl

PVLG – Polyvinyl-Lacto-Glycerol

$q\text{CO}_2$ – Metabolic coefficient

$q\text{PCR}$ – Quantitative polymerase chain reaction

RBS – Basal soil respiration

S – Sulfur

SB – Sum of bases

T-RFLP – Terminal restriction fragment length polymorphism

TTF – Triphenyl tetrazolium chloride

Uro – *Eucalyptus urophylla*

Zn - Zinc

1. INTRODUCTION AND JUSTIFICATIVE

Silviculture has been consolidated across the Brazilian territory and, nowadays, forest products are an important slice of exportations in agribusiness. The activity thrives for many reasons, due to the high adaptability of the genus *Eucalyptus*, which accounts for 75% of the Brazilian planted forests (ABRAF, 2013).

One of the challenges of agriculture is to keep high yields amid climate adversities, especially water deficit, which has been intensifying in the last years according to the global warming (Booth, 2013). Eucalypt plantations in Brazil are concentrated in the Southeast region, where the rainfall is relatively regular, but there is a trend for long dry periods (Gonçalves et al., 2013; Gonçalves et al., 2017). In order to obtain continued crop expansion in more arid regions, it is necessary to explore new means of adaptation to the changing environment (Cha-um et al., 2014).

Root colonization by arbuscular mycorrhizal fungi (AMF) or ectomycorrhiza (ECM) works as an extension of the roots, helping in the uptake of water and nutrients (Cardoso et al., 2016). Therefore, the study of how mycorrhizal fungi behave under different rainfall conditions and in association with different *Eucalyptus* species may enlighten our understanding of the phenomena and probably show interesting results related to the crop establishment in low rainfall regions or survival to long dry periods.

Evaluating the fungal community might result in the identification of species that can be helpful when inoculated into plants during dry periods, and being able to support the healthy development of the trees. Thus, there is a lack in our knowledge and it is urgent to obtain better information in literature correlating the eucalypt tree, mycorrhizal processes and water deficit, as a kickoff to further studies.

2. LITERATURE REVIEW

2.1 The genus *Eucalyptus*: origin and economic importance

The genus *Eucalyptus* comes originally from Australia and, despite of approximately 730 recognized species, only about 20 of them are in commercial use worldwide. The exact date of *Eucalyptus* introduction in Brazil is uncertain, probably between 1825 and 1868. However it is well documented that in 1904 the crop started to be studied by Edmundo Navarro de Andrade, at the Horto Florestal de Rio Claro, in São Paulo state, belonging to the former Companhia Paulista de Estradas de Ferro, a train company, which was the preferred mean of transportation. However, only after the proclamation of financial incentives in 1966 and the National Plan of Development in the decade of 1970 the eucalypt was considered as an important crop in the country (EMBRAPA, 2014).

According to IBGE, in 2015, the area occupied by planted forests in Brazil was of about 10 million hectares, almost 75% occupied by eucalypts. Southern Brazil has the greatest planted area, with 3.8 mi hectares, where 1.7 mi hectares are of eucalypt. Next comes the Southeast region, with around 3.3 mi hectares and more than 90% of the area occupied by eucalypt trees. The state with the larger planted area of eucalypt is Minas Gerais, with 1.9 mi hectares.

The importance of *Eucalyptus* is growing rapidly in an international scenario, and Brazil is one of the major producers of planted forests and the fourth major pulp producer. Today it accounts for over 16% of the Brazilian agribusiness exportations, only behind soybeans (34.49%) (MAPA, 2019). The major importing countries are the United States, China, Netherlands, Italy and Argentina (SNIF, 2015).

Most of these forest products come from eucalypt trees, and this predominance can be attributed to their fast growth, good adaptation, and economic potential. The national average of wood yield is $41 \text{ m}^3 \text{ ha}^{-1} \text{ year}^{-1}$, in rotation sequences of about seven years (EMBRAPA, 2014).

The Eucalypt wood provides quite versatile products, for sawmill, sheeting, charcoal and pulp. It also represents different options of diversification for farmers as pure plantations or integrated crop-livestock-forest systems (EMBRAPA, 2014).

2.2 The genus *Eucalyptus* and the water deficit in soil

Due to its origin from tropical regions, eucalypt suffers severe growth limitations in low temperatures, especially in the seedling stage. Only *Eucalyptus benthamii* and *Eucalyptus dunnii* are resistant or tolerant to high intensity frost (EMBRAPA, 2014).

If low temperatures are a limiting factor in temperate regions, in the tropics a limiting factor is water deficit (EMBRAPA, 2014). In Brazil, the rainfall varies across different regions, and the choice of species with some water stress tolerance may be necessary.

Nowadays, climate changes are becoming a very important issue in agriculture, as the increase of temperatures is one of its main causes in a global scale. This highlights the importance of investigations to clarify the efficiency of the water use by eucalypt plantations under climate and atmospheric changes (Booth, 2013).

However, water deficit is one of the worst abiotic factors, being accountable for damages in growth and development of plants and leading to yield losses (Cham et al., 2014), showing the necessity to develop new studies on the response of eucalypt to such conditions.

Naturally, plants can have or even develop certain mechanisms that are survival strategies under adverse conditions. Understanding those mechanisms as responses to physiological stresses is essential for drought tolerance (Navarrete-Campos et al., 2013). At least part of the harmful effects on plant performance of many different abiotic factors is due to the decrease of water availability in the environment (Verslues et al., 2006). The effects of water stress in plants can induce both morphological and physiological effects (Navarrete-Campos et al., 2013).

A strategy that can be used by the plant to cope with dry periods is the association between roots and soil microorganisms, such as arbuscular mycorrhizal fungi (Bowles et al. 2016).

2.3 Characterization of *Eucalyptus* species

Here, we present a brief characterization based on Flores et al. (2016) of the *Eucalyptus* species addressed in this work, especially regarding their climate requirements. The species described below were chosen due to their tolerance to drought and for being widely planted in Brazil.

Eucalyptus brassiana: annual temperature is between 21°C and 27°C and rainfall between 1000 and 3000 mm. Well adapted to water stress, it has potential to be grown across the Brazilian territory.

Eucalyptus camaldulensis: demands annual temperatures between 16°C and 27°C and rainfall from 500 to 1500 mm. It has tolerance to temporary floods and to high temperatures and long dry periods. Wood more adapted for sawmill, firewood and charcoal.

Eucalyptus citriodora: requires temperatures between 18°C and 25°C and annual rainfall between 800 and 2000 mm. Wood commonly employed for constructions, structures, firewood and charcoal. In addition, essential oil is obtained from leaves. In São Paulo state, presents susceptibility to frost and good resistance to water deficiencies.

Eucalyptus cloeziana: the main climatic requirements are annual temperatures from 18°C to 26°C and rainfall between 600 to 2500 mm. It has a low germination index and slower initial growth. It is susceptible to frost and water deficiency. High-density wood used for sawmills and structures.

Eucalyptus grandis: the species development is better in annual temperatures ranging from 15°C to 22°C and rainfall between 800 and 2000 mm. It is the main source of pulp production in São Paulo state and susceptible to water deficiency.

Eucalyptus urophylla: requires annual temperatures between 16°C and 27°C and rainfall from 1000 to 2000 mm. It can replace *E. grandis* in some situations. Presents lower tolerance to water deficiency.

2.4 Relations among mycorrhizal fungi, *Eucalyptus* and water deficit in soil

Eucalypt trees are able to form in their root system the two main types of mycorrhiza: ectomycorrhiza (ECM) and arbuscular mycorrhiza (AM) (Campos et al., 2011). These associations increase the area explored by the roots, leading to higher water and nutrient uptake by plants. They are also related to soil structure and resistance to some pathogens (Cardoso et al., 2016). Thus, they are essential for the maintenance of different ecosystems and extremely important under adverse conditions.

Essays carried out in *Eucalyptus* forests show that plant age has influence on the kind of mycorrhizal association and hence on diversity of mycorrhiza formed. Initially, in the seedling stage, there is colonization by arbuscular mycorrhizal fungi

(AMF), which is replaced gradually by ectomycorrhizal associations (ECM), as the plants get older (Santos et al., 2001; Pereira, 2015). The colonization by mycorrhizal fungi can be influenced by the tree species and the presence of both types of fungi probably can lead to competition and colonization reduction (Chilvers, Lapeyrie e Horan, 1987).

Most of the studies available on the effect of water deficit in eucalypt plantations do not refer to mycorrhizal associations. However, references of AMF on other crops have been reported. An article with tomato (*Solanum lycopersicum* L.), showed that the symbiosis with AMF increased the yield, what was associated to a greater water and nutrient uptake and higher photosynthesis rates (Bowles et al., 2016). That shows the importance of mycorrhizal fungi affecting plant response to watering deficiency in real conditions of the agroecosystem. Thus, any measure that minimizes great impacts on soil generally benefits the microbial community enhancing the efficiency of mycorrhizal associations (Bowles et al., 2016). De Sousa et al. (2015) found that sugarcane seedlings, under certain levels of water stress presented changes in AMF colonization. This suggests that abiotic factors also have influence on fungi diversity and colonization and these changes are strategies of adaptation to adverse conditions.

3. HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

- The colonization by AMF is different among the *Eucalyptus* species with different levels of drought tolerance.
- *Eucalyptus* species less tolerant to drought are more dependent on mycorrhizal fungi.
- Some AMF found in the area have potential to help trees with drought impacts.

3.2 Objectives

3.2.1 General objectives

To evaluate the root colonization of AMF in six *Eucalyptus* species with different levels of water stress.

3.2.2 Specific objectives

- To compare the AMF colonization between the *Eucalyptus* species.
- To evaluate the AMF root colonization at the end of the dry and the rainy period.
- To compare the number and species of AMF spores in soil among *Eucalyptus* species.
- To evaluate the soil fungal community structure by T-RFLP.
- To quantify the abundance of the ITS region in soil by quantitative PCR.

4. MATERIAL AND METHODS

4.1 Field Experiment

4.1.1 Local, climate, soil classification and history

The first experiment was implemented at the Experimental Station of Forest Sciences in Itatinga (23°02'S 48°38'W) - Department of Forest Sciences, "Luiz de Queiroz" College of Agriculture - University of São Paulo, Brazil. The climate of the region is Cfa (Köppen classification). The annual rainfall is 1350 mm, with 75% concentrated between March and October (Laclau et al., 2008). The soil was classified as red-yellow Latosol, of medium-sandy texture, low CEC (31.6 cmol_c dm⁻³) and typically dystrophic. The area is located at 830 m above sea level, where the experiment with *Eucalyptus brassiana*, *Eucalyptus camaldulensis*, *Eucalyptus citriodora*, *Eucalyptus cloeziana*, *Eucalyptus grandis* and *Eucalyptus urophylla* was installed in 2016. Previously, the area had been occupied by a clonal plantation of *Eucalyptus* sp., harvested in April 2015.

Table 1. Soil chemical characterization of the sampling site.

Treatments		Bra	Cam	Cit	Clo	Gra	Uro	Bra	Cam	Cit	Clo	Gra	Uro
Dry							Rainy						
pH	CaCl ₂	4,4	4,3	4,5	4,5	4,5	4,0	4,3	4,2	4,1	4,2	4,0	4,2
C	g dm ⁻³	18,36	20,67	16,69	20,16	17,53	17,40	19,58	21,70	22,41	19,84	14,51	23,43
NH ₄ ⁺	mg dm ⁻³	28,50	28,50	30,63	44,38	53,13	31,88	17,63	9,00	12,63	13,13	29,50	19,00
NO ₃ ⁻		13,75	9,88	16,63	21,75	25,00	19,38	16,38	9,50	14,13	15,00	12,88	13,88
P	mg dm ⁻³	4,65	2,10	2,07	3,88	2,26	2,18	3,10	3,93	3,26	8,76	4,54	6,26
S		3,78	3,84	3,96	2,50	3,71	4,14	4,23	5,18	3,42	3,35	4,19	3,48
Ca	mmol _c dm ⁻³	0,76	0,75	1,16	0,81	1,07	0,52	0,90	0,84	0,77	0,61	0,46	1,18
K		4,53	4,20	7,24	4,53	7,75	3,98	5,97	6,55	5,29	4,74	4,53	8,22
Mg		0,56	0,47	0,47	0,39	0,31	0,36	0,46	0,44	0,55	0,25	0,22	0,96
Al		4,6	5,5	4,6	4,7	2,8	8,3	4,2	5,8	5,3	5,3	6,1	4,4
H+Al		38	42	38	38	38	58	52	52	47	42	38	52
SB		5,85	5,42	8,87	5,72	9,13	4,86	7,33	7,83	6,61	5,60	5,20	10,36
CEC		43,85	47,42	46,87	43,72	47,13	62,86	59,33	59,83	53,61	47,60	43,20	62,36
V	%	13,335	11,436	18,925	13,089	19,367	7,727	12,356	13,084	12,326	11,757	12,040	16,611
m		86,66	88,56	81,07	86,91	80,63	92,27	87,64	86,92	87,67	88,24	87,96	83,39
B	mg dm ⁻³	0,21	0,20	0,21	0,14	0,15	0,20	0,23	0,17	0,20	0,18	0,16	0,19
Cu		0,44	0,39	0,39	0,29	0,35	0,44	0,38	0,22	0,38	0,40	0,35	0,41
Fe		193,96	227,53	189,02	169,75	158,22	301,22	194,20	123,52	224,55	242,10	206,09	224,46
Mn		1,84	2,98	1,36	3,42	3,09	2,18	2,31	2,14	3,59	3,10	2,28	4,46
Zn		0,12	0,11	0,03	0,16	0,04	0,04	0,05	0,00	0,01	0,05	0,05	0,13

4.1.2 Experimental design, planting and fertilization

The field experiment was installed in 2016, in which the treatments are the six *Eucalyptus* species with different levels of water stress tolerance mentioned above: *E. brassiana* and *E. camaldulensis* (high tolerance), *E. citriodora* and *E. cloeziana* (intermediate tolerance), *E. grandis* and *E. urophylla* (low tolerance). Each treatment (*Eucalyptus* species) was planted in an experimental plot of 38 lines, with 10 trees of the same species in line (total of 380 trees), with a double border and spacing of 3 x 3 m between plants, totalizing 2280 *Eucalyptus* plants. Each species occupies an area of 36 plants (highlighted in gray) for a periodic inventory. Soil and root samples were collected from the center of that area.

Figure 1. Field experiment design presenting the six treatments and the sampling area highlighted in grey. Source: Patrícia Andressa Ávila, 2017.

4.1.3 Epochs and sampling

There were two sampling periods in the field: in October 2017 and in May 2018. Due to the rain distribution, we called the first sampling “dry season”, at the end of the period with less accumulated precipitation and the second sampling “rainy season”, at the end of the period with more accumulated precipitation. Soil and root samples were collected 0-10 cm deep, close to five representative trees in each experimental plot totalizing 30 soil samples and 30 root samples.

Figure 2. Monthly rainfall, maximum and minimum temperature from June 2017 to May 2018 in Itatinga, São Paulo.

4.1.4 AMF spore extraction

The spore extraction was according to the wet sieving method and decantation of 50 g of soil (Gerdemann et al., 1963). Different sized meshes (0.71 e 0.045 mm) were used in the wet sieving process. Spores collected on the smallest mesh sieve (50 μ m) were transferred to 50 mL Falcon tubes, where 30 mL of 70% sucrose was added. Tubes were centrifuged for 4 minutes at 3600 g and the content was transferred into the same a small sieve and washed into a new tube.

The extracted spores were counted, separated and grouped according to color, size and shape, after viewing under a stereoscope (40 x). We considered only healthy spores, disregarding the empty ones. Slides were prepared using Polyvinyl-Lacto-Glycerol (PVLG) and Melzer's reagent (1:1). The spores were carefully broken to expose their internal walls.

The taxonomic classification was based on the guide of Blaszkowski (1990) and information found in the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM), available on <http://invam.wvu.edu>. The total number of spores and species was determined based on the number of spores found in a 50 g soil sample.

4.1.5 Evaluation of root colonization by AMF

The roots were washed with tap water and cut into segments of approximately 10 cm. 0.5 g of root segments about 1 cm in length were transferred into a 50 mL Falcon tube, covering the roots with potassium hydroxide (KOH) 10% solution. The tubes remained 1 hour in a water bath at 90°C. Additional bleaching was done with alkaline hydrogen peroxide 10% (H₂O₂) for 3 minutes at room temperature. After that, segments were colored with blue ink (Parker – Quink) (Vierheilig et al., 1998) during 15 seconds in a 90°C bath and kept in tubes with a Lacto-glycerol solution (1:1:1 lactic acid, glycerol and water). Segments of 1 cm were spread in a petri dish with a grid of 0.5 x 0.5 squares affixed to the base. Total number of intersects between lines (R1) and total number of intersects with mycorrhizal structures (R2) were counted. The rate R2/R1 was considered the percentage of AMF colonized roots.

4.1.6 DNA extraction from soil samples

The genomic DNA was extracted from 400 mg of each soil sample using the commercial set PowerSoil® DNA Isolation (MoBio, Carlsbad, EUA), following the manufacturer's instructions. After the extraction, nearly 5 µL of samples were evaluated by electrophoresis in 1.5% agarose gel with buffer TAE 1x [400 mM Tris, 20 mM acetic acid, 1 mM EDTA], stained with Gel RED™ (0.5 µg mL⁻¹), and viewed and photodocumented in a transilluminator under ultraviolet light (DNR – Bio Imaging Systems/MiniBis Pro).

4.1.7 ITS genes analysis through quantitative PCR (qPCR)

The analysis was performed using the StepOne™ Real-Time PCR System (Applied Biosystems®) and for the amplification reaction, we used the fluorescent marker SYBR® Green PCR Master Mix (Applied Biosystems®). The primers used were ITS1f (5' -CTTGGTCATTTAGAGGAAGTAA- 3') (Gardes and Bruns, 1993) and 5.8s (5' -CGCTGCGTTCTTCATCG- 3') (Gardes and Bruns, 1993), generating a 300 bp fragment. Conditions for the reaction were 94°C during 15 minutes, 40 cycles of 94°C for 60 seconds, 53°C for 30 seconds and 72°C for 60 seconds. The final volume of reactions was 25 µL, being 12,5 µL of SYBR® Select Master Mix (Applied Biosystems), 0,2 µL of each primer, 1 µL of DNA and 11,1 µL of ultrapure sterilized water to complete the reaction. The specificity of amplification was double-checked using the melting curve with a gradual increase of temperature, from 71°C to 96°C. Amplification efficiency (E) of 102% was calculated according to the equation $E = [10^{(-1/\text{slope})} - 1]$ and ITS copies (g soil⁻¹) were expressed in Log₁₀.

4.1.8 Fungal community structure by terminal restriction fragment length polymorphism (T-RFLP)

The DNA soil samples were submitted to amplification with ITS1F-FAM and ITS4. Conditions for PCR reaction were 94°C for 1 minute and 30 seconds, 94°C for 35 seconds, 55°C for 55 seconds and 72°C for 45 seconds 13 times, 94°C for 35 seconds, 55°C for 2 minutes and 72°C for 45 seconds 13 times, 94°C for 35 seconds, 55°C for 3 minutes and 72°C for 45 seconds 13 times and a final extension at 72°C for 10 minutes. We used 6 mM of MgCl₂, 4 mM dNTP (Invitrogen Corporation, USA) 0.5 U µL⁻¹ of Taq polimerase (Sinapse, São Paulo, Brazil), buffer for Taq polimerase 1X (Tris-HCl 1 mmol L⁻¹, pH 9,0 e KCl 5 mmol L⁻¹) (Sinapse, São Paulo, Brasil), 0.20 mmol µL⁻¹ of each primer, 1.5 mg mL⁻¹ of BSA, 2 µL of DNA template and Mili-Q water to complete the reaction volume. The final volume was 50 µl (48 µl of reaction + 2 µl of DNA). Amplification reactions were performed in a Thermal Cycler model Veriti 96-well Thermal Cycler (Applied Biosystems).

Samples were purified using 75% isopropanol and quantified in 2% agarose gel. To make the cleavages with the respective endonucleases we used nearly 100 mg of the product with 5 U of the enzyme HaeIII (Thermo Scientific, Massachusetts, USA), following the manufacturer's instructions. The products were precipitated with sodium acetate 3 M and EDTA 125 mM, resuspended in formamide HiDI with the

marker LIZ 600 (Applied Biosystems) and each sample was read in an automatic sequencer ABI Prism 3500As (Applied Biosystems). We adopted a threshold of 50 units of fluorescence to remove the “background” of samples and the results were transformed in a relative abundance matrix.

4.1.9 Enzymatic Activity

Dehydrogenase activity was performed according to Casida, Klein and Santoro (1964). 5 g of each soil sample was incubated with 5 mL of 1% triphenyl tetrazolium chloride (TTC) solution and kept at 37°C for 24 hours. After the incubation, the triphenyl tetrazolium formazan (TTF) formed was extracted with methanol, filtered and read in a spectrophotometer at 485 nm.

Acid phosphatase activity was determined by adding 4 mL of modified universal buffer (MUB) pH 6.5 in tubes with 1 g of each soil sample plus 1 mL of p-nitrophenyl-phosphate (PNP) 0.05 M. The content was homogenized and the tubes submitted to incubation at 37°C during 1 hour. Only control samples (blanks) received PNP after the incubation. After one hour, we added 1 mL of CaCl₂ and 4 mL of NaOH 0.5 M to samples that were quickly blended, filtered and then read in a spectrophotometer 410 nm (Tabatabai, 1994).

4.1.10 Microbial biomass carbon

Carbon from microbial biomass (C_{mic}) was estimated through fumigation-extraction following the method of Vance, Brookes and Jerkinson (1987). The calculation was based on the difference between C content of fumigated and non-fumigated samples.

4.1.11 Basal soil respiration

The estimation of soil respiration was performed according to Alef (1995). The CO₂ released was quantified by titulation of the remaining NaOH 0.5 mol L⁻¹ with HCl 0.5 mol L⁻¹ in the presence of phenolphthalein. This was repeated five times during 21 days of incubation.

4.1.12 Glomalin-related soil protein quantification

The glomalin-related soil protein (GRSP) was quantified following an adaptation of the method first proposed by Wright and Upadhyaya (1996). The samples were air-dried and sieved (2 mm mesh). Easily extractable protein (GRSP_{EE}) quantification was performed weighing 0.25 g of each soil sample in 5 mL Eppendorf tubes adding 2 mL of 20 mM sodium citrate pH 7.0 and autoclaving at 121°C for 30 min. The supernatant was removed and two sequential centrifugations of 10 and 5 minutes at 15000 x g were performed. After that, the supernatant was diluted into three concentrations: 1:2, 1:3 and 1:10, and quantified using the Bradford colorimetric assay, calibrated with solutions of bovine serum albumin (BSA), with concentrations of 0, 40, 80, 120, 160, 180 and 200 mg L⁻¹. All the measurements were performed in triplicate using different wells in the same microplate. In each well were added 20 µl of sample and 250 µl of Bradford dye reagent, while the sample blanks contained 20 µl of sample extract with 250 µl of 0.1 M HCl. Before running, the plate was covered and shaken in a Thermomixer for 1 minute, followed by 10 minutes of rest. We measured absorbance at 465, 595 and 665 nm with a ThermoScientific Multiskan GO spectrometer (Waltham, MA, USA). The protein concentration (equivalent BSA) was calculated, with or without the sample blank color correction, by fitting linear and non-linear functions to the calibration curves of the absorbance at 595 nm and of the ratio between absorbance values at 595 and 465 nm (Jorge-Araújo, 2014). Only the results from the direct method, with absorbance at 595 nm were taken into account, since Moragues-Saitua et al. (2018) found that the use of the absorbance ratios at 595 and 465 nm are not recommended because they are sensitive to pH and dilution-dependent at 465 nm.

4.2 Greenhouse experiment

The second experiment was carried out in a greenhouse at the Piracicaba campus of “Luiz de Queiroz” College of Agriculture. The six *Eucalyptus* species aforementioned were planted in pots, using autoclaved soil from the field experiment and submitted to different water levels: 50, 75 and 100% of the field capacity, inoculated and non-inoculated with mycorrhizal fungi, with four repetitions for each treatment.

4.2.1 Experimental design, planting and fertilization

The experimental design was a factorial with randomized blocks, 36 treatments and 4 replications, in 144 pots. The factors were the six *Eucalyptus* species, three water levels and inoculated or non-inoculated with mycorrhizal fungi.

First, the seedlings were obtained by sowing in trays with autoclaved substrate composed by vermiculite, rice husk, pine bark and coconut fiber, where they were maintained during 3 months, receiving daily fertilization and irrigation. Two weeks before the transplant to the pots, fertilization was suspended, especially because high contents of phosphorus can influence negatively the mycorrhizal colonization (Nogueira and Cardoso, 2000). A week after the transplant, half of the pots received, approximately, 58 spores/each of a mixture of *Gigaspora rosea*, *Glomus clarus*, *Acaulospora scrobiculata*, *Dentiscutata heterogama* and *Glomus etunicatum*. After inoculation, the plants were submitted to water control by weighing pots daily to keep 50, 75 or 100% of the field capacity. After three months in the pots, the plants were harvested separating soil, root and shoot systems.

4.2.2 Analysis

We determined spore numbers and root colonization following the same procedures aforementioned for the field experiment. Roots and shoots were dried in an oven at 65°C during 48 hours, and weighed to determine dry matter.

We also determined the P content in the shoots following the method proposed by IAC (1983).

4.3 Statistical Analysis

The data from the field essay was analyzed as a complete randomized design in a double factorial scheme (6x2). The first factor was species (Bra, Cam, Cit, Clo, Gra, Uro) and the second one was epochs (Dry and Rainy). Data were submitted to the normality test of Shapiro-Wilk followed by the homogeneity of variances test of Bartlett. Meeting the criteria of normality and homogeneity, a variance analysis was performed (ANOVA) and, when F was significant, a multiple comparisons test was done, Least Significant Difference (LSD) at 5%, with the statistical package *ExpDes* using the RStudio 1.1.453 software (R CORE TEAM, 2017). When one or both of criteria were not met, we adopted a non-parametric test of Friedman.

In addition, we submitted the data to an exploratory analysis of principal components (PCA). First, data was transformed into $\log(C+1)$ (except pH because is already given in log) using the package *FactoMineR*. Then, charts were plotted to verify the contribution of variables of samplings points using the package *factoextra*.

The data from the greenhouse essay was analyzed following the same procedure aforementioned, except by the design, which was a randomized block in a triple factorial scheme (6x3x2). The factors were species (Bra, Cam, Cit, Clo, Gra, Uro), water levels (50%, 75% and 100% of field capacity) and inoculation (inoculated or not). In this case, when necessary we submitted data to non-parametrical test of Kruskal.

T-RFLP data was analyzed calculating the relative abundance of the peak areas of terminal restriction fragments (T-RFs). Relative abundance was taken as a percentage of each T-RF in the sum of all peak areas of the T-RFLP profile. First, the data was transformed into $\log(C+1)$ and then, the differences between the profiles were proved with a dissimilarity analysis (ANOSIM) represented graphically in a Principal Coordinates Analysis (PCoA), supported by the *Vegan* package using the RStudio software (R CORE TEAM, 2017). We adopted the distance of “Bray-Curtis” to perform that analysis.

Redundancy analysis based on distance (dbRDA) was performed using T-RFLP profiles as response variables and the explanation variables were microbial analysis, genes abundance and chemical data. Differences were proved with the ADONIS test supported by the *Vegan* package using the RStudio software (R CORE TEAM, 2017).

5 RESULTS AND DISCUSSION

5.1 Field experiment

5.1.1 Spores number and roots colonization

In the dry season, the number of spores was higher in the rhizosphere of *E. brassiana* (Bra) when compared to the other treatments. *E. citriodora* (Cit) and *E. grandis* (Gra) presented a lower number of spores, while *E. camaldulensis* (Cam), *E. cloeziana* (Clo) and *E. urophylla* (Uro) did not present any spore. In the rainy season, the treatment with more spores was Uro, however, it was not significantly different from Bra, Cit and Clo. Except for Bra, all the treatments had an increase of spores from the dry to the rainy season (Figure 3).

Figure 3. Number of spores in 50g of soil for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).

AMF spores in soil are modulated by many factors such as developmental stage, climate, nutrients or toxic substances, soil pH, soil microorganisms and age of the host plant (Oehl et al., 2005). As the samplings were done when plants were 14 and 20 months old, the age may be related to the low number of spores, especially in the first sampling. In a study with pure *E. grandis* and mixed *E. grandis* with *Acacia*

mangium plantations, Bini et al. (2018) found a significant increase of spore numbers from two to 20 months after planting.

Sampling at the end of the dry period in Brazil also may have influenced spore number, since water deficit can stimulate sporulation of AMF species (Ferreira et al., 2012).

With a morphological analysis of AMF spores it was possible to identify genera and, in some cases, species present in the area. We found *Acaulospora mellea* and *Glomus* sp. in both epochs while *Ambispora leptoticha* and *Gigaspora* sp., only in the dry season and *Glomus glomerulatum* only in the rainy season (Table 2).

Table 2. Presence (+) or absence (-) of AMF spores in each treatment in both sampling periods.

FMA species	Bra	Cam	Cit	Clo	Gra	Uro	Bra	Cam	Cit	Clo	Gra	Uro
	Dry						Rainy					
<i>Acaulospora</i> sp.	+	-	+	-	-	-	-	+	-	+	-	-
<i>Ambispora leptoticha</i>	-	-	-	+	-	-	-	-	-	-	-	-
<i>Gigaspora</i> sp.	-	-	-	-	+	-	-	-	-	-	-	-
<i>Glomus glomerulatum</i>	-	-	-	-	-	-	-	-	+	+	+	-
<i>Glomus</i> sp.	-	-	+	-	-	-	-	-	-	-	-	-

Some AMF species (such as *Gigaspora margarita*, *Acaulospora scrobiculata*, *Racocetra coralloidea* and *Acaulospora laevis*) germinate well in soils with low pH, but in general, studies show a pH between 6.0 and 7.0 as being more favorable for germination and mycelium growth (Moreira et al., 2002). In our study, soil pH was between 4.0 and 4.5, what can have affected the low spore numbers overall.

Despite records of AMF symbioses being a critical factor in helping trees to acquire water from deep layers of the soil, the effect of soil moisture on mycorrhizal associations may vary with different plant species (Garcia and Mendoza, 2009).

In the dry season, Uro and Cam presented higher colonization rates however did not differ between each other. The smaller percentages of colonization were found in Bra and Gra. In the rainy season, Gra and Uro presented higher colonization rates, although they were not significantly different from Cit. The lowest colonization was found in Clo, but did not differ from Bra and Cam. All treatments differed between epochs, except Cit (Figure 4).

In our study, the root colonization rates in all treatments were close to those reported by Bini et al. (2018) in pure *E. grandis*. This may be due to the age of plants,

as in both studies trees were 20 months or less. It is recognized that AMF root colonization can be an advantage for the plant in its initial development due to the higher dependence on soil nutrients. When in advanced growth stages, the plants become more efficient in recycling nutrients, perhaps decreasing the demand for soil nutrients and fungal associations, what has been verified generally in forest environments (Dighton and Mason, 1985; Adams et al., 2006; Cheng et al., 2000).

Regarding the seasonal effect, in which we found an increase in root colonization from the dry to the rainy season for five of the six treatments, it may be related in this case, to the higher soil moisture having a positive effect on AMF and plant metabolism. Al-Karari et al. (2004), Bomfim et al. (2011) and Shukla et al. (2012) found similar results i.e., higher colonization rates when there was higher soil moisture.

In our study, we also did not find arbuscules in the roots of any species of *Eucalyptus*. Since the arbuscules are a fundamental structure for the chemical exchange between plant and fungi, the lack of arbuscules may be understood as not fulfilling their function. However, the same situation (of absence of arbuscules) has already been reported in other studies, such as Santana et al. (2018), but the reasons for this are not clear. It may be that, in certain host-endophyte combinations, nutrient exchange occurs through hyphal coils, for example.

Figure 4. Percentage of colonized roots for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).

5.1.2 Glomalin-related soil protein

None of the treatments in any sampling period presented different amounts of glomalin-related soil protein in both sampling periods, except for Uro, which had a significant increase from the dry to the rainy period (Figure 5).

Figure 5. Easily extractable Glomalin-related soil protein for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).

Glomalin-related soil protein is a glycoprotein first thought to be produced exclusively by AMF (Wright, 1996). The protein acts helping to bind soil particles into small aggregates. Hence, a well-aggregated soil can endure for long periods being resistant to erosion and presenting better water infiltration, what benefits plants and microbial growth (Rillig, 2006).

We did not find significant differences among treatments, probably also due to the age of plants and the short period between samplings. This may be related to the fact of GRSP and carbon having a slower turnover in soils (Rillig, 2004). Since the *Eucalyptus* seedlings were still very young, less than two years of age, when the samplings were done, a longer period of observation would probably be needed to find possible later and greater changes. Given the short period of *Eucalyptus* culture, the carbon and GRSP contents that we determined may have been due to previous land-use. However, after a longer growth period, the dominant mycorrhizal colonization would switch from AMF to ectomycorrhizal, and the latter is not reputed to produce GRSP.

Soil properties, climate and plant features also can influence glomalin contents in soil. In this study, we did not find great changes in soil properties and climate, but the litter composition due to the different features of each eucalypt species may change the quality of the organic matter.

Despite not being able of finding significant differences between species in the same epochs, once again, Uro had a particular behavior, increasing such values from the dry to the rainy period.

5.1.3 Enzymatic activity

None of the treatments presented significant differences for acid phosphatase in both time samplings. Only Cit presented significant difference between epochs, being lower in the rainy period (Figure 6).

Figure 6. Acid phosphatase activity for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).

The release of phosphatases by the roots is usually attributed to the reduction of P availability in soil (Raghothama and Karthikeyan, 2005). While plants are more dependent on soil nutrients and fungal associations in the first months, older plantations become more efficient in recycling nutrients (biochemical cycling) which can decrease their initial demand (Dighton and Mason, 1985). Our results show non-

significant differences between treatments and epochs what may be due the establishment of biochemical cycling.

In addition, the age of plants can influence the enzymatic activity. Lino et al. (2016) found that, in general, enzymatic activity was improved with the increasing age of *Eucalyptus grandis* plantations. Bini et al. (2018) also found higher values for acid phosphatase in pure *E. grandis* when trees were 14 and 20 months.

For dehydrogenase, none of the treatments were different in both samplings. Only Gra presented significant difference between epochs, being lower in the rainy period (Figure 7).

Figure 7. Dehydrogenase activity for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).

Dehydrogenase is an enzyme involved with transformation of organic matter and release of nutrients. As expected, Bini et al. (2018) found a correlation of that enzyme with microbial activity and litter composition, and in *E. grandis*, there were lower rates of dehydrogenase activity when compared to mixed plantations with *Acacia mangium*.

Following the same trend of the acid phosphatase activity, there were only non-significant differences between treatments within both epochs. Seasonality may change microbial and enzymatic activity in soil (Rigobelo and Nahas, 2004).

However, in this study, we did not observe great differences between the epochs of sampling.

Thus, due to the age of the trees, there is a lower carbon accumulation and the initial establishment of nutrient cycling may lead to a low enzymatic activity.

5.1.4 Microbial biomass carbon and soil basal respiration

There was no difference among the treatments in both samplings. However, between epochs, all species presented significant differences, with higher values in the rainy season (Figure 8).

Figure 8. Microbial biomass carbon for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).

There was no difference between the species regarding the microbial biomass carbon. Chabrierie et al. (2003) and Sinha et al. (2009) consider the quality and quantity of rhizodeposition of different species as a more important factor than climatic conditions on modulating the microbial community. However, our study confirmed that climate had a significant effect on plant and microbial metabolism in general between the dry and the rainy season, but never between species features.

Soil basal respiration, in the dry season, Bra, Cam, Cit, Clo and Gra had similar values and Cit, Gra and Uro did equally not present differences among each

other. In the rainy period, no difference was detected between the tree species, while all species had higher values in the rainy season (Figure 9).

Figure 9. Soil respiration for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).

Low basal respiration in the dry season may be a consequence of lack of moisture and low temperatures. In fact, Eucalypt is generally more sensitive to dry soil conditions than to other negative factors, as, for example, low soil nutrient contents, being especially sensitive to abrupt changes in the environment (Kaschuk, Alberton and Hungria, 2010). Despite we did not assess the litter, the different eucalypt species might have different compounds that influence microbial activity such as enzyme activity, microbial biomass carbon and soil basal respiration (Nogueira et al., 2006; Wang et al., 2010). Lower temperatures added to absence of rainfall can lead to decreases in microbial activity as well as high temperatures and rainfall can stimulate the opposite (Bini et al., 2013). Thus, it is important to consider the history of the area such as crops planted and land management to understand better the behavior of soil microorganisms (Bini et al., 2013; Varennes et al., 2011).

5.1.5 Abundance of ITS genes

No significant difference was found between the species in the dry season. In the rainy season, Cam showed higher values but these did not differ from those of Cit, Clo and Gra. Bra presented a lower value, but was not different from Cit, Gra and Uro. Only Clo showed a significant rise from the dry to the rainy period (Figure 10).

Figure 10. Number of copies from ITS regions for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).

In this study, the abundance of ITS genes was affected by the sampling time. Age of the trees and environmental conditions may have been more related to the changes, as occurred with the results previously presented.

The variation in the ITS abundance also can be due to an interaction or interference of other microorganisms present in the soil and in the plant. As the ITS region is specific for fungi, we cannot say which groups are present, but the fungi bacteria ratio (F/B) varies between plants as Carnovale et al. (2019) found in their study.

Concurrently, the microbial activity stimulated by the rainy epoch could be more specific for each treatment. Different plant species recruit different microorganisms because of the composition of their exudates.

5.1.6 Fungal community structure

Several authors suggest that soil attributes are the most important factors shaping the microbial community structure (Fierer et al., 2009, Girvan et al., 2003, Singh et al., 2008). Concurrently, many studies show that pH plays an important role in explaining variations in soil microbial communities, however we did not find great changes in soil attributes overall.

Nevertheless, our results show a variation between the sampling periods, in which the dry season treatments are more spreaded and those of the rainy season are more grouped (Figure 11). One hypothesis for this difference is a survival strategy because in non-favorable conditions, microorganisms tend to search for favorable spots in soil and hence they are scattered. On the other side, in more favorable conditions, the fungal community seems to behave in a similar way. Despite these differences, we could not observe similar behaviors or groupings of species with the same level of drought tolerance.

Carnovale et al. (2019) obtained similar results in which the fungal community of *Eucalyptus* area changed between periods only. In the same study, they found that the dominant tree genus was a significant factor for observed differences. Pereira et al. (2018) also obtained a clear differentiation in microbial community structures in presence of two plant genera: *Eucalyptus* and *Acacia*. These results lead us to consider that, even though our treatments being different species, they all belong to the same *Eucalyptus* genus, what can reduce the variation of the fungal community structure.

A)

B)

Figure 11. Diagram of Principal Coordinates (PCoA) of T-RFLP data of ITS genes in the dry (A) and in the rainy season (B).

5.1.7 Redundancy analysis

It is possible to see some overlapping of samples between epochs, but most of them showed a clear separation (Figure 12). In the dry season, Calcium (Ca) is the attribute that best explains the peak profile obtained by the T-RFLP. There are some studies reporting that calcium storage and mobilization are important in mycorrhizal symbiosis and response to aluminium stress (Sujkowska-Rybikowska and Znojek, 2018) and we found low values of calcium in the area. However, our data are not enough to explain coherently that relation. In the rainy season, more attributes explain the T-RFLP profile, such as easily extractable glomalin-related soil protein, soil phosphorus, spore number and root colonization.

Figure 12. Distance-based redundancy analysis (dbRDA) of fungal community structure obtained by T-RFLP, chemical and biological attributes comparing dry and rainy season. Only significant variables are shown, as Root colonization (Col); Spore number (Spores); Glomalin-related soil protein (EE-GRSP); Total Phosphorus in soil (P); Calcium in soil (Ca).

5.2 Greenhouse experiment

5.2.1 Spore numbers and root colonization

Our results show, in general, a low number of spores and low root colonization (Table 3). Cam and Clo presented significant spore numbers at all moisture levels when compared to non-inoculated treatments, while Bra did not present a significant value.

The three months in which pots were kept in the greenhouse may not have been enough for the establishment of AMF. In addition, the inoculum was not from the field area and some species of AMF chosen may not colonize eucalypt trees.

Table 3. Spore number and root colonization for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) inoculated with AMF and maintained at 50, 75 and 100% of the field capacity.

Species	Inoculated					
	Water levels					
	50		75		100	
Spore numbers (50g soil ⁻¹)						
Bra	1.00 ± 2.0	Ba	4.0 ± 5.7	Aa	2.0 ± 2,3	Ba
Cam	7.00 ± 5.8	Aa*	5.0 ± 3.5	Aa*	6.0 ± 2,3	Aa*
Cit	1.00 ± 2.0	Bb	5.0 ± 2.8	Aab*	8.0 ± 2,3	Aa*
Clo	9.00 ± 6.8	Aa*	7.0 ± 2.0	Aa*	6.0 ± 2,3	Aa*
Gra	1.00 ± 2.0	Ba	2.0 ± 2.0	Aa	6.0 ± 4,0	Aa*
Uro	4.00 ± 0.5	Aab*	3.0 ± 3.8	Aab	0 ± 0	Bb
Root colonization (%)						
Bra	0 ± 0	Ba	0 ± 0	Ca	0 ± 0	Ba
Cam	0 ± 0	Ba	1.0 ± 1.4	Ba	0 ± 0	Ba
Cit	0.25 ± 0.50	Ba	0 ± 0	Ca	0.5 ± 1.00	Ba
Clo	0.25 ± 0.50	Ba	0 ± 0	Ca	0 ± 0	Ba
Gra	0.25 ± 0.50	Ba	0 ± 0	Ca	0 ± 0	Ba
Uro	23 ± 9.3	Aa*	19.5 ± 10.8	Aa*	10.0 ± 3.4	Aa*

Uppercase letters compare different species at the same moisture level. Lowercase letters compare the same species at different moisture levels. Star (*) indicates when an inoculated treatment is significantly different of its similar non-inoculated by the test of Kruskal-Wallis.

For root colonization, Uro was differed significantly from all other treatments at the three moisture levels and was the only species different of the non-inoculated treatments. This result is similar to the field essay, in which Uro presented higher colonization when comparing to the other species. It may be an evidence that *E. urophylla* is more dependent on AMF colonization. Among the AMF spores

inoculated in the pots, we could only identify *Dentiscutata heterogama* at the end of the experiment.

5.2.2 P content in leaves and shoot dry matter

We correlated the leaf P with the shoot dry matter to have a better idea if AMF contributed to the P uptake and if this nutrient was converted into biomass.

Figure 13. Leaf P x Shoot dry matter for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) non-inoculated (A) and inoculated (B) at 50, 75 and 100% of the field capacity. Uppercase letters compare different species in the same moisture level. Lowercase letters compare the same species at different moisture levels. Star (*) indicates significant total P differences in the same species at different moisture levels.

In general, we did not find significant differences from inoculated to non-inoculated treatments. As we hypothesized for AMF spores and colonization, the greenhouse period may not have been enough to establish the symbiosis, as well as the AMF species inoculated may not have been efficient. Hence, the P uptake and shoot biomass was not significantly increased.

However, in Bra and Cam, lower values of total leaf P were obtained at 50% of the field capacity. It may be related to several factors, such as low soil moisture and low dependency of AMF. Unpublished data of Ávila et al. (2017) showed that *E. camaldulensis* mean fine roots density decreased when compared to *E. cloeziana* and *E. grandis*. It suggests that features like root system also should be considered when evaluating P uptake by trees.

6 CONCLUSION

Our study shows that the AMF colonization was different among *Eucalyptus* species, highlighting *Eucalyptus urophylla*, which is less tolerant to drought and presented more colonized roots, both in the field and in the greenhouse essays. However, the higher rates of colonization were observed in the rainy season and not under water stress as may have been expected.

Following the same trend, attributes such as soil basal respiration and microbial biomass carbon had a significant increase in the rainy season, showing again that soil moisture stimulates microbial activity overall.

The fungal community structure was not enlightening, as we could not identify groupings among the *Eucalyptus* species. To understand these processes we will need to set up new experiments with plants at different ages and with long-time follow-ups. Studies with other groups of microorganisms perhaps may help to get a better understanding of their behavior.

Besides soil moisture, other factors must be taken into account, such as the age of the plants and history of the area. *Eucalyptus* crops have a long cycle and the results we found are related to trees with less than two years of age, and this may change along the time.

It is important to highlight that this study is exploratory and we need further studies to understand better the role played by AMF in drought tolerance in *Eucalpytus* crops.

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ADDITIONAL CONTENT

A. Principal Components Analysis of the field experiment. Dehydrogenase (Dehyd), Spores (Spore number), Basal soil respiration (CO₂), Easily extractable Glomalin-related soil protein (EE-GRSP), Acid phosphatase activity (Phosph), ITS genes abundance (ITS), Microbial biomass carbon (C_{mic}), Roots colonization (Col).

B. Distance based redundancy analysis based on T-RFLP profiles of ITS genes and chemical and biological soil attributes.

Canonical axis	F		<i>p</i> -value	
	Dry and Rainy			
Model	1,4661		0.008	
Model variables	Dry		Rainy	
	F	<i>p</i> -value	F	<i>p</i> -value
Spores	0.24740	0.880	1,8503	0.168
Colonization	0.29706	0.824	0.4747	0.622
EE-GRSP	0.30652	0.809	2,7793	0.088
Phosphatase	0.29647	0.822	0.4748	0.624
Dehydrogenase	0.12581	0.951	0.4279	0.679
C _{mic}	0.18094	0.912	3,6503	0.060
SBR	0.61407	0.592	0.8949	0.402
ITS genes	0.17428	0.929	0.5825	0.532
Ca	0.80483	0.475	2,3443	0.131
Mg	0.15674	0.931	0.7543	0.459
K	0.27935	0.854	1,8938	0.175
Al	1,8391	0.163	1,1330	0.325
pH	1,2318	0.314	8,3275	0.004
P	0.82766	0.431	2,6731	0.113
C	1,0513	0.371	2,7330	0.111
B	0.32812	0.827	0.8433	0.352
S	0.01152	0.996	0.3605	0.718
Cu	0.9415	0.963	0.3831	0.692
Fe	0.23517	0.904	0.1041	0.924
Mn	0.23347	0.897	0.7465	0.471
Zn	0.26096	0.870	1,0962	0.305

Spore number (Spores); Root colonization (Colonization); Easily extractable glomalin-related soil protein (EE-GRSP), Acid phosphatase activity (Phosphatase); Dehydrogenase activity (Dehydrogenase); Microbial biomass carbon (C_{mic}); Soil basal respiration (SBR); Abundance of ITS genes (ITS).

C. Average per treatment of ITS copies in each sampling time.

	Dry	Rainy
	<i>log (ITS copies) g⁻¹</i>	
Bra	7,912	7,687
Cam	7,788	8,216
Cit	7,796	7,947
Clo	7,726	8,014
Gra	7,954	7,762
Uro	7,713	7,792

D. Values of ANOSIM correlations (R-value) obtained by the matrix of Bray-Curtis distance for the electropherograms of terminal restriction fragments (T-RFLP) of ITS genes between sampling times for the six *Eucalyptus* species.

		Bra	Cam	Cit	Clo	Gra	Uro	<i>p</i> -value
DRY SEASON	Bra		0.7308	0.535	0.6519	0.664	0.4454	
	Cam	-0.092		0.6447	0.348	0.6774	0.2877	
	Cit	-0.056	-0.1		0.2878	0.4492	0.4616	
	Clo	-0.068	0.036	0.076		0.3236	0.6621	
	Gra	-0.088	-0.08	-0.008	0.044		0.3632	
	Uro	-0.036	0.044	-0.012	-0.076	-0.004		
R-ANOSIM								
		Bra	Cam	Cit	Clo	Gra	Uro	<i>p</i> -value
RAINY SEASON	Bra		0.0474	0.0091	0.0153	0.0083	0.0539	
	Cam	0.276		0.0536	0.0071	0.1052	0.0163	
	Cit	0.472	0.16		0.008	0.4121	0.0065	
	Clo	0.34	0.484	0.488		0.2856	0.0392	
	Gra	0.288	0.132	0.008	0.06		0.0075	
	Uro	0.188	0.404	0.524	0.336	0.284		
R-ANOSIM								
DRY SEASON								
RAINY SEASON	Bra	0.592***						
	Cam		0.524***					
	Cit			0.148 ^{ns}				
	Clo				0.342**			
	Gra					0.176 ^{ns}		
	Uro						0.28**	

ns: non significant; ***: $p \leq 0.01$ **: $p \leq 0.05$; *: $p \leq 0.1$

A)

B)

E. Principal Components Analysis for the greenhouse experiment of *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) non-inoculated (A) and inoculated (B) with AMF at 50, 75 and 100% of the field capacity.

F. Metabolic coefficient (qCO_2) for *E. brassiana* (Bra), *E. camaldulensis* (Cam), *E. citriodora* (Cit), *E. cloeziana* (Clo), *E. grandis* (Gra) and *E. urophylla* (Uro) in the first sampling (Dry) and second sampling (Rainy). Lowercase letters compare the species in the Dry period and uppercase letters compare the species in the Rainy period. Bars indicate significant difference between the periods with a star (*) for the greatest one by the LSD test at 5% ($p \leq 0.05$).