

DYNAMICS OF ARBUSCULAR MYCORRHIZAL FUNGI IN *EUCALYPTUS GLOBULUS* PLANTATIONS

Marliane de Cássia Soares da Silva^(a); Igor Rodrigues Mendes^(a); Thiago de Almeida Paula^(a); José Maria Rodrigues da Luz^(a); Cristina Cruz^(b); Denise Mara Soares Bazzolli^(a); Maria Catarina Megumi Kasuya^(a)

^a*Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570-000, Brazil*

^b*Faculdade de Ciências da Universidade de Lisboa, Centro de Biologia Ambiental, Lisboa, Campo Grande, 1749-016, Portugal*

**Av. P. H. Rolfs, s/n, Campus UFV, Viçosa, Minas Gerais, 36570-000, Brazil,*

ABSTRACT: *Arbuscular mycorrhizal fungi (AMFs) interact with and benefit commercial cultures, such as Eucalyptus globulus. This crop occupies large areas in several countries, and can occupy up to one third of the reforested area in Portugal. The aim of this study was to evaluate and identify AMF groups in E. globulus plantations. Samples of soil and root systems were collected in the winter and the summer from fertilized or unfertilized plantations situated in three different regions of Portugal: Penafiel, Gavião and Odemira. The samples were subjected to DNA extraction, nested PCR-DGGE, sequencing and analysis. The data from DGGE and the soil pH, moisture, organic matter, acid and alkaline phosphatase and P content were used for principal component analysis. The AMF profile was influenced by season, winter and summer, N fertilization and region. Glomus sp. and Gigaspora sp. were the most frequently detected AMFs. We observed a greater number of AMF species in Penafiel than in the other two regions. Furthermore, Scutellospora heterogama was only found in Penafiel. Improved knowledge of AMF communities may facilitate better management of eucalypt plantations and further exploration of the potential and biodiversity of these partners.*

KEYWORDS: *Eucalypt, nested PCR-DGGE, Glomus, Gigaspora and Scutellospora heterogama*

INTRODUCTION

Arbuscular mycorrhizal fungi (AMFs) are ancient biotrophic fungi that belong to the Glomeromycota and form symbiotic associations with plants (Parniske, 2008). AMFs receive carbon from their hosts and in exchange, provide numerous benefits to plants, primarily by improving nutrition and growth, protecting against pathogens, increasing resistance to stress and heavy metals, and acting in nutrient cycling (Arriagada et al, 2009; Bonfante and Anca, 2009; Croll and Sanders, 2010). These fungi have hyphae 10 times thinner than the roots, and the cost for the growth of hyphae up to 100 times smaller (Helgason and Fitter, 2005).

Among the nutritional benefits of these fungi are increased absorption of nutrients, such as phosphorus (P), because these fungi possess transporters that absorb inorganic soil P and transfer this nutrient to the plant (Helgason and Fitter, 2005; Bonfante and Anca, 2009). The absorption of nitrogen (N) is also increased by AMFs because these fungi possess genes involved in the capture of inorganic and organic N that are active during mycorrhization (Parniske, 2008; Bonfante and Anca, 2009; Näsholm et al., 2009).

Eucalypt is a species of great interest to the forest industry that interacts with and receives the benefits of AMFs. *Eucalyptus globulus* belongs to the Myrtaceae, which include different species scattered throughout the world in widely varying environmental conditions (ABRAF, 2010). *Eucalyptus* sp. was introduced in Portugal in the 1950s and currently occupies 672,000 ha, which represents one third of the reforest area of the country. Due to the strong annual demand of industries, this area will reach 2.44 billion m³ in 2030, which represents a 45% increase over consumption in 2005 (FAO, 2009).

The interaction between AMFs and *E. globulus* has benefits for the plant, such as protection against heavy metals, including cadmium, lead, copper, zinc and aluminum (Arriagada et al., 2004, 2005, 2007a, 2007b, 2009, 2010). In addition, these authors observed an increase in the capacity to absorb nutrients, such as nitrogen, phosphate and potassium.

The objective of this work was to investigate the community of AMFs and identify the main groups present in the winter and summer seasons in three regions of Portugal that were reforested with *E. globulus* and either unfertilized or fertilized with nitrogen.

MATERIALS AND METHODS

Study site

This study was performed on *E. globulus* plantations belonging to the Portucel-Soporcel Group in three regions of Portugal: Penafiel in the northern region, Gavião in the central region and Odemira in the southern region. These plantations were approximately eight years old. The three regions were selected due to their distinct soil physicochemical properties, rainfall and climates (Table 1). In January (i.e., winter) and June 2011 (i.e., summer), samples of soil and root systems were collected from two plots within each plantation. Both plots had received an initial N fertilization (3.3 g of N per plant) at the time of planting, but only one plot from each plantation subsequently received N maintenance fertilization (60 kg of N ha⁻¹-187) in the period from 2004-2007.

Table 1- Characterization of soil of the Penafiel, Gavião and Odemira regions for the physicochemical properties, rainfall and climate.

Region	Textural Class	FAO Classification (1998)	Lithology	Stony (%)	Average Rainfall annual (mm)	Winter	Summer	Average Temperature annual (°C)			
Penafiel	Sandy loam	Regosols epileptic	Sediments	10%	1500	Fresh	Moderate	12.8			
Gavião	Sandy loam	Regosols epileptic skeletal	Shale and Grauvaques	5%	850	Moderate	Hot	9.7			
Odemira	Sandy loam	Umbrisol endoleptic arenic	Granite	50%	750	Moderate	Hot	14.6			
	N g Kg ⁻¹	P mg Kg ⁻¹	K -----cmol Kg ⁻¹ -----	Ca	Mg	Na	B	Cu	Zn	Fe	Mn
Penafiel	1.3	4.8	0.09	0.08	0.05	0.07	1.0	0.3	0.7	168	4.8
Gavião	1.12	0.4	0.6	0.77	0.4	0.03	0.5	1.8	1.3	52	0.6
Odemira	2.25	7.0	0.14	0.58	0.48	0.22	1.0	0.3	0.7	41	61.6

Sampling

Three composite samples were collected from each plot. Each composite sample consisted of soil or roots collected from three neighboring trees. In each tree, ten random points were sampled in a radius of 40 cm around the trunk at a depth of 0-10 cm. The root fragments were composed primarily of fine roots (0-1 mm in diameter). The samples were placed into plastic bags, stored on ice, transported to the laboratory, and subsequently stored at -20 °C for up to two months prior to analysis.

Soil characterization

Soils were classified according to the FAO/UNESCO (Table 1). Triplicate soil samples were weighed and placed in an oven at 60 °C for 48 h to determine the moisture content. The organic matter content was determined using the loss-on-ignition method. One gram of soil was weighed in a porcelain crucible and placed in a muffle furnace (Nabertherm 30-3000°C, model L3, Germany) at 500°C for 12 h (Schulte et al., 1987). For pH determination, soil extracts were prepared in 2 mol L⁻¹ potassium chlorate using 2 g of soil and completed by the addition of distilled water to achieve a final volume of 20 mL.

Phosphate content

A second soil extract was prepared using 2 g of each soil sample, with the volume increased to 20 mL with distilled water. The sample was incubated under agitation for 1 h. Then, a 10 mL sample of this extract was centrifuged and the supernatant was used to determine the phosphate concentration. This determination was performed using the colorimetric method, which involves the reaction of the Fiske reagent and ascorbic acid, which reacts with the phosphate present in the soil extract. For this assay, 50 µL of soil extract were used in triplicate. The absorbance was determined on a microplate reader (Spectra Rainbow - TECAN) at 700 nm.

Phosphatase activity

The phosphatase activity in the soil was determined using the colorimetric method, according to Tabatabai and Bremer (1969). Assays were performed in triplicate, with one gram of soil for each composite sample, and absorbances were determined on a microplate reader (Spectra Rainbow - TECAN) at 405 nm. The control readings were performed in the same manner, except for the addition of the substrate PNP-phosphate.

Nested PCR of the 18S rDNA gene

The denaturing gradient gel electrophoresis (DGGE) technique was used to assess the profile of microbial communities of AMFs in eucalypt plantations in Portugal. Total DNA was extracted from 250 mg of the root system or soil samples using an *UltraClean PowerSoil* kit (Mobio Laboratories, Solana Beach, CA, USA), according to the manufacturer's instructions.

The total DNA was used as the template in a PCR reaction to amplify the 18S rDNA gene. The primers used to amplify the 18S rDNA fragments corresponding to the first round included the AM1 primer (5'-GTTTCCCGTAAGGCGCCGAA-3') (Helgason et al., 1998) in combination with the universal primer for eukaryotes, NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3') (Simon et al., 1992). The PCR mixture consisted of

20 ng of total DNA, 0.2 μ M of each oligonucleotide, 200 μ M dNTP, 2 mM MgCl₂, 0.5 mg mL⁻¹ of bovine serum albumin (BSA) and 1.25 units of GO Taq DNA polymerase (Promega, Madison, USA) in a total reaction volume of 50 μ L. The negative controls consisted of MilliQ water to replace the DNA sample to evaluate the presence of possible contaminants. All materials used in the preparation of the reactions were previously sterilized and nuclease-free.

The PCR amplifications were performed in a thermocycler (Mastercycler epgradient, Eppendorf) using the following steps: an initial cycle of 1 min at 94 °C, 1 min at 66 °C and 1 min 30 s at 72 °C, followed by an additional 30 cycles of 30 s at 94 °C, 1 min at 66 °C and 1 min 30 s at 72 °C and a 10 min final extension at 72 °C. To confirm the presence of the amplified product, aliquots of the products from PCR reactions were submitted to electrophoresis in agarose gels (0.8% w:v, at 80 V for 80 min) stained with ethidium bromide and visualized under a UV light photodocumentation imaging system (Loccus Biotechnologic L-Pix Chemi).

The amplification of the DNA fragment corresponding to the 18S rDNA of AMFs from the first two primers described (i.e., AM1 and NS31) resulted in DNA fragments of approximately 560 bp. To obtain a smaller DNA fragment to perform the DGGE technique and for the careful observation of the bands in the gel, a second round of PCR reactions was performed (i.e., nested PCR) using the primers NS31-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGTTGGAGGGCAAGTC TGGTGCC-3') (Kowalchuk et al., 2002) and Glo1 (5'-GCCTGCTTTAAACTCTA-3') (Cornejo et al., 2004) and the same reaction mixture utilized in the first round of PCR. An initial denaturation of 5 min at 94 °C was performed, followed by 35 cycles of 45 s of denaturation at 94 °C, 45 s at 52 °C, 1 min at 72 °C and the extension of the fragments at 72 °C for 30 min. To confirm the presence of the product, 5 μ L of the PCR product was verified by electrophoresis on agarose gels (1.5% w:v, 80 V, 80 min) stained with ethidium bromide and visualized under a UV light photodocumentation imaging system (Loccus Biotechnologic L-Pix Chemi).

DGGE

The DNA fragments obtained from the soil and root samples using the nested PCR technique were analyzed by DGGE (DCode System, Bio-Rad Inc., California). The reference markers were *Rhizophagus clarus* from an *in vitro* collection (Laboratory of Mycorrhizal Associations, Universidade Federal de Viçosa -Viçosa, MG), *Acaulospora koskei* SCT406A, *Acaulospora tuberculata* SCT250B, *Gigaspora albida* PRN201A, *Gigaspora decipiens* SCT304A and *Dentiscutata heterogama* (= *Scutellospora heterogama*) PNB102A. Fungal isolates were obtained from the International Culture Collection of Glomeromycota (CICG) (www.furb.br/cicg) at the Universidade Regional de Blumenau in Blumenau, SC, Brazil. The references were treated as described for the field samples, and a volume of 15 μ L of the DNA mixture of these species was used as a marker for the DGGE analysis. For this analysis, a 20 μ L sample of the nested PCR products, ranging from 150 to 200 ng of DNA, was loaded onto an 8% (w/v) polyacrylamide gel in 1 X Tris-acetate-EDTA (TAE) buffer. The gel was

prepared with a denaturing gradient ranging from 35% to 50% (where 100% denaturation indicates a concentration of 7 mol L⁻¹ urea and 40% formamide). The gel was subjected to vertical electrophoresis at 60 V for 20 h at 60°C, then stained for 40 min with 1x *Sybr Gold* (Molecular Probes, Leiden, The Netherlands); the gel was then photographed under UV light on a molecular imaging system (Loccus Biotechnologic L-Pix Chemi). The bands of interest were excised, eluted, used as templates in a new PCR reaction using the same primers but without the addition of the GC-clamp, and then sequenced by Macrogen, Inc. (Korea). All sequences obtained in the present study were edited using Sequencher software (Version 4.1, Gene Codes Corp Ann Arbor, USA). The results were analyzed by comparing the obtained sequences with those deposited in the GenBank database using the BLASTn search tool (Autshul et al., 1997).

Statistical analyses

The data were subjected to variance analysis (ANOVA), and mean values were compared by the Saeg software (version 9.1, Universidade Federal de Viçosa) using Tukey's test ($p < 0.05$). The DGGE profiles, which were aligned based on the external markers, were analyzed and compared using BioNumerics software (Version 5.1, Applied Maths NV). Correlations between the environmental data and the occurrence and intensities of DGGE bands were determined using principal component analysis (PCA) and canonical analysis (CA) with Canoco software (version 4.5, Biometris, Wageningen, Netherlands). Distinct bands observed in the DGGE gels were considered different species, and the relative intensities of these bands were considered to represent the frequency at which these species occur. The environmental variables considered in the analysis were: organic matter, moisture, phosphate, phosphatase activity and pH.

RESULTS

Soil characterization

The soils were acidic, and moisture varied greatly with season and region, reaching very low values in summer, particularly in the Gavião region (Table 2). The phosphate concentrations in the soil were higher in summer than in winter, except for the Gavião region, which had similar values in both seasons (Table 2). The soil from Odemira exhibited a higher concentration of phosphates than the soil from the other regions, regardless of the evaluation period (Table 2). In all regions, the acid phosphatase activity was higher than the alkaline phosphatase activity (Table 2). The highest alkaline phosphatase and acid phosphatase activities were quantified in the winter and in the summer, respectively, except for the Gavião region, where the acid phosphatase activity was greatest in the winter. The soils from Odemira exhibited high phosphatase activity in both seasons (Table 2).

Table 2- Physicochemical analysis of the soils of planted forests of *Eucalyptus globulus* in the Penafiel, Gavião and Odemira regions.

Region	Treatment	Organic matter (%)		pH		Moisture (%)		Acid phosphatase $\mu\text{g P-PO}_4 \text{ g}^{-1} \text{ dry soil h}^{-1}$		Alkaline phosphatase $\mu\text{g P-PO}_4 \text{ g}^{-1} \text{ dry soil h}^{-1}$		Phosphate $\mu\text{g P-PO}_4 \text{ g}^{-1} \text{ dry soil}$	
		winter	summer	winter	summer	winter	summer	winter	summer	winter	summer	winter	summer
Penafiel	WNF	7.57±0.98	9.02±1.56	3.76±0.08	3.85±0.08	16.53±1.95	9.36±0.99	248.32 ±155.59	319.30±200.3	74.51±29.41	19.80±32.7	5.79±0.16	7.92±1.90
	NF	9.42±0.87	9.46±0.76	3.79±0.07	4.04±0.38	14.68±3.91	8.34±2.32	398.60±100.10	448.58±125.3	76.75±48.56	40.26±33.06	5.80±0.43	6.87±1.09
Gavião	WNF	5.24±1.38	4.82±0.07	4.17±0.07	4.08±0.10	11.64±2.20	5.75±2.04	244.71±43.43	190.10±57.00	115.02±21.56	24.61±12.07	5.25±0.70	5.18±4.59
	NF	7.66±3.12	6.05±0.06	3.75±0.06	3.79±0.17	10.50±1.17	4.49±0.85	441.41±134.69	176.70±86.30	213.46±67.37	29.27±7.03	6.58±1.38	6.22±3.45
Odemira	WNF	12.74±0.40	17.50±3.98	3.43±0.17	4.74±1.32	18.22±1.08	15.20±0.75	478.73±56.30	675.60±221.3	127.37±67.01	30.27±32.26	10.10±2.58	13.72±1.3
	NF	12.85±0.95	14.09±1.90	3.20±0.06	3.52±0.03	21.04±1.61	15.66±2.73	537.09±133.96	541.35±72.17	260.33±66.84	26.99±7.08	8.28±1.44	12.01±1.38

Without nitrogen fertilization (WNF); with nitrogen fertilization (NF)

Arbuscular mycorrhizal fungi

The 18S rDNA gene was successfully amplified, enabling analysis of the amplicon by DGGE. This technique allowed the analysis of culture-independent AMFs directly from the soil and roots of the sampled plots (Figures 1, 2 and 3). The banding patterns generated by DGGE revealed different profiles, especially when each region was assessed in relation to nitrogen fertilization and seasons of the year. In Gavião and Odemira, a greater number of bands was observed in the soil samples than in the root system samples. In the region of Penafiel, a greater number of bands was found in the area that received nitrogen fertilization in the winter. Root system samples collected from the areas that received nitrogen fertilization had a larger number of bands than unfertilized areas (Figures 1A-B, 2A-B, and 3A-B), suggesting that the diversity of these AMF communities is distinct.

The AMF communities were grouped primarily according to season. However, AMFs in some soil and root system samples were also grouped according to nitrogen fertilization (Figures 1C-D, 2C-D, and 3C-D). In the soils and roots from Penafiel, a difference was observed between winter and summer, with the formation of two groups with 30% similarity. The winter group exhibited 76% similarity (Figure 1C). In the root system samples, differences were also observed between winter and summer, with the formation of two groups with 35% similarity. The summer group exhibited 50% similarity (Figure 1D).

Season impacted the AMF communities in the soil from Gavião, where the formation of two groups with 40% similarity was observed. The first group within the summer samples exhibited 53% similarity, and the second group in the winter samples exhibited 60% similarity (Figure 2 C). The root system samples exhibited two main groups of AMF communities, according to N fertilization (Figure 2 D).

In the soil from Odemira, one main group of AMFs with 50% similarity to samples that did not receive maintenance N was observed (Figure 3 C). In the root system samples, differentiation was observed between winter and summer, with the formation of two groups with 40% similarity. The summer group exhibited 50% similarity (Figure 3 D).

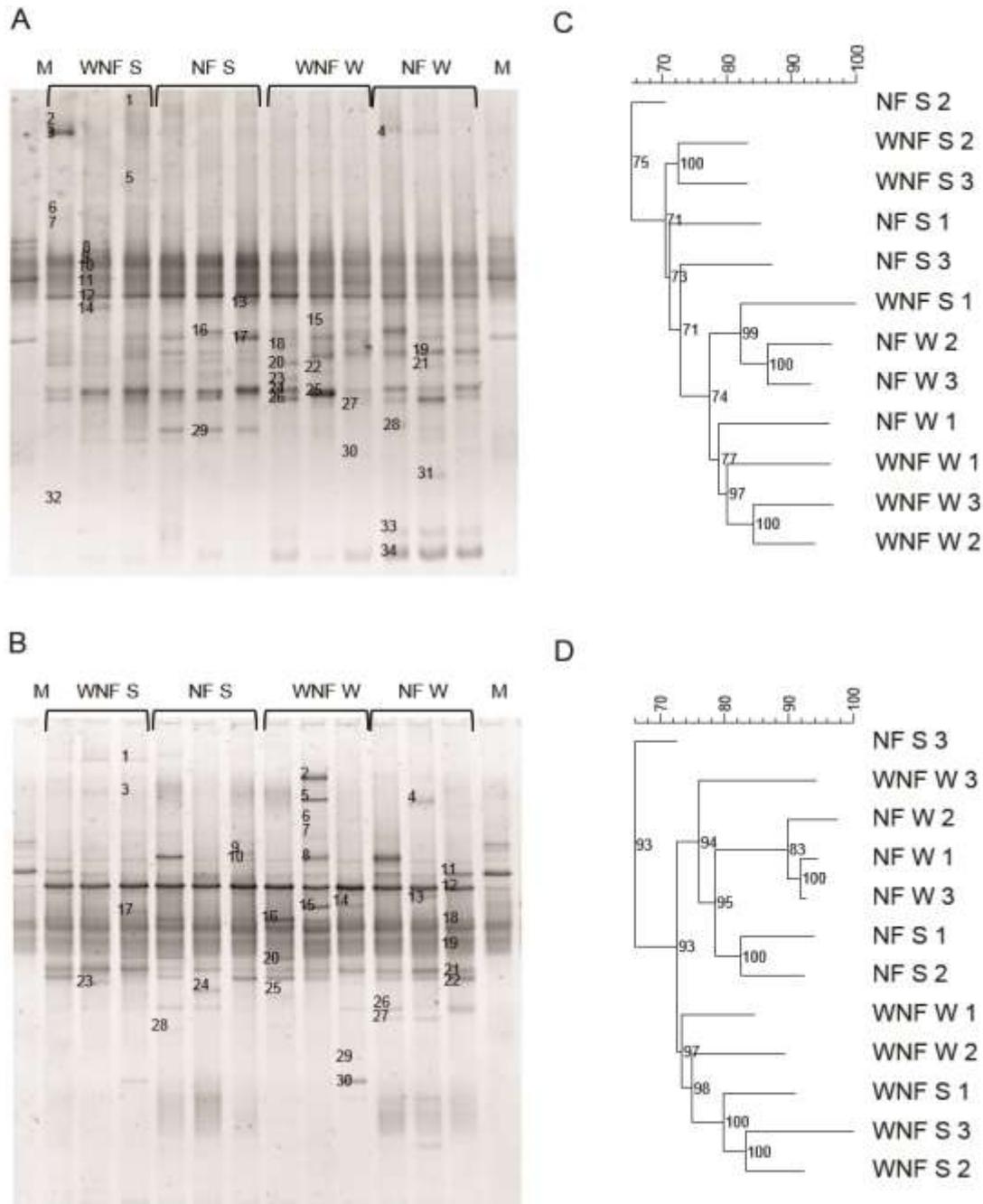


Figure 1 - Analysis of the cluster obtained from the DGGE banding patterns of the AMF communities in *E. globulus* plantations in the Penafiel region of northern Portugal. A) DGGE band pattern of soil samples obtained using the 18S rDNA gene. B) DGGE band pattern of root system samples obtained using the 18S rDNA gene. C) Dendrogram of soil samples (*UPGMA*). D) Dendrogram of root system samples (*Neighbor Joining*). NF: with maintenance nitrogen fertilization; WNF: without maintenance nitrogen fertilization; W: winter; S: summer; M: Marker. Scale = similarity (%).

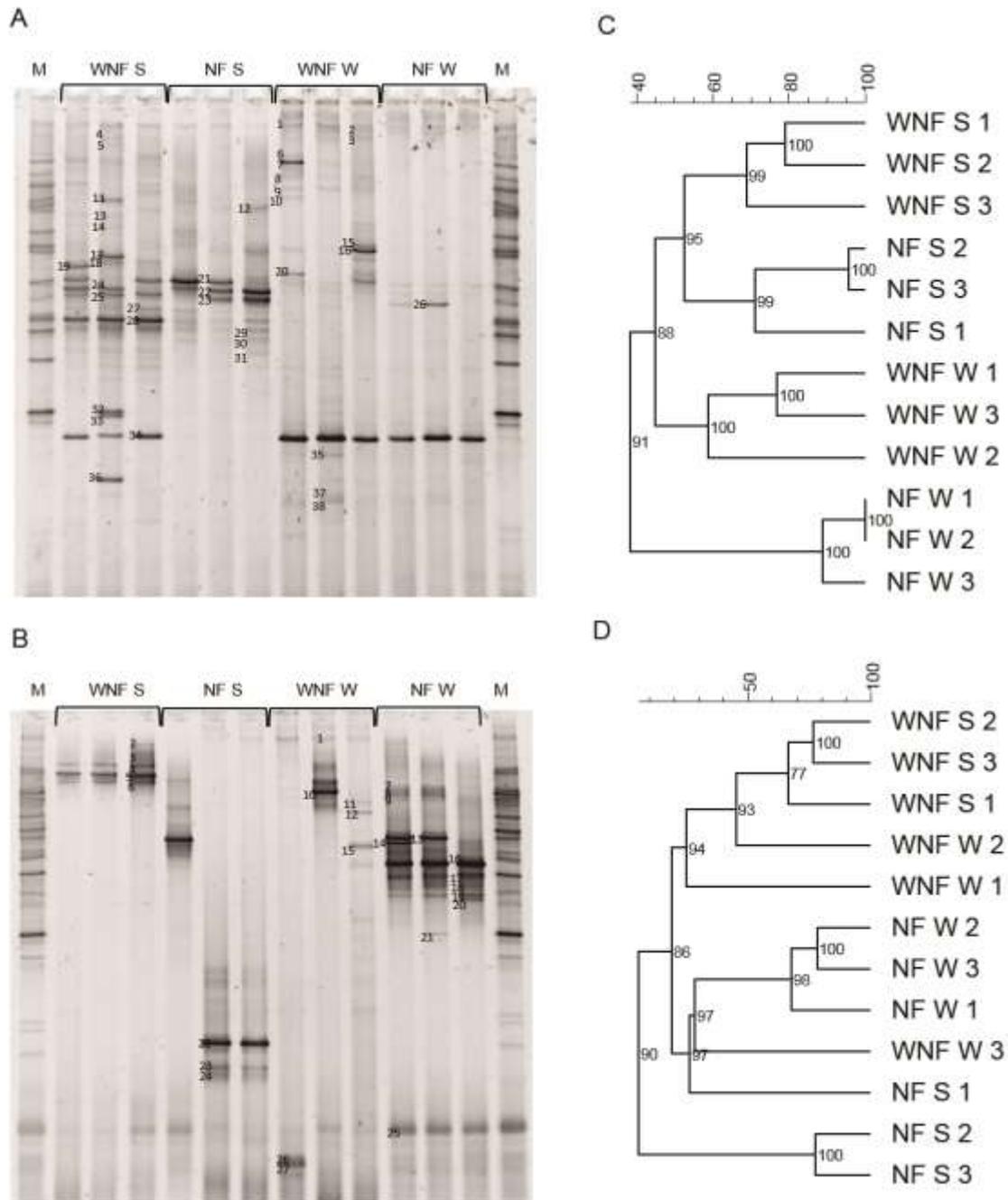


Figure 2 – Analysis of the cluster obtained from the DGGE banding patterns of the AMF communities in *E. globulus* plantations in the Gavião region of northern Portugal. A) DGGE band pattern of soil samples obtained using the 18S rDNA gene. B) DGGE band pattern of root system samples obtained using the 18S rDNA gene. C) Dendrogram of soil samples (*UPGMA*). D) Dendrogram of root system samples (*UPGMA ing*). NF: with maintenance nitrogen fertilization; WNF: without maintenance nitrogen fertilization; W: winter; S: summer; M: Marker. Scale = similarity (%).

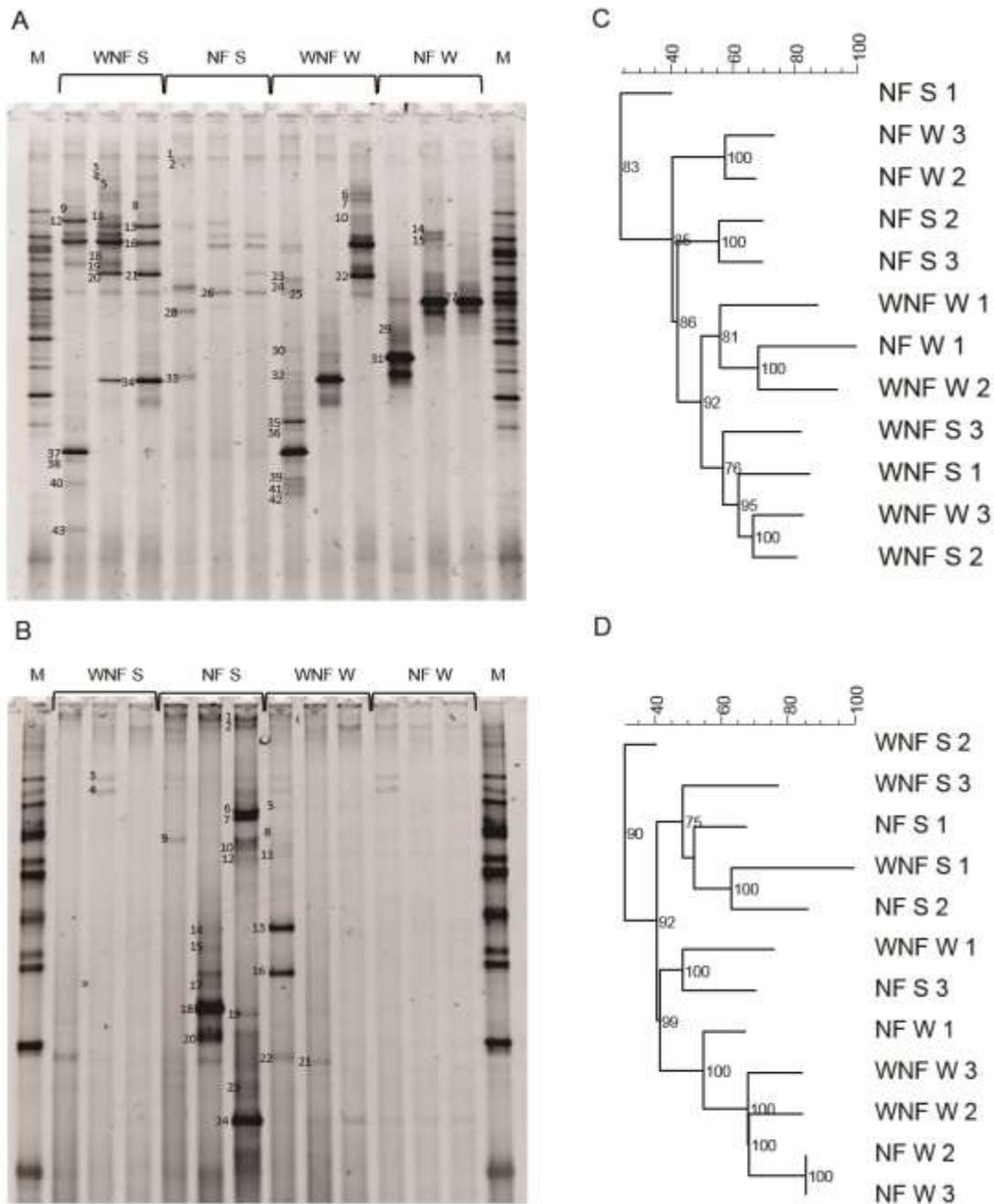


Figure 3 – Analysis of the cluster obtained from the DGGE banding patterns of the AMF communities in *E. globulus* plantations in the Odemira region of northern Portugal. A) DGGE band pattern of soil samples obtained using the 18S rDNA gene. B) DGGE band pattern of root system samples obtained using the 18S rDNA gene. C) Dendrogram of soil samples (*Neighbor Joining*). D) Dendrogram of root system samples (*Neighbor Joining*). NF: with maintenance nitrogen fertilization; WNF: without maintenance nitrogen fertilization; W: winter; S: summer; M: Marker. Scale = similarity (%).

Effect of environmental factors on AMF communities

Multivariate analysis demonstrated that in the soils from Penafiel, there was an explanation for 38.5% of the variation in the AMF community (Figure 4). The first axis explained 25.7% of the variability, and the differentiation in the microbial community was primarily associated with the season, winter and summer. In the root system samples, there was an explanation for 33.4% of the variation in the AMF communities and the groups were primarily associated with the season, winter and summer. In both cases, the winter samples had a stronger relationship with moisture and alkaline phosphatase than the summer samples, which exhibited a stronger relationship with the concentration of phosphorus and the acid phosphatase activity (Figure 4 A-B). The root system was also influenced by om (Figure 4 A-B).

In the soils and root systems from the Gavião region, the AMF communities were grouped according to the seasons, with an explanation for 61.7 and 41.1% of the variability, respectively (Figures 4 C-D). In both cases, the winter samples exhibited a stronger relationship with acid and alkaline phosphatase activity, om, moisture and phosphorus concentration (Figure 4 C-D).

In the soils and root systems from the Odemira region, the AMF communities were grouped according to the seasons, with an explanation for 55.8 and 45.5% of the variability, respectively. In both cases, the winter samples exhibited a stronger relationship with alkaline phosphatase activity and moisture than the summer samples.

In the samples from soils that received maintenance N fertilization in the summer, the strongest relationships were observed with pH, om and the concentration of phosphorus (Figure 4 E-F).

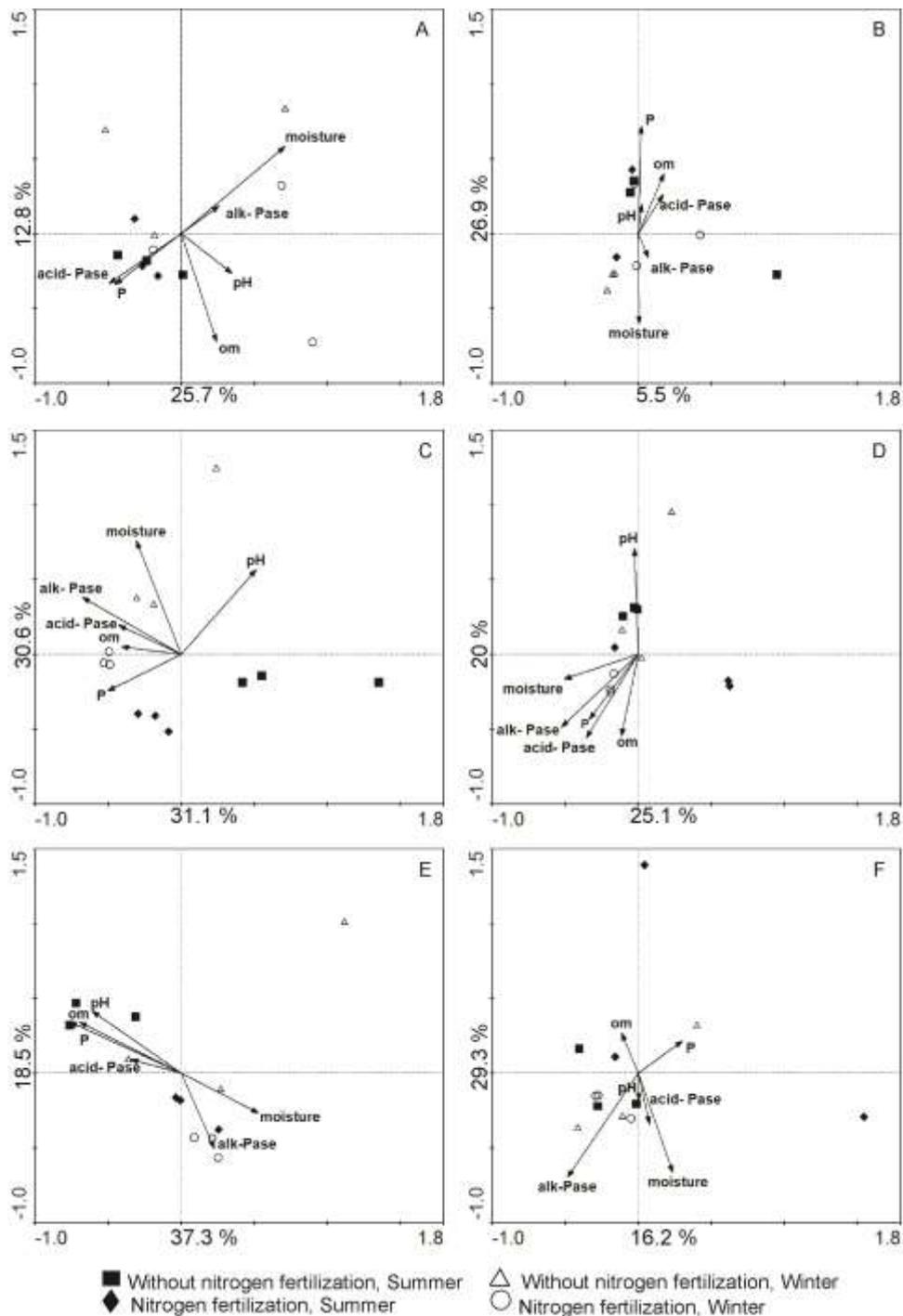


Figure 4 - PCA and CA based on PCR-DGGE profiles of the 18S rDNA gene from soil and root system samples from E. globulus plantations in Penafiel (northern), Gavião (central) and Odemira (southern) regions of Portugal, overlaid with environmental data. A and B - soil and root systems (Penafiel); C and D - soil and root systems (Gavião); E and F - soil and root systems (Odemira); moisture, pH, phosphorus concentration (P), organic matter (om), acid phosphatase (acid-Pase) and alkaline phosphatase (alk-Pase).

Identification of AMF communities

The DGGE gel bands of the AMFs from the three regions were sequenced, and the results were compared using the BLASTn algorithm (NCBI). Band sequencing indicated that most of the bands that belonged to the AMFs in the soil samples corresponded to *Gigaspora* sp. and *Glomus* sp. (Figure 5). This latter genus was also predominant in the root samples (Figure 5). We also found many bands that belonged to non-arbuscular mycorrhizal fungi, especially in samples from the Odemira region.

The results of the band sequencing revealed that the samples of soil from Penafiel had a large number of species. *Scutellospora heterogama* was only found in this region. In the root samples, *Gigaspora rosea* and *Glomus* sp. were found. In the soil samples from Gavião, the greatest percentage (40%) of sequences corresponded to *Gigaspora margarita*, followed by *Glomus* spp. The opposite was observed in samples from the root system. In the soil samples from Odemira, *Glomus irregulare* predominated, followed by *Gigaspora margarita*; in the root samples, only *Glomus* spp. were found (Figure 5).

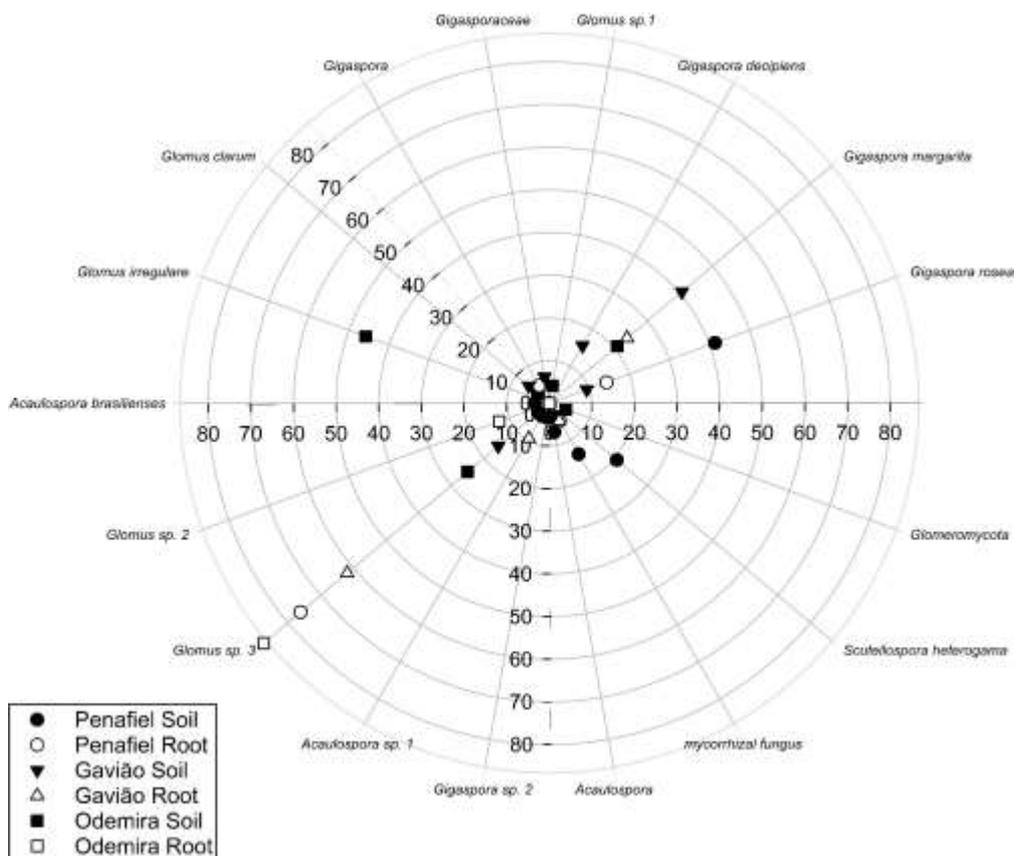


Figure 5 - Distribution of bacterial genera found in the soil and root systems of *E. globulus* plantations in three regions of Portugal (Penafiel, Gavião and Odemira) obtained by sequencing the DGGE bands of the 18S rDNA gene.

DISCUSSION

The influence of the rainy winters associated with a Mediterranean climate was decisive in the regions studied, especially in view of the reduction of moisture that occurs in the summer, which is characterized by the absence of rainfall (Table 1). The alkaline phosphatase activity increased in the winter compared to the summer. This difference may be due to high moisture, which is very important to the metabolism of the fungi and bacteria responsible for alkaline phosphatase production (Dakora and Phillips, 2002; Caldwell, 2005) (Table 1). Another determining factor for the activity of this phosphatase is soil pH, which is acidic for eucalypts, favoring the synthesis and activity of phosphatases (Cao et al., 2010). The Odemira soils exhibited high phosphatase activity in both winter and summer; this activity can be related to the large amount of organic matter and moisture and the low phosphorus concentrations observed in the soil (Table 1).

All of the evaluated parameters may interfere with the AMF community in eucalypts. According to the literature, the composition of the microbial community correlates strongly with soil pH (Bardgett et al, 1993; Högberg et al, 2007). Seasonal changes in soil microbial communities have been reported, and these communities are susceptible to change with variations in temperature and soil moisture (Moore-Kucera and Dick 2008). Similar to observations reported by Cao et al., (2010), the AMF communities assessed using the DGGE technique were influenced by region, season and nitrogen fertilization (Figures 2, 3 and 4).

The larger number of AMF bands that was observed in soil samples compared to root system samples may be due to the smaller number of AMFs in adult eucalypt plants (Figures 1, 2 and 3), where the succession of AMFs can be occurred by ectomycorrhizal fungi (Bellei et al, 1992; Yinglong et al, 1999, 2000). However, this sequence is not a rule because under certain conditions, this sequence does not occur (Campos et al., 2011).

A larger fungal biomass is found in N-poor soils, and the increased supply of nitrogen to plants decreases the C supplied to the fungi (Högberg et al., 2007), resulting in increased fungal diversity in areas that have not received nitrogen fertilization (Figures 1, 2 and 3). Moisture is a crucial factor for microbial activity, and in the dry seasons, the plant's need for water promotes a stronger interaction between the plant and the AMF, favoring the emergence of a more diverse profile of AMFs in the summer season because the AMFs increase the water absorption of the area (Helgason and Fitter, 2005).

The three Portuguese regions studied have different characteristics (Table 1). In Penafiel, a greater number of species was observed in the winter in the fertilized area than in the other regions. This difference may be due to the sandy soil and to the more severe winters that occur in Penafiel than in the other regions; these conditions require a stronger plant/AMF interaction that results in increased strength and protection for the plant (Figure 1).

The interaction between environmental factors and the data obtained via DGGE highlights moisture as the factor that exerts the most influence on the community of AMFs. Soil moisture is one of the main limiting factors for the accumulation of biomass and increases the

efficient use of N in terms of accumulated carbon, which is reduced with the addition of fertilizer (Wood et al., 2002).

AMFs of the *Glomus* genus have different spreading and survival strategies (Liang et al, 2008), which can justify their presence in a higher percentage in this study. The interactions between *Glomus mosseae* and *Glomus deserticola* and *E. globulus* has been studied, and the benefits of this interaction are of great importance for the cultivation of this plant (Arriagada et al., 2004, 2005, 2007a, 2007b, 2010). Studies have also been conducted with *Gigaspora rosea*, which was present in significant proportions in this community (Arriagada et al., 2009).

The strategy of nested PCR, together with the partial specificity of the primer AM1 and the resolving power of the DGGE gel with the primer pair NS31-GC/Glo1, allows the observation of the profile of the AMF community (Cornejo et al., 2004). Understanding the community structure of AMFs in the soil and roots of plants is necessary for adequate management, particularly in acidic soils with low availability of P and other nutrients.

CONCLUSIONS

The profile of the AMF community is influenced by the winter and summer seasons and nitrogen fertilization. Soil moisture was the environmental factor most strongly related to the variation observed in the AMF community. Regardless of the region analyzed, the main genera of AMFs that are associated with eucalypts are *Glomus* and *Gigaspora*.

ACKNOWLEDGEMENTS

The authors are very grateful to the following Brazilian Financial Institutions: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do estado de Minas Gerais (FAPEMIG). We are also grateful to the Institute of Forest and Paper Research (RAIZ Institute) of the Portucel Soporcel Group, especially to the researchers Antonio Sergio Fabres and Daniela Ferreira, for providing assistance during field sampling and for providing data concerning the collection areas. We are also very grateful to Edynei Miguel Cristino, for all help during the field sampling and enzymatic analysis.

REFERENCES

- ABRAF, Associação Brasileira de Produtores de Florestas Plantadas. Anuário estatístico da ABRAF 2010 ano base 2009/ ABRAF. – Brasília, 2010. 140p.
- Altshul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Miller, W.; Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25 (17), 3389.
- Arriagada, C.A., Herrera, M.A., Garcia-Romera, I., Ocampo, J.A., 2004. Tolerance to Cd of Soybean (*Glycine max*) and *Eucalyptus* (*Eucalyptus globulus*) inoculated with arbuscular mycorrhizal and saprobe fungi. *Symbiosis* 36, 1-15.

- Arriagada, C.A., Herrera, M.A., Ocampo, J. A., 2005. Contribution of arbuscular mycorrhizal and saprobe fungi to the tolerance of *Eucalyptus globulus* to Pb. *Water, Air, and Soil Pollution* 166, 31–47.
- Arriagada, C.A., Herrera, M.A., Borie, F., Ocampo, J. A., 2007. Contribution of arbuscular mycorrhizal and saprobe fungi to the aluminum resistance of *Eucaiyptus globulus*. *Water, Air, Soil Pollut* 182, 383-394.
- Arriagada, C.A., Herrera, M.A., Ocampo, J.A., 2007. Beneficial effect of saprobe and arbuscular mycorrhizal fungi on growth of *Eucalyptus globulus* co-cultured with *Glycine max* in soil contaminated with heavy metals. *Journal of Environmental Management* 84, 93–99.
- Arriagada, C.A., Aranda, E., Sampedro, I., Garcia-Romera, I., Ocampo, J.A., 2009. Interactions of *Trametes versicolor*, *Corioloopsis rigida* and the arbuscular mycorrhizal fungus *Glomus deserticola* on the copper tolerance of *Eucalyptus globules*. *Chemosphere* 77, 273–278.
- Arriagada, C.A., Pereira, G., Garcia-Romera, I., Ocampo, J.A., 2010. Improved zinc tolerance in *Eucalyptus globulus* inoculated with *Glomus deserticola* and *Trametes versicolor* or *Corioloopsis rigida*. *Soil Biology & Biochemistry* 42, 118-124.
- Bardgett, R.D., Frankland, J.C., Whittaker, J.B., 1993. The effects of agricultural practices on the soil biota of some upland grass lands. *Agriculture, Ecosystems and Environment* 45, 25-45.
- Bellei, M.M., Garbaye, J., Gill, M., 1992. Mycorrhizal succession in young *Eucalyptus viminalis* plantations in Santa Catarina (southern Brazil). *Forest Ecology and Management* 54, 205-213.
- Bianciotto, V., Bonfante, P., 2002. Arbuscular mycorrhizal fungi: a specialized niche for rhizospheric and endocellular bacteria. *Antonie Van Leeuwenhoek*, Wageningen 81, 365-371.
- Bianciotto, V., Mumini, E., Bonfante, P., Vandamme, P., 2003 “Candidatus Glomeribacter Gigasporarum,” an endosymbiont of arbuscular mycorrhizal fungus. *International Journal of Systematic and Evolutionary Microbiology* 53, 121-124.
- Bonfante, P., 2003. Plants, mycorrhizal fungi and endobacteria: a dialog among cells and genomes. *Biol. Bull* 204, 215-220.
- Bonfante, P., Anca, L.A., 2009. Plants, Mycorrhizal Fungi, and Bacteria: A Network of Interactions. *Annual Review of Microbiology* 63, 363–383.
- Caldwell, B.A., 2005. Enzyme activities as a component of soil biodiversity: A review. *Pedologia* 49, 637-644.
- Campos, D.T.S., Da Silva, M.C.S., Da Luz, J.M.R., Telesfora, R.J., Kasuya, M.C.M., 2011. Colonização micorrízica em plantios de eucalipto. *Revista Árvore* 35, 965-974.
- Cao, Y., Fu, S., Zou, X., Cao, H., Shao, Y., Zhou, L., 2010. Soil microbial community composition under *Eucalyptus* plantations of different age in subtropical China. *European Journal of Soil Biology* 46, 128-135.
- Cornejo, P., Azcon-Aguilar, C., Barea, J.M., Ferrol, N., 2004. Temporal Temperature Gradient Gel Electrophoresis (TTGE) as a tool for the characterization of arbuscular mycorrhizal fungi. *FEMS Microbiology Letters* 241, 265-270.
- Dakora, F.D., Phillips, D.A., 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil* 245, 35-47.

- Douhan, G.W.; Petersen, C.; Bledsoe, C.S.; Rizzo, D.M., 2005. Contrasting root associated fungi of three common oak-woodland plant species based on molecular identification: host specificity or non-specific amplification? *Mycorrhiza* 15: 365-372.
- FAO, 2009. Global demand for wood products. State of the World's Forests 62-96
- Helgason, T., Fitter, A.H., Young, J.P.W., 1998. Ploughing up the wood-wide web? *Nature* 394-431.
- Helgason, T., Fitter, A.H., 2005. The ecology and evolution of the arbuscular mycorrhizal fungi. *Mycologist* 19, 96-101.
- Högberg, M.N., Högberg, P., Myrold, D.D., 2007. Is microbial community composition in boreal forest soils determined by pH, C-to-N ratio, the trees, or all three. *Oecologia* 150, 590-601.
- Kowalchuk, G.A., De Souza, F.A., Van Veen, J.A., 2002. Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in dutch coastal sand dunes. *Molecular Ecology* 11, 571-581.
- Liang, Z., Drijber, R.A., Lee, D.J., Dwiekat, I.M., Harris, S.D., Wedin, D.A., 2008. A DGGE-cloning method to characterize arbuscular mycorrhizal community structure in soil. *Soil Biology & Biochemistry* 40, 956-966.
- Ma, W.K., Siciliano, S.D., Germida, J.J., 2005. A PCR-DGGE method for detecting arbuscular mycorrhizal fungi in cultivated soils. *Soil Biology & Biochemistry* 37, 1589-1597.
- Madeira, M.V., Fabião, A., Pereira, J.S., Araújo, M.C., Ribeiro, C., 2002. Changes in carbon stocks in *Eucalyptus globulus* Labill. plantations induced by different water and nutrient availability. *Forest Ecology and Management* 171, 75-85.
- Moore-Kucera, J., Dick, R., 2008. PLFA profiling of microbial community structure and seasonal shifts in soils of a Douglas-fir chronosequence. *Microbial Ecology* 55, 500-511.
- Näsholm, T., Kielland, K., Ganeteg, U., 2009. *Transley review*: Uptake of organic nitrogen by plants. *New Phytologist* 182, 31-48.
- Parniske, M., 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology* 6, 763-775.
- Sanders I.R., Croll, D., 2010. Arbuscular Mycorrhiza: The challenge to understand the genetics of the fungal partner. *Annual Review of Genetics* 44, 271-292.
- Simon, L., Lalonde, M., Bruns, T.D., 1992. Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. *Applied and Environmental Microbiology* 58, 291-295.
- Tabatabai M.A., Bremner J.M., 1969. Use of p-nitrophenyl phosphate in assay of soil phosphatase activity. *Soil Biology & Biochemistry* 1, 301-307.
- Yinglong, C., Mingqin, G., Fengzhen, W., Dell, B., 1999. Mycorrhizal succession and inoculant efficiency of dual inoculation on *Eucalyptus urophylla*. *Forestry Studies in China* 1,16-21.
- Yinglong, L.C., Brundrett, M.C., Dell, B., 2000. Effects of ectomycorrhizas and vesicular-arbuscular mycorrhizas, alone or in competition, on root colonization and growth of *Eucalyptus globulus* and *E. urophylla*. *New Phytologist* 146, 545-556.