

Full Length Research Paper

## Arbuscular mycorrhizal fungi diversity in revegetated areas after bauxite mining

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Arbuscular mycorrhizal fungi (AMF) are obligatory biotrophs that have a symbiotic evolutionary relationship with about 80% of all terrestrial plant species. The fungus mainly supplies water and nutrients to the plant and receives photoassimilates. The AMF diversity affects both the competition among species and floristic composition of an area. Fluctuations in the population of this group of microorganisms can cause fluctuations in plant populations above ground. In this work, the AMF community profile in areas with different ages of revegetation was evaluated by morphological identification of spores and denaturing gradient gel electrophoresis (DGGE) techniques. We found 12 AMF species and dominance of the species *Glomus macrocarpum* determined by spore density. Since the richness level observed in each plot was low and there was predominance of one species of AMF, it can be concluded that these areas still present a high degree of disturbance. It was possible to detect complex band profiles by DGGE analyses for the two plant species studied, *Visnia latifolia* and *Cecropia hololeuca*. No relationship between AMF diversity and revegetation time was observed in these areas.

**Key words:** Denaturing gradient gel electrophoresis (DGGE), symbiosis, succession, survey.

### INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) belong to the Glomeromycota phylum. It is abundant and diverse in the soil of the majority of terrestrial ecosystems. These fungi

are obligatorily biotrophic and establish mutualistic symbiotic relationships with the majority of land plant species, acting as an extension of the root systems

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through the hyphae that they form. They influence the nutrition of plants through two basic mechanisms: by increasing the root absorption surface and increasing the soil volume exploited by the root system. These mechanisms increase the absorption of nutrients, especially those that have lower mobility in the soil, such as phosphorus, and facilitate the uptake of water under drought conditions.

The beneficial effects of AMF for many plant species have been reported in the literature, both for agricultural crops like *Manihot esculenta* and forest species such as *Trema micrantha*, *Schinus terebinthifolius*, *Senna macranthera* and *Caesalpinia ferrea* (de Souza et al., 1999; Siqueira and Saggin-Júnior, 2001). AMF have a synergetic effect on biological nitrogen fixation (BNF) in some triple interactions (fungus-plant-bacteria), increasing this fixation because leguminous species are better nourished in association with AMF or when the fungi influence the radicular infection process (Jesus et al., 2005).

Recently the role of AMF in complex ecosystems has been studied (Siqueira et al., 1998; Zangaro et al., 2007; 2008). Besides the benefits mentioned above, these fungi increase plants' tolerance for toxic elements, which is particularly important in contaminated environments. For this reason, they are fundamental to improve the survival of seedlings in mined areas in the process of revegetation (Chaer et al., 2011; Soares and Siqueira, 2008).

The diversity of AMF has been evaluated by identifying morphological characteristics of their spores or by using molecular biology tools. Some authors urge the use of these tools because the population of spores present in the soil may have little relationship with the AMF colonizing the roots (Husband et al., 2002a, b). In this case, improved molecular techniques can help directly identify the AMF population in root systems

The aim of this work was to assess the AMF diversity using morphological characterization of the spores obtained from soil samples and to evaluate the profile of the AMF community colonizing the roots of *Visnia latifolia* and *Cecropia hololeuca* by means of denaturing gradient gel electrophoresis (DGGE), in areas with different revegetation ages after bauxite mining.

## MATERIALS AND METHODS

### Study area

The company Mineração Rio do Norte (MRN) operates the Saracá, Almeidas and Avisos mines (all within the Saracá-Taquêra National Forest, located in the municipality of Oriximiná, Pará state, at 1° 21' S - 56° 22' W, 180 m elevation). In these mines, the ore is found at an average depth of 8 m, covered by dense vegetation and a layer called overburden, composed of organic soil, nodular bauxite and ferruginous laterite. To mine the reserves, it is necessary to remove the overburden to reveal the economically exploitable bauxite ore.

This operation is done in sequence, where the overburden is deposited in the adjacent pit that was previously mined. In these areas, the replanting is done on the overburden. The replanting of the overburden areas investigated in this study was done by the company using available seeds of various species (*Parkia multijuga*, *Parkia pendula*, *Parkia oppositifolia*, *Ormosia holerytra*, *Ormosia excelsa*, *Sclerolobium paniculatum* and *Acosmium nitens*) in 1982, 1983, 1984, 1985, 1986, 1987, 1992, 1993, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005 and 2006.

### Evaluation of the AMF community by morphological characteristics of spores

Twenty simple samples (0 – 0.20 m) were harvested to compose a compound sample in each plot measuring 250 m<sup>2</sup> in each area. Thirty-two compound soil samples were collected in 2007. For the years 1987, 2004, 2005 and 2006 soil samples were not collected because in these years only one plot was revegetated.

Spores were extracted from 50 mL soil samples by wet sieving and decanting (Gerdemann and Nicolson, 1963) then centrifuging in water and 45% sucrose solution (Jenkins, 1964). The spores recovered were counted and grouped according to their size and color. Representatives of each group were mounted on slides for microscopic examination using polyvinyl-lacto-glycerol (PVLG) and PVLG plus Melzer reagent (1:1) as mounting. The AMF species were identified by comparison with descriptions found in Schenck and Perez (1990) and at the site of the International Culture Collection of Arbuscular Mycorrhizal Fungi (<http://invam.caf.wvu.edu>).

The species richness, shannon-weiner diversity and evenness (J') values were determined using the Past program (PAleontological STatistics) (Hammer et al., 2001).

### Evaluation of the AMF community by DGGE

Twenty-nine and 17 root samples of *V. latifolia* and *C. hololeuca* were collected, respectively. These species were chosen because of the native occurrence in the region and are often found in areas in the early stages of succession. Specimens of *V. latifolia* were found in areas revegetated in 1982, 1984, 1986, 1987, 1999, 2004 and 2006 (1 individual for each year); 1993, 1995, 1996, 1997, 1998, 2000, 2001, 2003 and 2005 (2 individuals each) and 2002 (4 individuals). In turn, *C. hololeuca* specimens were found in areas revegetated in 1986, 1998, 2005 and 2006 (1 individual each); 1999, 2002 and 2003 (3 individuals each); and 2001 and 2004 (2 individuals each). The roots were dehydrated in silica gel for the transport and then stored in 80% ethanol (v/v) at 4°C in the laboratory. Before DNA extraction, some samples were randomly selected to confirm the root colonization and all samples showed fungal structures.

For DNA extraction, the root samples were taken from alcohol, dried quickly on absorbent paper at 65°C to eliminate residual alcohol and macerated in liquid nitrogen. The DNA extraction was done with the DNeasy Mini Plant kit (Qiagen). The 18S rDNA gene was amplified, in two PCR reactions. In the first PCR, the primers used were AML1 (5'-ATCAACTTTCGATGGTAGGATAGA-3') and AML2 (5'-GAACCCAAACACTTTGGTTCC-3') (Lee et al., 2008). The final volume of the reaction was 15 µL and the amplification conditions were: 1X buffer; 200 µM of each dNTP; 1.625 mM of MgCl<sub>2</sub>; 0.2 µM of each primer; 0.14 µg µL<sup>-1</sup> of BSA (bovine serum albumin); 0.78% v/v of formamide and 1 U of DNA polymerase (GoTAq<sup>®</sup> Flexi DNA Polymerase - Promega). Two µL of a 1:20 dilution of the extraction product was used as template DNA. The reaction was carried out in an Eppendorff thermocycler with the

following steps: 94°C for 3 min for initial denaturing; 30 cycles at 94°C for 45 s for denaturing; 58.5°C for 1 min for annealing; 72°C for 55 s for extension; and 72°C for 5 min for final extension.

The PCR product was verified in 1% (w/v) agarose gel. For the samples presenting visible bands, a 1:1000 dilution was made in water, while the dilution for the other samples was 1:500.

The second reaction used the primers NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3', Simon et al., 1992), containing the GC clamp NS31GC (5'-CGCCCGGGCGCGCCCCGGGCGGGCGGGGCGGGGACGGGGGTGGAGGGCAAGTCTGGTGCC-3', Kowalchuk et al., 2002), and AM1 (5'-GTTTCCCCTAAGGCGCCGAA-3', Helgason et al., 1998). In this reaction, the final volume was 35 µL and the amplification conditions were: 1X buffer; 200 µM of each dNTP; 2.5 mM of MgCl<sub>2</sub>; 0.2 µM of each primer; 0.135 µg µL<sup>-1</sup> of BSA; and 1 U of DNA polymerase (GoTaq® Flexi DNA Polymerase - Promega). The template DNA consisted of 2 µL of the dilution of the first PCR product. The reaction was performed in the same thermocycler, with the following steps: 94°C for 3 min for initial denaturing; 34 cycles at 94°C for 1 min for denaturing; 60°C for 1 min for annealing; 72°C for 1 min for extension; and final extension at 72°C for 5 min.

The products obtained with the primers NS31GC and AM1 were separated by polyacrylamide gel (6% w/v) electrophoresis (37.5:1 acrylamide:bisacrylamida) with a chemical denaturing gradient composed of formamide and urea, varying from 30 to 45% (100% denaturant corresponds to 7M of urea and 40% formamide), in 0.5X TAE at 60°C under constant voltage of 75 V for 15 h. The gels were stained with ethidium bromide and photographed under UV light.

The band profiles were used to construct similarity dendrograms with the Jaccard index and the UPGMA clustering method, available in the GelCompar II program (Applied Maths).

## RESULTS

### Evaluation of the AMF community by spore occurrence

Twelve AMF species were identified, one of them belonging to the *Glomus* genus (*G. macrocarpum*), one to the *Claroideoglossum* (*C. etunicatum*), one to the *Scutellospora* (*S. scutata*), one to the *Gigaspora* (*G. margarita*), three to the *Rhizophagus* (*Rhizophagus* sp., *Rhizophagus clarus*, *Rhizophagus fasciculatus*) and five to the *Acaulospora* genus (*A. foveata*, *A. mellea*, *A. scrobiculata*, *A. tuberculata* and *Acaulospora* sp.).

*G. macrocarpum* spores were found in all the soil samples and these spores accounted for 97% of all the spores found. Besides this, *G. macrocarpum* was the only AMF species found in eight of the soil samples and the only one found in the samples from the plots revegetated in 1995 and 2000. Spores of this species represented at least 82% of the total for each year analyzed (Figure 1A). *A. mellea*, *G. margarita* and *S. scutata* were found in 11, 8 and 7 soil samples, respectively, while *A. scrobiculata* and *R. clarus* were only found in one sample each.

The mean species richness varied between 1 and 4 and was higher in the areas revegetated in 1982, 1983, 1993, 1997, 1998, 2001, 2002 and 2003 (Figure 1B). The

Shannon index and evenness index values were higher in the plots revegetated in 1983, 1985, 1998, 2002 and 2003 (Figure 1B).

### Evaluation of the AMF community by DGGE

The number of bands ranged from 2 to 5 for *C. hololeuca* and 0 to 7 for *V. latifolia* by the DGGE technique. For *V. latifolia* there were two principal clusters formed both by DGGE (Figure 2). The DGGE analysis revealed that the two samples from 1998-A1 (1 and 2); 1997-A1-1 and 1997-A2-1; 1995-A2-1 and 1987-A2-1; 2001-A1-1 and 2002-A1-1; 2000-A2 (1 and 2) and 1996-A13-1 presented 100% similarity (Figure 2). For *C. hololeuca*, two samples from 2003-02 (1 and 2); two 2004-FP samples (1 and 2); 1999-A2-2 and 2001-A2-2; 2002-A1-3 and 1986-A1-1; and 1999-A2-1 and 2001-A1-1 presented 100% similarity (Figure 3).

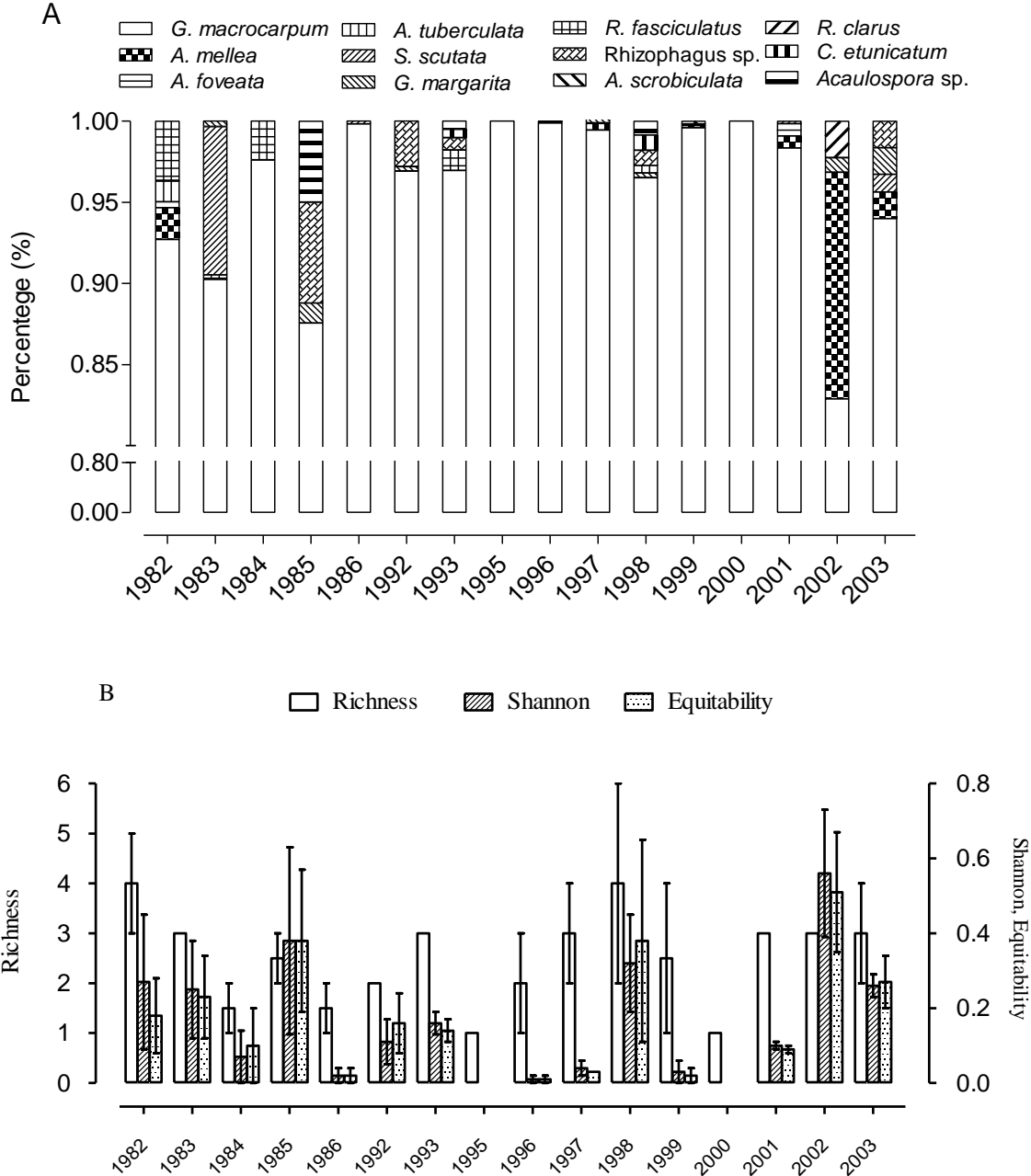
## DISCUSSION

The predominance of spore and AMF species belonging to the *Acaulospora*, *Rhizophagus* and *Glomus* genera observed in this study is in accord with other studies in various ecosystems (Carrenho et al., 2001; Leal et al., 2009; Lovelock et al., 2003), including studies of reclaimed mining areas (Caproni et al., 2003, 2005; Mergulhão et al., 2010).

Caproni et al. (2003) identified 57 species, belonging to six genera, with the greatest number belonging to the *Glomus* genus, followed by *Acaulospora*, *Entrophospora*, *Scutellospora*, *Gigaspora* and *Archeospora*. Caproni et al. (2005) identified a total of 18 AMF species belonging to the genera *Glomus* (7 species), *Acaulospora* (6 species), *Scutellospora* (2 species), *Archeospora*, *Entrophospora* and *Gigaspora* (1 species each).

Carrenho et al. (2001) evaluating the effect of corn monoculture for three consecutive years on the composition of the AMF community, reported that *G. macrocarpum* was recovered from 100, 90 and 60% of the samples in the first, second and third years, respectively. Caproni et al. (2003) found *G. macrocarpum* in 93% of the samples analyzed. According to them, the high frequency and relative density observed for this species indicate a wide range of adaptation. They stressed that it is important to study more details of the ecology of this species because it has potential for use in programs to restore degraded areas.

The diversity of AMF species can be influenced by the composition of plant species and environmental and edaphic factors (Carrenho et al., 2001; Mergulhão et al., 2009). This fact can also be related to the characteristics inherent to the life strategy of these species. Species of these genera usually produce small spores, in great



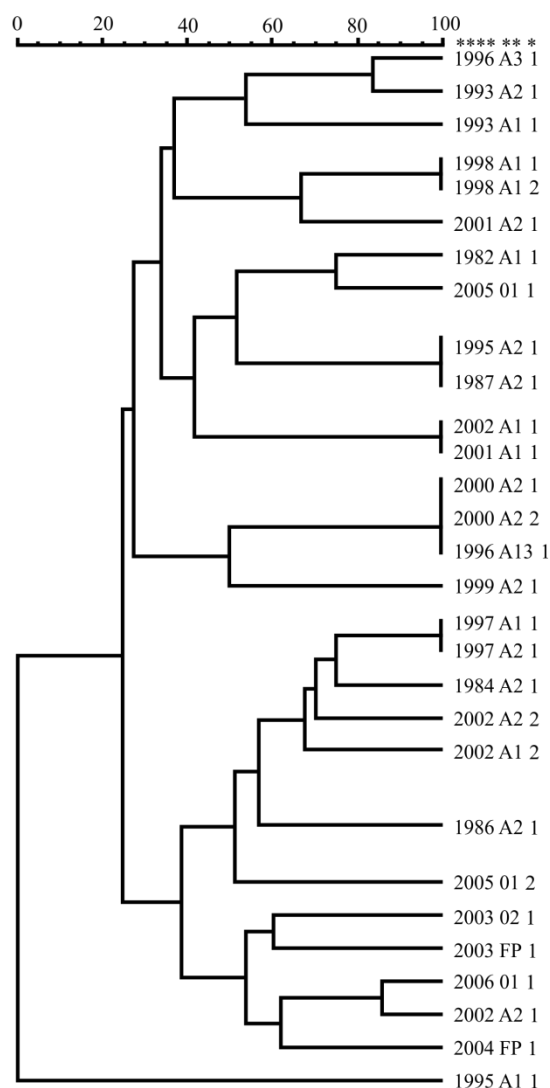
**Figure 1. A.** Percent of spores of each AMF species. **B.** mean and standard error for species richness, Shannon index and evenness index in function of year of revegetation.

abundance, and colonize the roots plentifully, while *Gigasporaceae* species generally produce larger spores, in smaller number, with less intense colonization.

Although there are limitations to the technique, some authors have used the number of bands in DGGE gel as an estimate of the species richness (Zhang et al., 2009). Considering that each band in the gel represents an AMF taxon, there was greater richness of the samples from the

areas planted with *V. latifolia* than in the samples from the areas planted with *C. hololeuca*. This situation can indicate differences in the degree of specificity of these species in relation to mycorrhizal colonization.

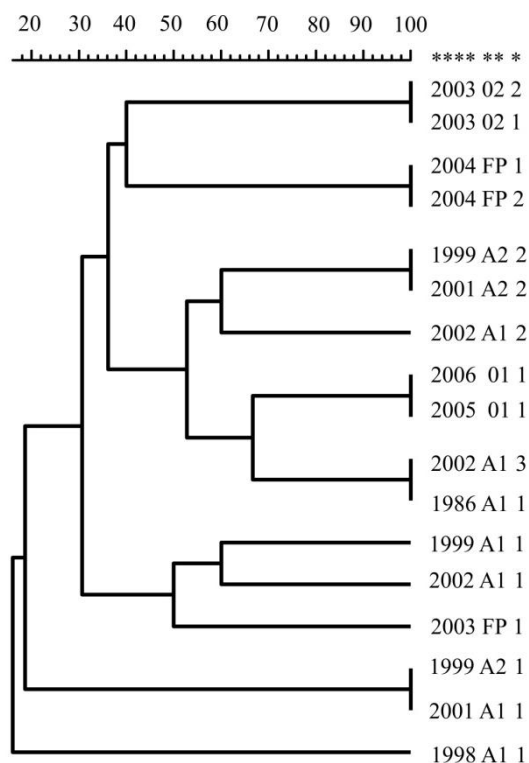
The DGGE analysis indicated 100% similarity between some root samples (Figures 2 and 3). Interestingly, in some cases, the samples were from the same revegetated plot and other cases where they were



**Figure 2.** Genetic similarity dendrograms of the AMF community for samples from roots of *Visnia latifolia* evaluated by denaturing gradient gel electrophoresis (DGGE) of the 18S rDNA gene. \*\*\*\* Year of revegetation; \*\* Plot sampled; \* Plant specimen.

collected from different plots.

Melloni et al. (2003) concluded that in areas being restored after mining, the diversity of AMF species is more closely related to the type of vegetation introduced than to the time since revegetation. In the present study we did not observe any relationship between the profile of the AMF community, analyzed both by spore presence and by molecular tools, and time since the areas were revegetated. We also did not observe any progressive alteration of the relative density of the different species in function of time revegetation, which if present would have characterized succession within the AMF community.



**Figure 3.** Genetic similarity dendrograms of the AMF community for samples from roots of *Cecropia hololeuca* evaluated by denaturing gradient gel electrophoresis (DGGE) of the 18S rDNA gene. \*\*\*\* Year of revegetation; \*\* plot sampled; \* plant specimen.

The results obtained show that the AMF community in the areas analyzed might be determined by varied combinations of environmental and edaphic factors; composition of plant species; spatial variability of the AMF community; and specificity of the host species in each area analyzed.

In general, the results show that despite the degree of degradation caused by mining activity, the revegetation practices followed are enabling the reestablishment of the AMF community in the soil. However, the areas studied still present a high level of disturbance, because the AMF richness and number of spores found were both low and there was overwhelming predominance of a single species, *G. macrocarpum*.

### Conflict of Interests

The authors have not declared any conflict of interests.

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