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Arbuscular mycorrhizal fungi associated with *Araucaria angustifolia* (Bert.) O. Ktze

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Abstract Specimens of *Araucaria angustifolia* from a native forest reserve and a reforested area in the State Park of Campos do Jordão were studied to determine the number and diversity of spore populations of arbuscular mycorrhizal fungi (AMF) and root colonization. Six randomly chosen plots (planted with 8- to 12-year-old plants) were delimited, four in the native forest and two in the reforested area. Rhizosphere and root samples were collected during two periods of the year corresponding to the rainy and dry seasons. A greenhouse experiment was set up for multiplication of field propagules (from the native forest and reforested area) for two consecutive generations. *Araucaria* leaves from the experimental plots were collected during the first sampling for nutrient analysis. Twenty-four AMF taxa were found and percent AM colonization was determined in all plots. Not all AMF species observed in the field were re-isolated through the recovery pot cultures, even after a second cultivation cycle. The foliar nutrient analysis showed higher nutrient levels in plants from the native forest than the reforested area. Generally, spore richness and diversity were highest during the warmer and more humid period and in the native forest plants.

Keywords Arbuscular mycorrhiza · Brazilian pine · Diversity · Symbiosis

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Introduction

Araucaria angustifolia (Bertoloni) Otto Kuntze, known as Brazilian pine, is an endangered native species. Preservation is justified by its economic value as timber, resin and food, as well as the need to maintain biological diversity. The soils chosen for forestation programs in Brazil generally have low nutrient availability, making establishment of the new plants difficult. Therefore, the presence of arbuscular mycorrhizal fungi (AMF) may be essential for ecosystem sustainability (Janos 1980) as they favor the establishment of plants (Allen and Allen 1980). The use of vigorous seedlings in revegetation programs is important. However, seedlings of forest species are generally weak, often phosphorus deficient and have high mortality rates after transplanting (Rifle and Boosalis 1979). Such problems may be reduced by AMF inoculation in nurseries, if efficient AMF species are available (Menge 1983).

There are few reports of AMF in the rhizosphere of *A. angustifolia* in the literature (Milanez and Monteiro 1950; Oliveira and Ventura 1952; Bononi et al. 1989; Breuninger et al. 2000) and virtually nothing is known about the diversity of such fungi or the benefits that this symbiosis may bring to *Araucaria*. Moreira-Souza and Cardoso (2002) recently verified that *Araucaria* is a mycotrophic plant that relies on AMF. The present work aims at understanding the diversity, abundance and root colonization of the AMF associated with *A. angustifolia* in native forest conditions, as well as in reforested areas.

Material and methods

Samples were taken from a native forest of *Araucaria*, which also contains over 200 other plant species, such as *Podocarpus lambertii*, *Ilex paraguariensis*, *Clethra scabra*, *Weinmannia pinnata*, *Cryptocarya aschersoniana*, *Prunus myrtifolia*, *Symplocus aegrota*, *Drymys winterii*, and from areas reforested with *A. angustifolia* and *Pinus eliotti*.

Both areas are located in the State Park of Campos do Jordão (PECJ) 210 km from the city of San Paulo at 22°44'S and 45°30'W in Campos do Jordão, San Paulo, Brazil. The local climate is

subtropical, mesothermic and humid, with an average rainfall of 245 mm and 88 mm per month in February (rainy season) and September (dry season), respectively. Average air temperatures range from 17.5°C in the hottest month (February) to 11.5°C in the coldest month (June), occasionally falling below zero.

The samples were collected in six randomly chosen plots, each containing five *Araucaria* trees ranging in age from 8 to 12 years and 4 to 6 m high. Plots 1, 2, 3 and 4 were in the native forest area, while plots 5 and 6 were located in a replanted area.

Rhizosphere soil and *Araucaria* roots were collected in February and September 1998. The rhizosphere soil was collected at depths of 0–20 cm around each tree, at four different points for each plant, giving a combined sample of approximately 300 g of soil per plant. The sample was analyzed for nutrients and pH and was used to determine the number and diversity of AMF spores. Soil analysis was done according to the procedures of van Raij et al. (1987). The pH as well as H and Al were determined using a pH meter in a solution of 0.01 M $CaCl_2$ (dilution factor 1:2.5) (van Raij et al. 2001). Organic matter was measured photometrically after dichromate/sulfuric acid digestion. Ca, P, Mg and K were extracted with an ion-exchange resin. P was quantified colorimetrically using ammonium molybdate. Ca and Mg were measured by atomic absorption spectrophotometry and K by flame photometry. The chemical constitution of the soil from analyzed areas was, for the native forest area, pH 3.7, organic matter 61.7 g dm^{-3}, P 10.0 mg dm^{-3}, and K^+ 2.3, Ca^{2+} 11.5, Mg^{2+} 3.3, $H^+ + Al^{3+}$ 122 (mmol dm^{-3}). For the reforested area, the results were pH 3.4, organic matter 54.0 g dm^{-3}, P 4.5 mg dm^{-3} and K^+ 2.0, Ca^{2+} 1.0, Mg^{2+} 1.0, $H^+ + Al^{3+}$ 122 (mmol dm^{-3}).

Spores were extracted by wet sieving (Gerdemann and Nicolson 1963) a 100-g soil aliquot from the combined sample for each tree, followed by centrifugation in sucrose (Jenkins 1964). Spores were quantified under a stereoscopic microscope at ×40 magnification in Petri dishes with concentric divisions. After determination of the total number, spores were separated into groups according to their morphology and mounted on semi-permanent slides with polyvinyl alcohol and glycerol resin (PVLG) (Morton et al. 1993). Spores were identified at the species level with the aid of an optical microscope at ×100–400 magnification using Schenck and Pérez (1990) and descriptions provided by the INVAM 1999 database (http://invam.caf.wvu.edu).

The total number of spores (TN) for each morphologically distinct AMF group was evaluated in 100 g of air-dried soil. The number of spores (NS) parameter is related to the number of AMF spores for each species. The relative frequency of occurrence (RF) was determined by RF= NA/TA × 100, where NA is the number of samples in which each AMF species appeared and TA the total number of analyzed samples. Other indices were estimated as follows: abundance of species as the number of species occurring in 100 g of air-dried soil; diversity as a Whittaker (1975) index.

Roots were collected from the same four points surrounding each plant to a total of nearly 50 g per tree. *Araucaria* roots differ from others due to their red color and pseudonodules (Oliveira and Ventura 1952), which made it easier to find them in the forest soil. The roots were cleared with KOH and stained in acid glycerol and trypan blue solution (Phillips and Hayman 1970) for determining the root colonization level according to Giovannetti and Mosse (1980).

In February 1998, *A. angustifolia* leaves for chemical analysis were collected at heights of 3–4 m at points oriented to north, south, east and west.

AMF spores found in the rhizosphere of *Araucaria* were multiplied under greenhouse conditions. The substrate was a mixture of a sandy soil and washed river sand (2:3 v:v). The mixture was dried, sieved and autoclaved at 121°C for 2 h. Subsamples were analyzed for plant nutrients and amended as needed. The substrate was placed in 3-l pots and amended with 50 g of inoculum containing AMF spores, propagules and colonized roots from the *Araucaria* forest. The inoculum consisted of a mixture of soils sampled from around the different trees, collected during the first sampling. Thirty pots planted with sorghum (*Sorghum bicolor* Moench. L.) were used, following the method of Stutz and Morton (1996). The sorghum seeds were surface sterilized with 2% sodium hypochlorite solution for 5 min. Ten days after sowing, seedlings were thinned, leaving only the most vigorous plant per pot, which grew for 160 days. The same procedure was repeated for an additional cycle and, after determining the number of spores from the first cycle, new pots were planted as described above, each receiving 50 g of soil inoculum from the pots of the previous cycle. Spore counting and identification followed the methodology used for spores collected in field conditions.

All variables were submitted to ANOVA, with the factors or the interaction of the factors being isolated; the Tukey test was used for comparison of means. For analysis of variance, the root colonization percentage data were arcsine transformed to $(x/100)^{1/2}$ and the number of spores to $(x + 0.5)^{1/2}$.

Results and Discussion

The number of AMF spores (Table 1) tended to be higher in the native forest than in the replanted area. Spore density data can be useful when evaluating AMF reproductive potential. Some AMF species produce more spores than others, with environmental factors significantly influencing spore reproduction and root colonization rate (Saif and Khan 1975; Ferguson and Menge 1982).

For the native forest, 732 spores per 100 g soil were found on average during the hot and humid season (February) and 810 spores per 100 g soil during the cold and dry season (September). In comparison, in the replanted area, 385 spores per 100 g soil were found in the first sampling and 423 spores per 100 g soil in the second sampling (Table 1). Studies of *Araucaria* areas in Rio Grande do Sul found on average 600 spores per 100 g soil, while 360 spores per 100 g soil were found in campo conditions in the rhizosphere of adult plants (Breuninger et al. 2000). These results are similar to those found in the present study. In agricultural areas, spore densities (corn monoculture) varied between 26 and 251 spores per 100 g soil (Maia and Trufem 1990). Read et al. (1976) reported in forest ecosystems in temperate regions that the number of spores ranged from 11 to 384 spores per 100 g soil. Compared with these data, there was a large number of spores in *Araucaria* native forest areas whilst the AMF species diversity in the rhizosphere was similar (Table 2).

Table 1 Number of spores (*NS*) per100 g dried soil and percent root colonization (*RC*) of *Araucaria angustifolia* by arbuscular mycorrhizal fungi (AMF) in February and September 1998 in the State Park of Campos do Jordão, San Paulo, Brazil. Plots 1–4 correspond to native forest and plots 5 and 6 to a replanted area (mean of 5 replicates). Different letters show significant differences (Tukey test, $P<0.05$)

Plot	NS	RC	NS	RC
	February		September	
1	730 a	28.8 a	982 a	20.6 a
2	1034 a	21.2 ab	866 a	19.5 a
3	406 b	21.2 ab	612 a	22.0 a
4	758 a	28.2 a	782 a	21.4 a
5	358 b	13.9 b	338 b	10.3 b
6	412 b	17.3 b	508 b	9.4 b

Table 2 Number of AMF spores (*NS*) per 100 g dried soil and the relative frequencies (*RF*) of different species in an *A. angustifolia* native forest and reforested area of the State Park of Campos do Jordão, San Paulo, Brazil during February and September 1998 (mean of 5 replicates)

AMF species	Native forest				Reforested			
	February		September		February		September	
	NS	RF	NS	RF	NS	RF	NS	RF
Acaulospora bireticulata Rothwell & Trappe	42	20	–	–	–	–	–	–
A. gerdemannii Schenck & Nicol.	216	40	114	35	–	–	–	–
A. laevis Gerd. & Trappe	272	65	136	65	90	30	–	–
A. scrobiculata Trappe	78	25	162	50	–	–	–	–
A. spinosa Walker & Trappe	26	20	184	75	–	–	36	40
A. rehmii Sieverding & Toro	–	–	–	–	16	30	–	–
Acaulospora sp. 1	64	25	54	15	36	20	–	–
Acaulospora sp. 2	50	20	16	10	–	–	–	–
Entrophospora colombiana Spain & Schenck	120	20	100	50	86	70	44	40
Gigaspora margarita Becker & Hall	318	65	576	50	160	50	–	–
G. decipiens Hall & Abbott	80	25	–	–	14	30	–	–
Glomus agreggatum (Schenck & Smith) Koske	136	25	–	–	–	–	–	–
G. clarum Nicol. & Schenck	–	–	38	25	–	–	54	50
G. diaphanum Morton & Walker	–	–	74	20	340	30	328	60
G. etunicatum Becker & Gerd.	530	75	596	50	–	–	30	100
G. fasciculatum Gerd. & Trappe (Walker & Koske)	186	40	202	25	–	–	–	–
G. geosporum (Nicol. & Gerd.) Walker	–	–	88	15	–	–	–	–
G. macrocarpum Tul. & Tul.	252	40	494	75	256	100	348	90
G. microcarpum Gerd. & Trappe	–	–	276	45	–	–	–	–
G. pansihalos Berch & Koske	220	35	–	–	172	40	–	–
Scutellospora gilmorei (Trappe & Gerd.) Walker & Sanders	–	–	–	–	–	–	30	30
S. nigra (Redhead) Walker & Sanders	50	20	–	–	–	–	–	–
S. pellucida (Nicol. & Schenck) Walker Sanders	282	15	–	–	–	–	–	–
Scutellospora sp.	–	–	–	–	–	–	20	20
Not identified	–	–	10	10	–	–	–	–
Total number of spores	2922	–	3354	–	1170	–	890	–
Richness index	17	–	16	–	9	–	8	–
Diversity (Whittaker)	4.9	–	4.5	–	2.9	–	2.7	–

This confirms the data of Sieverding (1991), who found that AMF spore population diversity in natural ecosystems is higher (25–30 species) than in agro-ecosystems (5–15 species).

Twenty-one AMF species were found in the *Araucaria* rhizosphere of the native forest, together with a non-identified taxon, while in the replanted area a total of 14 species was found in both sampling seasons (Table 2), with 11 AMF species occurring in both areas. Bononi et al. (1989) found 15 AMF species in the *A. angustifolia* rhizosphere at the Botanical Garden of San Paulo, six of which were also found in our studies: *S. pellucida*, *A. gerdemannii*, *Glomus macrocarpum*, *Glomus etunicatum*, *E. colombiana* and *A. scrobiculata*. Schenck and Siqueira (1987) reported 16 AMF species in the central Brazilian natural "cerrado" ecosystems. *Glomus agreggatum*, *Glomus fasciculatum*, *Glomus geosporum*, *Glomus microcarpum*, *Acaulospora* sp., *A. bireticulata*, *A. gerdemannii*, *A. scrobiculata*, *Scutellospora nigra*, *S. pellucida* and a non-identified taxon were identified only in the native forest area, whereas *A. rehmii*, *Scutellospora sp.* and *S. gilmorei* were found exclusively in the replanted area (Table 2).

In February, *Glomus macrocarpum* and *Entrophospora colombiana* were the most common AMF species present in such an environment, while in September the most common species were *Glomus etunicatum* and *Glomus macrocarpum*. Certain species of Glomales are adapted to acid soils and, generally, dominate the AMF community (Sieverding 1991; Siqueira et al. 1989); they may be dominant due to their high competitiveness and/or reproductive capability (Sieverding 1991). The abundance of AMF species and the diversity index showed the same tendency, i.e., they were higher in the native forest area than in the replanted area in the two seasons. It has been shown that AMF species diversity can be improved by an increase in host plant species; the productivity of these ecosystems will then be higher and more stable (van der Heidjen et al. 1998). In field conditions, the maintenance or introduction of a diversified AMF community is desirable in order to promote efficient combination of fungi and host plants.

As regards root colonization, all analyzed segments of *A. angustifolia* roots were colonized by AMF, with hyphae, vesicles, arbuscules and auxiliary cells in the interior of roots and/or attached to them. These results are similar to those of Breuninger et al. (2000) for *A. angustifolia* areas in the south of Brazil. Several spores of *Gigaspora* and/or *Scutellospora* spp. were found in *Araucaria* rhizospheres in the native forest, and many root samples had only auxiliary cells attached to them, confirming that Gigasporaceae can sometimes colonize

Table 3 Macronutrient concentrations (g per kg) in *A. angustifolia* leaves collected in February 1998 from native forest and reforested areas of the State Park of Campos do Jordão (mean of 5 replicates). Different letters show significant differences (Tukey test, $P<0.08$)

Site	N	P	K	Ca	Mg	S
Native forest	19.8 a	1.9 a	19.1 a	4.9 a	2.8 a	1.4 a
Reforested	13.3 b	1.3 b	18.4 a	3.2 a	2.2 a	1.0 a

arboreal species with a certain degree of exclusivity. However, in replanted *Araucaria*, small and round vesicles were found in almost all root segments, suggesting colonization by Glomaceae.

The roots of native forest plants consistently had higher colonization (RC) than the plants in the replanted area (Table 1). *Araucaria* root colonization ranged from 7.2 to 48.3% for native forest plants and from 4.4 to 15.8% for replanted plants. The average RC was 22% in February and 17% in September.

Several factors may contribute to these results, such as seasonal sporulation, seasonal variation in the development of host plants (Sutton and Baron 1972) and nutrient availability (Louis and Lim 1987). Fontenla et al. (1998) studied two *Austrocedrus* (coniferous) forests in Argentina and found no difference in the number of spores or percent root colonization between the two areas. However, there was an influence of the season, i.e., there was little RC in September, when the rainy season is at an end and the weather is hot. On the other hand, the number of spores was higher in September and December at the beginning of the dry season.

There were significant differences in N and P in *Araucaria* leaves (Table 3) between the two areas studied, with higher values for the native forest than for the replanted area. Nutrient concentration, pH and soil humidity level can influence fungal distribution, root colonization and mycorrhizal efficiency (Koide and Li 1990). It is possible that the higher RC found in the native forest was responsible for the higher N and P concentrations in the leaves. Very often, well-colonized plants with the correct AMF are more vigorous and have higher nutrient contents. Also, the nutrient content of the soil was somewhat higher in native forests than in replanted areas.

Another aspect that may be related to soil pH is the presence of AMF species, such as *Gigaspora margarita*, that are only found at pH <5.5 (Schenck and Siqueira 1987); in contrast, *Acaulospora scrobiculata*, *A. spinosa*, *Glomus aggregatum* and *Glomus etunicatum* were found in tropical soils with pH values ranging from 3.8 to 8.0 (Schenck and Siqueira 1987). All these species were also found in the present study at soil pH values of 3.4 to 4.1.

In both spore multiplication cycles, 11 species of AMF and a non-identified taxon were recovered, but some of the spores present in the *A. angustifolia* rhizosphere were not recovered in sorghum (Table 4). This indicates that not every propagule was adapted to the new substrate and/or to the new environmental conditions, or that more sporulation cycles are necessary. Of the 22 species found in the native forest, 12 AMF species were recovered from the pots, and 8 of 14 species found in the replanted area were recovered, i.e., 54 and 57% recovery, respectively. *Glomus macrocarpum*, *Glomus diaphanum* and *Entrophospora colombiana* were the most frequent AMF species found in the native forest in the first multiplication cycle. In the second cycle, the most frequent species were *Scutellospora pellucida* and *Acaulospora gerdemannii*. In addition, *Acaulospora foveata*, which was not found in field conditions, was recovered from the pots representing the native forest, and infrequent species, such as *Glomus diaphanum*, showed enhanced sporulation. *Gigaspora margarita*, a widely found species in field conditions, showed lower sporulation. Stimulated sporulation of *Glomus diaphanum* and inhibited sporulation of

Table 4 Number of spores (*NS*) of different AMF per 100 g of dried soil and their relative frequencies (*RF*) in multiplication pots with sorghum (*Sorghum bicolor* Moench. L.) as the host plant& inoculated with 50 g of rhizosphere soil from a native forest and a replanted area (mean of 5 replicates)

AMF species	Native forest				Reforested			
	1st cycle		2nd cycle		1st cycle		2nd cycle	
	NS	RF	NS	RF	NS	RF	NS	RF
Acaulospora foveata Janos & Trappe	8	15	–	–	–	–	–	–
A. gerdemannii Rose, Daniels & Trappe	–	–	212	65	–	–	28	30
A. laevis Gerd. & Trappe	24	20	–	–	4	10	–	–
A. scrobiculata Trappe	18	15	50	25	22	60	16	40
A. spinosa Walker & Trappe	6	15	–	–	–	–	–	–
Entrophospora colombiana Spain & Schenck	50	25	50	40	–	–	10	30
Gigaspora margarita Becker & Hall	–	–	6	10	–	–	14	30
G. diaphanum Morton & Walker	82	25	22	10	38	50	74	90
G. etunicatum Becker & Gerd.	20	90	–	–	–	–	76	60
G. macrocarpum Tul. & Tul.	98	60	18	20	78	100	94	100
Scutellospora pellucida (Nicol. & Schenck) Walker & Sanders	–	–	434	65	–	–	–	–
Not identified	–	–	42	15	–	–	–	–
Total number of spores	306	–	834	–	142	–	312	–
Richness index	8	–	8	–	4	–	7	–

Gigaspora margarita were also verified by Colozzi-Filho (1999) under greenhouse conditions.

Acaulospora rehmii, *Glomus decipiens*, *Glomus clarum*, *Glomus pansihalos* and *S. gilmorei* did not multiply in the pots representing the replanted area, while *Glomus macrocarpum*, *Glomus diaphanum* and *A. scrobiculata* sporulated most during the first cycle. In the second cycle, *Glomus macrocarpum*, *Glomus diaphanum* and *Glomus etunicatum* sporulated most. Strubble and Skipper (1988) found that sorghum was not a good host for sporulation of *Glomus macrocarpum*, *Glomus etunicatum* and *Glomus claroideum*, in contrast to the results of our study. The number of AMF spores and species was higher in the second cycle for both areas studied (Table 4).

These results suggest that the native forest offers better environmental conditions for development of the plants when all studied aspects are considered. Thus, the natural habitats of *Araucaria* must be preserved in order to lessen the risk of extinction for this species.

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