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Ectomycorrhizal fungal communities in *Nothofagus nervosa* (*Raulí*): A comparison between domesticated and naturally established specimens in a native forest of Patagonia, Argentina

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ABSTRACT

Due to its overexploitation during the past century, *Nothofagus nervosa* is currently included in conservation and domestication programs, in which ectomycorrhizas play an important role. We aimed to describe the abundance and diversity of ectomycorrhizal fungi (EcMF) in both domesticated and naturally established *N. nervosa* specimens, and to analyse the influence of age, seasonality and forest management on EcMF communities. The occurrence of arbuscular mycorrhizas (AM) and dark septate endophytes (DSE) was also investigated. Fungal diversity and taxonomic identification were assessed by morphotyping and subsequent ITS-rDNA sequencing. Plant age, seasonality and forest management influenced EcMF communities. Colonization rates were higher than 90 % in all the specimens, and were significantly higher in mature trees and in autumn. The highest EcMF richness and diversity values were registered in domesticated specimens and in autumn. Most EcMF were basidiomycetes, belonging mainly to the Cortinariaceae and Tricholomataceae. Arbuscular mycorrhizas were not detected, while DSE were present within *N. nervosa* roots. Our results and previously published reports showed that some EcMF are capable of colonizing different *Nothofagus* species. In addition, the EcMF described in natural ecosystems are different from those colonizing *N. nervosa* during its cultivation in the nursery. These results improve our understanding of key factors affecting EcMF communities associated with *Nothofagus* in native forests and nurseries (age, season, forest management, cultivation techniques), and this information is relevant for improving domestication programs.

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1. Introduction

Some of the most important fungal groups in soil, in terms of biomass and the ecosystem processes they perform, are those which form mutualistic associations with plants, the mycorrhizas. These associations usually benefit host plants by enhancing water and nutrient uptake (especially with regard to phosphorous and nitrogen) and by increasing host resistance to pathogens and other biotic and abiotic stresses. Consequently, mycorrhizas have a direct bearing on plant community diversity, structure and productivity (Van der Heijden et al., 1998; Smith and Read, 2008). In addition, the establishment, growth and survival of different forestry species that occupy large areas worldwide are usually highly dependent on ectomycorrhizas (EcM) (Smith and Read, 2008). Most of these tree species are economically important, including those belonging to the Nothofagaceae (Wang and Qiu, 2006; Smith and Read, 2008). *Nothofagus* species constitute the main component of South American temperate forests. Soils in these forests are mostly derived from volcanic ash (Andisols). They are characterized by elevated P content and a high capacity to stabilize organic matter, store water and buffer pH. The main limitation of this type of soil is high P retention (Mazzarino and Gobbi, 2005). The fact that *Nothofagus* roots are extensively colonized by EcM (Tedersoo et al., 2009; Nouhra et al., 2013) with rates usually higher than 70% (Diehl et al., 2008; Longo et al., 2011; Fernández et al., 2013), and that these tree species are not proficient at P uptake, suggest a strong dependence on this symbiosis, and that EcM constitute an effective adaptation mechanism to soils with low P availability (Mazzarino and Gobbi, 2005; Diehl et al., 2008).

Plants belonging to the same genus usually have the same type of mycorrhiza, but some species can harbour more than one type (Wang and Qiu, 2006; Smith and Read, 2008). This phenomenon was observed among important forestry species of the genera *Eucalyptus* (Adjoud-Sadadou and Halli-Hargas, 2000), *Quercus* (Dickie et al., 2001), *Pinus*, *Tsuga* and *Pseudotsuga* (Wang and Qiu, 2006), in which both EcM and arbuscular mycorrhizas (AM) were described. Arbuscular mycorrhizas have been registered only in one unique *Nothofagus dombeyi* tree (Bidartondo et al., 2002), which raises the question of whether both types of mycorrhiza (EcM + AM) can be found in *Nothofagus* specimens growing in natural ecosystems.

Dark septate endophytes (DSE) are conidial or sterile fungi that colonize living plant roots without causing any apparent negative effects. They have been reported worldwide in diverse plant species and habitats (Jumpponen, 2001; Newsham, 2011), including different Patagonian environments (Fernández et al., 2008, 2010, 2012; Bruzone, 2008). Dark septate endophytes were also detected in nursery cultivated seedlings of different *Nothofagus* species (Salgado-Salomón et al., 2013). These fungi could provide important benefits to the host plant, such as protection against pathogens or abiotic stresses (water availability, extreme temperatures, frosts, etc.), and could, therefore, act as pioneering colonizers of these plants in secondary successional environments (Salgado-Salomón et al., 2013). Nevertheless, the occurrence of DSE within *Nothofagus* roots in natural environments has not been well documented.

Nothofagus nervosa is one of the most ecologically and economically important species of South American temperate forests. Due to its extremely valuable wood, suitable for regional and international commercialization, this species was overexploited in the past and natural populations were drastically reduced. This situation led to the implementation of conservation and domestication programs, to optimize seedling cultivation techniques, in order to improve forestry production as well as afforestation and restoration activities (Marchelli and Gallo, 1999; Gallo et al., 2004). Since inoculation with mycorrhizal fungi during nursery cultivation increases plant growth and improves subsequent performance under natural conditions when they are outplanted to the field (Pera et al., 1999; Quoreshi, 2003; Rincón et al., 2005, 2007; Oliveira et al., 2010), mycorrhizal colonization of root systems (abundance, diversity, occurrence of specific EcMF) is currently considered an important factor for determining seedling vigour and quality (Quoreshi, 2003; Pera and Parladé, 2005; Missanjo and Thole, 2014). Therefore, the mycorrhizas associated with *N. nervosa* are one of the factors that should be considered during domestication programs. However, there is little information concerning the composition or community dynamics of the ectomycorrhizal fungi (EcMF) colonizing *Nothofagus* roots in South American natural environments (Nouhra et al., 2013), and the influence of age, seasonality and/or domestication practices on mycorrhizal fungal communities is not known. The main objective of this study was to compare the abundance and diversity of EcM associated with domesticated *N. nervosa* specimens outplanted in a native forest with naturally established trees, and to analyse the influence of various factors (age, seasonality, domestication practices) on this symbiosis. The occurrence of AM and DSE within *N. nervosa* roots was also analysed.

2. Materials and methods

2.1. Study site and sampling

The study was conducted in a native forest within the Yuco region (40° 07′ 48″ S–71° 34′ 48″ W) of Patagonia, Argentina. This area holds the largest *N. nervosa* forests in Argentina, both in terms of continuity and density as well as the size and vigour of the trees (Sabatier et al., 2011), and is registered in the INASE (National Seed Institute) as *N. nervosa* seed producing area (http://www.inase.gov.ar). A provenance trial, established in 1999 by the Unidad de Genética Ecológica y Mejoramiento Forestal, INTA EEA Bariloche, is being carried out in this forest. *N. nervosa* seedlings were cultivated in the forest nursery following the procedures commonly carried out for domestication programs, which consisted of producing containerized seedlings in a porous substratum consisting of peat bog and volcanic sand (1:1 v/v) using a fertigation technique (Fernández et al., 2013). Plants were not artificially inoculated with EcM fungi, but according to previous studies seedlings cultivated under these nursery conditions are colonized by naturally established EcM after 1 yr (Fernández

et al., 2013). Consequently, it is highly possible that seedlings used in this study had EcM when they were outplanted to the native forest when they were 2 yr old. The provenance trial plot (40 × 70 m) was surrounded by a fence to keep animals out and was maintained by basic management practices (e.g. part of the forest understory was removed before planting the seedlings, branches and fallen trees were removed).

For the analysis of EcM fungal communities in *N. nervosa* roots, four categories were considered, depending on plant age and origin: young domesticated plants cultivated in the nursery and included in the provenance trial (Yd) and seedlings (Sn), young individuals (Yn) and adults (An) established naturally in the forest. Shoot length and diameter of the seedlings (Sn) were less than 50 cm and 3 cm, respectively. The height of the young specimens (Yn and Yd) ranged between 2 and 4 m, and diameter at breast height (DBH) between 5 and 12 cm. Adult trees (Ad) were over 25 m in height and DBH exceeded 70 cm. Two samplings were carried out in 2008, one in autumn and the other in spring. Trees corresponding to the Yd category, which spent 9 yr in the native forest, were sampled from the trial plot described above, and naturally established specimens from the native forest surrounding the plot. Five individuals per plant category (Yd, Sn, Yn, Ad) and season were randomly selected from the trial plot and the native forest. Specimens grew at least 25 m distant from each other. Before collecting root samples, the surface layer (leaf litter) was removed. In the case of seedlings (Sn), the complete root system was removed, while the other plants (Yd, Yn, An) were sampled by taking three cores (7 cm diameter × 15 cm depth) per tree from the topsoil layer. Samples were wrapped in plastic bags and stored at 4 °C until used for further procedures. Roots were sieved from soil cores and carefully cleansed under a stereoscopic microscope (Olympus SZ30). A fraction of the root system was fixed in 70% v/v ethylic alcohol for AM and DSE description and EcM were examined in fresh roots.

2.2. Ectomycorrhiza (EcM) morphotyping and quantification

Ectomycorrhizal root tips (= ectomorphotypes) were separated from the roots and classified into morphological groups according to the following characteristics: ramification and shape, mantle texture and colour, and the presence and colour of emanating hyphae and/or rhizomorphs. The description of each morphological group was complemented with microscopic features (Olympus BX40), such as colour of the hyphae forming the mantle, mantle pattern (plectenchymatus or pseudoparenchymatous), and presence of cystidia and clamp connections (Agerer 1987-2006, Agerer 2001). For the molecular analyses, individual ectomorphotypes corresponding to each morphological group were kept in 1.5 ml plastic tubes and dried at 25 °C for 24 hr.

Total percentage of EcM and the relative frequency of each ectomorphotype were quantified according to Grand and Harvey (1982). Colonization percentages were calculated by dividing the total number of ectomycorrhizal tips by the total number of tips quantified. Relative frequency of the different ectomorphotypes was obtained by dividing the number of tips belonging to each ectomorphotype by the total number of ectomycorrhizal tips.

2.3. EcM molecular identification

The ITS region was amplified by nested PCR using the universal primers NSI1 (5′-GATTGAATGGCTTAGTGAGG-3′) and NLB4 (5′-GGATTCTCACCCTCTATGAC-3′) for the first PCR, and ITS1F (5′-CTTGGTCATTTAGAGGAACTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Sigma) for the second reaction (Kendall and Rygiewicz, 2005). PCR were performed in 20 μl (Thermocycler MultiGene, Labnet) containing: ultra-pure water, 2× PCR buffer, 0.5 μM of each primer and 0.5 U μl^{-1} Phire polymerase (Phire Plant Direct PCR Kit, Thermo Scientific, Germany). In the first PCR (NSI1/NLB4) a dot sized piece of an ectomycorrhizal root tip was used as template DNA. The PCR program used in this case consisted of: initial denaturation at 98 °C for 5 min followed by 28 cycles of denaturation at 98 °C for 5 s, annealing at 54 °C for 5 s and extension at 72 °C for 20 s, and a final extension at 72 °C for 1 min. For the second reaction (ITS1F/ITS4), PCR products from the first were diluted to a final concentration of ~2 ng μl^{-1} DNA and used as template DNA, and PCR were carried out according to the following conditions: initial denaturation at 98 °C for 5 min followed by 30 cycles of denaturation at 98 °C for 5 s, annealing at 52 °C for 5 s and extension at 72 °C for 20 s, and a final extension at 72 °C for 1 min. After purification of final PCR products (QIAquick® PCR Purification Kit, Qiagen), they were sequenced by LGC Genomics (Berlin, Germany) using ABI 3730 XL platforms for traditional Sanger sequencing. The sequences obtained were compared with those available in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) using the BLASTn algorithm. Sequences were assigned to species based on ≥97% ITS sequence similarity threshold (Nouhra et al., 2013). The PubMed database (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) was used for nomenclature and classification of the species. Nucleotide sequences obtained in this work were deposited in the NCBI GenBank database and are available for comparative purposes under the accession numbers KJ701290–KJ701324.

2.4. EcM community analyses

Different community parameters (richness, diversity, similarity) were calculated and compared between plant categories (Yd, Sn, Yn, An) and seasons (autumn, spring). Ectomycorrhizal fungal richness was calculated as the number of EcMF associated with *N. nervosa* roots. The Simpson index (*D*) was used for analysing EcMF diversity, and the modified Jaccard index (J_{mod}) for comparing similarity among EcMF communities (Chao et al., 2005). For calculating these community parameters information corresponding to individual trees was used. Diversity and similarity indices were calculated with EstimateS 8.2 (http://viceroy.eeb.uconn.edu/EstimateS).

2.5. EcM statistical analyses

Colonization percentages and EcMF richness were compared between plant categories (Yd, Sn, Yn, An) and seasons

(autumn, spring) using two-way-ANOVA followed by Holm-Sidak *post hoc* tests. Two Chi-square tests were performed to determine dependence between different pairs of variables: (a) EcMF and plant categories (Yd, Sn, Yn, An) and (b) EcMF and seasons (autumn, spring). To analyse the relationship between these variables (plant category, season, EcMF) a Multiple Correspondence Analysis (MCA) was conducted. In addition, the heatmap function in R (V3.1.2) was used for evaluating similarities between EcMF communities corresponding to different plant categories in both seasons.

2.6. *Arbuscular mycorrhizas (AM) and dark septate endophytes (DSE)*

The occurrence of AM and DSE was analysed in roots of every *N. nervosa* specimen. Roots were stained using a modified Phillips & Hayman method (Phillips and Hayman, 1970; Fernández et al., 2012). Once stained, ten root pieces of approximately 1 cm length were randomly selected from each sample and mounted on a microscope slide. For each specimen, three slides were made and examined with a light microscope (Olympus BX40). On each slide no less than 100 fields were observed. The criterion used in this study for the determination of AM was the presence of at least one arbuscule in one individual. The occurrence of DSE within *N. nervosa* roots was determined by the presence of usually dark and regularly septate hyphae, which sometimes stained blue, and intracellular microsclerotia (intracellular groups of rounded, closely packed, thick-walled and usually darkly pigmented fungal cells).

3. Results

All *N. nervosa* individuals analysed had EcM colonization values higher than 90% (Fig. 1). Significant differences in mean colonization values were detected for plant categories ($p < 0.001$; $f = 13.857$) and seasons ($p = 0.003$; $f = 10.668$). The interaction between these two factors was not significant ($p = 0.528$; $f = 0.755$). For plant categories, mean colonization values were significantly higher in adults ($p < 0.001$) and young specimens ($p_{Yn} < 0.001$; $p_{Yd} = 0.009$) than in seedlings, and were also higher in adults than in young domesticated trees ($p = 0.002$). Colonization percentages were significantly higher ($p = 0.003$) in autumn ($X_A = 97.3 \pm 0.9$) than in spring ($X_S = 96.1 \pm 1$). Significant differences in ectomycorrhizal colonization levels were also registered when plant categories were compared within each season, with seedlings showing the lowest values and adults the highest (Fig. 1).

Different ectomorphotypes were registered in each of the specimens examined, and 25 EcMF species were identified (Table 1), most of which belong to the Basidiomycota (88%). Only the Cortinariaceae and the Tricholomataceae were represented by more than two species (seven and three, respectively). Some of the most abundant species belonged to these two families, such as *Cortinarius* sp. 1 and Tricholomataceae sp. 1 in autumn, *Cortinarius* sp. 6 in spring, and *Cortinarius* sp. 2, which was the most abundant species in both seasons (Table 1, Fig. 2).

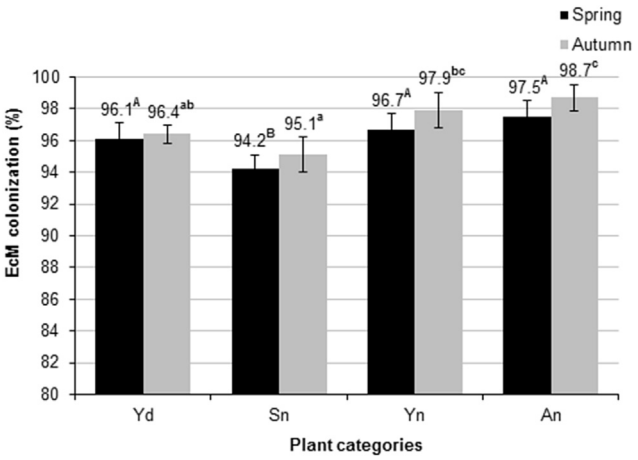

Fig. 1 – Ectomycorrhizal colonization percentages in *Nothofagus nervosa* specimens corresponding to different plant categories in spring and autumn. Sn = native seedlings, Yn = young native individuals, An = native adults, Yd = young domesticated specimens outplanted in the native forest. Significant differences in colonization values registered in spring (uppercase) and autumn (lowercase) are indicated with different letters. Error bars represent ± standard deviation.

Occurrence and abundance of each EcMF varied between individuals, plant categories and seasons (Table 1, Fig. 2). Some EcMF were found in all plant categories and in both seasons (e.g. *Cenococcum geophylum*, *Cortinarius* sp. 2, *Genea* sp. 1, *Inocybe* sp. 1), while others were observed in few individuals and/or only in one season (e.g. *Clavulina* sp. 1 and Tricholomataceae sp. 2 were registered only in autumn) (Fig. 2). Chi-square tests indicated that EcMF distribution was significantly related to plant categories ($Chi = 3499.419$, $df = 66$, $p < 0.001$) and seasons ($Chi = 1629.092$, $df = 26$, $p < 0.001$), and four groups were defined after the Multiple Correspondence Analysis (MCA) (Table 1, Fig. 3). Group 1 included the young domesticated specimens (Yd) and those EcMF mostly associated with this plant category (*Cortinarius* sp. 1, Tricholomataceae sp. 2 and Sebacinaceae sp. 1). Group 2 was characterized by the seedlings (Sn) and the EcMF which were not present or very scarce in these plants (Clavulinaceae sp. 2, *Cortinarius* sp. 1, *Genea* sp. 1, Tricholomataceae sp. 1). Groups 3 and 4 comprised the EcMF that were only, or mainly, registered in autumn (Clavulinaceae sp. 1, *Cortinarius* sp. 7, *Descolea* sp. 2) and spring (*Laccaria* sp. 1, Thelephoraceae sp. 2), respectively (Fig. 3). Plant categories corresponding to adults (An) and young native specimens (Yn) were not well represented on these axes.

Taking into account the total number of EcMF (richness) registered in each *N. nervosa* specimen (Table 2), values corresponding to young domesticated specimens (Yd) were significantly higher than those registered in adults ($p_{An} = 0.008$) and young individuals established naturally in the forest ($p_{Yn} = 0.041$). Ectomycorrhizal richness was significantly lower in the seedlings than in the other plant categories ($p_{An} = 0.034$, $p_{Yn} = 0.008$, $p_{Yd} = 0.001$). This tendency was also observed for the total number of EcMF registered per plant category, with young domesticated specimens and seedlings

Table 1 – Ectomycorrhizal fungi recorded in roots of naturally established and domesticated *Nothofagus nervosa* specimens of different ages in autumn and spring, based on GenBank blast searches. EcMF = fungi forming ectomycorrhizas in *N. nervosa* roots, Sn = native seedlings, Yn = young native individuals, An = native adults, Yd = young domesticated specimens outplanted to the native forest, A = autumn, S = spring, * = EcMF previously described in *Nothofagus* forests of Patagonia [7].

EcMF	Best NCBI BLASTn match			Plant category and season							
				Sn		Yn		An		Yd	
	Identity	Accession number	Identity (%)	A	S	A	S	A	S	A	S
Cenococcum geophylum	*Cenococcum geophylum*	AY394919.1	99	2	4	4	5	4	3	5	3
Clavulina sp. 1	/clavulina	JX316347.1*	100	0	0	2	0	0	0	2	0
Clavulinaceae sp. 2	Clavulinaceae sp. 1	JX316386.1*	98	0	0	2	5	0	4	2	1
Cortinarius sp. 1	*Cortinarius viscoviridis*	JQ282167.1	96	0	0	3	2	3	1	5	2
Cortinarius sp. 2	/cortinarius	JX316427.1*	100	1	4	3	4	4	2	4	4
Cortinarius sp. 3	*Cortinarius picoides*	GU233371.1	96	0	1	1	1	1	0	1	0
Cortinarius sp. 4	*Cortinarius stephanopus*	AY669603.1	96	0	0	2	0	0	1	3	1
Cortinarius sp. 5	*Cortinarius balteatocumatilis*	AY174801.1	96	0	1	0	2	2	1	2	3
Cortinarius sp. 6	*Cortinarius* sp. 4	JX316382.1*	99	0	2	0	1	1	3	2	3
Cortinarius sp. 7	/cortinarius	JX316440.1*	99	0	0	0	0	1	0	1	0
Descolea sp. 1	/descolea	JX316303.1*	99	3	2	4	0	3	1	1	1
Descolea sp. 2	*Descolea* sp. 1	JX316348.1*	99	1	0	0	0	1	0	0	0
Genea sp. 1	*Genea* sp. 1	JX316365.1*	99	1	2	3	2	4	2	5	4
Hebeloma sp. 1	*Hebeloma* sp. 2	JX316416.1*	99	0	1	1	2	1	1	2	0
Inocybe sp. 1	*Inocybe* sp. 13	JX316403.1*	99	3	1	3	2	5	4	4	5
Inocybe sp. 2	*Inocybe* sp. 5	JX316250.1*	99	1	0	2	2	0	2	2	2
Laccaria sp. 1	*Laccaria* sp. 1	JX316292.1*	99	0	1	0	5	1	2	2	5
Russulaceae sp. 1	Russulaceae sp. 1	JX316375.1*	99	0	0	2	0	1	1	1	0
Sebacinaceae sp. 1	*Sebacina incrustans*	AF490395.1	91	0	1	1	1	0	0	3	2
Pyrenomataceae sp. 1	Pyrenomataceae sp. 7	JX316346.1*	100	0	1	2	0	3	0	3	4
Thelephoraceae sp. 1	*Pseudotomentella larsenii*	AF326981.1	93	3	0	1	0	1	0	0	1
Thelephoraceae sp. 2	/tomentella-thelephora	JX316237.1*	100	1	3	3	4	4	5	4	5
Tricholomataceae sp. 1	Tricholomataceae sp. 1	JX316261.1*	99	0	0	2	1	2	2	3	1
Tricholomataceae sp. 2	*Tricholoma pessundatum*	FJ845446.1	93	0	0	0	0	0	0	4	0
Tricholomataceae sp. 3	Tricholomataceae sp. 3	JX316322.1*	99	0	0	3	0	1	0	1	2
Total N° EcMF per plant category and season				**9**	**13**	**19**	**15**	**19**	**16**	**23**	**18**

having the highest and lowest values for both seasons, respectively (Table 1). Ectomycorrhizal fungal richness tended to be higher in autumn for all plant categories, except in the seedlings, in which it was higher in spring (Table 1). With respect to EcMF diversity, it was observed for both seasons that young domesticated trees (Yd) had the highest Simpson index (*D*) values, while seedlings (Sn) had the lowest (Table 2). When similarity of EcMF communities was compared between age categories using the modified Jaccard index (J_{mod}), the lowest values for both seasons were associated with the seedlings (Table 3).

According to the clustering analysis performed in R (heatmap), EcMF communities associated with seedlings in autumn and spring (Sn.A and Sn.S) were similar to one another, and distinct from those corresponding to the other plant categories (Yn, An, Yd) (Fig. 4A). This analysis also showed that seasonality was an important factor influencing EcMF communities, since young (Yn and Yd) and adult (An) *N. nervosa* were clustered by season instead of by plant category (Fig. 4B and C). It is also remarkable that within each season (autumn or spring), EcMF communities in native *N. nervosa* plants (Yn and An) were more similar to each other than to those associated with young domesticated specimens (D). Fungal species were clustered in two main groups, corresponding to the most abundant (Fig. 4E) and the least represented EcMF species (Fig. 4F).

Arbuscular mycorrhizas were not detected in any of the specimens analysed in this study. On the other hand, DSE were observed within *N. nervosa* roots (Fig. 5). These fungi were present in 39% of the specimens analysed, and no differences between seasons or plant categories were observed. They were characterized by frequently melanised and regularly septate intracellular hyphae, which occasionally became stained with Trypan blue. Microsclerotia, which sometimes occupied the entire volume of the cell, were also observed. A distinguishing feature of the DSE present in these plants was the occurrence of "cerebriform microsclerotia" (Fig. 5A and B). The root stele was never colonized by these fungi.

4. Discussion

4.1. *Occurrence and abundance of mycorrhizas*

All the *N. nervosa* specimens were more than 90% colonized by EcMF (Fig. 1), which is in agreement with reports from different *Nothofagus pumilio* forests (Longo et al., 2011). Diehl et al. (2008) reported that mean colonization rates in five *Nothofagus*

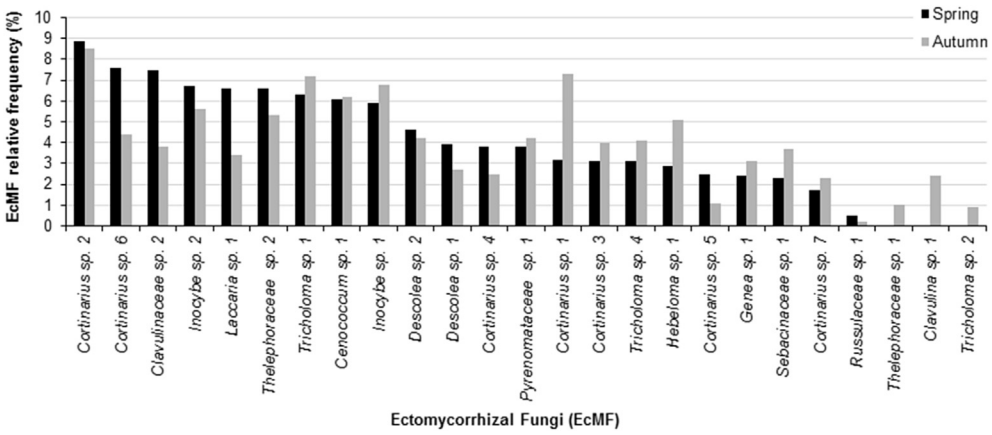

Fig. 2 – Relative frequency of the different ectomycorrhizal fungal species (EcMF) recorded in *Nothofagus nervosa* roots in spring and autumn.

species studied in summer varied between 75% and 79%, 74% being the value corresponding to *N. nervosa*. This difference is probably related to the different sampling seasons. Colonization percentages above 90% were also observed in other forestry species, such as *Picea sitchensis* (Palfner et al., 2005), *Quercus rubra* (Gebhardt et al., 2007), *Pseudotsuga menziesii* and *Betula papyrifera* (Twieg et al., 2007).

Nothofagus species growing on the Andisols of Patagonia, like most of the tree species in this region, are not proficient at P uptake, mycorrhizas being considered one of the mechanisms that enhance P absorption in these plants (Mazzarino and Gobbi, 2005; Diehl et al., 2008). The high EcM percentages found in this study support this statement, and highlight not only the importance of this symbiosis in nutrient dynamics and forest ecology, but also the need to take it into account during *Nothofagus* domestication programs.

Despite the fact that all colonization rates were high (>90%), the highest values corresponded to adults and the lowest to seedlings (Fig. 1). Similar tendencies have been described for other tree species. For example, Gebhardt et al. (2007) determined that EcM colonization in young (5-yr-old) *Q. rubra* specimens was significantly lower than in adults (21- to 46-yr-old), and Palfner et al. (2005) found that *Picea sitchensis* seedlings had lower colonization rates than older trees. This variation in EcM abundance could be related to the time of exposure of the plant to the mycorrhizal inoculum, so that longer exposure to the inoculum results in higher colonization rates. Concerning seasonality, it was determined that colonization values were higher in autumn than in spring. The same tendency was registered for *N. pumilio* in other Patagonian forests (Longo et al., 2011).

The lack of AM in *N. nervosa* specimens is in agreement with other studies (Fontenla et al., 1998; Fernández et al., 2013), and also with the general trend followed by most of the tree species in North America and Europe, which usually have only one type of mycorrhiza (Wang and Qiu, 2006; Smith and

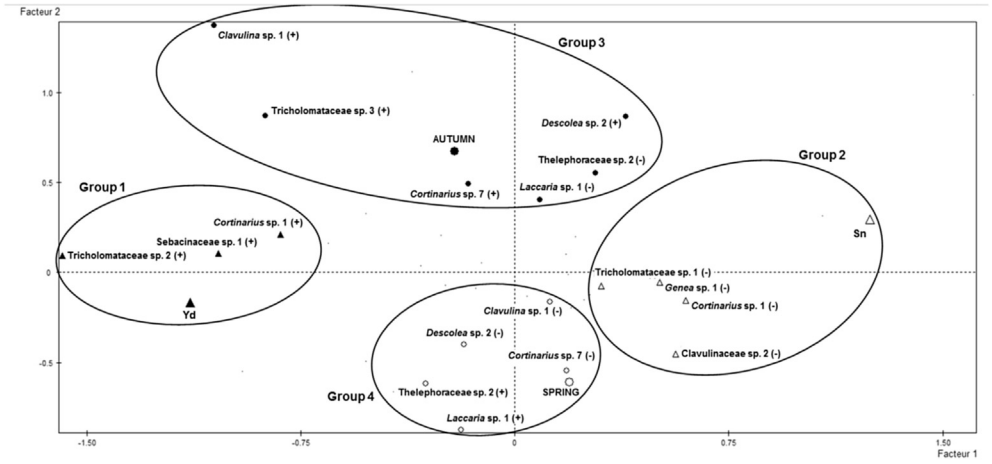

Fig. 3 – Multiple Correspondence Analyses indicating the ectomycorrhizal fungal species (EcMF) associated with plant categories and seasons. Yd = young domesticated specimens outplanted in the native forest, Sn = native seedlings (only variables that were well represented are shown), (+) = presence or high occurrence, (−) = absence or low occurrence. Bigger symbols represent plant categories (filled triangles = Yd, open triangles = Sn) and seasons (filled circles = Autumn, open circles = Spring) and smaller symbols represent the EcMF associated with each of them.

Table 2 – Mean number of ectomycorrhizal fungal species (EcMF richness) and Simpson diversity indices in *Nothofagus nervosa* per plant category and season. Sn = native seedlings, Yn = young native individuals, An = native adults, Yd = young domesticated specimens outplanted to the native forest, (a–b) = the lowest and the highest number of EcMF recorded for the specimens analysed per plant category and season, [a] = Different superscript letters indicate statically significant differences between richness values registered per season.

Plant category	EcMF richness		Simpson diversity index		
	Spring	Autumn	Spring	Autumn	Total
Sn	5 (3–6)[a]	6 (5–9)[a]	10.36	12.28	14.34
Yn	8 (6–11)[b]	9 (8–11)[b]	14.04	16.52	17.23
An	7 (3–11)[b]	8 (3–11)[b]	16.16	17.98	18.53
Yd	11 (8–13)[c]	13 (11–15)[c]	17.42	18.69	19.09

Read, 2008). On the other hand, the occurrence of DSE in *N. nervosa* roots demonstrates that these fungi are capable of colonizing *Nothofagus* roots in natural ecosystems. The presence of "cerebriform microsclerotia" is noteworthy, since as far as we are aware they had previously been observed only in pteridophytes growing in a Valdivian temperate rainforest (Fernández et al., 2008, 2010, 2012) and in high-Andean plant species (Bruzone, 2008) from Patagonia, Argentina. It has been suggested that DSE may function as "mycorrhizal fungi" (Jumpponen, 2001; Newsham, 2011), but further studies are necessary to determine the importance of these fungi for *Nothofagus* establishment and development, and even for production or restoration activities (Salgado-Salomón et al., 2013).

4.2. *Ectomycorrhizal fungal diversity and community dynamics*

In all the specimens analysed, more than one ectomorphotype was observed. A total of 25 fungal species were identified, most (88%) belonging to the Basidiomycota. This result is in agreement with previous studies in *Nothofagus* (Tedersoo et al., 2009; Nouhra et al., 2013) and other important forestry species. The Cortinariaceae and Tricholomataceae were the only families represented by more than two species (Table 1).

Table 3 – Modified Jaccard similarity indices corresponding to the different *Nothofagus nervosa* plant categories in spring and autumn (in bold). Sn = native seedlings, Yn = young native individuals, An = native adults, Yd = young domesticated specimens outplanted to the native forest.

Season	Spring			Autumn		
Plant categories	Yn	An	Yd	Yn	An	Yd
Sn	0.56	0.39	0.71	**0.40**	**0.45**	**0.43**
Yn		0.78	0.74		**0.8**	**0.93**
An			0.82			**0.77**

These taxa were described as abundant in *Nothofagus* roots (Tedersoo et al., 2009; Nouhra et al., 2013). Our results were in accordance with Nouhra et al. (2013), who stated that there is a high level of similarity in the EcMF communities associated with different *Nothofagus* species. However, most of the sequences could not confidently be matched to reliably named specimens in GenBank. This is related to the low number of fungal sequences corresponding to the ITS region existing for South America compared to other parts of the world (North America, Eastern Europe, China, Japan) (Ryberg et al., 2009). The *Suillus/Rhizopogon*, *Boletus* and *Pisolithus/Scleroderma* lineages were not detected in any of the *N. nervosa* specimens analysed. According to Tedersoo et al. (2012) and Nouhra et al. (2013) this is a unique global pattern related to the fact that the distribution of many EcM fungal lineages is restricted to the northern and/or southern temperate ecosystems. Altogether, this information suggests the co-evolution of ectomycorrhizal symbionts and their hosts.

The number of fungal species forming EcM in *N. nervosa* was significantly higher in the native forest than previously registered in nursery-cultivated seedlings, where only six EcMF were found. Species composition was also completely different in these two sites: no fungal species were detected in common and species belonging to the Cortinariaceae or Tricholomataceae were not identified in the nursery-cultivated plants (Fernández et al., 2013). Similar results were reported for natural and nursery cultivated plants of *Pinus radiata* (Walbert et al., 2010). Such a big discrepancy in EcMF diversity between the native forest and the nursery could be explained by the marked differences in environmental conditions and fungal inoculum. In the nursery, plants are cultivated in pots, under artificial conditions, and are changed every year. These practices make the nursery a selective environment, which is expected to limit ectomycorrhizal diversity. In contrast, in native forests plants can grow for a long time in the same environment and in contact with different fungal species, thus favouring high ectomycorrhizal diversity. Mycorrhizal fungi adapted to greenhouse conditions often remain in the root system during a time after transplantation, but they are gradually replaced by native mycorrhizal species, specific to the place where seedlings were established and better adapted to natural environmental conditions (Dahlberg and Stenstrom, 1991; Quoreshi, 2003). For example, *T. terrestris* is very common in nursery systems, but uncompetitive in natural ecosystems, so it is usually easily displaced by other EcMF (Colpaert, 1999; Menkis and Vasaitis, 2011). Consequently, it is expected that ectomycorrhizal fungal communities associated with young domesticated specimens that spent 9 yr growing in the native forest are completely different from those colonizing their roots in the forest nursery.

Our results showed a high variability in EcMF occurrence, some species being abundant or present in most of the specimens in both seasons, while others were rare and/or exclusive to one season (Figs. 2 and 3). This is a common pattern that has been described in several ecosystems and taxonomic groups worldwide (Jonsson et al., 1999; Horton and Bruns, 2001; Izzo et al., 2005; Gebhardt et al., 2007; Courty et al., 2008; Wang et al., 2012). In addition, all the EcMF registered in the seedlings were also found in the other plant categories (Table 1), as observed in *Quercus liaotungensis* (Wang et al.,

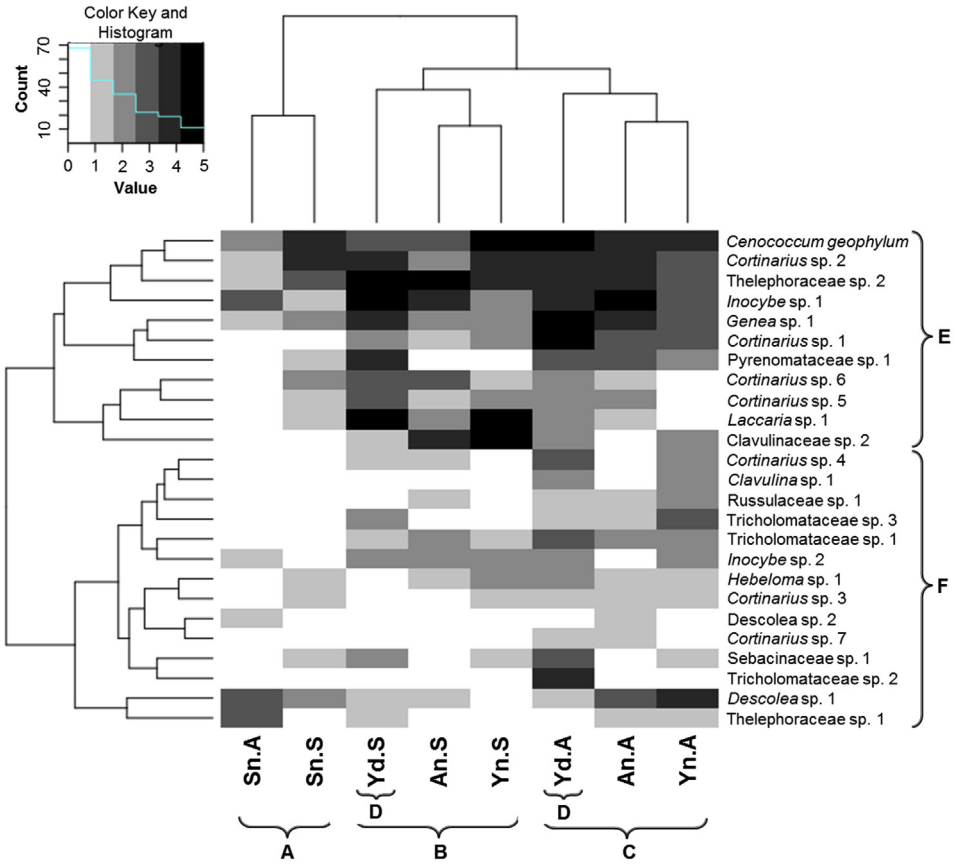

Fig. 4 – Heatmap displaying the composition of ectomycorrhizal fungal communities associated with *Nothofagus nervosa* across plant categories in both seasons. Plant categories and corresponding EcMF communities were first split into seedlings (A) and older plants (B and C). Young and adult plants were then grouped by season (B: spring, C: autumn), and within each season young domesticated specimens (D) were separated from naturally established plants (Yn and An). Fungal species were clustered into two main groups, one of them including the most abundant species (E) and the other the less represented and rare fungal species (F).

2012), *Pinus sylvestris* (Jonsson et al., 1999) and *P. menziesii* (Bingham and Simard, 2011). These findings support the fact that the EcMF colonizing naturally established seedlings are largely the same species which are present in the surrounding tree roots, which constitute a fungal inoculum source. These mycelial networks are important not only for interconnections between ectomycorrhizal host trees and for the continuity or perpetuation of EcMF communities (Wang et al., 2012), but also for seedlings' survival and fitness (Simard et al.,. 1997; Bingham and Simard, 2011). Due to these hyphal connections seedlings form mycorrhizas quickly and capture resources early. Besides, hyphal links to larger and previously established trees might benefit neighbouring seedlings through direct transfer of organic nutrients and increased access to inorganic nutrients or water. Consequently, seedling fitness is usually increased as they are more likely to survive (Simard et al., 1997; Bingham and Simard, 2011).

At community level, EcMF richness and diversity were higher in adults and young specimens than in seedlings (Tables 2 and 3), in agreement with findings in other tree species, such as *Picea sitchensis* (Palfner et al., 2005), *Q. rubra* (Gebhardt et al., 2007), *P. menziesii* (Twieg et al., 2007) and *Pinus kesiya* (Rao et al., 1997). A plausible explanation for this pattern is that as the canopy develops, tree growth rates are rapid and leaf area increases significantly with correspondingly high potential for carbon allocation to both roots and mycobionts. In addition, host roots are also more abundant and evenly distributed so they are able to be in contact with a higher diversity of EcMF (Twieg et al., 2007). Linking all these results together, plant age seems to be an important factor in determining mycorrhizal diversity (Gebhardt et al., 2007; Courty et al., 2008).

It is interesting to note that when EcMF communities were compared between domesticated and naturally established plants, the highest EcMF richness and diversity values were registered in young domesticated specimens (Tables 1 and 2). This could be related to management practices (e.g. it is surrounded by a fence that keeps animals out, part of the understory was removed before plantation, fallen branches and trees were removed), which could modify microenvironmental conditions, thus influencing fungal communities forming EcM. These types of changes in the structure

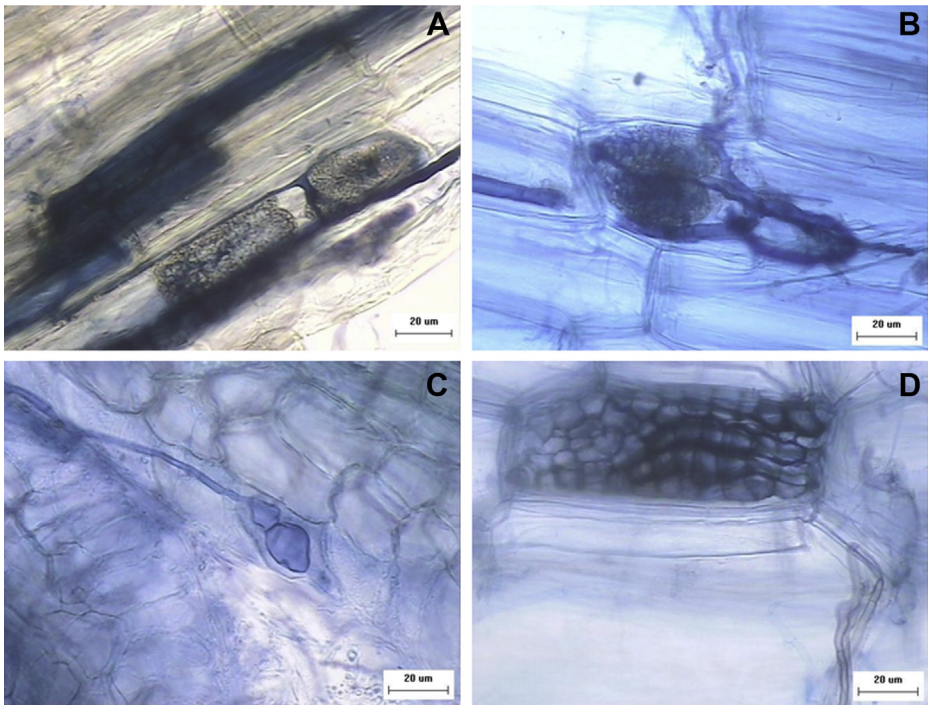

Fig. 5 – Colonization structures corresponding to dark septate fungi (DSE) recorded in *Nothofagus nervosa* roots. (A,B) Cerebriform microsclerotia associated with regularly septate hyphae, (C) DSE hyphae starting to form a microsclerotium. (D) Microsclerotia formed by rounded, closely packed, fungal cells that occupy the entire volume of the root cell.

and/or composition of ectomycorrhizal fungal communities have been also described in other forests subjected to different management or exploitation practices. For instance, Jones et al. (2003) established that the major impact of clearcut logging is a change in the species composition of the ectomycorrhizal fungal community, rather than a reduction in the percentage of roots colonized. They also suggested that these shifts in fungal species composition are driven by changes in the biology and chemistry of the soil environment as much as they are by loss or change in fungal inoculum. In addition, Durall et al. (1999) analysed the richness of EcMF (sporocarps and belowground EcM) in forests where 30% and 60% of the stand volume was removed by cutting, and observed that the number of fungal species forming sporocarps and EcM in roots of *Pinus contorta* and *Tsuga heterophylla* seedlings decreased as gap size increased. Timber harvest and site preparation are the most widespread forestry activities that alter both the aboveground and belowground environments. For example, changes in aboveground plant community composition alter quality and quantity of root exudates and litter leachates, and decaying wood modifies moisture content in soil, thus potentially impacting EcMF communities. However, the degree of ectomycorrhizal shifts varies widely depending on the environment and on the type and intensity of the disturbance (Amaranthus, 1991).

Ectomycorrhizal fungal richness and diversity tended to be higher in autumn, with three species registered in this season alone. The relative frequency of most EcMF changed seasonally (e.g. some EcMF were more abundant in spring while others were in autumn – Table 1; Fig. 3), except for *Cortinarius* sp. 2 which was the most abundant species for both seasons (Table 1; Fig. 2). Different *Quercus* species have also been described as having EcMF communities with significant seasonal shifts in diversity and dominance (Walker et al., 2008; Jumpponen et al., 2010). In Patagonia, Longo et al. (2011) observed that EcMF diversity in *Nothofagus antarctica* and *N. pumilio* was higher in spring. In contrast, Nouhra et al. (2012) found that richness of EcMF forming hypogeous sporocarps in native *Nothofagus* forests tended to be higher in autumn and that fungal biomass during this season was significantly higher than in spring. Based on this information, it seems that EcMF communities may be affected by seasonal patterns, which are not regular. A possible mechanism for these temporal shifts in EcMF assemblages might be related to root turnover, stochasticity, fine scale disturbance and mycelia dieback, as well as differences in EcMF environmental tolerance or in EcM foraging strategies (Agerer, 2001; Izzo et al., 2005; Courty et al., 2008; Walker et al., 2008). In this study it is most likely that EcMF richness and diversity were higher during the autumn, which corresponds to the rainy season with moderate temperatures, environmental conditions which usually increase fungal diversity (Courty et al., 2008). The fact that the highest number and diversity of epigeous fruit bodies within Yuco native forest (*unpublished data*) as well as hypogeous fruit bodies in *Nothofagus* forests of Patagonia (Nouhra et al., 2012) were also registered during this season

supports this hypothesis. However, long-term studies are needed to confirm these seasonal patterns and to be able to explain them in detail.

In conclusion, *N. nervosa* is extensively colonized by different EcMF, mostly basidiomycetes. According to our results, plant age and seasonality influence belowground EcMF communities, so that abundance, richness and diversity tended to increase with plant age and during the autumn. In addition, the highest richness and diversity of EcMF corresponded to young domesticated specimens, suggesting that forest management also impacts these fungal communities. All of this information was summarized in the clustering analysis performed (Fig. 4), in which it was observed that taking into account EcMF communities associated with native and domesticated *N. nervosa* in both seasons, plant categories were first clustered by age (seedlings were similar to one another and distinct from the other plant categories), then young and adults were grouped by season (spring *vs* autumn), and within each season young domesticated plants (Yd) were separated from native specimens (Yn and An). It was also noticed that fungal species colonizing *N. nervosa* roots in the forest and in the nursery are completely different. Because most tree species require EcM for nutrient and water uptake, the importance of understanding the relationship between biotic and abiotic factors, forest management and/or disturbance, and mycorrhizas cannot be emphasized enough. The information presented in this work is relevant from an ecological perspective, and should also be taken into account in *Nothofagus* domestication programs. For example, we have described which of the EcMF registered in this study are most abundant in *N. nervosa* roots and which are common to other *Nothofagus* species, information which is important for the selection of target fungal species to be used in nursery inoculation experiments. Finding EcMF species capable of improving seedling fitness and/or establishment when they are outplanted into the field is crucial for domestication programs. Additional research is needed to completely describe the ecological importance and the dynamics of this symbiosis in *Nothofagus* forests and to be able to use these fungi in sustainable forestry practices.

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