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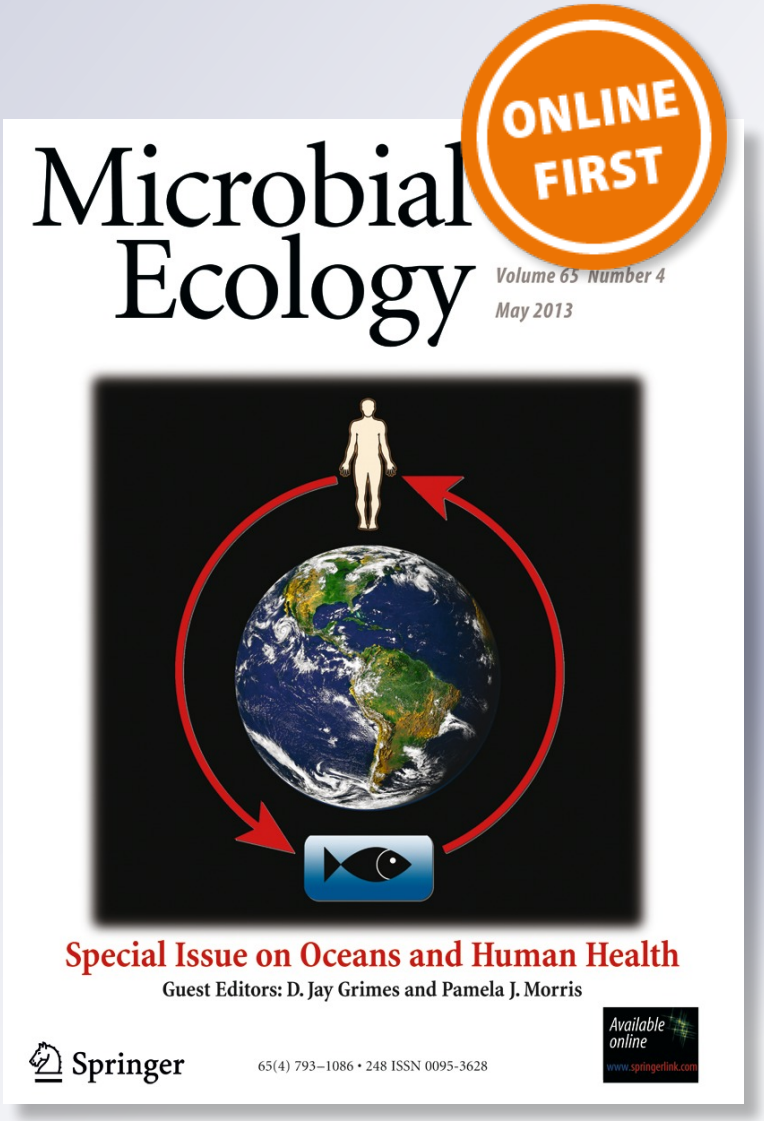

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PLANT MICROBE INTERACTIONS

Ectomycorrhizas Naturally Established in *Nothofagus nervosa* Seedlings Under Different Cultivation Practices in a Forest Nursery

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Abstract Mycorrhizas are mutualistic associations between soil fungi and plant roots which usually improve water and nutrient uptake, influencing plant fitness. *Nothofagus nervosa* (Raulí) is an ecologically and economically important species of South American temperate forests. Since this native tree species yields valuable timber, it was overexploited and its natural distribution area was critically reduced, so it is currently included in domestication and conservation programs. Among the factors that should be considered in these programs are the ectomycorrhizas (EcM), which would be important for the successful establishment and survival of outplanted seedlings. The aim of this work was to analyze the abundance and diversity of EcM in *N. nervosa* nursery-cultivated seedlings assessed by morphotyping, fungal isolation, and DNA sequencing. Arbuscular mycorrhiza (AM) occurrence was also studied. A 2-year trial was conducted following the cultivation conditions used for domestication programs. Seedlings were cultivated under two different cultivation practices (greenhouse and nursery soil) without artificial inoculation of mycorrhizal fungi. Seedlings' roots were examined at different times. It was observed that they developed EcM between 6 and 12 months after germination and AMs were not detected in any plant. The most abundant ectomycorrhizal fungi present in seedlings' roots were *Tomentella ellisii* (Basidiomycota) and an unidentified fungus named Ascomicetous EcM sp. 1. Abundance and diversity of EcM varied between the two cultivation techniques analyzed in this study, since seedlings that continued growing in the greenhouse had higher colonization values, but those transplanted to the nursery soil were colonized by a higher diversity of fungal taxa.

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Introduction

In temperate and boreal forests, the establishment, growth, and survival of different tree species are usually dependent on the mycorrhizas present in their roots. Mycorrhizas usually benefit host plants by enhancing water and nutrient acquisition, especially with regards to P, and by increasing host resistance to pathogens and other biotic and abiotic stresses [1]. Species belonging to the genus *Nothofagus* constitute the main component of South American temperate forests, which soils are predominantly derived from volcanic ashes (Andisols) and are characterized by a high capacity to stabilize organic matter, store water, and buffer pH. The main limitation of this type of soils is the high P retention, which determines that this nutrient is slightly available to plants, although total values are high [2]. It has been described that root systems of *Nothofagus* species are extensively colonized by ectomycorrhizas (EcM) [3–5], having colonization rates higher than 70 % [6–9], which suggests a high dependence of *Nothofagus* species on the ectomycorrhizal symbiosis [6, 7, 10]. This phenomenon together with the fact that these forestry species do not have

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P limitation suggests that EcM promote absorption of this nutrient and that this symbiosis constitutes an effective adaptation mechanism of *Nothofagus* species to P-deficient soils [6, 7, 10]. Arbuscular mycorrhizas (AM) were also found in association with *Nothofagus* roots, but they were solely described in a unique adult tree of *Nothofagus dombeyi* (Mirb.) Oerst. (Coihue) situated in an urban zone within the Andean–Patagonian region [11]. This raises the question of the possibility of finding both types of mycorrhizas (EcM + AM) in *Nothofagus* specimens, a phenomenon that has been described in other species of vascular plants [12].

Nothofagus nervosa (Phil.) Dim. et Mil. (Raulí) is one of the most ecologically and economically important species of South American temperate forests. It yields a highly valuable wood, resembling that of *Fagus sylvatica* L., suitable for regional and international commercialization. Consequently, this species was overexploited and its natural populations were drastically reduced. This critical situation led to the implementation of conservation and domestication programs. One of the main purposes of domestication programs is to develop cultivation techniques of seedling production for afforestation and restoration activities [13, 14].

Several studies carried out in different important forestry species, such as *Larix* [15], *Picea* [16], *Pinus* [17, 18], *Pseudotsuga* [19], and *Quercus* [20], have described that inoculation with mycorrhizal fungi during their cultivation in the nursery increases both plant growth and its subsequent performance under natural conditions when they are outplanted to the field. Therefore, mycorrhizal colonization of root systems is currently considered as an important factor for determining seedling vigor and, consequently, their quality [1, 16, 21, 22]. According to this, one of the aspects that should be considered in *N. nervosa* domestication programs are the mycorrhizas associated with this important forestry species.

Mycorrhizas naturally occurring in nurseries may be diverse and their establishment depends on several factors, such as host–fungus interaction, cultivation system, and nursery conditions [23–26]. During cultivation, the nursery environment is usually modified by fertilization, irrigation, and weed and pest control. These management practices can either adversely affect the beneficial mycoflora present in seedling roots or promote colonization by particular EcM species [21, 27]. For example, the cosmopolitan fungal species *Thelephora terrestris* (Basidiomycota) and *Cenococcum geophilum* (Ascomycota) are well adapted to cultivation conditions and have been described in several forest nurseries worldwide [16, 20, 22, 26, 28], including one situated in the Andean–Patagonian region in which *Nothofagus* and *Pinus* seedlings are produced [29]. In addition, naturally established mycorrhizas usually influence the success of inoculated mycorrhizal fungi, since the introduced species may persist alone, coexist with, or be out competed by the fungal species naturally occurring in the nursery [23, 29]. Consequently, the origin and identity of the symbiotic fungi colonizing the roots of the species of interest in cultivating remain a permanent concern for nursery and mycorrhiza managers, being necessary to know which cultivation conditions promote mycorrhizal colonization and to identify the fungal taxa associated with mycorrhizas naturally established in nurseries [23, 24]. This information would be important for allowing further EcM management practices in forest nurseries and for the optimization of domestication programs.

In Argentina, *N. nervosa* seedlings are not inoculated with mycorrhizal fungi during their cultivation in nurseries, and any study has been conducted on their mycorrhizal status throughout this important stage of this species domestication. The objective of the present study was to describe the diversity of mycorrhizas naturally established on *N. nervosa* seedlings and to compare species composition and abundance in plants cultivated under two different techniques. Fungal diversity and taxonomic identification were determined by morphotyping, fungal isolation, and DNA sequencing.

Methods

Seedling Cultivation

The present study was carried out in the forest nursery (greenhouse and outdoor) of Instituto Nacional de Tecnología Agropecuaria, Argentina (INTA), situated in San Carlos de Bariloche, Northern Patagonia. For cultivating *N. nervosa*, fruits[1] were collected from a native forest situated in the Yuco Region (Lacar lake watershed, 40°07′48″S–71°34′48″W, Neuquén Province, Patagonia, Argentina). This region comprises the largest *N. nervosa* forests, both in terms of continuity and density and in size and vigor of the trees [30]. The Yuco Region is registered in the INASE (National Seed Institute) as an *N. nervosa* seed-producing area (http://www.inase.gov.ar).

Fruits were collected by placing nets below *N. nervosa* canopy at approximately 1.5 m above the ground. For germination, seed dormancy was broken by soaking the fruits in cold running water (~5 °C) for 5 days [31]. Containerized seedlings were produced in plastic trays by sowing the fruits no more than 1 cm below the surface in a porous substrate constituted by peat bog and volcanic sand (1:1 *v*/*v*), which allows hydration and avoids compaction. Plastic trays were placed in the greenhouse under controlled conditions [32,

[1] *Nothofagus* species have indehiscent dry fruits containing only one seed (nuts). Since the pericarp does not split open, seedlings are cultivated directly from these fruits.

33]. They were irrigated by a micro-sprinkler system only with water until germination. After seeds had germinated, seedlings were irrigated using a fertigation technique which consisted in applying different nutrient concentration according to three growing stages (Table 1): (a) *Establishment:* This begins once the seeds germinate and ends when the first pair of true leaves appears (~45 days). The aim of this stage is to promote root development. (b) *Fast growth*: During this stage, plants develop most of the aerial biomass and ends when seedlings reach 80 % of the desired stem length (~75 days); and (c) *Rustification*: This stage promotes plant resistance to environmental conditions (e.g., low temperatures, lack of water, wind) in order to improve its adaptation in the field. During this last stage, frequency of fertigation, nutrient concentration (mainly N), and protection from wind and solar radiation are reduced. The rustification stage (~60 days) is crucial for plant quality and finishes when seedlings achieve the desired height (~40 cm) and are ready to be transplanted to the nursery soil (outside the greenhouse) or the field [32, 33]. In this work, both fruit collection and cultivation practices were the same as those used in *Nothofagus* domestication programs. Plants were not artificially inoculated with ectomycorrhizal fungi.

Seedlings were analyzed at different times for 2 years (October 2007–October 2009). During the first month after germination, they were collected every 10 days. Then, seedlings were monthly analyzed until they were 6 months old. After that moment, the seedlings were examined every 6 months until 2 years (12, 18, and 24 months). When seedlings were 1 year old, half of them were outplanted to the nursery soil outside the greenhouse, without fertigation and under natural environmental conditions. Therefore, there were two observations for 18- and 24-month-old seedlings: one for plants that continued growing in the greenhouse and the other to those that were transplanted to the nursery soil. In each sampling time, 15 plants were randomly selected and kept in individual plastic bags until further procedures.

For analyzing the occurrence of mycorrhizas (AM and EcM), roots were separated from the rest of the plant and carefully cleansed under a stereoscopic microscope (Olympus SZ30). A fraction of the root system was fixed in 70 % *v/v* alcohol for AM description, while the rest of it was kept at 4 °C for analyzing EcM in fresh roots.

Table 1 Nutrient concentration applied in each growing stage during *Nothofagus nervosa* seedling production in the greenhouse

Nursery growing stages	Nutrient concentration (ppm/week)[a]		
	N	P	K
Establishment	87–196	100–309	68–204
Fast growth	93–319	15–54	72–240
Rustification	8–9	50–54	100–105

[a] Ranges indicate nutrient concentration applied at the beginning and at the end of each growing stage, respectively

Morphometric Measures

The following measures were taken from each seedling at all observation times: stem length, root collar diameter, and stem collar diameter. For seedlings up to 1 year old, root length, total length, and fresh plant weight were also registered.[2]

Ectomycorrhizas

Morphotyping and Quantification

When ectomycorrhizal morphotypes (= ectomorphotypes) were detected, they were characterized and classified into morphological groups according to the following characteristics: ramification and shape, mantle texture and color, and presence and color of emanating hyphae and/or rhizomorphs. The description of each morphological group was complemented with microscopic features (Olympus BX40), such as color of the hyphae forming the mantle, mantle pattern (plectenchymatus or pseudoparenchymatous), and presence of cystidia and clamp connections [34]. Representatives from each morphological group were excised under a stereoscopic microscope and kept in 1.5 mL plastic tubes at −20 °C for molecular analysis.

Total percentage of EcM and relative abundance of each ectomorphotype were quantified according to the method described by Grand and Harvey [35]. It consisted in placing pieces of the root system unevenly in a Petri dish on which a grid of 1 × 1 cm was drawn. The plate was observed under a stereoscopic microscope (Olympus SZ30) and where each root portion overlapped with vertical lines of the grid; root tips colonized by ectomycorrhizal fungi were quantified and classified in one of the previously mentioned groups. Ectomorphotypes that did not match any of these morphological groups and appeared infrequently were classed as "unclassified." At least 300 intersections per sample were quantified, excepting those individuals where the root system was not sufficiently large to reach this number, in which case all the root system was measured (i.e., 10-day-old seedlings). Colonization percentages were calculated dividing the number of ectomycorrhizal tips by the total number of tips quantified. Relative abundance of the different ectomorphotypes was obtained by dividing the number of

[2] It was not possible to measure these variables in plants at 18 and 24 months because the complexity of the root system impeded to extend it properly and to separate the substrate from the roots.

tips belonging to each group by the total number of ectomycorrhizal tips.

Isolation of Fungi Associated with Ectomycorrhizal Root Tips

Pure cultures of EcM-associated fungi were attempted from individual mycorrhizal root tips corresponding to 2-year-old seedlings. Fifteen ectomycorrhizal root tips of each of the morphological groups observed in plants growing in the greenhouse and other 15 from each of the groups registered in seedlings transplanted to the nursery soil were selected for fungal isolation. Before isolation, root tips were pretreated with a 0.2 % Tween aqueous solution (*v*/*v*) and surface sterilized in a 30 % hydrogen peroxide aqueous solution (*v*/*v*) for 20 s and then rinsed three times in sterile water. Tips were plated onto PDA medium supplemented with 0.01 % chloramphenicol [36] and incubated at room temperature (~20 °C) in the dark. Dishes were checked daily and emerging cultures were transferred onto fresh medium. Fungal cultures were grouped accordingly to morphological characteristics. For identification, one to three representative cultures from each group were selected for DNA extraction and sequencing.

Molecular Identification of Ectomycorrhizal and Associated Fungi

DNA was extracted from fresh and preserved (−20 °C) ectomorphotypes and from fresh fungal mycelia using a modified Bent and Taylor method [37]. It consisted in placing one to five ectomycorrhizal root tips or a ~25 mm^2 of fungal mycelia in 38 μL Tris-EDTA (TE) buffer and 12 μL of a Proteinase K solution (3.24 mg/mL TE). An equivalent of 0.15 g of glass beads (2 mm diameter) was added to each tube and all samples were vortexed at a maximum speed for 2 min. Samples were incubated at 55 °C for 3 h and at 75 °C for 1 h, and then, they were centrifuged at 14,000×*g* for 15 min and the supernatant was transferred to a clean tube. For purification, an equal volume of chloroform–isoamyl (24:1 *v*/*v*) was added to all samples, which were gently agitated by inversion and centrifuged for 15 min at 14,000×*g*. The aqueous phase containing the DNA was transferred to a sterile tube, added with one volume of cold isopropanol, agitated by inversion, and centrifuged at 14,000×*g* for 5 min. The supernatant was discarded, 1 mL of cold ethanol 70 % *v*/*v* was added to each sample, gently agitated, and centrifuged at 14,000×*g* for 5 min. Finally, the supernatant was discarded, and to evaporate residual ethanol, tubes were placed at room temperature for 30 min. DNA was resuspended in 20 μL of deionized sterile water and stored at −20 °C.

The fungal-specific primer ITS1F (5′-CTTGGTCA TTTAGAGGAACTAA-3′) and the universal primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Sigma) were used for PCR amplification of the ITS region of each sample (Thermocycler MultiGene, Labnet). The PCR reaction was performed in 50 μL according to the following concentrations: 6 mM $MgCl_2$, 0.32 mM dNTPs, 0.2 μM of each primer, 0.06 mg/mL bovine serum albumin, 1 U Taq polymerase (Invitrogen), 1× reaction buffer, and 5 μL of DNA dilution 1:50, 1:100, and 1:200 depending on the sample. The reaction was carried out according to the following PCR conditions: an initial denaturing step at 94 °C for 4 min, 35 cycles of 30 s at 94 °C, 45 s at 55 °C, and 70 s at 72 °C and a final extension for 10 min at 72 °C. Amplified DNA fragments were separated by electrophoresis in 1.5 % (*w*/*v*) agarose gels, stained with GelRed (Biotium), and observed under a UV transilluminator (DNR MiniBIS Pro). Considering that PCR products that presented a single band contained fungal DNA, they were purified (Bioneer purification kit) and sequenced by Macrogen (Seoul, South Korea) with the primer ITS1F. Sequences were manually corrected, aligned, and subjected to phylogenetic analysis using the Molecular Evolutionary Genetics Analysis software (MEGA4). Two phylogenetic trees, one for Ascomycetes and the other for Basidiomycetes, were constructed using the neighbor-joining algorithm. Bootstrap values were calculated from 1,000 replicates and the Kimura two-parameter model to estimate evolutionary distance. If several strains of a single species were obtained, only four were taken into account for constructing the tree. In the phylogenetic tree, all the species were clustered with their nearest phylogenetic relatives according to the NCBI database (http://www.ncbi.nlm.nih.gov). The PubMed database was used for nomenclature and classification of the species (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). All the nucleotide sequences obtained in this work were deposited in the NCBI GenBank database and are available for comparative purposes under the accession numbers KC759473–KC759517.

Arbuscular Mycorrhizas

For studying the occurrence of AM in *N. nervosa* seedlings, root samples were stained using a modified Phillips and Hayman [38] method [see 39]. Once stained, ten root pieces of approximately 1 cm length were randomly selected from each sample and mounted in glycerin on a microscope slide. For each specimen, three slides (30 root pieces=~30 cm) were made and examined with a light microscope (Olympus BX40). In 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. Typical AM structures (intra- or intercellular hyphae, vesicles, coils, and arbuscules) were used for quantifying mycorrhizal colonization [39]. Root length colonized by AM fungi was

estimated according to the intersect method described by McGonigle et al. [40].

Statistical Analyses

One-way ANOVAs followed by Tukey tests for analyses a posteriori were performed for comparing morphometric measures between seedlings that continued growing in the greenhouse and those that were outplanted to the nursery soil (stem length, root collar diameter, stem collar diameter) at each observation time (18 and 24 months).

For comparing ectomycorrhizal colonization percentages between seedlings in the greenhouse and those placed in the nursery soil, a two-way ANOVA and a Tukey test were performed (factor 1=location: greenhouse vs nursery soil and factor 2=age: 18 vs 24 months). Data were normalized using the quadratic exponential function.

To evaluate if there were significant correlations between the different morphometric measures registered and colonization percentages, the Pearson coefficient (r) was used for normally distributed variables (stem length, root collar diameter, stem collar diameter, fresh plant weight) and the Spearman coefficient (r_s) for those that showed no normal distribution (total length and root length).

Results

Plant Growth and Mycorrhizal Colonization

Seedlings lacked EcM during the first 6 months. Ectomycorrhizas were recorded in 14 out of the 15 one-year-old plants analyzed (corresponding the only non-colonized specimen to the smallest observed at that time) and in all the seedlings of 18 and 24 months growing in the greenhouse and in the nursery soil. Mean colonization values were significantly higher ($p<0.001$) in seedlings growing in the greenhouse than in those outplanted to the nursery soil (Fig. 1). Significantly higher values of stem length, root collar diameter, and stem collar diameter were also observed in 18- ($p<0.001$, $p<0.001$, and $p=0.001$, respectively) and 24-month-old seedlings ($p=0.012$, $p=0.036$, $p=0.041$, respectively) situated in the greenhouse with respect to those transplanted to the nursery soil (Table 2). There were no significant correlations between colonization percentages and any of the morphometric measures registered.

Two morphological groups of ectomorphotypes were defined based on macroscopic and microscopic features. They were the same for seedlings which remained in the greenhouse than for those outplanted to the nursery soil. According to molecular results, one of the groups was formed by the species *Tomentella ellisii* (Basidiomycota) and the other by an unidentified fungus named Ascomicetous EcM sp. 1

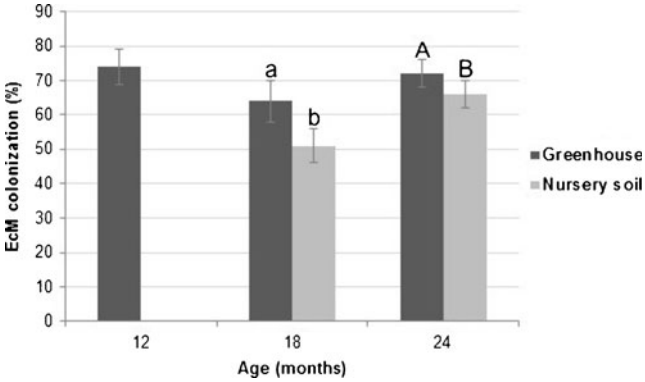


Fig. 1 Ectomycorrhizal colonization percentages in *N. nervosa* seedlings growing in the greenhouse and in the nursery soil. Significant differences in colonization values are indicated with different *superscript letters* in seedlings of 18 months (*lowercase*) and 24 months old (*uppercase*)

(Ascomycota) (Fig. 2). In the first group, white to brownish, simple straight, or monopodial-pinnate ectomorphotypes, with smooth mantle, without or with sparse, short and hyaline emanating hyphae, and lack of rhizomorphs, were included. The pseudoparenchymatous mantle of the ectomorphotypes comprised in this group was formed by colorless hyphae, clamps were observed, and cystidia were not registered (Fig. 3). The second group corresponded to mostly simple, straight, and dark brown to black ectomorphotypes, with abundant emanating hyphae emerging in all directions and without rizomorphs. In ectomorphotypes included in this last group, the plectenchymatus mantle was formed by dark brown hyphae, and no clamps or cystidia were registered (Fig. 4).

Colonization percentages corresponding to the species *T. ellisii* were significantly higher than those recorded for the fungus Ascomicetous EcM sp. 1 at all observation times and in both cultivation places (greenhouse and nursery soil). Ectomorphotypes that could not be classified in any of the two described groups (unclassified) were found in 24-month-old seedlings placed in the greenhouse as well as in 18- and 24-month-old seedlings located in the nursery soil, but the proportion of root tips corresponding to this group was very low, being higher in seedlings growing in the soil (Table 3). Sequences corresponding to the fungi forming unclassified ectomorphotypes belonged only to 2-year-old seedlings situated in the nursery soil and were taxonomically related to four species, two included in the Phylum Basidiomycota (*Hebeloma cavipes* and *Rickenella* sp. 1) and the other two in the Phylum Ascomycota (*Peziza* sp. 1 and *Peziza* sp. 2) (Fig. 2). Three fungal species that were clearly non-mycorrhizal were also identified from healthy-looking ectomorphotypes. They were: *Ilyonectria radicicola*, *Dendryphion nanum*, and the yeast *Cryptococcus victoriae*. Arbuscular mycorrhizas were not detected in any of the 195 seedlings analyzed in this study.

Table 2 Morphometric measures registered in 18- and 24-month-old seedlings situated in the greenhouse and in the nursery soil

Morphometric measures	Greenhouse		Nursery soil	
AGE	18 months	24 months	18 months	24 months
Stem length	44.5±10.2	56.1±16	20.9±6.7	35.1±5.5
Root collar diameter	7.7±1.9	9.1±2.6	4.9±1.3	7.4±1
Stem collar diameter	5.9±1.5	7±2.2	4±1	5.6±0.7

X±SD=mean colonization percentages and standard deviation

Fungal Isolation

Nine fungal species were isolated from surface-sterilized ectomorphotypes corresponding to 2-year-old seedlings growing in the greenhouse and in the nursery soil (Fig. 2). Most of them (89 %) were included in the Phylum Ascomycota (*Allantophomopsis lycopodina*, *Cylindrocarpon* sp. 1, *Cylindrocarpon* sp. 2, *Ilyonectria macrodidyma*, *I. radicicola*, *Leptosphaeria* sp. 1, *Paraconiothyrium sporulosum*, *Penicillium* sp. 1), excepting for *Stereum* sp. 1, which is a basidiomicetous species. All the isolated fungi corresponded to non-ectomycorrhizal species, and there was only one species in common with those identified from ectomorphotypes (*I. radicicola*).

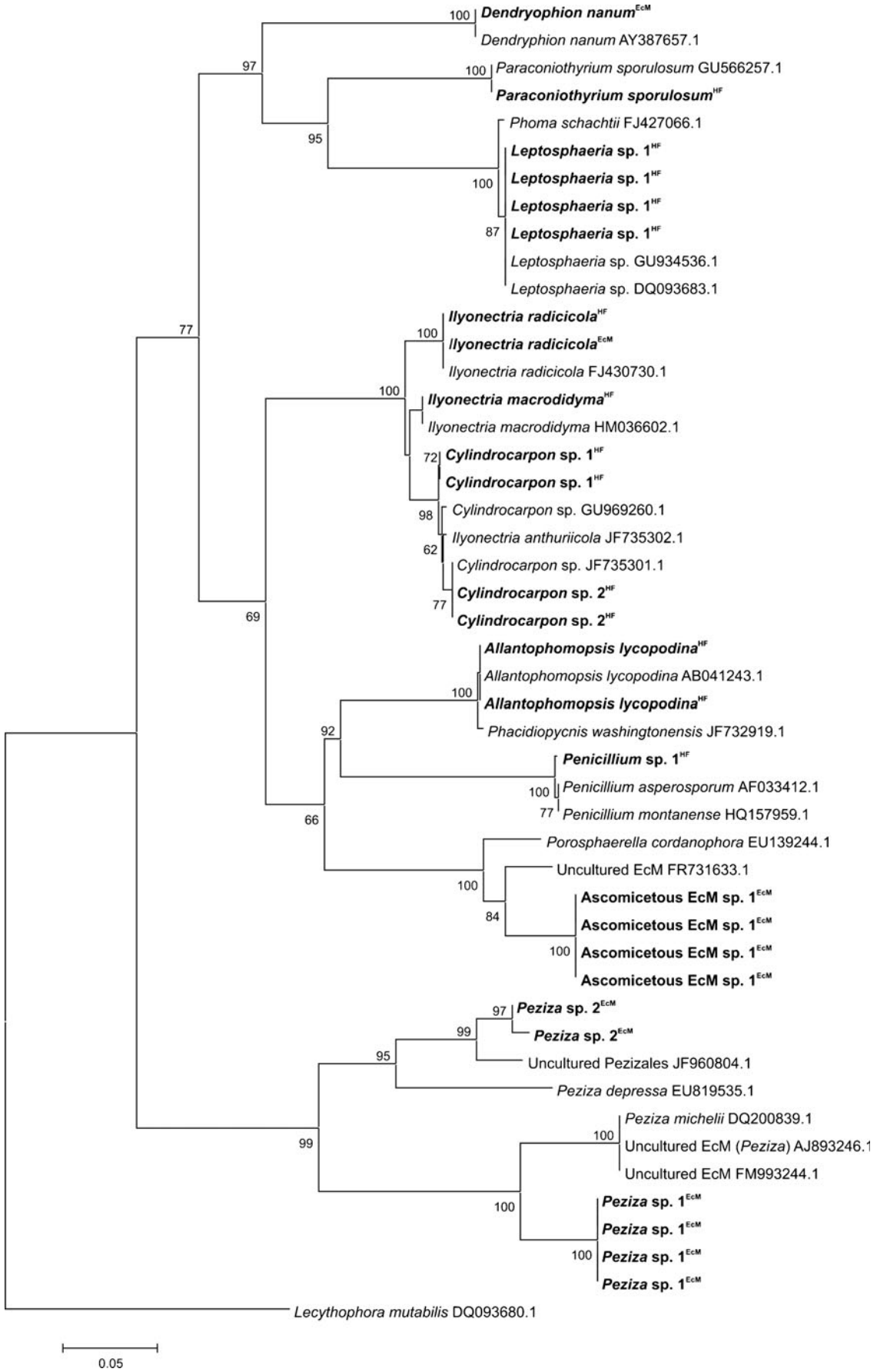
Discussion

Plant Growth and Mycorrhizal Occurrence

In the greenhouse, *N. nervosa* seedlings grew according to the three stages described previously (establishment, fast growth, and rustification) [33]. Establishment of the ectomycorrhizal symbiosis occurred naturally (without intentional inoculation) between 6 and 12 months after germination, after the rustification stage, in which nutrient supply (mainly N and P) and irrigation frequency were significantly reduced. A similar tendency was reported by Barroetaveña et al. [29], who conducted a trial in which three *Nothofagus* species (*N. nervosa*, *Nothofagus obliqua*, and *Nothofagus pumilio*) were cultivated under greenhouse conditions similar to those presented in this work (substrate, fertigation, nutrient concentration) during 6 months (until the rustification stage ended) and observed that after this period of time seedlings were scarcely colonized (2.8 to 5.9 %), which could indicate that this symbiosis was recently established. Several authors have described that high fertilizer concentration usually used in nurseries inhibits or at least reduces ectomycorrhizal colonization, since it can be toxic to the fungus [16] or because plants grown under nursery conditions apparently do not need a symbiotic association for water and nutrient uptake since they are in high availability [1, 15]. For example, Rincón et al. [18] found in pine seedlings inoculated with different ectomycorrhizal fungi that colonization percentages varied significantly among different fertilization methods, being some fungal species most adversely affected by fertilizers than others. Similarly, Martinez et al. [41] observed that the introduction of a dormant period (2 months with low fertilization) significantly stimulates ectomycorrhizal colonization in *Pinus ponderosa* seedlings inoculated with *Rhizopogon roseolus*, and that percentages of EcM increased with decreasing amount of fertilizers. This trend has been reported by other authors and in different forestry species, such as *Pinus contorta*, *Picea glauca*, and *Picea mariana* [1, 42]. In a study carried out by Fernández et al. (unpublished), it was observed that 3-month-old *N. nervosa* seedlings cultivated in the same substrate than the one used in this work but added with forest soil were already colonized by ectomycorrhizal fungi. Based on these studies, it can be suggested that during cultivation in nurseries, *N. nervosa* seedlings are naturally colonized after rustification by ectomycorrhizal fungi present in the cultivation system, and that this phenomenon is more related with the available inocula and external nutrient supply than to plant age. The fact that AM were not registered in any of the seedlings analyzed in this study is consistent with many authors who established that *Nothofagus* species are ectomycorrhizal and do not harbor mixed colonization (EcM + AM) [3–10].

The occurrence of EcM in non-inoculated nursery plants is a phenomenon that has been described worldwide [23, 43] and even in the Patagonian region [29]. A common question that arises from this fact is the mycorrhizal inoculum source. It is possible that the input of ectomycorrhizal fungi spores into the cultivating system may be related with water, the irrigation system, wind [29], or resistant propagules remaining in the greenhouse from one season to the next (mycelium, spores, rhizomorphs, and mycorrhizal root

Fig. 2 Phylogenetic tree constructed by neighbor-joining and the Kimura two-parameter analysis of the ITS region sequences obtained from ectomorphotypes and associated fungal isolates. The *numbers* on the branches are the frequencies with which a given branch appeared in 1,000 bootstrap replications (values smaller than 50 % are not shown). Sequences determined in the present study are in *bold*. Additional sequences were retrieved from the GenBank. *Lecythophora mutabilis* and *Trichosporon porosum* were used as outgroups for the fungi belonging to the **a** Phylum Ascomycota and **b** Phylum Basidiomycota. Fungal sequences obtained from ectomorphotypes (*EcM*) and from fungal cultures (*HF*) are differentiated



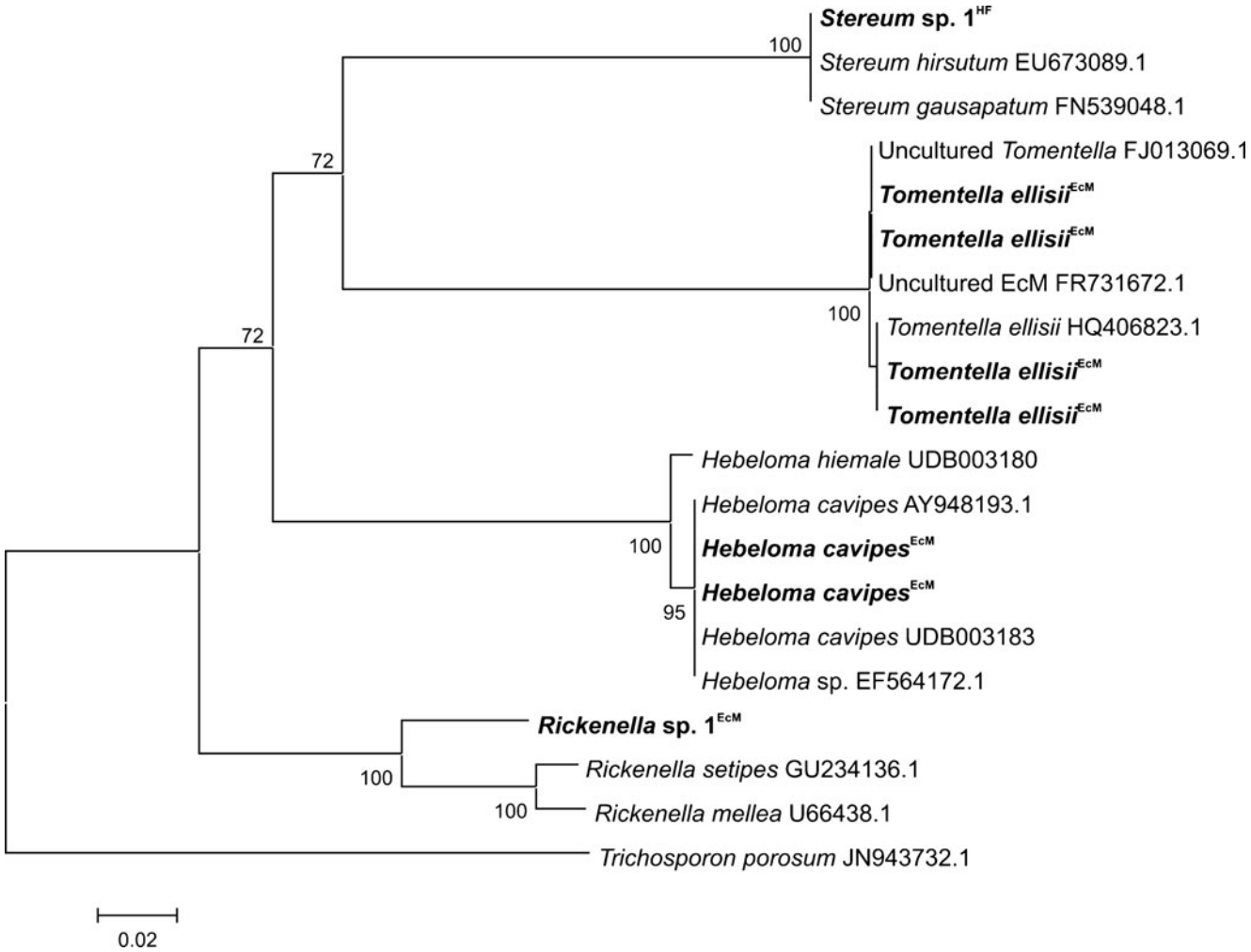


Fig. 2 (continued)

fragments present in seedlings trays, tools, or in the infrastructure) [23]. In this study, it is also possible that the fungal inoculum was associated with the peat bog used for making the cultivation substrate, since it is extracted from Tierra del Fuego (Patagonia, Argentina), where *Nothofagus* forests occur and therefore fungal species associated with these tree species. The cultivation substrate was disinfected before use but not sterilized, making it possible that resistant fungal propagules capable of forming EcM in *N. nervosa* remained in it. It is also possible that, given the small size of the spores, they entered in the greenhouse with the fruits used for cultivating this species.

At the end of our experiment, seedlings that were transplanted to the nursery soil were smaller (Table 2) and had lower colonization rates (Fig. 1) than those that continued growing within the greenhouse. This is probably related with transplant stress and changes in environmental conditions, since these plants went from being in a controlled environment and with high nutrient availability to be outdoors, without fertigation and exposed to natural conditions (radiation, frost, wind, wide temperature variation). This is in agreement with other authors who have observed that under stressful conditions plants usually redirect the carbon flow into the tissues or organs of greatest need, resulting in a smaller contribution to the symbiotic fungi and therefore in a reduction of mycorrhizal colonization [1, 44].

Ectomycorrhizas and Associated Fungi

In this study, it was observed that *N. nervosa* seedlings cultivated under nursery conditions are mainly colonized by two ectomycorrhizal species. One of them is *T. ellisii* (Basidiomycota) (Figs. 2 and 3), a fungal species that has been previously found in forest nurseries. For example, Minchin et al. [45] observed that *T. ellisii* was the dominant species colonizing *Pinus radiata* root tips during its cultivation in a glasshouse. The other fungal species found in this work belongs to the Phylum Ascomycota and probably corresponds to a new fungal species (Ascomicetous EcM sp. 1), since its closest phylogenetic relative corresponded to an

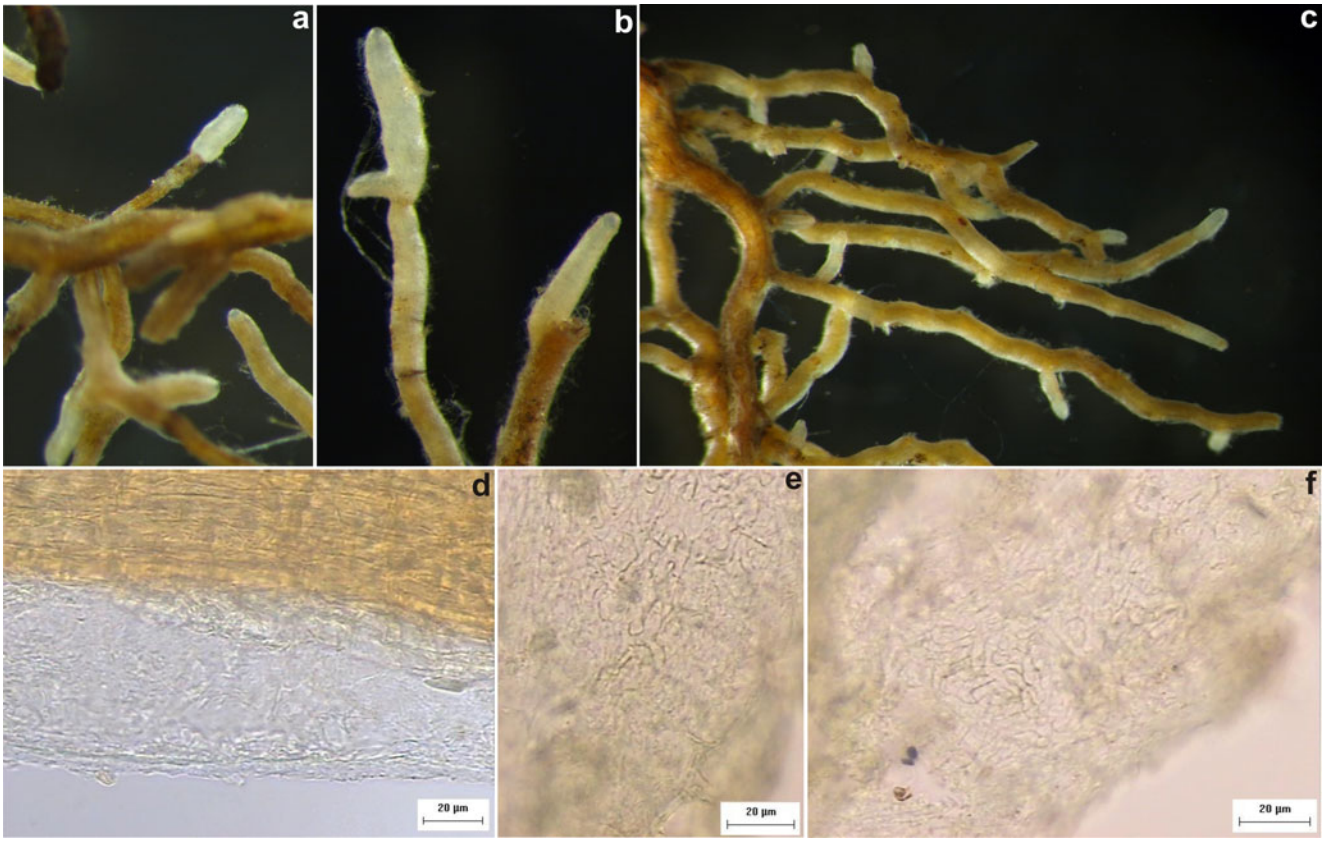


Fig. 3 Characteristic morphological features of the ectomorphotypes formed by *T. ellisii*. **a**–**c** White to brownish simple straight or monopodial-pinnate root tips. **d** Thick mantle surrounding the root. **e**–**f** Pseudoparenchymatous mantle formed by colorless hyphae in surface view

unidentified mycorrhizal fungus (Figs. 2 and 4). The former species was more abundant than the last in all the observation times and both in the greenhouse and in nursery soil (Table 3). Similar trends, in which one or a few ectomycorrhizal fungal species are dominant over the rest of the fungi forming EcM in a forest nursery, have been described. For example, Barroetaveña et al. [29] observed in three *Nothofagus* species colonized by two ectomycorrhizal species that *T. terrestris* was significantly more abundant than *C. geophilum*. Similarly, Menkis and Vassaitis [26] found nine ectomorphotypes in nursery-cultivated *Pinus sylvestris* seedlings, but only those formed by *T. terrestris* (39.7 %) and *Hebeloma* sp. (17.8 %) were abundant. These authors attribute the low diversity of EcM to the predominance of *T. terrestris*, which has been considered as an excellent competitor under nursery conditions.

The number of fungal species forming EcM in nursery-cultivated *N. nervosa* individuals was significantly lower than those observed in seedlings corresponding to the same species growing in a native forest, in which 18 ectomycorrhizal fungal species were found [9]. Differences in ectomycorrhizal diversity between the nursery and the native forest could be explained by changes in environmental conditions and fungal inoculum [46]. For example, in the nursery, host plants are cultivated in containers, 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, host plants can grow for many decades in the same environment and in contact with different fungal species, thus favoring high ectomycorrhizal diversity [23]. Altogether, this information indicates that nursery conditions (fertigation, mechanical and chemical weed control, containerized root systems) may negatively affect some mycorrhizal fungi, then favoring colonization by a few species tolerant to these conditions [18, 23, 26, 29], such as *T. terrestris* and *C. geophilum* which are cosmopolitan species that have been described in seedlings of different plant species grown in nurseries with fertigation worldwide [16, 20, 22, 25, 26, 28].

In 2-year-old seedling growing in the soil, four ectomycorrhizal species that were not registered in the greenhouse were identified (*Rickenella* sp. 1 and *H. cavipes* and *Peziza* sp. 1 and *Peziza* sp. 2). These results indicate that after being transplanted to the nursery soil, *N. nervosa* seedlings are exposed to a natural inoculum and form symbiotic associations with fungi different from those that colonized their roots in the greenhouse (*T. ellisii* and Ascomicetous EcM sp. 1). This is in agreement with other authors, who have observed that mycorrhizal fungi adapted to greenhouse conditions often remain in the root system

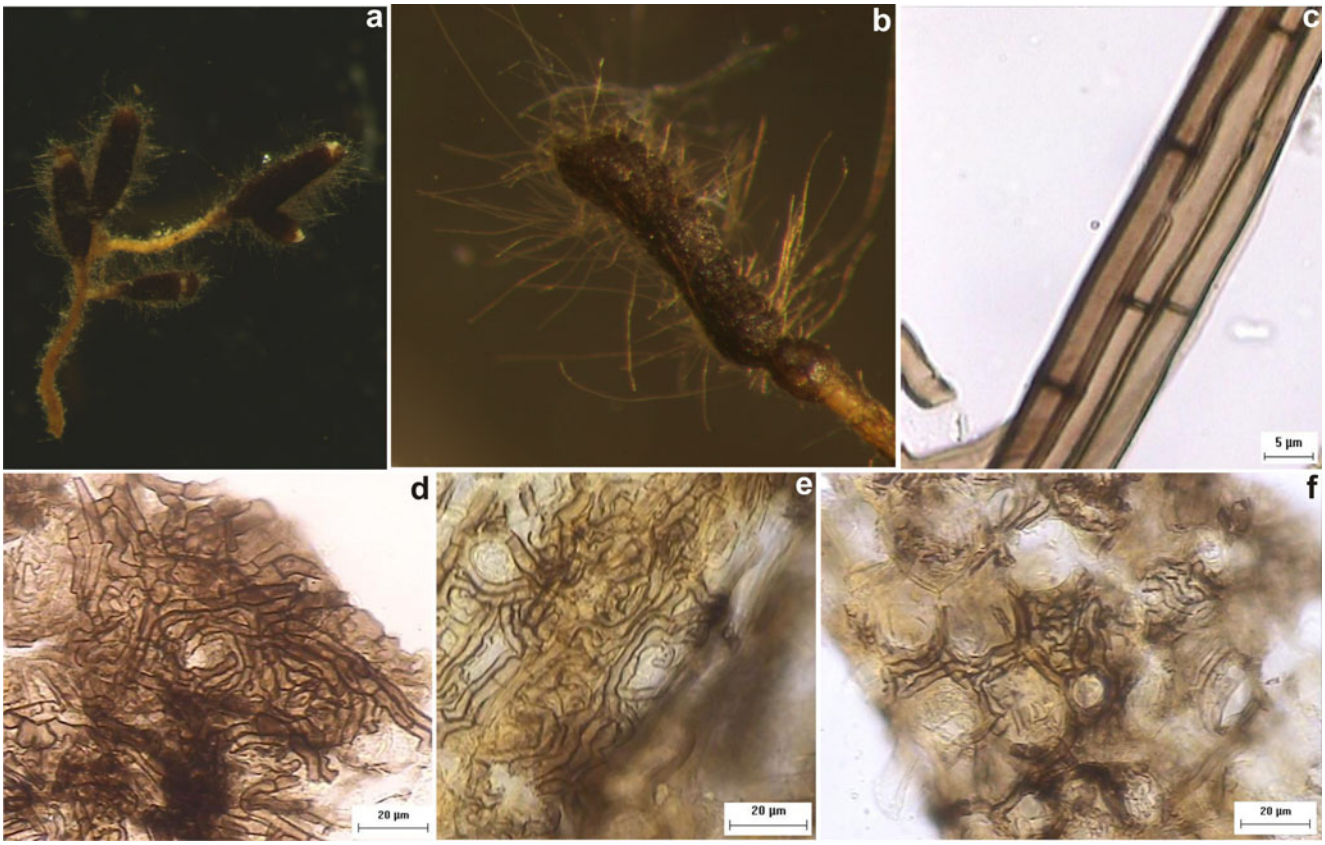


Fig. 4 Morphological characteristics of the ectomorphotypes corresponding to the fungus Ascomicetous EcM sp. 1. **a**, **b** Dark brown to black simple ectomorphotypes. **c** Dark emanating hyphae tabicated and without clamps. **d**, **e** Plectenchymatus mantle formed by dark hyphae. **f** Hartig net in surface view

during a time after transplantation, but they are gradually replaced by native mycorrhizal species, characteristic of the place where seedlings have been established and better adapted to natural environmental conditions [16, 43]. For example, the fungus *T. terrestris* is very common in nursery systems, but uncompetitive in natural ecosystems, being easily displaced by other ectomycorrhizal fungi [26, 42, 47].

There are several fungi associated with plant roots that coexist with mycorrhizal fungi. In this study, three clearly non-mycorrhizal fungal species were identified from colonized root tips. One of them was *C. victoriae*, a yeast that has been registered as abundant in the Nahuel Huapi Lake [48], and that is likely to have been incorporated into the cultivating system through irrigation. The other two were *N. radicicola* and *D. nanum*, which have been described as pathogenic in different plant species [49, 50]. In addition, all the fungal species isolated from *N. nervosa* healthy-looking ectomycorrhizal root tips corresponded to non-mycorrhizal fungi, indicating that these species were actually coexisting with those fungi forming the ectomorphotypes. Similar results were obtained by Menkis and Vasaitis [26], who found out that the majority of the EcM presented in nursery cultivated *Pinus* seedlings were formed by basidiomycetes, but most of the fungi isolated from colonized root tips corresponded to

Table 3 Relative abundance of the different ectomorphotypes registered in *Nothofagus nervosa* seedlings during their cultivation in the nursery

EcM	Greenhouse			Nursery soil	
AGE	12 months	18 months	24 months	18 months	24 months
Tomentella ellisii	67±14	71±16	68±13	59±19	54±14
Ascomicetous EcM sp. 1	33±14	29±16	30±14	36±18	37±14
Unclassified	0	0	2±2	5±7	9±4

X±SD=mean colonization percentages and standard deviation

EcM ectomycorrhizal fungal species forming the ectomorphotypes

non-mycorrhizal saprotrophs and necrotrophs mainly corresponding to the Phylum Ascomycota. Such bias towards these groups of fungi can be explained by their higher in vitro growing rates [24, 26].

Conclusion

According to this study, *N. nervosa* seedlings cultivated in the present forest nursery are colonized by naturally established ectomycorrhizal fungi when they are transplanted to the field. Moreover, these EcM remain in their root system at least during plant establishment, since the same fungal species that colonize their roots within the greenhouse are present in 2-year-old seedlings placed in the soil for a year. The fact that the seedlings that remain in the greenhouse were more highly colonized than those transplanted to the nursery soil but that the last were associated with a higher diversity of fungal taxa demonstrates that abundance and diversity of EcM depend on the cultivation technique used, as it has been reported by other researchers [16, 17, 20, 24]. In addition, these findings indicate that changes in environmental conditions alter mycorrhizal communities and initiate a succession of fungal species that is determined by the biotic and abiotic factors characteristic of the place where the seedlings are established. The information presented in this study is relevant for setting down management guidelines including the employment of ectomycorrhizal fungi for cultivation during domestication of *N. nervosa* and other important native forestry species in Patagonia.

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