Co-occurrence of *Paris*- and *Arum*-type endomycorrhiza in invasive *Pseudotsuga menziesii* seedlings in Patagonia, Argentina

María Eugenia Salgado Salomón[1,3], Carolina Barroetaveña[1,2,3,*] and Mario Rajchenberg[1,2,3]

[1] Ingeniería Forestal, Universidad Nacional de la Patagonia S.J. Bosco, Sarmiento 849, Esquel, Chubut, Argentina

[2] Centro Forestal CIEFAP, CC 14, 9200 Esquel, Chubut, Argentina

[3] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)

With 3 figures and 1 table

Abstract: *Pseudotsuga menziesii*, one of the most widely planted exotic Pinaceae in NW Patagonia (Argentina), has been introduced in different environmental conditions showing an invasive behavior on neighboring *Nothofagus* forests. Recent researches have shown high abundance of ecto- (EM) and ectendomycorrhiza in *Ps. menziesii* seedlings from Patagonia but the presence, type and abundance of endomycorrhiza (arbuscular mycorrhiza, AM) in the context of invasion processes remain unknown. We studied the AM status of invading *Ps. menziesii* seedlings from *Nothofagus-Ps. menziesii* invasion matrices in Patagonia, including AM colonization percentages and AM types, and we analyzed the evolution of AM colonization with seedlings' age in relation with EM colonization. Twenty-five, up to 5 years old seedlings from each six *Nothofagus-Ps. menziesii* invasion matrices were collected to measure AM and EM colonization percentages and to determine AM root colonization types (*Arum* or *Paris*). AM colonization varied from 23.7–29.4% among sites, with different proportions of *Arum*- and *Paris*-types. Also, the co-existence of both AM types was found in the same plant in all studied sites. Dark Septate Endophytes were found widely distributed, abundant, and coexisting with AM and EM in roots. A clear AM-EM colonization succession was not found, as both AM and EM colonization coexist from the beginning of seedlings' establishment. A great plasticity regarding the mutualistic associations established by *Ps. menziesii* revealed a highly beneficial strategy to succeed in the invasion process.

Keywords: DSE, invasion, mycorrhizas, *Nothofagus* forests.

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*Corresponding author: cbarroetavena@ciefap.org.ar

Introduction

Pseudotsuga menziesii (Mirb.) Blanco is one of the most widely planted exotic Pinaceae in NW Patagonia, Argentina, valued for its timber quality and growth rate (Andenmatten et al. 2002). Previous studies have shown the predominance of non-native ecto- (EM) and ectendo- (ECM) mycorrhiza associated with *Ps. menziesii* in Patagonia (Barroetaveña 2004, Barroetaveña & Rajchenberg 2003). All the reported species belong to the large EM biota associated with this tree in its original distribution range (Barroetaveña et al. 2007), confirming the essential role of the EM symbiosis in the establishment and growth of *Ps. menziesii* (Trappe & Strand 1969, Wright 1971). On the other hand, the presence of AM has been reported for *Ps. menziesii* in native forests (Cázares & Trappe 1993) and it has been postulated that AM fungi colonize cortical cells before EM fungi (Cázares & Smith 1996), although no evidence was presented that such succession occurs.

AM fungi use different colonization strategies in root tissues, defined as *Arum-* and *Paris*-types (Dickson et al. 2007, Gallaud 1905) and, generally, the plant genome, and not the AM fungal genome, has main control over AM morphology (Barrett 1958, Gerdemann 1965, Jacquelinet-Jeanmougin & Gianinazzi-Pearson 1983, Smith & Smith 1997, Bedini et al. 2000, Kubota et al. 2005). Nevertheless, there are cases where different AM fungi produced variations in structure in the same host (Abbott 1982, Demuth et al. 1991, Cavagnaro et al. 2001a, Smith et al. 2004, Harikumar & Potty 2009). In *Arum*-type, development of hyphae occurs along cortical and intercellular air spaces, followed by penetration and formation of individual and terminal intracellular arbuscules. In *Paris*-type the fungus grows entirely intracellularly, forming irregular coiled hyphae and 'arbusculate coils' (Gallaud 1905, Smith & Read 2008).

Given the invasive characteristics of *Ps. menziesii* (Núñez et al. 2008; Orellana & Raffaele 2010, 2012; Richardson et al. 2008; Sarasola et al. 2006), afforestation with this tree in Patagonia begins to generate negative impacts on native vegetation by displacement or invasion of neighboring native forests, largely dominated by *Nothofagus* spp. Among all the factors that govern this process in native forests and grasslands of Patagonia, the role and dynamics of the mutualistic associations (mycorrhizas) have received little attention (Núñez et al. 2009, Salgado Salomón et al. 2011). Few studies on the presence, abundance, and colonization strategies of AM in invading *Ps. menziesii* seedlings have been conducted (Salgado Salomón et al. 2013).

The aim of this work was to evaluate the AM status of invasive *Ps. menziesii* seedlings growing in *Nothofagus-Ps. menziesii* matrices considering AM colonization percentage and AM types, and to analyze the evolution of AM colonization with seedlings' age in relation to EM colonization. Clarifying this issue will help us to understand the role of AM during the invasion process.

Materials and methods

Sampling sites: The study was conducted in six *Ps. menziesii* plantation - *Nothofagus* forest matrixes in NW Patagonia, Argentina, located in the deciduous forest District, Sub-Antarctic Province, Sub-Antarctic Domain (Cabrera & Willink 1980) as follows: one study site with *Nothofagus alpina* (Phil.)

Krasser - *N. obliqua* (Mirb.) Oerst. - *Ps. menziesii* (Est. **Quechuquina,** GPS point: lat.: -40.15, long.: -71.59), two study sites with *N. dombeyi* (Mirb.) Oerst. - *Ps. menziesii* (**Isla Victoria**, GPS point: lat.: -40.97, long.: -71.53; Est. **Newmeyer,** GPS point: lat.: -40.12, long.: -71.33), and three study sites with *N. antarctica* (G.Forster) Oerst. - *Ps. menziesii* (**Corcovado**, GPS point: lat.: -43.63, long.: -71.44; **Foyel,** GPS point: lat.: -41.67, long.: -71.45; **ENFORSA,** GPS point: lat.: -41.23, long.: -71.42). The area has perennial forests dominated by *Nothofagus* species, mixed with *Luma apiculata* (DC.) Burret., *Chusquea culeou* E.Desv., *Berberis* sp. *and Schinus patagonicus* (Phil.) I.M.Johnst. ex Cabrera as main accompanying species. For more details see Salgado Salomón et al. (2013).

Sᴇᴇᴅʟɪɴɢs sᴀᴍᴘʟɪɴɢ: Each site was considered as a block. A transect was established at each site, along the Effective Recruitment Area (ERA) (Sarasola et al. 2006). Twenty five invasive, between <1 to 5 years old seedlings were selected, sampled and kept in brand-new plastic bags. Seedlings were separated by age in classes <1, 1, 2, 3, 4 and 5 years old, counting rings at the neck when processed. Seedlings measurements included stem height (H, from apex to collar) and stem collar diameter (CD, measured at ground level), seedling root system length (RL, sum of principal and lateral root lengths).

Cʟᴇᴀʀɪɴɢ ᴀɴᴅ sᴛᴀɪɴɪɴɢ: Within a week after being sampled, *Ps. menziesii* seedlings were carefully washed in running tap water. Root samples were cut into 10 mm lengths (approx. 600 mg per seedling) to fit in Tissue-Tek plastic capsules (Fisher Scientific Co., Pittsburgh, PA), cleared in 10% KOH for 30 min at 100°C under water bath and 15% H_2O_2 for 12 h at room temperature. Cleared samples were immersed 60 min at 4°C in a staining solution of 0.05% trypan-blue in lactoglycerol, rinsed with tap water and stored in lactoglycerol at 4°C until microscopic examination (Cázares & Smith 1996, Cázares & Trappe 1993).

Mʏᴄᴏʀʀʜɪᴢᴀʟ sᴛᴀᴛᴜs ᴇᴠᴀʟᴜᴀᴛɪᴏɴ: Percentage of AM colonization for each seedling was estimated following Brundrett et al. (1996), using the complete root system. Each positive intersection root fragment was microscopically analyzed in order to check the presence of intercellular/intracellular hyphae, arbuscules (dichotomously branched, haustorium-like structures arising from intercellular hyphae), hyphal coils, arbuscular coils (hyphal coils with fine arbuscule-like branches on their surfaces), spores and vesicles. AM types were classified following Brundrett et al. (1996), Oehl et al. (2008) and Smith & Smith (1997). Seedlings with *Arum*- and *Paris*-types structures in the same root system and/or with intermediate structures between both types (following Dickson 2004) were called "Both". The incidence of AM types was quantified as the proportion of presence / absence of *Paris*-, *Arum*- and Both types by age class.

EM-ECM colonization percentages were jointly quantified in order to relate their abundance with AM colonization percentage and types. Complete, washed root systems from all seedlings were evaluated under a dissecting microscope following Brundrett et al. (1996).

Sᴛᴀᴛɪsᴛɪᴄᴀʟ ᴀɴᴀʟʏsɪs: AM and EM colonization percentages (AM%, EM%) and AM types between age classes did not meet the assumptions of normal distribution and equal variances using Shapiro Wilk and Levenne tests, respectively (Everitt 2005). Therefore, differences in AM% and AM types incidence between age classes were analyzed by generalized linear mixed models (GLMM) with a restricted maximum likelihood estimation method, and sites were treated as blocks and incorporated as a random effect; subsequent comparison with DGC test (exclusive groups formation test) were performed in R for R-DCOM (Di Rienzo et al. 2002, 2010).

All the analyses were performed at 0.05 significance level, with the statistical package InfoStat for Windows version 2011 (Di Rienzo et al. 2011).

Results

AM type incidences showed significant differences between age classes (p=0.0015, GLMM, Fig. 1). Two or less years old seedlings showed significantly higher values of *Arum*-type incidence. Three and five years old seedlings showed significantly higher *Paris*-type incidence, but 5 years old seedlings did not present Both-type. Four years old seedlings showed a significantly higher Both-type incidence (Fig. 1). Diversity
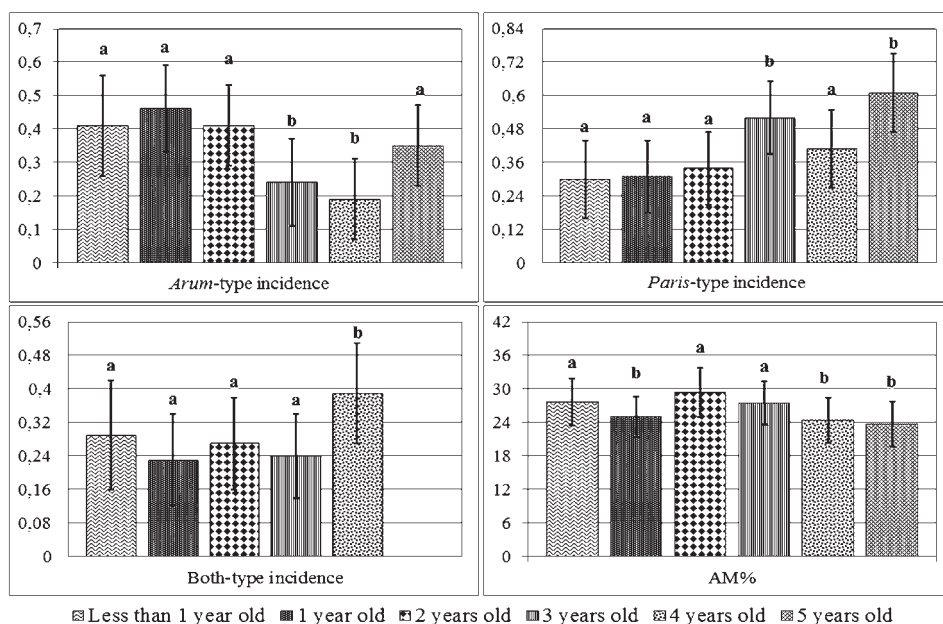
Fig. 1. Statistical analysis of AM% and AM types incidence in *Ps. menziesii* seedlings. Bars indicate the standard error. Different letters indicate significant differences (p<0.05, GLMM).

of structures, i.e. vesicle sizes, presence/absence of arbuscules and 'coils', intra- vs. extracellular hyphae, and microsclerotia (Fig. 2), could easily be separated into *Arum*- and *Paris*-types (Table 1).

Seedlings showed AM colonization percentages (AM%) with values ranging from 23.7–29.4% (Table 1). Significant differences in AM% between seedlings from different age classes were found (p<0.0001, GLMM). Less than one year old, 2 and 3 years old seedlings had higher AM% compared with 1, 4 and 5 years old (Fig. 1). One year old seedlings had the lowest values, possibly because the samples showed very small and atypical root systems.

Ecto- + ectendo-mycorrhizal colonization percentages ranged from 74–87% (Table 1). There were significant differences in EM+ECM% between seedlings from different age classes (p=0.04, GLMM). Less than one year old seedlings had the lowest EM + ECM% compared with 1, 2, 3, 4 and 5 years old seedlings (Fig. 3). EM and ECM presented the same morphotypes as reported in previous studies (Barroetaveña 2004, Barroetaveña & Rajchenberg 2003), and we do no further deal with the later data here.

At all sites and ages there was a constant presence of Dark Septate Endophytes (DSE), with different structures and colonization forms. Typical DSE hyphae were 2–3 µm wide, and DSE microesclerotia of different sizes (50–120 µm in diam.) were observed.

Table 1. Seedling measurements, AM and EM colonization percentage (AM%, EM%) and incidence of different AM-type by age classes.

Sites	Seedling age	CD[1]	SH[2]	RL[3]	AM%	*Arum*-type (%)	*Paris*-type (%)	Both-type (%)	EM%
	<1	4	25.5	89.3	41.4	100.0	0.0	0.0	74.12
	1	6.67	32.3	101	30.8	33.33	33.33	33.33	77.65
	2	7.33	37.6	176	41.7	83.0	17.0	0.0	78.16
Corcovado	3	9.17	59.8	272	38.6	17.0	83.0	0.0	79.66
	4	10.9	52.9	456	37.8	60.0	40.0	0.0	81.62
	5	17.4	61.3	600	32.1	100.0	0.0	0.0	83.08
	<1	2.5	16.5	28	31.9	50.0	50.0	0.0	68.54
	1	5.5	24.8	103	31.3	50.0	0.0	50.0	93.8
	2	7.2	32.7	106	24.5	20.0	60.0	20.0	87.73
ENFORSA	3	7.83	43.2	146	22.3	33.0	67.0	0.0	92
	4	10	54.2	125	32.8	0.0	33.0	67.0	91.5
	5	10.7	58.8	206	26.9	0.0	100.0	0.0	88.53
	<1	2	14	30	33.3	0.0	0.0	100.0	68.18
	1	4.86	26.5	72	33.5	67.0	33.0	0.0	80.56
	2	5.33	36.3	59.7	37.7	50.0	17.0	33.0	84.31
Foyel	3	7.6	45.6	433	42.3	40.0	0.0	60.0	92.94
	4	10	65.1	202	15.1	0.0	20.0	80.0	84.95
	5	16	86.5	221	5.77	0.0	100.0	0.0	96.45
	<1	2.5	14.8	28	16.1	0.0	100.0	0.0	68.75
	1	3.88	19.9	74.8	19.3	25.0	75.0	0.0	84.94
	2	5.83	30	97.8	22.5	17.0	67.0	17.0	76.84
Isla Victoria	3	8.25	37.9	134	26.2	25.0	75.0	0.0	83.96
	4	9.33	51.3	152	22.7	0.0	67.0	33.0	89.23
	5	8.67	50.5	185	23.4	0.0	100.0	0.0	78.94
	<1	4	36.4	98	28.5	33.0	0.0	67.0	80.29
	1	5	45.7	109	18.3	50.0	0.0	50.0	82.38
Est.	2	6	53.2	91	39.7	0.0	0.0	100.0	85.19
Newmeyer	3	6.17	50.5	135	18.7	33.0	17.0	50.0	87.35
	4	7.33	57.6	157	29.8	33.33	33.33	33.33	88.41
	5	8.33	74.5	221	25.6	67.0	33.0	0.0	74.63
	<1	2.5	21.7	70.7	14.7	33.33	33.33	33.33	75.52
	1	3.5	32.9	86.3	15.9	33.0	17.0	50.0	87.08
Est. Quechu-	2	4.67	40.3	155	17.1	33.33	33.33	33.33	96.94
quina	3	6.67	65.2	195	20.5	0.0	67.0	33.0	79.39
	4	7.75	64.7	164	15	25.0	50.0	25.0	86.71
	5	9.17	85.8	209	14.7	0.0	100.0	0.0	88.28

[1]Stem collar diameter (mm). [2]Stem height (cm). [3]Root system length (cm).
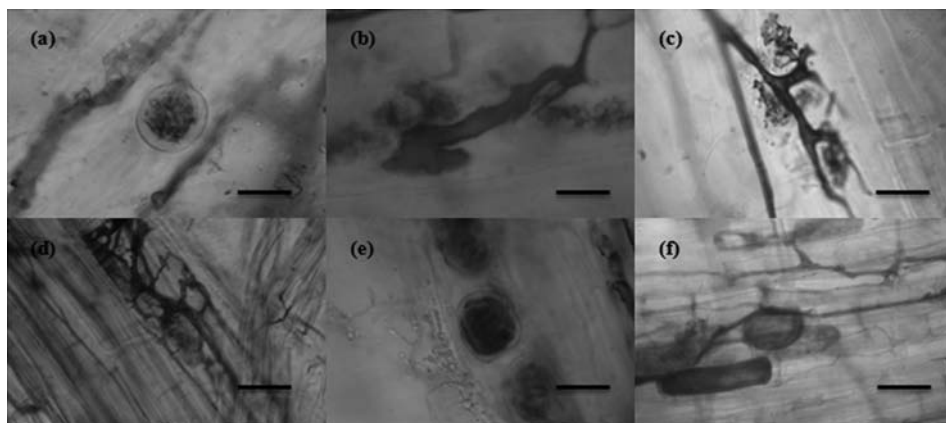
Fig. 2. AM structures. a, b and c: *Arum*-type. a. arbuscule and intercellular hyphae. b. arbuscules, irregular vesicles and intercellular hyphae. c. arbuscule. d, e and f: *Paris*-type. d. irregular coiled hypha and intracellular hyphae. e. Non-terminal arbuscules, named 'arbusculate coils' (Cavagnaro et al. 2001a). f. Intracellular hyphal net and vesicles. (Bars = 10 μm). Pictures b and e belong to the same root system.

All seedlings presented up to 4 types of mutualistic associations in the same plant: AM, EM, ECM and DSE.

Discussion

AM colonization of *Ps. menziesii* seedlings showed the co-occurrence of *Paris*- and *Arum*-types in the same root system and the presence of intermediate structures. Hitherto, this has only been reported for tomatoes, squash, potatoes and some herbs (Matekwor Ahulu et al. 2005, Cavagnaro et al. 2001b, Harikumar & Potty 2009, Kubota et al. 2005, Urcelay et al. 2005) and, so far, it has not been reported for conifers. Dickson et al. (2007) showed that, except few cases, gymnosperms typically form *Paris*-type structures that are also abundant in angiosperms in the wild. They reported *Arum*-type and 'intermediate' structures as rare in tree species and frequent only in grasses and legumes.

Previous studies dealing with AM fungi associated with *Ps. menziesii* only reported *Arum*-type colonization (Cázares & Smith 1996, Cázares & Trappe 1993, Smith et al. 1998). These studies also indicated that AM colonization might have a secondary role in the mycorrhizal assemblage, as nutrient exchange structures such as arbuscules were lacking (Smith & Read 2008). In this work we report the presence of coils, intracellular hyphae and arbuscules, which indicates that an active symbiosis occurs.

From an ecological point of view it can be underlined that seedlings from all sites developed *Paris*-, *Arum*- and Both-types, indicating that these structures can be formed and coexist in a wide range of situations. Arbuscular mycorrhiza types seem to have
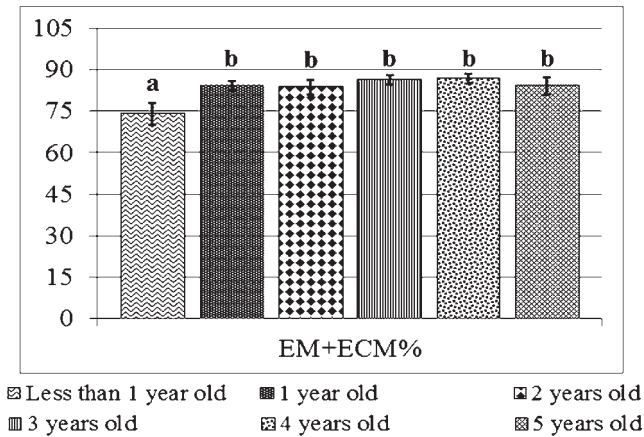
Fig. 3. Statistical analysis of EM + ECM% in *Ps. menziesii* seedlings. Bars indicate the standard error. Different letters indicate significant differences (p<0.05, GLMM)

different strategies in nutrient transfer (Dickson & Kolesik 1999), possibly different rates in P uptake efficiency (Peterson & Massicotte 2004) or C allocation (Dickson et al. 2007), and also different enzymatic activities (van Aarle et al. 2005) or plant growth-related responses (Peterson & Massicotte 2004). For tomatoes it was reported that *Paris*-type can increase the expression of some defense-related genes, which are suppressed in presence of *Arum*-type structures (Gao 2002; Gao et al. 2004). Dickson et al. (2007) proposed various factors that might determine which AM type is formed. While recognizing that the plant genome could have most prominence in this event, a possible role of AM fungal genome has to be included, along with plant/AM fungus/ site interactions (e.g. light, temperature, soil compaction, soil temperature, water-content, pH and nutrient supply). Although the identity of AM fungi is unknown, the observed differences in AM colonization may confer further strategies to invade *Nothofagus* forests.

The fact that 2 or less years old seedlings showed significantly higher values of *Arum*-type incidence compared with 3–5 years old seedlings could indicate that younger seedlings form *Arum*-type and then gradually, through hybrid structures (Intermediate), form *Paris*-type. In this sense, Twahir & Tibuhwa (2013) had hypothesized that *Arum*-type was dominant in the fast growing vegetation and may be useful in reforestation compared to *Paris*-type.

Both EM and AM were always present in seedlings of all age classes and sites. It is possible that, together with some EM species, AM fungi have a role similar to "early-stage" EM species (Agerer 2001), at least during the first 5 years. The facts that AM% was significantly higher in seedlings less than 2 years old and that EM% was significantly lower in seedlings less than 1 year old, suggest a pioneering role of AM fungi in invasive seedlings, based on their greatest contribution in the seedlings' early establishment phase.

Cázares & Smith (1996) proposed that AM fungi that colonize typically EM hosts do it at early plant stages, in areas where EM inoculum is scarce or absent and that later on, by succession, the EM association is established. In this work, however, seedlings from all age classes showed high EM% (over 68.8%), including those less than one year old. Endomycorrhizas presented moderate colonization percentages (over 23.7%), varying in a very narrow range, although with values significantly lower for more than 3 years old seedlings. Thus, and contrary to our expectations, we did not find a clear AM-EM colonization succession of *Ps. menziesii* seedlings invading *Nothofagus* forests in Patagonia, as both AM and EM colonization coexist from the beginning of seedlings' establishment. The coexistence seems more associated with *Ps. menziesii* plasticity than with a succession process.

Kropp & Trappe (1982) suggested that some tree species could adapt to the mycorrhizal fungi from the understory (shrubs and herbs) to establish new seedlings. This fits perfectly with *Ps. menziesii* in Patagonia where, in order to invade new environments, the species would be able to associate with mycorrhizal fungi from native understory herbs and shrubs, typically AM fungi (Fontenla et al. 1998).

Dark Septate Endophytes were reported previously for *Ps. menziesii* (Cázares & Smith 1996) and other conifers, mainly *Pinus* spp. (Cázares & Trappe 1993, Grünig et al. 2008, Peterson et al. 2008, Usuki & Narisawa 2007, Wagg et al. 2008), and in various environmental types (Mandyam & Jumpponen 2005). It has been suggested that DSE might be nutritionally important for plants (Jumpponen 2001, Barrow & Osuna 2002). Newsham (2011) showed that DSE promote mineralization of organic N to inorganic N forms available for plants. Moreover, DSE may increase plant biomass and N and P tissue contents (Alberton et al. 2010, Newsham 2011, Newsham et al. 2009).

Overall, this study shows that invasive *Ps. menziesii* seedlings are able to develop a great plasticity regarding mutualistic associations, which certainly is related with the capability to succeed in the invasion process. Integrative studies aiming to determine the specific AM and DSE species associated are needed in order to establish their origin, either native or exotic, for this exotic tree species in Patagonia.

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