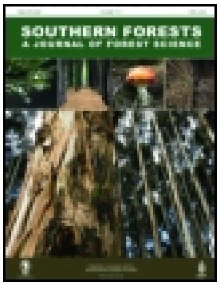

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María E Salgado Salomón [a c], Carolina Barroetaveña [a b c] & Mario Rajchenberg [a b c]
[a] Ingeniería Forestal UNPSJB, Sarmiento 849, Esquel, Chubut, Argentina
[b] Centro Forestal CIEFAP, CC 14, 9200, Esquel, Chubut, Argentina
[c] Concejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, C1033AAJ, Buenos Aires, Argentina
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Occurrence of dark septate endophytes in *Nothofagus* seedlings from Patagonia, Argentina

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

[1] *Ingeniería Forestal UNPSJB, Sarmiento 849, Esquel, Chubut, Argentina*
[2] *Centro Forestal CIEFAP, CC 14, 9200 Esquel, Chubut, Argentina*
[3] *Concejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, C1033AAJ Buenos Aires, Argentina*
* *Corresponding author, e-mail: mesalgadosalomon@gmail.com*

Temperate forests of southern Argentina and Chile cover a wide latitudinal and altitudinal range on both sides of the Andes, with *Nothofagus* species being one of the main tree species. Most of the larger remnants are protected within the limits of national parks and forest reserves. However, some of these are invaded by exotic conifers such as *Pseudotsuga menziesii*. In order to better understand the mycorrhizal condition within the *Nothofagus*–*P. menziesii* interaction, transects were established at six study sites along the effective recruitment area. Three treatments were established: *Nothofagus* forest without *P. menziesii* invasion (Forest), *Nothofagus*–*P. menziesii* matrix (Interface) and *P. menziesii* plantation lacking *Nothofagus* specimens (Plantation). A 2 kg mixed soil sample was taken at each treatment site and kept in brand-new plastic bags. A soil bioassay with *Nothofagus* seedlings acting as baits was set to evaluate the mycorrhizal inoculum potential and soil-fungi composition at each sampling treatment. Forty percent of the evaluated seedlings were found to be colonised by dark septate endophytes (DSE) fungi. Ectomycorrhizal colonisation percentage was significantly higher in plants growing in Forest soils (mean = 57.77%) when compared with Interface (mean = 42.53%) and Plantation (mean = 44.65%). The high incidence of DSE in young roots of *Nothofagus* seedlings in this study supports the hypothesis that DSE might be pioneering colonisers of young tree seedlings in secondary successional environments. For future interventions in the forest, either with productive or protective intentions, it should be taken into account the incidence of these fungi, which may have an important positive role in *Nothofagus* forest post-invasion recovery.

Keywords: dark septate endophytes, invasion, mycorrhizae, *Pseudotsuga menziesii*

Introduction

Temperate forests of southern Argentina and Chile cover a wide latitudinal and altitudinal range on both sides of the Andes, with several species of *Nothofagus* Blume (Nothofagaceae) as the main components (Donoso et al. 2004). Since 1922 these endemic species have been protected in Argentina because of their high economic and ecological value (Veblen et al. 1996, Gallo et al. 2009), although their original area has been dramatically reduced because of uncontrolled logging and burning regimes (Kitzberger and Veblen 1997). Many of these altered stands present small to large invaded areas with exotic conifers such as *Pseudotsuga menziesii* (Mirb.) Blanco.

Patagonian *Nothofagus* form symbiotic associations with ectomycorrhizal (EM) fungi, but neither arbuscular mycorrhizae (AM) nor other root–fungal associations have been reported (Singer and Morello 1960, Garrido 1985, Peredo 1987, Palfner and Godoy 1996a, 1996b, Godoy and Palfner 1997, Fontenla et al. 1998, Diehl 2005). On the other hand, the largely planted *P. menziesii* forms EM and AM associations at the seedling stage (Cázares and Trappe 1993, Cázares and Smith 1996, Horton et al. 1998).

Fungi forming dematiaceous, septate hyphae in plant roots and usually referred to as dark septate endophytes (DSE) have been reported in a wide range of terrestrial ecosystems (Newsham et al. 2009) associated with c. 600 plant species (Jumpponen and Trappe 1998, Jumpponen 2001), including EM tree taxa such as many conifers (Cázares and Trappe 1993, Usuki and Narisawa 2007, Grünig et al. 2008, Peterson et al. 2008, Wagg et al. 2008). As DSE prevail in highly stressed environments (Mandyam and Jumpponen 2005), *Nothofagus* forests from Patagonia exposed to severe water stress during summer are potential candidates to form this association. On the other hand, the influence of invasive *P. menziesii* could produce a stressful environment for *Nothofagus*, modifying the soil community and microenvironment and creating suitable conditions for the association in the roots (Klironomos 2002, Niu et al. 2007, Dehlin et al. 2008).

The aims of this work were to jointly assess the incidence of DSE, AM and EM in *Nothofagus* spp. seedlings from Patagonia, Argentina, and to compare their incidence in seedlings grown in soils from pure *Nothofagus* forests, *Nothofagus* forests invaded by *P. menziesii*, and *P. menziesii* plantations.

Materials and methods

Soil bioassay setup

A soil bioassay with seedlings acting as baits was set up to evaluate mycorrhizal inoculum potential and DSE presence at six sites with *P. menziesii* plantations adjacent to *Nothofagus* spp. forests. Soil sampling at each site was carried out along a transect including three treatments: *Nothofagus* forest without *P. menziesii* invasion (Forest), *Nothofagus–P. menziesii* matrix (Interface) and *P. menziesii* plantation lacking *Nothofagus* specimens (Plantation). A mixed soil sample of about 2 kg was taken at each sampling unit and kept in brand-new plastic bags. The study sites were located in north-western Patagonia, Argentina, in habitats belonging to the Deciduous Forest District, Sub-Antarctic Province, Sub-Antarctic Domain (Cabrera and Willink 1980) as follows: one study site with *Nothofagus alpina* (Phil.) Krasser–*N. obliqua* (Mirb.) Oerst. (Quechuquina, 40.15° S, 71.59° W), two study sites with *N. dombeyi* (Mirb.) Oerst. (Isla Victoria, 40.97° S, 71.53° W; Newmeyer, 40.12° S, 71.33° W), and three study sites with *N. antarctica* (G.Forst.) Oerst. (Corcovado, 43.63° S, 71.44° W; Foyel, 41.67° S, 71.45° E; ENFORSA, 41.23° S, 71.42° W).

Nothofagus dombeyi, *N. antarctica*, *N. alpina* and *N. obliqua* seeds of commercial quality were cleaned and conditioned in accordance with Willan (1991), and stratified for 24 h in water and then at 4 °C for 60 d in a refrigerator. Sterile seedlings were obtained in a growth chamber at 17/19 °C, 48–55% relative air humidity, and a 16 h-photoperiod with 1 400 lux radiation for 30 d.

Clean, 250 cm^3 flower pots were filled with a 1:1 (v/v) mix of soil obtained from each sampling unit and sterilised pumicite (oven, 120 °C for 48 h). As a control, 10 flower pots for each *Nothofagus* species were filled with sterilised soil (mixing soil from each treatment from all sites having that species) autoclaved at 1.2 Atm for 30 min., mixed (1:1, v/v) with sterilised pumicite (oven, 120 °C for 48 h; van der Heijden et al. 1998) in order to check for inoculum contamination through watering or other sources. Seedlings grown in sterilised pumicite (oven, 120 °C for 48 h) were transplanted into flower pots comprising 10 replicates per treatment, and three treatments per site (Forest, Interface and Plantation); in total, 180 seedlings were planted. Seedlings were grown for 12 months in a greenhouse devoted exclusively to this experiment and watered regularly according to weather conditions with water obtained directly from a well through pipes, in order to minimise mycorrhizal inoculum contamination.

Clearing and staining

Seedlings were carefully washed with running tap water. Complete root systems from all surviving seedlings were examined under a stereoscopic microscope to quantify EM colonisation, and each EM root tip was excised and studied. The remaining portions of each root system (approximately 400 mg per seedling) were cut into 20-millimetre-long segments to fit into Tissue-Tek plastic capsules (Fisher Scientific Co., Pittsburgh, PA, USA), and cleared in 10% KOH for 15 min at 100 °C in a water bath and then in 15% H_2O_2 for 3 h at room temperature. Cleared samples were immersed in a staining solution of 0.5% trypan-blue in lactoglycerol for 30 min at 4 °C, rinsed with tap water and stored in lactoglycerol at 4 °C until microscopic examination for DSE and AM colonisation (Cázares and Trappe 1993, Cázares and Smith 1996).

Mycorrhizal status evaluation

Percentage EM colonisation (EM%) was calculated as the proportion of EM root tips over total root tips from complete root systems. Percentage AM colonisation (AM%) was estimated using the gridline interception method (Brundrett et al. 1996). Incidence of DSE was estimated as the proportion of plants per treatment presenting diagnostic DSE structures (microsclerotia and melanised, septate hyphae, both using all of the remaining root system).

Statistical analysis

Percentages of AM colonisation (AM%), EM% and DSE incidence between treatments did not meet the assumptions of normal distribution and equal variances using the Shapiro-Wilk and Levenne tests, respectively (Everitt 2005). Differences in EM% between treatments were analysed by non-parametric analysis of variance (ANOVA) with the Friedman test. Sites were treated as blocks and different plant cover (Plantation, Interface and Forest) as treatments (Di Rienzo et al. 2010). Differences in DSE incidence between treatments were analysed with generalised linear mixed models (GLMM) with a restricted maximum likelihood estimation method, with sites treated as blocks and incorporated as a random effect; subsequent comparison with the DGC test (exclusive groups formation test) was performed (Di Rienzo et al. 2002) in R with R-DCOM (Di Rienzo et al. 2010). Sites with less than three seedlings per treatment (because of mortality, see below) were not included in the statistical analysis.

Table 1: Incidence of dark septate endophytes (DSE) and ectomycorrhizal colonisation percentage (EM%) of *Nothofagus* seedlings. NS = No surviving seedling per treatment

Site	Treatment	No. of surviving seedlings	Incidence of DSE (%)	EM%
Corcovado	Forest	7	42.86	45.26
	Interface	3	33.33	29.58
	Plantation	9	0.00	40.29
Foyel	Forest	6	66.67	43.73
	Interface	1	100.00	73.66
	Plantation	8	25.00	50.61
ENFORSA	Forest	9	44.44	59.12
	Interface	7	57.00	48.65
	Plantation	10	60.00	45.96
Isla Victoria	Forest	9	44.40	63.00
	Interface	3	100.00	40.81
	Plantation	4	50.00	37.37
Quechuquina	Forest	1	100.00	69.21
	Interface	7	85.71	60.09
	Plantation	0	NS	NS
Newmeyer	Forest	8	12.50	63.68
	Interface	6	0.00	51.08
	Plantation	8	50.00	54.96
Control seedlings	*N. antarctica*	4	25.00	20.74
	N. alpina	0	NS	NS
	N. dombeyi	8	0.00	34.52
	N. obliqua	0	NS	NS

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

Results

Forty-four percent of the evaluated seedlings were colonised by DSE fungi (Table 1). Melanised, septate, intercellular hyphae and microsclerotia were observed in fine, fresh roots (Figure 1), but never in senescent roots. Incidence of DSE structures was variable between sites (Table 1) and significantly different between treatments (DGC test, $p = 0.0002$), Interface and Plantation being significantly higher than Forest. Only one control seedling showed presence of DSE (Figure 2).

Ectomycorrhizal colonisation percentages varied significantly between treatments (Friedman test, $T^2 = 9$, $p = 0.0156$), Forests showing significantly higher values (mean = 57.77%) than Interface (mean = 42.53%) and Plantation (mean = 44.65%) (Figure 2, Tables 1 and 2). Arbuscular mycorrhizal colonisation was not detected in any treatment or control seedlings.

There was a high mortality of seedlings that was unevenly distributed, with some treatments and sites more affected than others (see Table 1). Plantation at the Quechuquina site and the *N. alpina* and *N. obliqua* controls were the most extreme cases with no surviving seedlings recorded.

Discussion

This is the first record of DSE presence in *Nothofagus* spp. DSE have been reported in only a few Fagales (Jumpponen and Trappe 1998), and are suggested to be pioneering colonisers of young tree seedlings in secondary successional environments (Horton et al. 1998, Kernaghan et al. 2003) and in the earliest stages of primary succession (Jumpponen 2003, Cázares et al. 2005). This could explain the high incidence of DSE in young roots of *Nothofagus* seedlings in our study.

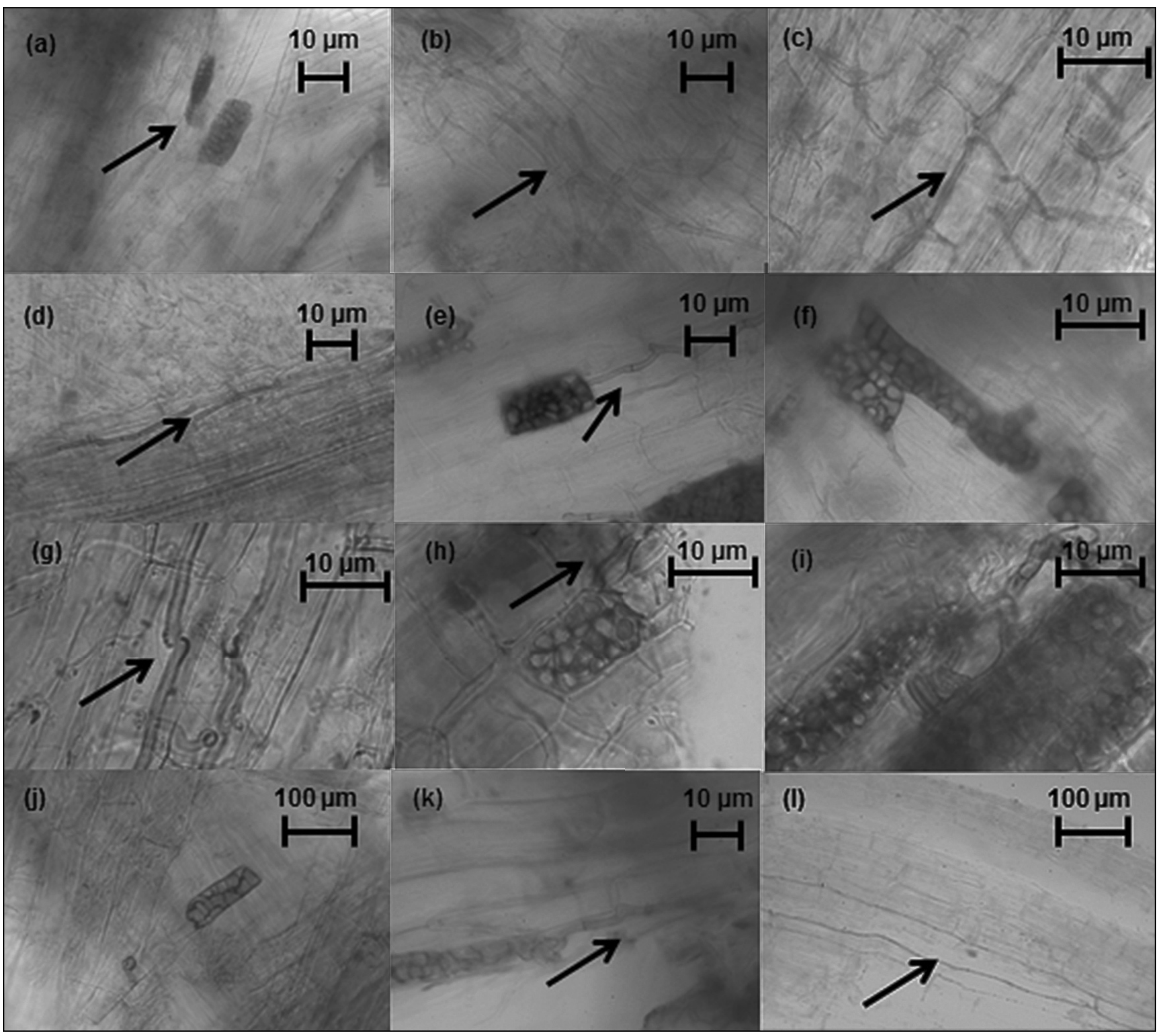

Figure 1: Dark septate endophyte (DSE) structures. (a–c) Typical DSE hyphae and microsclerotia in *Nothofagus antarctica*. (d–f) Typical DSE hyphae and microsclerotia in *N. dombeyi*. (g–i) Typical DSE hyphae and microsclerotia in *N. alpina*. (j–l) Typical DSE hyphae and microsclerotia in *N. obliqua*. Arrows indicate DSE hyphae

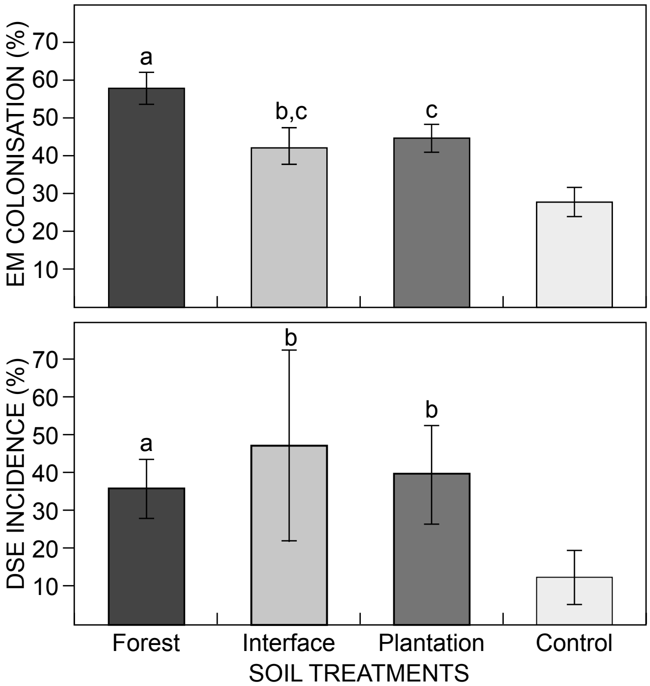

Figure 2: Percentage ectomycorrhizal mycorrhizae (EM) colonisation and dark septate endophyte (DSE) incidence in *Nothofagus* seedlings growing in different soil treatments. Bars represent the SE. Different letters indicate a significant difference between treatments ($p = 0.05$)

When the distribution of DSE incidence between treatments was analysed, a pattern of higher incidence in Interface and Plantation than in Forest was detected. This tendency agrees with previous reports showing that stressful sites, including high latitudes or altitudes, increase the presence of DSE in plants (Read and Haselwandter 1981, Gardes and Dahlberg 1996, Newsham et al. 2009, Roberts et al. 2009). In this study, *Nothofagus* seedlings growing in areas surrounded by invading *P. menziesii* showed an increased association with DSE, which could be hypothesised to be a response to a stressful situation. In contrast, EM colonisation showed a decreasing pattern from its own soil (Forest) to invaded (Interface) or planted (Plantation) with *P. menziesii* sites, which suggested that *Nothofagus* seedlings do not share EM fungi with this conifer, as Dickie et al. (2010) reported for *Nothofagus* growing in a *Pinus radiata* D.Don invaded forest in New Zealand. In agreement with all previous studies, our results could indicate that the stress produced by the lack of EM symbionts or by a presumed allelopathic effect produced by the invasive *P. menziesii* (see below) promotes a higher DSE incidence in *Nothofagus* seedlings growing together with that conifer.

High seedling mortality, concentrated in some specific treatments but present in all *Nothofagus* species, could be due to (a) soil treatment deleterious effects, either chemical (through toxicity) or biological (through pathogens), (b) low colonisation by the studied DSE/EM fungi, or (c) an interactive effect between (a) and (b). In this sense, a hypothesis of an allelopathic effect produced by the presence of *P. menziesii* on *Nothofagus* seedling development could be supported by the fact that regeneration of *Nothofagus* in

Table 2: Non-parametric ANOVA (Friedman test) results. Means with the same letters are not significantly different ($p = 0.050$)

Treatment	Sum (ranks)	Mean (ranks)	*n* (sites)	Significance
Plantation	6	1.5	4	a
Interface	6	1.5	4	a,b
Forest	12	3.0	4	c

these invaded areas was never registered during a 4-year sampling (2008–2011) performed for a separate study. On the other hand, the fact that surviving seedlings from treatments with high mortality (cf. Table 1, Isla Victoria and Foyel Interfaces and Quechuquina Forest) were all intensively colonised by DSE could indicate that these fungi provide an important set of benefits as recorded for other species, such as protection against water, heat or cold stress (Rodriguez et al. 2008, Newsham et al. 2009, Newsham 2011), protection against root pathogens (Smith and Read 2008), and increased shoot of P and N (Haselwandter and Read 1982, Jumpponen et al. 1998) which could have helped to prevent their mortality.

Conclusions

This is the first report of DSE presence in *Nothofagus* seedling roots. A high incidence in Interface seedlings was detected, which indicated that DSE fungi may have a protective role against possible deleterious effects by invasive *P. menziesii* and also may have an important role in *Nothofagus* forest post-invasion recovery. However, this study is a preliminary approach and future research needs to include a higher number of samples, identification of fungal species and an increased number of sites.

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