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Effect of ectomycorrhizal colonization and drought on reactive oxygen species metabolism of *Nothofagus dombeyi* roots

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Summary Infection with ectomycorrhizal fungi can increase the ability of plants to resist drought stress through morphophysiological and biochemical mechanisms. However, the metabolism of antioxidative enzyme activities in the ectomycorrhizal symbiosis remains poorly understood. This study investigated biomass production, reactive oxygen metabolism (hydrogen peroxide and malondialdehyde concentration) and antioxidant enzyme activity (superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) in pure cultures of the ectomycorrhizal fungi *Descolea antartica* Sing. and *Pisolithus tinctorius* (Pers.) Coker & Couch, and non-mycorrhizal and mycorrhizal roots of *Nothofagus dombeyi* (Mirb.) roots under well-watered conditions and drought conditions (DC). The studied ectomycorrhizal fungi regulated their antioxidative enzyme metabolism differentially in response to drought, resulting in cellular damage in *D. antartica* but not in *P. tinctorius*. Ectomycorrhizal inoculation and water treatment had a significant effect on all parameters studied, including relative water content of the plant. As such, *N. dombeyi* plants in symbiosis experienced a lower oxidative stress effect than non-mycorrhizal plants under DC. Additionally, ectomycorrhizal *N. dombeyi* roots showed a greater antioxidant enzyme activity relative to non-mycorrhizal roots, an effect which was further expressed under DC. The association between the non-specific *P. tinctorius* and *N. dombeyi* had a more effective reactive oxygen species (ROS) metabolism than the specific *D. antartica–N. dombeyi* symbiosis. We conclude that the combination of effective ROS prevention and ROS detoxification by ectomycorrhizal plants resulted in reduced cellular damage and increased plant growth relative to non-mycorrhizal plants under drought.

Keywords: ascorbate peroxidase, catalase, glutathione reductase, pristine forest, superoxide dismutase, volcanic soil.

Introduction

A changing environment induces a series of plant stresses and associated responses to adapt to the newly established conditions. As such, shifting soil water contents from well-watered conditions (WC) to drought conditions (DC) causes a series of reactions in plant cells; for example, stomatal closure that limits CO_2 fixation and reduced $NADP^+$ generation in plant cells (Satoh and Murata 1998, Wilkinson and Davies 2002). Under these conditions, oxygen acts as an electron acceptor, resulting in the formation of superoxide radical ($O_2^{-\bullet}$) (Cadenas 1989). This superoxide radical, and its reduction product hydrogen peroxide (H_2O_2), can subsequently combine by the Haber–Weiss reaction to form the hydroxyl radical ($^{\bullet}OH$) (Sairam et al. 1998). Hence, drought generates excess reactive oxygen species (ROS) in plants (i.e., oxidative stress), which may cause lipid peroxidation, membrane injury, protein and nucleic acid degradation, ultimately leading to cell death (Apel and Hirt 2004). Notwithstanding the adverse effects of excess ROS on plant growth and survival under severe stress conditions, it should be noted that ROS is also produced during normal plant metabolism processes. As such, ROS is formed as a by-product during photosynthesis and respiration (Foyer and Harbinson 1994, Mittler 2002), and certain processes referring to root biology such as rhizobial interaction, gravitropism, root growth and root hair elongation (Joo et al. 2001, Foreman et al. 2003, Matamoros et al. 2003, Liszkay

et al. 2004). Moreover, ROS also participates in the plant defence processes such as programmed cell death (Jacobson 1996) and acts as a signalling molecule to control defence reactions in intermediate stress conditions (Karpinski et al. 1999, Morita et al. 1999). Because of these multiple functions, ROS in plant cells should be carefully regulated by a set of mechanisms to ensure appropriate plant functioning under a broad range of environmental conditions.

Main enzymatic ROS scavenging mechanisms in roots include superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Mittler 2002, Bian and Jiang 2008). SOD acts as the first line of defence against ROS, dismutating superoxide to H_2O_2. CAT and APX subsequently detoxify H_2O_2. While CAT directly forms H_2O and O_2, APX requires an ascorbate and glutathione regeneration system, the ascorbate–glutathione cycle (Apel and Hirt 2004). Detoxifying H_2O_2 to H_2O by APX occurs by oxidation of ascorbate to monodehydroascorbate, which can be regenerated by monodehydroascorbate reductase using NAD(P)H as reducing equivalents. Monodehydroascorbate can spontaneously dismutate into dehydroascorbate. Ascorbate regeneration is mediated by dehydroascorbate reductase driven by the oxidation of glutathione to oxidized glutathione. Finally, glutathione reductase (GR) can regenerate glutathione from oxidized glutathione using NAD(P)H as a reducing agent to close the ascorbate–glutathione cycle. The balance between SOD, CAT and the ascorbate–glutathione cycle is crucial for determining the steady-state level of ROS in plant roots (Apel and Hirt 2004).

Infection with mycorrhizal fungi can increase the ability of plants to resist environmental stress such as drought (Smith and Read 2008). As indicated by Breda et al. (2006), water deficit of plants can be alleviated by mycorrhizal associations due to a variety of morphophysiological and biochemical strategies. Ectomycorrhizal symbioses might improve the water status of seedlings under drought, by means of increased absorbing surface, efficient water conduction through mycelial strands, enhanced hydraulic conductivity at the soil–root interface or hormonal and nutritional effects modifying stomatal regulation of the tree (Boyd et al. 1986, Guehl and Garbaye 1990, Guehl et al. 1992, Querejeta et al. 2003, Marjanovic et al. 2005). However, the role of antioxidative enzyme activity in ectomycorrhizal functioning is poorly understood. Both ectomycorrhizal fungal and plant cells have been shown to express the activity of a specific set of enzymes involved in ROS scavenging mechanisms (Ott et al. 2002, Apel and Hirt 2004, Langenfeld-Heyser et al. 2007). If fungal and plant cells have distinctive pathways of suppressing ROS, it is important to determine how these are controlled in the ectomycorrhiza–plant symbiosis.

Among the nine *Nothofagus* species that prevail in south Chile, *Nothofagus dombeyi* (Mirb.) Oerst is highly resistant to a variety of ambient stress factors, such as drought (Veblen et al. 1996, Donoso and Lara 1999). This endemic tree species is often associated with specific ectomycorrhizal fungi in the field (Valenzuela et al. 2000). Recently, we indicated that ectomycorrhizal incubation of *N. dombeyi* improved the plant photosynthetic metabolism in plant roots as a response to water-limited conditions (Alberdi et al. 2007).

We studied the effect of ectomycorrhizal colonization of *N. dombeyi* on the antioxidative enzyme metabolism in plant responses to drought. Both the non-specific ectomycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker & Couch and the specific ectomycorrhizal fungus *Descolea antartica* Sing. were investigated. The term specific refers to ectomycorrhizal fungi that interact exclusively with *Nothofagus*, while non-specific refers to fungi that show a wide host range. We hypothesized that (1) the ectomycorrhizal colonization of *N. dombeyi* has a positive influence on the expression of antioxidative enzyme activities under DC; (2) the up-regulation of the antioxidative enzyme metabolism under drought reduces cellular damage and enhances plant growth in mycorrhizal *N. dombeyi* roots; and (3) the colonization by the *Nothofagus* specific ectomycorrhizal fungus *D. antartica* is more effective to attenuate oxidative stress than *N. dombeyi* colonization by the non-specific *P. tinctorius*.

Materials and methods

Pure ectomycorrhizal fungal cultures: maintenance, growth and water treatments

Inoculation experiments were carried out with ectomycorrhizal inocula of *D. antartica* and *P. tinctorius* (isolate 441). Cultures of *D. antartica* were obtained from fruiting bodies collected from a *N. dombeyi* temperate forest, Puyehue National Park, Antillanca, Chile. *Pisolithus tinctorius* was obtained from cultures maintained at the Institute of Environmental Science and Technology, University of Bremen, Germany. The fungi were grown on solid Melin–Norkrans culture media (agar agar 20 g l^{-1}, malt extract 5 g l^{-1}, D-glucose 10 g l^{-1}, $(NH_4)_2HPO_4$ 0.25 g l^{-1}, KH_2PO_4 0.5 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.15 g l^{-1}, $CaCl_2$ 0.05 g l^{-1}, Fe-EDTA 0.022 g l^{-1}, $NaCl_2$ 0.025 g l^{-1}, thiamine HCl 1×10^{-4} g l^{-1}) (Molina and Palmer 1982). Fungal stock cultures were grown and maintained in the dark, at 25 °C and pH 5. To expose the fungus to water potential treatments, ectomycorrhizal fungal colonies were suspended on the surface of a liquid Melin–Norkrans culture medium (25 ml; pH 5; T = 24 °C) (Coleman et al. 1989). Polyethylene glycol (PEG-3640, Carbowax, Union Carbide Corp., CT) was used to adjust water potential (osmometer analysis, 3D3, Advanced Instruments Inc., MA) in the culture medium to −0.16 and −0.96 MPa, respectively, for WC and DC (Coleman et al. 1989). A glass bead – Petri dish (60 mm, Merck, Chile) unit was used to grow fungi under different levels of water stress. The unit consisted of a 5-mm diameter agar inoculums plug placed on a nylon mesh disc that was supported at the liquid medium surface by glass beads. Each nylon disc was pre-weighed and fungal dry weight (DW) was obtained by the difference. After inoculation, the Petri dish unit was sealed with Parafilm and

maintained in the dark at 24 °C for 2 weeks, after which the DW was measured (Coleman et al. 1989). Each isolate in each treatment was replicated five times.

Non-mycorrhizal and mycorrhizal plant seedlings: cultivation, growth, ectomycorrhizal colonization and water treatments

Nothofagus dombeyi seeds (surface sterilized, 30% H_2O_2 solution for 3 min) were germinated in germination trays (50 × 30 × 8 cm). The soil used for germination was collected from the mineral soil layer from a *N. dombeyi* forest in the Puyehue National Park at 725 m a.s.l. (X Region, Chile, 41° S and 72° W). Soils are derived from a recent coarse volcanic ash with allophane as the dominating clay mineral, and classified as Umbric Vitric Andosols. The main physical and chemical soil characteristics are given in Table 1. The collected soils were sieved (2 mm) and autoclaved at 120 °C for 50 min. Further cultivation of *N. dombeyi* plants took place in a phytotron (humidity: 80%; irradiation: 15.7 klx; day/night cycle: 13/11 h; temperature: 25/20 °C). Plants were fertilized every 2 weeks with the Knop solution (1:3) (Ziegler 1998).

Individual seedlings of *N. dombeyi* were transferred to pots (18.5 cm depth, 13 cm diameter, filled with 13.5 cm of autoclaved soil) 3 months after germination. Pots contained the same soil as described for germination trays. Six months after the transfer, plants were separated into three groups ($n = 3 \times 10$): (i) *N. dombeyi* grown without mycorrhizal inoculum (*Nd*), (ii) *N. dombeyi* inoculated with *D. antartica* (*Nd*/*Da*) and (iii) *N. dombeyi* inoculated with *P. tinctorius* (*Nd*/*Pt*). For (ii) and (iii), each seedling was inoculated with six plugs of mycelium, placed in the pots close to young short roots according to Kottke et al. (1987). The plugs had been isolated from the leading edge of 1-month-old stock cultures with a sterile cork borer (diameter = 7 mm) and placed on fresh agar medium. After 1 week, those plugs showing actively growing hyphae were used for inoculation.

Four weeks after the inoculation, plugs had infected the neighbouring main and lateral short roots. For anatomical verification of ectomycorrhizal formation, plants were removed from the pots and three root tips per plant (length = 1.5–3.5 mm) were cut at their bases under a binocular (Stemi SV 11 Apot, Zeiss, Germany). Afterwards, the root tips were embedded in 4% agar and cross-sections of 100 μm were cut with a Vibratom (model 1000 Classic, GaLa Gabler Labor Instumente, Bad Schwalbach, Germany). Light microscopy revealed a para-epidermal Hartig net and a plectenchymatous mantle (results not shown). Finally, *N. dombeyi* plants were grown for 10 months under WC before starting water treatment (see further).

Table 1. Physico-chemical properties of the *N. dombeyi* forest soil (adopted from Alvarez et al. 2009).

Soil property	Value
Soil depth (cm)	0–20
Bulk density (g cm^{-3})	0.51
pH (H_2O)	5.03
Total carbon (%)	13.8
Total nitrogen (%)	0.74
C/N	18.6
P (μg g^{-1})	7.0
Na^+ (μg g^{-1})	91
K^+ (μg g^{-1})	195
Ca^{2+} (μg g^{-1})	1010
Mg^{2+} (μg g^{-1})	125

A randomized block design with two factors and five replications was set up. The first factor had three levels: no inoculation of *N. dombeyi* (*Nd*), inoculation with *D. antartica* (*Nd*/*Da*) or inoculation with *P. tinctorius* (*Nd*/*Pt*). The second factor had two levels: the volumetric water content (θ_v) of the soil was set to 8.5–19.5% for WC and 1–3% for DC.

During 4 weeks, inoculated and non-inoculated *N. dombeyi* plants were subjected to the two irrigation levels until roots and shoots were harvested. Under WC, the plants were watered regularly to maintain the volumetric water content (θ_v) of the soil between 8.5% and 19.5% (soil water potential (Ψ) of −0.07 to −0.09 MPa). Under DC, θ_v of the soil was maintained in the range from 1% to 3% (Ψ of −1.0 to −1.5 MPa). θ_v was determined using time domain reflectometry (TDR, IMKO Micromodultechnik GmbH, Germany).

After the application of water treatments for 4 weeks, plants were uprooted and their root systems were gently washed under running tap water. Fresh weight (FW) was determined shortly after the leaves were excised from the plants. Saturated weight (SW) was determined after rehydration for 12 h in the dark and the DW was determined after oven drying at 70 °C for at least 48 h. Relative water content (RWC) was determined as RWC = (FW − DW)/(SW − DW) × 100. Ectomycorrhizal colonization (number of ectomycorrhizal short roots/total number of short roots × 100) was determined under a stereomicroscope (DM 1000, Leica, Germany) at 40× magnification for a random sample of at least 100 short roots per root system.

Antioxidant enzyme activities

The extraction procedure was carried out at 0–4 °C. Ectomycorrhizal fungal, and non-mycorrhizal and mycorrhizal root tissues (1 g FW) were ground using a chilled homogenizer (Jürgens Type RM 90, Bremen, Germany) with 20 ml of extraction buffer consisting of 50 mM K-phosphate buffer pH 7.8 to which 0.1 mM EDTA was added for SOD (EC 1.15.1.1), CAT (EC 1.16.1.6) and APX (EC 1.11.1.11) (Gogorcena et al. 1995). The same medium supplied with 10 mM β-mercaptoethanol was used for GR (EC 1.6.4.2) (Moran et al. 1994). Extracts were filtered through four layers of nylon cloth and centrifuged at 20,000*g*, 20 min (Mikro 22R, Hettich, Germany). The supernatants were kept at −80 °C for subsequent enzymatic assays. Soluble protein

was determined using the dye binding microassay (Bio-Rad, CA) using bovine serum albumin (Merck, Chile) as standard. SOD activity was determined using the method described in Beyer and Fridovich (1987) which is based on the ability of SOD to inhibit the reduction of ρ-nitro blue tetrazolium chloride by photochemically generated superoxide radicals. One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of reduction measured at 560 nm (S-22, Boeco, Germany). CAT activity was assayed by monitoring the decrease in absorbance at 240 nm (S-22, Boeco, Germany) for 3 min as a consequence of H_2O_2 consumption (Aebi 1984). The reaction mixture (3 ml) contained 10.6 mM H_2O_2 and 200 μl of enzyme extract. APX activity was determined by following the decrease in absorbance at 290 nm (S-22, Boeco, Germany) for 1 min after adding H_2O_2 to ascorbate. The reaction mixture (1 ml) contained 200 μl of enzyme extract, 50 mM K-phosphate buffer (pH 7.0), 0.5 mM ascorbate and 0.1 mM H_2O_2 (Sigma–Aldrich, Chile) (Amako et al. 1994). GR activity was assayed as previously described in Carlberg and Mannervik (1985). This method is based on the determination of the NADPH oxidation rate, at 340 nm for 2 min (S-22, Boeco, Germany), catalysed by GR activity present in the sample. The reaction mixture (1 ml) contained 150 μl of enzyme extract, 0.1 M HEPES pH 7.8, 1 mM EDTA, 3 mM $MgCl_2$, and 0.5 mM oxidized glutathione, 0.2 mM NADPH (Sigma–Aldrich, Chile).

Hydrogen peroxide determination and oxidative damage to lipids

Hydrogen peroxide concentration was evaluated by the 4-aminoantipyrine method as described in Frew et al. (1983). Ectomycorrhizal fungal, non-mycorrhizal and mycorrhizal root tissues (1 g FW) were homogenized with an ice-cold potter in HCl (25 mM) and filtered through four layers of nylon cloth. The supernatants were adjusted to pH 7.0 for subsequent H_2O_2 quantification (absorbance measurements at 505 nm (S-22, Boeco, Germany)). For the measurement of lipid peroxidation in tissue, the thiobarbituric acid test was adopted which determines malondialdehyde (MDA) as an end product of lipid peroxidation (Halliwell and Gutteridge 1989). Tissues (0.5 g) were homogenized using a chilled homogenizer (Jürgens Type RM 90, Bremen, Germany) in 6 ml of 100 mM K-phosphate buffer pH 7.0 as described in Ruiz-Lozano et al. (2001). The reaction mixture contained 200 μl of enzyme extract, 15% (w/v) trichloroacetic acid, 0.375% (w/v) 2-thiobarbituric acid, 0.1% (w/v) butyl hydroxytoluene and 0.25 N HCl (Sigma–Aldrich, Chile) in a 1 ml volume (Minotti and Aust 1987). The absorbance at 532 nm was measured (S-22, Boeco, Germany).

Statistical analysis

Normality of the data was assessed with the Kolmogorov–Smirnov test. The significance of effects of water treatment and fungal species, and their interaction, on measured variables studied for pure ectomycorrhizal fungal cultures, were tested by a two-way analysis of variance (ANOVA). Likewise, the significance of effects of water treatment and mycorrhizal inoculation, and their interaction, on measured variables related to the study of mycorrhizal and non-mycorrhizal plants, were tested by a two-way ANOVA. Statistical differences ($P < 0.05$) between mean values were assessed by a Student's *t* test (comparison between two variables) or a one-way ANOVA (comparison between three variables, Bonferroni post hoc test). Statistical procedures were carried out with the software package SPSS (Statistical Package for the Social Science, IL) 16.0 for Windows.

Results

Pure cultures of ectomycorrhizal fungi

Ectomycorrhizal fungal species selection and water treatment had a significant effect on the fungal biomass production (Table 2). For both water treatments, a greater fungal biomass was observed for *D. antartica* than for *P. tinctorius* (Figure 1). Shifting water contents from WC to DC significantly decreased biomass of both ectomycorrhizal fungi (−22% and −19%, respectively, for *D. antartica* and *P. tinctorius*).

Ectomycorrhizal species selection and water treatment had a significant effect on SOD, CAT and GR activities (Table 2). APX, on the other hand, was not affected by water treatment or ectomycorrhizal species selection (Table 2). SOD activity was greater in *P. tinctorius* than in *D. antartica* under WC (Table 3). Shifting water contents from WC to DC significantly increased SOD activity in *P. tinctorius* (+49%), while no significant changes were observed for *D. antartica* (+2%). Significant differences in CAT activity were observed between both ectomycorrhizal fungi under WC and DC (Table 3). Decreasing water contents (WC to DC) decreased CAT activity in *D. antartica* (−69%), while a large increase was observed for *P. tinctorius* (+536%). No significant differences in APX activity were observed between both ectomycorrhizal fungi under WC (Table 3). Shifting water contents from WC to DC significantly increased APX activity for *P. tinctorius* (+76%), while a decrease in the APX activity was observed for *D. antartica* (−50%). As such, APX activity was significantly greater for *P. tinctorius* than for *D. antartica* under DC. GR activity was greater for *P. tinctorius* than for *D. antartica* under WC (Table 3). For none of the ectomycorrhizal fungi, we detected significant differences in the GR activity between WC and DC. The GR activities under DC were not significantly different between both ectomycorrhizal fungi.

Mycorrhizal species selection and water treatment had a significant effect on the ectomycorrhizal fungal H_2O_2 concentration (Table 2). Decreasing water contents (WC to DC) increased H_2O_2 concentration in both ectomycorrhizal

Table 2. Statistical significance of the effects of water treatment and fungal species, and their interaction, on all parameters studied for pure ectomycorrhizal cultures as determined using two-factor ANOVA; statistical significance of the effects of water treatment and mycorrhizal inoculation, and their interaction, on all parameters studied for non-mycorrhizal and mycorrhizal plants as determined using two-factor ANOVA.

Source of variation	Ectomycorrhizal fungi			Mycorrhizal and non-mycorrhizal plants		
	Fungal species	Water treatment	Interaction	Mycorrhizal inoculation	Water treatment	Interaction
Fungal biomass	< 0.001	0.003	0.595	–	–	–
Plant shoot dry biomass	–	–	–	< 0.001	< 0.001	< 0.001
Plant root dry biomass	–	–	–	< 0.001	< 0.001	0.001
Relative water content	–	–	–	< 0.001	< 0.001	0.001
Ectomycorrhizal colonization	–	–	–	< 0.001	< 0.001	< 0.001
SOD	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
APX	0.102	0.963	0.002	< 0.001	< 0.001	0.046
CAT	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
GR	0.017	0.029	0.724	< 0.001	< 0.001	0.055
MDA	0.490	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
H_2O_2	< 0.001	< 0.001	0.031	< 0.001	< 0.001	< 0.001

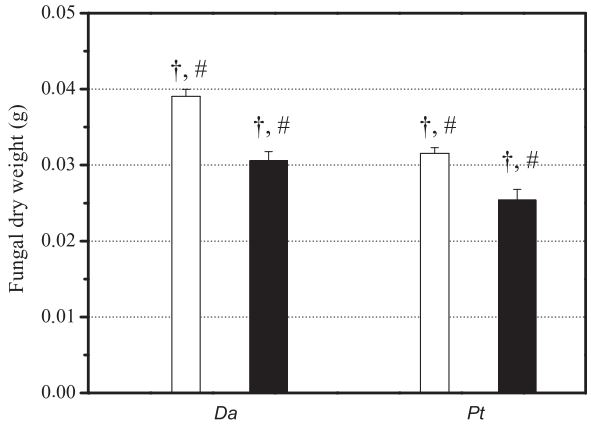

Figure 1. Biomass content of *D. antartica* (*Da*) and *P. tinctorius* (*Pt*) grown under WC (□) and DC (■). Columns represent mean values and standard errors ($n = 5$); †, significant differences between ectomycorrhizal species for the same water treatment (Student's t test, $P < 0.05$); #, significant differences between water treatments for the same ectomycorrhizal fungal species (Student's t test, $P < 0.05$).

fungi. Under WC, no significant differences in H_2O_2 concentrations were observed between both ectomycorrhizal associations (Figure 2). A differential increase in H_2O_2 concentration in response to drought resulted in a greater H_2O_2 concentration for *P. tinctorius* than for *D. antartica* under DC. Only water treatment had a significant effect on MDA concentration (Table 2). Under WC, the MDA concentration was greater for *P. tinctorius* than for *D. antartica* (Figure 2). Under DC, the MDA concentrations were significantly greater for *D. antartica* than for *P. tinctorius*. Shifting water contents from WC to DC significantly increased MDA concentrations only for *D. antartica*, while no differences were observed for *P. tinctorius*.

Mycorrhizal and non-mycorrhizal plants

Mycorrhizal inoculation and water treatment had a significant effect on the following studied parameters: root and shoot biomass, RWC, ectomycorrhizal colonization, antioxidative enzyme activities, and MDA and H_2O_2 colonization (Table 2).

No significant differences in root and shoot dry biomass were observed between non-mycorrhizal and mycorrhizal plants under WC (Table 4). Under DC, mycorrhizal plants had 72% more shoot DW and 76–77% more root DW than non-mycorrhizal plants (Table 4). Shifting water contents from WC to DC decreased shoot and root DWs in mycorrhizal and non-mycorrhizal plants. No significant differences between *P. tinctorius* and *D. antartica* were observed. The RWC, an indicator for plant water status, showed only significant differences among mycorrhizal treatments under DC (*Nd*/*Pt* > *Nd*/*Da* > *Nd*, Table 4). Ectomycorrhizal colonization was depressed under DC (Table 4). *Nothofagus dombeyi* plants inoculated with *P. tinctorius* showed significantly greater percentages of root colonization than the plants colonized by *D. antartica*. The non-inoculated roots showed negligible levels of mycorrhizal colonization under both water regimes (WC and DC).

No significant differences in SOD activity were observed between non-mycorrhizal and mycorrhizal *Nd* under WC (Table 5). Shifting soil water contents from WC to DC significantly increased SOD activities in all treatments. The relative increase was greatest for *Nd*/*Pt* (+418%), followed by *Nd*/*Da* (+209%) and *Nd* (+185%).

Under WC, both ectomycorrhizal associations showed a greater CAT activity than non-mycorrhizal *Nd* (*Nd*/*Pt* > *Nd*/*Da* > *Nd*) (Table 5). Shifting soil water contents from WC to DC significantly increased CAT activities in all treatments. The increase was greater for *Nd*/*Da* (+123%) than for *Nd*/*Pt* (+23%) and *Nd* (+14%). As such, the CAT activities

Table 3. SOD, CAT, APX and GR activity in the mycelium of *D. antartica* and *P. tinctorius* under WC or DC, and their relative increases/decreases when shifting soil water contents from WC to DC. Standard errors given in brackets ($n = 5$); †, significant differences between ectomycorrhizal species for the same water treatment (Student's t test, $P < 0.05$); #, significant differences between water treatments for the same ectomycorrhizal fungal species (Student's t test, $P < 0.05$).

	WC	DC	WC → DC (%)
SOD (U min^{-1} mg^{-1} protein)			
D. antartica	14.2 (0.8) †	14.5 (0.5) †	+2
P. tinctorius	20.1 (1.2) †, #	30.1 (2.5) †, #	+49
CAT (μmol min^{-1} mg^{-1} protein)			
D. antartica	11.0 (0.6) †, #	3.4 (0.4) †, #	−69
P. tinctorius	4.9 (0.7) †, #	31.4 (1.9) †, #	+536
APX (mmol min^{-1} mg^{-1} protein)			
D. antartica	2.4 (0.3) #	1.2 (0.2) †, #	−50
P. tinctorius	1.7 (0.1) #	3.0 (0.4) †, #	+76
GR (nmol min^{-1} mg^{-1} protein)			
D. antartica	0.1 (0.0) †	0.2 (0.0)	+42
P. tinctorius	0.2 (0.0) †	0.3 (0.0)	+18

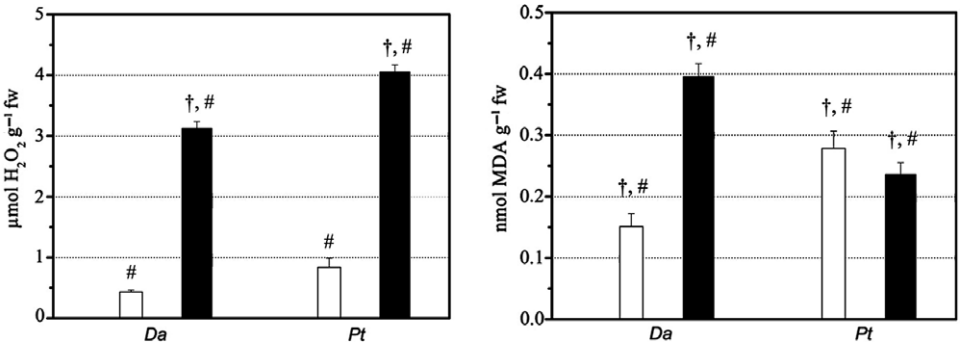

Figure 2. H_2O_2 and MDA in *D. antartica* (*Da*) and *P. tinctorius* (*Pt*) mycelium under WC (□) or DC (■). Columns represent mean values and standard errors ($n = 5$); †, significant differences between ectomycorrhizal species for the same water treatment (Student's t test, $P < 0.05$); #, significant differences between water treatments for the same ectomycorrhizal fungal species (Student's t test, $P < 0.05$).

under DC significantly decreased in the following order: *Nd*/*Da* > *Nd*/*Pt* > *Nd*.

Under WC, APX activities were significantly greater for the ectomycorrhizal associations *Nd*/*Pt* than for the non-mycorrhizal *Nd*, while *Nd*/*Da* showed an intermediate APX activity (Table 5). Shifting soil water contents from WC to DC significantly increased APX activities in all treatments. Under DC, the APX activities significantly decreased in the following order: *Nd*/*Da* > *Nd*/*Pt* > *Nd*.

The ectomycorrhizal associations showed a greater GR activity than non-mycorrhizal *Nd* under WC (no significant differences between *Nd*/*Da* and *Nd*/*Pt*) (Table 5). Shifting soil water contents from WC to DC significantly increased GR activities in all treatments. Under DC, the GR activities significantly decreased in the following order: *Nd*/*Pt* > *Nd*/*Da* > *Nd*.

Under WC, no significant differences were observed in H_2O_2 concentrations between non-mycorrhizal and mycorrhizal *Nd* (Figure 3). Shifting soil water contents from WC to DC significantly increased H_2O_2 concentrations in all treatments. Under DC, the H_2O_2 concentrations were significantly greater for *Nd* than for *Nd*/*Da* and *Nd*/*Pt*.

Under WC, no significant differences were observed in MDA concentrations between non-mycorrhizal and mycorrhizal *Nd* (Figure 3). Shifting soil water contents from WC to DC significantly increased MDA concentrations in *Nd*/*Da* and *Nd*, but not in *Nd*/*Pt*. As such, the MDA concentrations under DC were significantly greater for *Nd* than for *Nd*/*Da* and *Nd*/*Pt*.

Discussion

Sustained ectomycorrhizal fungal growth under DC has previously been observed in some ectomycorrhizal fungi by Coleman et al. (1989) and Hutton et al. (1996). Antioxidative

Table 4. Shoot and root dry biomass, shoot water content and ectomycorrhizal colonization in non-mycorrhizal and mycorrhizal plants of *N. dombeyi* inoculated with *D. antartica* and *P. tinctorius* under WC or DC. Standard errors given in brackets ($n = 5$); values in the same column that differ significantly are not followed by a common letter (Bonferroni, $P < 0.05$); #, significant differences between water treatments for the same ectomycorrhizal treatment (Student's t test, $P < 0.05$).

	WC	DC
Shoot dry weight (g)		
N. dombeyi/*D. antartica*	5.80 (0.48) #, a	4.25 (0.18) #, b
N. dombeyi/*P. tinctorius*	5.87 (0.46) #, a	4.20 (0.13) #, b
N. dombeyi	5.74 (0.24) #, a	1.20 (0.14) #, a
Root dry weight (g)		
N. dombeyi/*D. antartica*	3.96 (0.35) #, a	2.65 (0.10) #, b
N. dombeyi/*P. tinctorius*	4.00 (0.12) #, a	2.54 (0.12) #, b
N. dombeyi	3.65 (0.27) #, a	0.62 (0.07) #, a
Relative water content (%)		
N. dombeyi/*D. antartica*	94.2 (1.1) #, a	90.7 (1.1) #, c
N. dombeyi/*P. tinctorius*	94.6 (1.0) a	93.3 (1.1) b
N. dombeyi	95.7 (1.4) #, a	83.3 (1.7) #, a
Ectomycorrhizal colonization (%)		
N. dombeyi/*D. antartica*	64.8 (1.1) #, b	53.5 (0.8) #, b
N. dombeyi/*P. tinctorius*	73.5 (0.7) #, c	58.9 (0.6) #, c
N. dombeyi	2.9 (0.1) #, a	1.4 (0.2) #, a

Table 5. SOD, CAT, APX and GR activity in *N. dombeyi–D. antartica* and *N. dombeyi–P. tinctorius* ectomycorrhizas and non-mycorrhizal roots of *N. dombeyi* under WC or DC, and their relative increases/decreases when shifting soil water contents from WC to DC. Standard errors given in brackets ($n = 5$); values in the same column that differ significantly are not followed by a common letter (Bonferroni, $P < 0.05$); #, significant differences between water treatments for the same ectomycorrhizal treatment (Student's t test, $P < 0.05$).

	WC	DC	WC → DC (%)
SOD (U min^{-1} mg^{-1} protein)			
N. dombeyi/*D. antartica*	137.7 (8.4) #, a	425.1 (12.2) #, b	+209
N. dombeyi/*P. tinctorius*	118.8 (6.0) #, a	615.3 (25.9) #, c	+418
N. dombeyi	114.1 (7.1) #, a	325.7 (11.4) #, a	+185
CAT (μmol min^{-1} mg^{-1} protein)			
N. dombeyi/*D. antartica*	49.1 (0.9) #, b	109.7 (1.3) #, c	+123
N. dombeyi/*P. tinctorius*	66.5 (0.9) #, c	81.8 (1.1) #, b	+23
N. dombeyi	40.7 (0.9) #, a	46.3 (0.9) #, a	+14
APX (mmol min^{-1} mg^{-1} protein)			
N. dombeyi/*D. antartica*	1.2 (0.1) #, ab	3.9 (0.3) #, c	+217
N. dombeyi/*P. tinctorius*	1.7 (0.1) #, b	3.2 (0.1) #, b	+89
N. dombeyi	0.8 (0.1) #, a	1.8 (0.1) #, a	+135
GR (nmol min^{-1} mg^{-1} protein)			
N. dombeyi/*D. antartica*	0.36 (0.00) #, b	0.43 (0.01) #, d	+20
N. dombeyi/*P. tinctorius*	0.38 (0.01) #, b	0.51 (0.01) #, c	+33
N. dombeyi	0.29 (0.00) #, a	0.37 (0.01) #, a	+26

enzymes play an important role in detoxifying ROS, produced by many fungi in response to drought (Apel and Hirt 2004). While Ott et al. (2002) and Langenfeld-Heyser et al. (2007) indicated the involvement of antioxidative enzyme system in response to metal- and saline-induced oxidative stress, no information is available on ectomycorrhizal fungal antioxidative responses to drought stress. The ROS concentrations can damage fungal cells and form MDA, a product of lipid peroxidation by a thiobarbituric acid reaction. Hence, high activities of antioxidant enzymes and low MDA concentrations during drought are linked to drought tolerance (Bowler et al. 1992). A clear difference in ROS metabolism in response to drought can be observed between both ectomycorrhizal fungi investigated here. *Descolea*

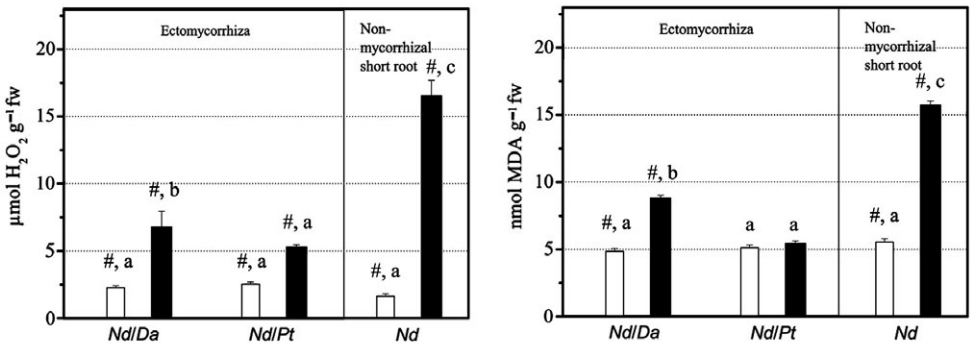

Figure 3. H_2O_2 and MDA in *N. dombeyi–D. antartica* (*Nd*/*Da*) and *N. dombeyi–P. tinctorius* (*Nd*/*Pt*) ectomycorrhizas and non-mycorrhizal roots of *N. dombeyi* (*Nd*) under WC (□) or DC (■). Columns represent mean values and standard errors ($n = 5$); significant differences between mycorrhizal treatments for the same water treatment are indicated by a different letter (Bonferroni, $P < 0.05$); #, significant differences between water treatments for the same ectomycorrhizal treatment (Student's t test, $P < 0.05$).

antartica kept SOD activity at a steady-state level, but was not able to maintain a sustained activity of other ROS scavenging enzymes such as CAT and APX. This resulted in cellular damage to *D. antartica* cells. On the contrary, *P. tinctorius* showed increased activity of all ROS scavenging enzymes, which effectively reduced MDA production. Hence, it can be concluded that *P. tinctorius* disposes of a more effective antioxidative system. The observation that H_2O_2 increases in the fungal cells of *P. tinctorius* can potentially be related to H_2O_2 functions such as cell wall reinforcement or signalling (Yang et al. 1997, Park et al. 1998). Despite their difference in ROS metabolism, both ectomycorrhizal fungi showed a similar reduction in fungal growth in response to drought (−22% and −19%, respectively, for *D. antartica* and *P. tinctorius*). This observation may relate to differential nutrient mobilization and uptake rates of the studied ectomycorrhizal fungi (Colpaert et al. 1999).

In addition to chloroplasts (Foyer and Noctor 2000), root peroxisomes and mitochondria are considered to be the major sites of ROS generation in plants subjected to stress (Corpas et al. 2001, del Rio et al. 2001, Mittova et al. 2004). The importance of antioxidative root enzymes in regulating plant metabolism under drought has been shown by Shvaleva et al. (2006). The balance between SOD and APX or CAT activities is critical for suppressing toxic ROS levels in a cell (Mittler 2002, Apel and Hirt 2004). However, little information is available on how ectomycorrhizal colonization affects ROS metabolism in plant roots, and whether or not this symbiosis attenuates stress events by the regulation of ROS scavenging enzymes. Hence, it remains unclear whether the drought tolerance of fungi in pure culture is transmitted to associated host plants. This stands in contrast with the literature on arbuscular mycorrhizal fungi where this symbiosis has been documented to alleviate drought-induced oxidative stress and reduce oxidative damage to lipids and proteins in plant roots (e.g., Ruiz-Lozano et al. 1996, 2001, Porcel et al. 2003, Wu et al. 2006). This study indicated that ectomycorrhizal treatment and soil water treatment had a significant effect on fungal and plant biomass production, plant water status, antioxidative enzyme activities and ROS metabolism.

In non-mycorrhizal *N. dombeyi* roots, reduced soil water content clearly induced H_2O_2 accumulation and cellular damage. Though antioxidative enzyme activities augmented in response to drought, this increase was not able to attenuate drought stress and detoxify ROS species. This observation explains the large increase in H_2O_2 concentration. As such, drought-induced oxidative stress significantly affected root and shoot growth of *N. dombeyi* plants. However, one should note that drought was applied in an abrupt manner in this study, inducing suddenly a severe stress to plants. In a field situation, where drought is induced more gradually, changes in ROS metabolism and antioxidative enzyme activities in response to drought could be less pronounced.

In both ectomycorrhizal associations of *N. dombeyi*, root and shoot growth were relatively enhanced under drought. Ectomycorrhizal inoculation had a positive effect on plant water status. In this respect, the symbiosis of *N. dombeyi* and *P. tinctorius* improved plant water status even more than the association between *N. dombeyi* and *D. antartica*. In addition, ectomycorrhizal inoculation also increased the activity of many antioxidative enzymes relative to non-mycorrhizal plants. Hence, it seems that ectomycorrhizal plants obtain a high resistance to drought by the prevention of oxidative stress and effective elimination of ROS (Bartels 2001).

Ectomycorrhizal associations of *N. dombeyi* with *D. antartica* and *P. tinctorius* significantly increased their antioxidant enzyme activities to eliminate ROS. In this study, the symbiosis between *D. antartica* and *N. dombeyi* increased SOD, CAT and APX activity relative to non-mycorrhizal *N. dombeyi* roots under DC. Since SOD activity, as a measure for H_2O_2 production, only slightly increased compared to non-mycorrhizal *N. dombeyi*, cellular damage and H_2O_2

accumulation were mainly reduced by the simultaneous action of H_2O_2 detoxification enzymes. CAT has an extremely high maximum catalytic rate (Willekens et al. 1995). The scavenging of H_2O_2 by CAT may also be an efficient mechanism to elicit plant defence responses, facilitating intraradical fungal growth and differentiation (Wu et al. 1997). An alternative route for H_2O_2 destruction is through the action of APX, which has a much higher affinity for H_2O_2 than CAT (Willekens et al. 1995, Jiménez et al. 1997). The ascorbate–glutathione cycle might be enhanced under DC by sustained ascorbate production (Bartoli et al. 2005) and/or GR activity (Foyer et al. 1995). While CAT is mainly located in root peroxisomes, APX has different isoforms that are specifically targeted to chloroplasts, mitochondria, peroxisomes, as well as to the cytosol and apoplast (Mittova et al. 2000). The synchronized action of all ROS scavenging enzymes in the symbiosis between *P. tinctorius*/*N. dombeyi* largely reduced H_2O_2 concentrations, and fully prevented MDA accumulation. The latter symbiosis further increased the activities of SOD and GR relative to non-mycorrhizal *N. dombeyi* and the symbiosis between *N. dombeyi* and *D. antartica*. These responses to drought can be ascribed to de novo synthesis of enzymatic proteins when both partners are in symbiosis (Baisak et al. 1994). The effective ROS detoxification, in combination with the improved plant water status, provides *N. dombeyi*/*P. tinctorius* even a greater tolerance to resist drought than the symbiosis between *N. dombeyi* and *D. antartica*.

The enzymatic ROS metabolism in liquid ectomycorrhizal fungal cultures is differently regulated to that of plants. Summing the antioxidant activities of individual ectomycorrhizal fungi and *N. dombeyi* plants, it becomes clear that the expression of antioxidative enzymes is up-regulated when both partners are in symbiosis. Clear shifts in ROS production and consumption pathways suggest the activation of novel pathways to cope with drought-induced oxidative stress in the symbiosis. Unfortunately, our methodological design does not allow a clear separation between fungal and plant antioxidative activity when both partners are in symbiosis. Hence, we cannot exclude the possibility that increases of the measured enzymatic activity only reflect the activity in the fungus but not in the plant root. Further research, focusing on the exact localization of the antioxidative enzymes and metabolic indicators, should reveal this research gap. Our results complement observations by Baptista et al. (2007), who demonstrated that a coordinated activation of SOD and CAT could prevent host cell damage during ectomycorrhizal establishment between *Castanea sativa* Mill. (chestnut tree) and *P. tinctorius*. In contrast to our study, Langenfeld-Heyser et al. (2007) found that ectomycorrhizal colonization was unable to suppress salt-induced oxidative stress. In their study, SOD and H_2O_2 detoxification enzyme activities did not increase when ectomycorrhizal plants were subjected to salt stress. This observation may indicate that drought-induced and salt-induced antioxidant enzyme activities induce a different response in the regulation of antioxidative enzymes, or that specific fungal/plant species combinations could show a dissimilar behaviour in response to environmental stress.

In conclusion, this study indicates that mycorrhizal plants show a high tolerance against drought. We believe that this effect may be attributed to a reduction in ROS production as a result of the improved plant water status, in combination with the effective ROS detoxification through the synchronized action of antioxidative enzymes such as SOD, CAT, APX and GR. In this respect, the non-specific ectomycorrhizal fungus *P. tinctorius* was more compatible with *N. dombeyi* than the *Nothofagus* specific *D. antartica*.

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