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ORIGINAL ARTICLE

Ectomycorrhizal inoculation with *Pisolithus tinctorius* reduces stress induced by drought in cork oak

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Abstract

We investigated whether the performance of cork oak under drought could be improved by colonization with the ectomycorrhizal fungus *Pisolithus tinctorius*. Results show that inoculation alone had a positive effect on plant height, shoot biomass, shoot basal diameter, and root growth. Under drought, root growth of mycorrhizal plants was significantly increased showing that inoculation was effective in increasing tolerance to drought. In accordance, mycorrhizal plants subjected to drought showed less symptoms of stress when compared to non-mycorrhizal plants, such as lower concentration of soluble sugars and starch, increased ability to maintain fatty acid content and composition, and increased unsaturation level of membrane lipids. After testing some of the mechanisms suggested to contribute to the enhanced tolerance of mycorrhizal plants to drought, we could not find any by which *Pisolithus tinctorius* could benefit cork oak, at least under the drought conditions imposed in our experiment. Inoculation did not increase photosynthesis under drought, suggesting no effect in sustaining stomatal opening at low soil water content. Similarly, plant water status was not affected by inoculation suggesting that *P. tinctorius* does not contribute to an increased plant water uptake during drought. Inoculation did increase nitrogen concentration in plants but it was independent of the water status. Furthermore, no significant mycorrhizal effect on drought-induced ROS production or osmotic adjustment was detected, suggesting that these factors are not important for the improved drought tolerance triggered by *P. tinctorius*.

Keywords Cork oak · Ectomycorrhiza · Symbiosis · Drought · Physiological response · Biochemical response

Introduction

Drought is one of the most important abiotic stresses that plants have to cope with, limiting survival, growth, and productivity. The severity of drought is dangerously increasing due to global climate change which is adding extra heat to the climate system that will translate into more intense and longer periods of drought (Trenberth et al. 2014). One major limitation in

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reforestation practices is the low survival rate and poor initial growth of soil-planted seedlings, mainly because of drought stress. In Mediterranean forests, dominant tree species, such as oaks and pines, live in association with symbiotic ectomycorrhizal (ECM) fungi. In this association, established at the root level, the fungal partner delivers mineral nutrients to the host plant, which in return transfers sugars produced during photosynthesis to the fungal cells. Thus, ECM plants are generally reported to have increased growth ability and higher tolerance to both biotic and abiotic stresses making this symbiotic relation very interesting to forestry practices. Besides increased plant nutrient acquisition, improved water uptake has also been cited as one of the benefits of the ECM symbiosis. However, the role of ECM on plant water uptake is not yet clear with several studies reporting reduced root hydraulic conductivity and reduced water uptake in ECM roots (for a review see Lehto and Zwiazek 2011). Nevertheless, in most cases, the ECM symbiosis is reported to alleviate the negative effects of drought stress making the host plant more tolerant to drought (Kivlin et al. 2013, Mohan et al. 2014). Several mechanisms by which symbiotic mycorrhizal fungi could promote drought tolerance have been proposed (Fig. 1). Direct mechanisms include the increased extension of the root system due to greater lateral root formation in mycorrhizal plants, and the uptake and transport of water by the extensive extraradical mycelia of ECM fungal species which develop specialized vessel hyphae that absorb and transport water to the mycorrhizal roots (Duddridge et al. 1980; Plamboeck et al. 2007). This increased absorbing surface could result in higher root conductance and hence increased carbon assimilation under stress. An indirect way could be by facilitated nutrient acquisition. Adequate mineral nutrition is known to improve efficiency of the photosynthetic machinery promoting plant performance and recovery after stress. In *Pinus* seedlings, ECM fungal hyphae were shown to take up N from an isolated compartment during drought, promoting plant resistance to drought (Wu et al. 1999), and in *Nothofagus dombeyi* ECM inoculation promoted N and P accumulation during drought, which was accompanied by an enhanced activity of N and P assimilatory enzymes (Alvarez et al. 2009a). Another proposed mechanism includes the activation of the plant antioxidant system that would reduce reactive oxygen species (ROS) generated during drought, mitigating cellular damage (Porcel and Ruiz-Lozano 2004; Alvarez et al. 2009b). Additionally, the capacity of mycorrhizal plants to tolerate drought by osmotic adjustment related to elevated sugar levels has also been reported in several studies (Wu and Xia 2006; Yooyongwech et al. 2013). Since sugar levels in mycorrhizal plants depend on the rate of CO_2 fixation versus sugar export to the fungus (sink effect), higher CO_2 fixation under drought would result in higher sugar levels. Studies have also suggested that improved photosynthesis under drought could be attributed to the increased sugar sink activity of the ECM symbiosis (Dosskey et al. 1991).

In this study, we intended to characterize the response of ECM plants when subjected to drought stress. For that we choose cork oak (*Quercus suber* L.), a drought-resistant species well adapted to the hot and dry summer conditions of the western Mediterranean region. The ECM fungus *Pisolithus tinctorius*, which is known to provide extensive extraradicular mycelium that could transport water into the host roots, was used for cork oak inoculation. *P. tinctorius* is a stress-resistant species which can benefit its hosts even under severe conditions, such as drought stress (Cairney and Chambers 1997). The *P. tinctorius* strain used in our study is native to a very arid region in the south of Portugal, where it naturally associates with cork oak trees (Sebastiana et al. 2013). Our hypothesis was that ECM inoculation with *P. tinctorius* would increase the drought tolerance of cork oak plants growing in the nursery. We therefore measured growth in inoculated and non-inoculated plants under two soil moisture conditions, well water (WW) and water stress (WS). Furthermore, to help uncover the contribution of *P. tinctorius* to the oak's drought tolerance, we tested some of the mechanisms by which ECM fungi can improve drought tolerance in host plants described above. To test whether *P. tinctorius* inoculation can improve water availability that would increase photosynthetic activity during drought, we measured plant water status parameters, leaf gas exchange, chlorophyll a fluorescence, and photosynthetic pigments. To investigate whether *P. tinctorius* could improve plant nitrogen uptake during drought, we measured nitrogen content in the leaves. We also measured lipid peroxidation, protein carbonylation, and electrolyte leakage in order to verify whether *P. tinctorius* could attenuate the negative effects from ROS produced during drought. To detect whether *P. tinctorius* could promote an osmotic adjustment by increased sugar accumulation in the host plant during drought we analyzed soluble sugars. Since the ECM symbiosis is known to alter sugar metabolism in the host plant, we investigated alterations in non-structural carbohydrates and total carbon percentage. Finally, since studies have shown that drought alters plant membrane lipids (Yordanov et al. 2000), we analyzed fatty acids in roots and leaves to test if inoculation with *P. tinctorius* could contribute to shifts in fatty acid content and composition which would increase/maintain cellular membrane fluidity during drought stress.

Materials and methods

Biological material and experimental setup

P. tinctorius (strain Pt23 in the collection of the Plant Functional Genomics Group, Faculty of Sciences, University of Lisbon) was grown on BAF agar medium and subsequently in a peat-vermiculite mixture moistened with liquid BAF medium as described previously (Sebastiana et al. 2013).

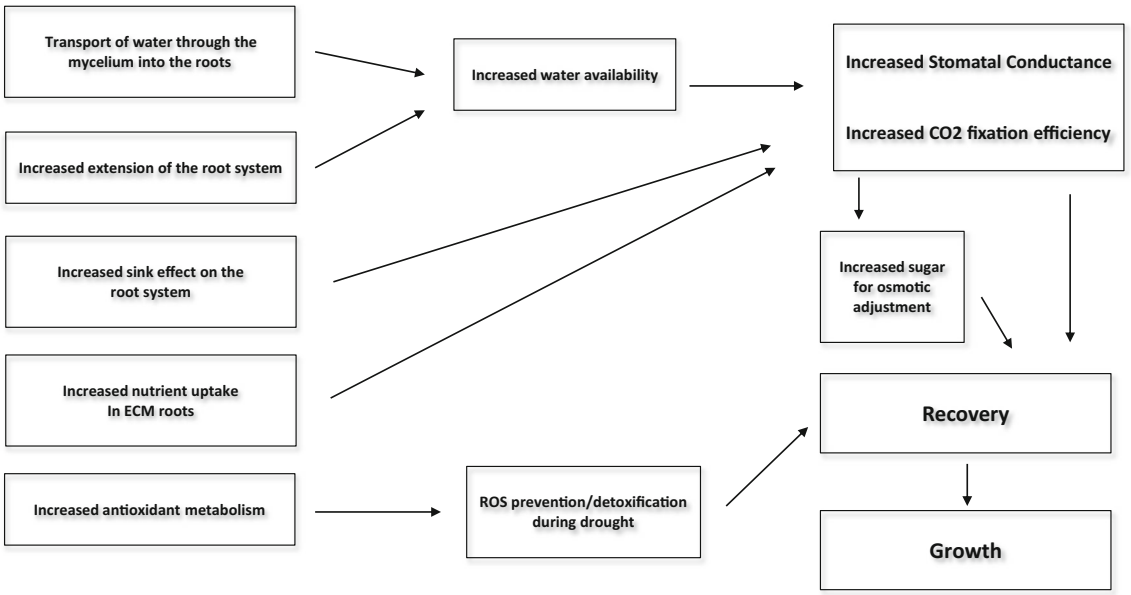

Fig. 1 Mechanisms by which symbiotic mycorrhizal fungi could promote plant drought tolerance

Q. suber seeds were germinated in a greenhouse, in plastic trays containing soil acquired from a gardening store (Siro® Universal, Portugal; 80–150 mg/L N, 80–150 mg/L P_2O_5, 300–500 mg/L K_2O, pH ($CaCl_2$) 5.5–6.5, organic matter > 70%). After germination, 3-month-old plantlets were transferred to 10-L pots and simultaneously inoculated with the *P. tinctorius* peat-vermiculite inoculum, according to Sebastiana et al. (2013). Control plants were treated with a non-inoculated peat-vermiculite mixture. Plants were grown in a greenhouse and watered once a week with 500 mL of tap water. No fertilization was applied. Sixteen months after *P. tinctorius* inoculation, plants were subjected to the drought treatment. Each group of ECM and non-ECM plants was divided into two groups of 6–7 individuals arranged in a randomized plot. One group was irrigated as before (WW), whereas the other group was subjected to drought by withholding water (WS). ECM and non-ECM plant size was similar at the onset of the experiment. Plants were subjected to the two irrigation regimes for 6 weeks during the summer. After that period plants were collected for analysis.

Plant biomass

After the application of the two different water treatments, plants were uprooted and roots were rinsed with tap water to eliminate soil particles. For each plant, shoot and root length, shoot basal diameter, and total leaf number were recorded. Fresh weight (FW) of the roots and leaves was recorded shortly after excision from the plants. For dry weight (DW) determination, a subset of leaves and roots from each plant were dried in an oven at 70 °C for 72 h. Leaf area was measured in five leaves per plant using a McMaster Biophotonics Facility ImageJ for Microscopy software program (Maryland, USA).

Plant and soil water status

Root and leaf water content (WC) were determined for each plant as a weight fraction: WC (%) = 100*(FW − DW)/FW. Leaf relative water content (RWC) was calculated as RWC (%) = [(FW − DW)/(TW − DW)] (Čatský 1960) using one mature leaf per plant. TW denotes the turgid weight (TW) of a leaf sample obtained by floating the leaves in a closed petri dish with deionized water for at least 48 h in the dark.

Soil water content (SWC) was determined for each plant and expressed as a weight fraction: SWC (%) = 100*(FW − DW)/DW, where FW denotes the fresh weight of a soil portion of the internal area of each pot and DW the dry weight of the same soil portion after being oven-dried at 105 °C during 4 days.

Leaf water potential was determined with a HR-33T Dew Point Microvoltmeter (Wescor, USA) equipped with a Wescor C-52 leaf chamber. Psychrometric measurements were performed in 6-mm diameter leaf discs after 20 min of equilibrium with the atmosphere of the leaf chamber, in accordance with Nunes et al. (2008).

Leaf gas exchange and chlorophyll *a* fluorescence

Gas exchange measurements were performed using a portable infrared gas analyzer (IRGA, LCpro+ ADC BioScientific, Herfordshire, UK) in a controlled environment (50 ± 5% relative humidity, 370 ppm CO_2, 25 ± 2 °C, and 750 μmol m^{-2} s^{-1} irradiance). One fully expanded leaf per plant was used. Photosynthetic rate (A, μmol CO_2 m^{-2} s^{-1}) and transpiration rate (E, mol H_2O m^{-2} s^{-1}) were measured every minute until stabilization (around 10 min). All parameters were corrected to the analyzed leaf area. Instantaneous

water use efficiency (WUE, μmolCO_2/molH_2O) was calculated as the ratio of photosynthetic rate to transpiration rate (*A*/*E* ratio).

Chlorophyll *a* (Chl a) fluorescence was measured using a Handy Plant Efficiency Analyzer (PEA) – Chlorophyll Fluorimeter (Hansatech Instruments, Kings Lynn, UK), according to Silvestre et al. (2014). Non-detached, young fully expanded leaves were allowed a 10 min dark adaptation, after which they were exposed to a saturating light pulse of 3500 μmol m^{-2} s^{-1} for 1 s. The kinetics of the fluorescence rise was recorded, and the maximum quantum efficiency of the photosystem II (Fv/Fm) was calculated according to Strasser and Strasser (1995).

Photosynthetic pigment analysis

Pigments were extracted from leaf disks (0.6-cm diameter) with 2 ml of methanol (99.9%, Sigma) in the dark at 4 °C until full extraction. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid (Carot) concentrations were calculated according to Lichtenthaler (1987) after the respective spectrophotometric measurements (Helios Beta UV/Vis spectrophotometer, Thermo Electron Corporation, USA). One fully expanded leaf per plant was used.

Determination of starch and soluble sugars

Soluble sugars and starch were extracted from each plant using liquid N_2-grounded leaves and roots (0.1 g FW) according to Guy et al. (1992). Soluble sugar content was determined by enzymatic assay using the sucrose/D-glucose/D-fructose UV method test kit (Boehringer Mannheim/R-Biopharm) at 340 nm. Sucrose, glucose, and fructose concentrations were expressed as glucose equivalents. The insoluble fraction was assayed for starch after acid hydrolysis with 30% HCl at 90 °C for 20 min, followed by measurement of released D-glucose at 340 nm using the D-glucose HK, UV method test kit (Nzytech), after neutralization with KOH 5M. Starch concentration was expressed as glucose equivalents. Non-structural carbohydrate concentration was defined as the total amount of soluble sugars (glucose, sucrose, and fructose) plus starch content.

Analysis of oxidative damage to lipids and proteins

Malondialdehyde (MDA), the end-product of lipid peroxidation was quantified with the thiobarbituric acid (TBA) test (Hodges et al. 1999). Frozen leaf powder (approx. 0.2 g FW from each plant) was homogenized with 2 ml 80% ethanol and centrifuged 140,000*g*, 5 min at 4 °C. A 0.8 ml aliquot was added to a test tube with either (i) 0.8 ml of − TBA solution comprised of 20% (*w*/*v*) trichloroacetic acid and 0.01% butylated hydroxytoluene or (ii) + TBA solution containing the above plus 0.65% thiobarbituric acid. Samples were then mixed, heated at 95 °C for 30 min, cooled on ice, and centrifuged at 3000*g* for 10 min. Absorbances were read at 440, 532, and 600 nm and MDA equivalents were calculated as described by Hodges et al. (1999).

Protein carbonylation was determined by the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al. 1994). First, frozen leaf powder was homogenized with mortar and pestle in 0.1 M Phosphate buffer (pH 7.8) containing 1 mM EDTA, 2% (*w*/*v*) PVPP, 1% Triton X100, in an ice bath (6 ml per g FW). Samples were centrifuged for 15 min at 15000*g* and 4 °C. Aliquots of the supernatant containing 0.5 mg protein were used for determination of carbonyl content using the protein carbonyl content assay kit (Sigma) and expressed as nanomoles per milligram protein. Protein content was measured using the bicinchoninic acid (BCA) assay (Sigma).

Carbon and nitrogen elemental analysis

For the carbon and nitrogen elemental analysis, frozen leaf material from each plant was dried at 70 °C for 72 h and grounded in a mill (Retsch Germany) to a homogenous fine powder for isotopic analysis. After grinding, samples were used for carbon (C) and (N) percentage calculation, according to Rodrigues et al. (2010), on a EuroEA 3000 Elemental Analyzer (EuroVector, Milan), with a TDC detector, at the Stable Isotopes and Instrumental Analysis Facility, Faculty of Sciences, Lisbon University. C and N concentrations were defined as % of dry weight.

Electrolyte leakage measurements

One set of five leaf discs (0.5-cm diameter) or five pieces of root (circa 0.5-cm length) *per* plant were rinsed (three times) with deionized water to remove solutes from damaged cells and veins, and floated on 5 mL of deionized water for 15 h in the dark, at room temperature. After this period (T0), the conductivity of the solutions was monitored using a conductimeter (Con 5- EcoScan, Eutech Instruments, Singapore). Total conductivity (T1) was measured in the solution at room temperature after heating the samples at 90 °C for 1 h. The in vivo electrolyte leakage was determined as the conductivity in T0 expressed as percentage of total conductivity (T1).

Lipid and fatty acid analysis

Fatty acid analysis was performed by direct transesterification of leaf or root tissues, grounded in liquid N_2. Incubation in methanol-sulfuric acid (97.5:2.5, *v*/*v*) was carried at 70 °C for 60 min, as previously described (Gameiro et al. 2016). Heptadecanoic acid (C17:0) was used as internal

standard. Fatty acid methyl esters were rescued using petroleum ether, dried under a N_2 flow, re-suspended in hexane, and analyzed in a gas chromatograph (Varian 430-GC gas chromatograph) equipped with a hydrogen flame ionization detector set at 300 °C. The temperature of the injector was set to 270 °C, with a split ratio of 50. The fused-silica capillary column (50 m × 0.25 mm; WCOT Fused Silica, CP-Sil 88 for FAME; Varian) was maintained at a constant nitrogen flow of 2.0 mL/min and the oven temperature set at 190 °C. Fatty acids were identified by comparison of their retention times with standards (Sigma-Aldrich) and chromatograms analyzed by the peak surface method, using the Galaxy software. Double Bond Index (DBI) was calculated as follows: $2 \times [(1 \times \%$ monodienoic acids) + $(2 \times \%$ dienoic acids) + $(3 \times \%$ trienoic acids)]/100.

Statistical analysis

Statistical procedures were carried out with the IBM SPSS v. 22 statistics software (SPSS® Inc., Chicago, IL, USA). The data were analyzed by a two-way factorial ANOVA (general linear model) to determine the effects of *P. tinctorius* inoculation, watering regime, and their interaction on the parameters investigated. Before two-way ANOVA, data were tested for normality with Shapiro-Wilk's test and for equality of variance with Levene's test. When variances were not homogenous, log10 transformation was used to ensure homogeneity of variances. In cases where interaction between mycorrhizal inoculation and water stress were significant, a Tukey's HSD post hoc test was used to compare means.

Results

After inoculation, plants were periodically checked for root colonization with *P. tinctorius* or other fungi which could contaminate the roots during the experimental period. In fact, five plants, one inoculated and four non-inoculated, were discarded because their roots were colonized by an unknown fungus. At harvest, all plants from the inoculated group showed the mycorrhizas typical of the association between *P. tinctorius* and cork oak—bright yellow mycorrhizal root tips with a thick fungal mantle and visible fungal hyphae spreading into the soil (Online Resource 1). Non-inoculated plants did not show any signs of *P. tinctorius* or other fungi in their root systems.

The water stress treatment decreased shoot biomass (Table 1). Inoculation with *P. tinctorius* increased plant height, shoot biomass (DW), shoot basal diameter (Table 1), and root biomass (DW) (Fig. 2). A significant interaction between ECM inoculation and drought was found for root biomass at 0.1 significance level, with ECM plants showing 49.5% more root biomass under WS conditions, when compared to non-ECM plants (Fig. 2). No interaction between inoculation and water stress was found for the other plant growth parameters analyzed.

Concerning the parameters related to photosynthesis, both net CO_2 assimilation rate (A) and transpiration rate (E) were significantly decreased by the water stress treatment (Table 2). The maximum quantum yield of photosystem II (Fv/Fm) was increased by the water stress treatment and by the *P. tinctorius* inoculation treatment. No interaction between water stress and inoculation was found for any of the photosynthesis-related parameters.

The water stress treatment had no effect on most of the plant water status parameters analyzed, except for leaf water potential which was decreased in water stressed plants. (Online Resource 2). *P. tinctorius* inoculation treatment did not affect any of the plant water status parameters analyzed. No interaction between inoculation and water stress was found.

Decreased water availability led to a lower leaf nitrogen and carbon percentage (Table 3). ECM inoculation significantly increased leaf N and C percentage but no interaction with the water stress treatment was found.

The water stress treatment did not increase parameters related to oxidative stress (Online Resource 3). In fact, the water stress imposed had no effect on lipid peroxidation, and a significant decrease in protein carbonylation levels was even detected on WS plants compared with WW plants. Inoculation with *P. tinctorius* increased lipid peroxidation but had no effect on protein carbonylation levels in cork oak plants. Cell membrane stability, estimated by measuring electrolyte leakage, showed no alterations with the mycorrhizal status or water treatment in the leaves of the plants (Online Resource 3). In roots, the water stress treatment increased significantly electrolyte leakage levels. However, no interaction between inoculation and water stress was observed for any of the parameters related to oxidative stress or membrane stability.

Concerning non-structural carbohydrates, a significant interaction between ECM inoculation and water stress was found for all the sugars analyzed in roots and leaves (Fig. 3), except for root glucose (Online Resource 4). Under WW conditions, ECM plants showed lower levels of soluble sugars in the roots, especially sucrose, when compared with non-ECM plants (Fig. 3). Shifting soil water content from WW to WS increased significantly the soluble sugar content in roots and leaves of both ECM and non-ECM plants. However, leaves of ECM plants accumulated significantly lower amounts of soluble sugars, when compared to non-ECM plants. The analysis of starch revealed that, under WW conditions, ECM plants accumulated significantly higher amounts of starch in their root system, when compared with non-ECM plants (Fig. 3). However, under WS, a significant accumulation of starch in the roots of non-ECM plants was recorded, while ECM plants did not alter their root starch content when changing from

Table 1 Effect of ECM inoculation (M) and water regime (W) on growth parameters of cork oak seedlings

Parameter	ECM inoculation (M)		Water regime (W)		Significance		
	+ M	− M	WW	WS	M	W	M × W
Height (cm)	54.4 ± 3.8a	39.1 ± 3.7b	49.0 ± 3.7a	44.5 ± 5.5a	**	n.s	n.s.
Shoot dry weight (g)	10.4 ± 0.8a	7.9 ± 0.7b	10.4 ± 0.9a	8.1 ± 0.5b	*	*	n.s.
Shoot diameter (mm)	6.8 ± 0.2a	6.0 ± 0.2b	6.7 ± 0.3a	6.1 ± 0.2a	*	n.s.	n.s.
Number leaves	180 ± 15a	162 ± 11a	178 ± 15a	154 ± 10a	n.s.	n.s.	n.s.
Root length (cm)	41.7 ± 1.2a	37.8 ± 2.3a	40.2 ± 1.6a	39.3 ± 2.1a	n.s.	n.s.	n.s.
Leaf area (cm^2)	7.4 ± 0.5a	6.5 ± 0.6a	6.9 ± 0.6a	7.0 ± 0.5a	n.s.	n.s.	n.s.

+ *M*—inoculation treatment, − *M*—non-inoculation treatment, *WW*—well water treatment, *WS*—water stress treatment. Data are presented as mean ± SE. Different letters within the same line indicate significant differences between treatments according to 2-way ANOVA. Significance levels are shown (*$P \leq 0.05$; **$P \leq 0.01$ n.s. no significant effect)

WW to WS conditions. This resulted in a significantly higher root starch content in the non-ECM plants under WS, compared with the ECM ones. In the leaves, WS resulted in a significant increase in the level of starch in non-ECM plants, while ECM plants showed no alteration with WS.

A significant interaction between ECM inoculation and water stress was observed for total fatty acid content in both roots and leaves (Fig. 4). When changing from WW to WS, total root fatty acid (FA) content increased significantly in both ECM and non-ECM plants, but non-ECM plants showed significantly higher levels of FA when compared to ECM plants (Fig. 4). In the leaves, WS resulted in a decrease in FA accumulation but only in non-ECM plants. ECM plants were able to maintain the same level of total leaf FA content regardless of the water regime.

Root fatty acid composition was not affected by the interaction between ECM inoculation and water stress (Online Resource 4), except for myristic acid (C14:0), which was decreased by the WS conditions, non-ECM plants showing a more pronounced decrease than ECM plants (Fig. 5). However, under WS the root myristic acid percentage of ECM and non-ECM plants was not significantly different. Compared to roots, the leaf fatty acid composition was more affected by the interaction between inoculation and water stress treatments, with significant alterations in myristic acid (C14:0), trans-delta 3-hexadecenoic acid (C16:1 t), stearic acid (C18:0), and oleic acid (C18:1) (Fig. 5). Leaf C16:0, C18:2, and C18:3 were not affected by the interaction between ECM and water stress (Online Resource 4). In general, the water stress treatment led to significant alterations in leaf fatty acid composition of non-ECM plants, whereas ECM plants maintained their fatty acid composition unaltered upon water stress (Fig. 5). The double bond index (DBI) of leaves was significantly decreased by the water stress treatment in ECM and non-ECM plants. However, the reduction in DBI was more pronounced in non-ECM plants compared with ECM plants which had increased DBI under WS.

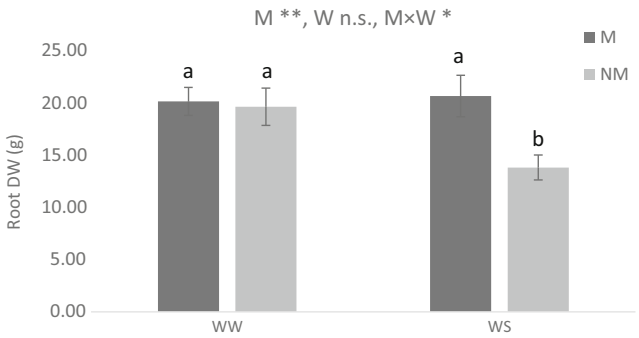

Fig. 2 Influence of ECM symbiosis and drought stress on cork oak root biomass (DW) of cork oak plants (means ± SE). *M* ECM treatment, *NM* non-ECM treatment, *WW* well water treatment, *WS* water stress treatment. Bars with different letters are significantly different according to Tukey's HSD test ($n = 6–7$). Significance levels of the effect of ECM treatment (M), water stress treatment (W), and their interaction (M × W) are indicated: *$P < 0.1$, **$P < 0.05$, n.s. no significant effect

Discussion

Several studies have reported that the ECM symbiosis improves drought tolerance of host plants (Alvarez et al. 2009a, b; Beniwal et al. 2010). However, there are also studies that found no effect of ECM symbiosis on plants' ability to cope with drought stress (Lehto 1992; Coleman et al. 1990). In addition, there is a lack of information on how ECM symbiosis could contribute to alleviate the negative effects of water stress on drought-tolerant species, such as cork oak. In the present study, we investigated if inoculation with the ECM fungus *P. tintorius* could be an advantage to cork oak plants growing under drought conditions. Furthermore, we studied several mechanisms by which symbiosis with *P. tinctorius* could increase cork oak drought tolerance. To accomplish this we analyzed growth, photosynthetic performance, water status, oxidative stress, nitrogen concentration, sugar levels and fatty acid content, and composition of cork oak plants colonized or not by *P. tinctorius* under WW and WS conditions.

Table 2 Effect of ECM inoculation (M) and water regime (W) on photosynthesis, transpiration, maximum quantum efficiency of PSII (Fv/Fm), and photosynthetic pigments (total chlorophylls and carotenoids) of cork oak seedlings

Parameter	ECM inoculation (M)		Water regime (W)		Significance		
	+ M	− M	WW	WS	M	W	M × W
Photosynthesis ($\mu moleCO_2 m^{-2}\ s^{-1}$)	4.6 ± 0.8a	4.7 ± 0.8a	6.5 ± 0.8a	3.1 ± 0.5b	n.s.	**	n.s.
Transpiration ($mmoleH_2Om^{-2}\ s^{-1}$)	2.5 ± 0.3a	2.4 ± 0.3a	3.2 ± 0.3a	1.8 ± 0.2b	n.s.	**	n.s.
Fv/Fm	0.838 ± 0.002a	0.831 ± 0.002b	0.830 ± 0.001a	0.839 ± 0.002b	*	**	n.s.
Chlorophyll ($\mu g\ cm^{-2}$)	39.5 ± 2.2a	40.5 ± 2.4a	42.6 ± 2.4a	36.2 ± 1.2a	n.s.	n.s.	n.s.
Carotenoids ($\mu g\ cm^{-2}$)	5.7 ± 0.2a	5.3 ± 0.3a	5.6 ± 0.2a	5.8 ± 0.2a	n.s.	n.s.	n.s.

+ *M*—inoculation treatment, − *M*—non-inoculation treatment, *WW*—well water treatment, *WS*—water stress treatment. Data are presented as mean ± SE. Different letters within the same line indicate significant differences between treatments according to 2-way ANOVA. Significance levels are shown (*$P \le 0.05$; **$P \le 0.01$ n.s. no significant effect)

The water stress imposed in our study did not result in a strong response on the growth of the cork oak. The only growth parameter negatively affected by the WS treatment was shoot biomass, accompanied by a significant decrease in % C. This is consistent with the high cork oak tolerance to drought (Grant et al. 2010). Previous studies have shown that inoculation with *P. tinctorius* increases the performance of cork oak plants under normal irrigation conditions, both in the nursery and in the field (Sebastiana et al. 2013). The present study confirms those results by showing that *P. tinctorius* inoculation increases plant height, shoot basal diameter, shoot biomass, and root biomass. Increased growth of the above-ground parts in *P. tinctorius*-inoculated plants is in accordance with the increase in % C detected in leaves of mycorrhizal plants. In the present study, we detected a significant interaction between *P. tinctorius* inoculation and WS for root biomass, illustrating that *P. tinctorius* could improve cork oak root growth under conditions of limited water availability. Thus, ECM symbiosis could alleviate drought stress allowing plants to maintain a higher root growth under these unfavorable conditions.

As expected, the drought stress imposed significantly decreased CO_2 fixation and transpiration rate, which is in accordance with the reported cork oak response to summer drought, characterized by the closure of stomata, ensuring optimal use of carbon and water resources without compromising survival (Otieno et al. 2007). In accordance, WS also decreased leaf % C of cork oak plants. Downregulation of photosynthesis constitutes an effective control mechanism for protecting the photosynthetic apparatus from photodamage due to drought (Valladares and Pearcy 1997). ECM inoculation had no effect on photosynthesis and no interaction between inoculation and WS was found, suggesting that symbiosis with *P. tinctorius* was not effective in inducing stomata opening for sustaining photosynthesis under conditions of low water availability, as suggested in other studies (Morte et al. 2001; Ortega et al. 2004). Concerning photochemical efficiency, the significant increase in the maximum quantum efficiency of the photosystem II (Fv/Fm) in *P. tinctorius*-colonized cork oak plants reveals an increased photochemical potential of mycorrhizal plants. However, the positive effect on Fv/Fm was independent of the drought treatment showing that *P. tinctorius* did not provide any advantage in terms of photochemical efficiency under water stress conditions. In our experiment, Fv/Fm was increased by the WS treatment, which constitutes another indication of the resilience of this plant species to intense drought stress conditions.

It is often suggested that fungal species with extensive external fungal mycelium, like *P. tinctorius*, are better in taking up water for the host plant (Lamhamedi et al. 1992; Garbaye

Table 3 Effect of ECM inoculation (M) and water regime (W) on nitrogen and carbon percentage in leaves of cork oak seedlings

Parameter	ECM inoculation (M)		Water regime (W)		Significance		
	+ M	− M	WW	WS	M	W	M × W
N (%)	1.74 ± 0.04a	1.66 ± 0.03b	1.80 ± 0.02a	1.59 ± 0.02b	*	***	n.s.
C (%)	46.27 ± 0.06a	45.70 ± 0.07b	46.08 ± 0.08a	45.90 ± 0.11b	***	*	n.s.

+ *M*—inoculation treatment, − *M*—non-inoculation treatment, *WW*—well water treatment, *WS*—water stress treatment. Data are presented as mean ± SE. Different letters within the same line indicate significant differences between treatments according to 2-way ANOVA. Significance levels are shown (*$P \le 0.05$; **$P \le 0.01$, ***$P \le 0.001$ n.s. no significant effect)

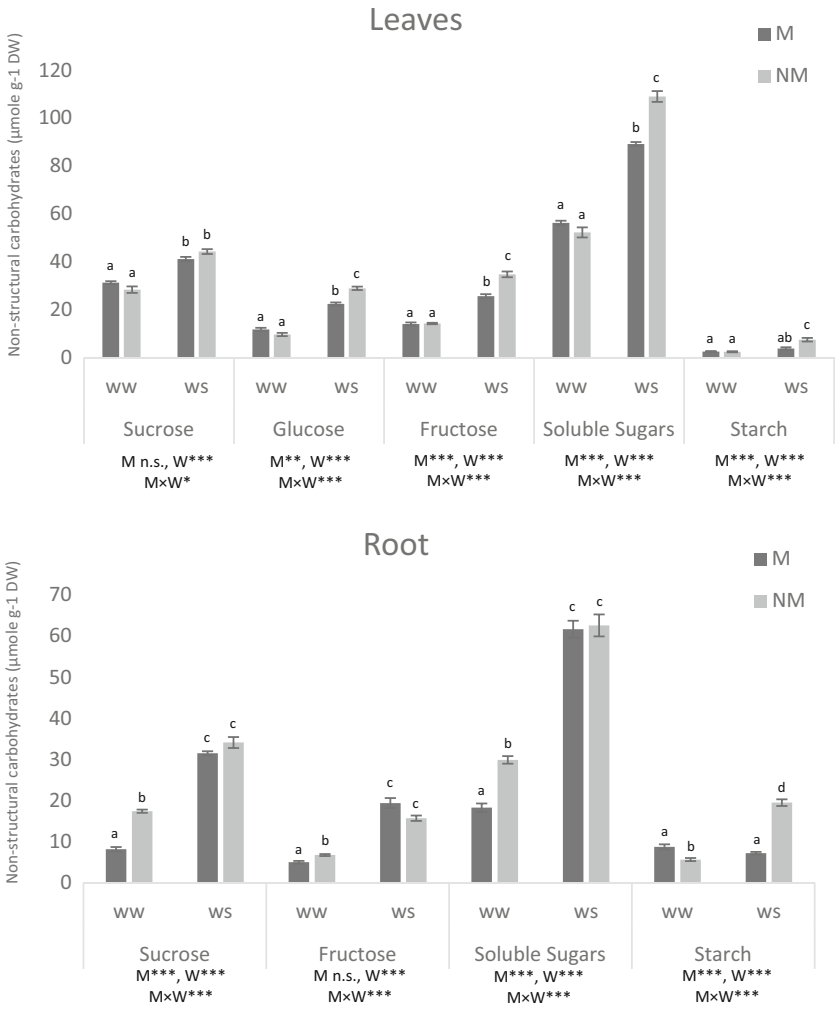

Fig. 3 Influence of ECM symbiosis and drought stress on the content of non-structural carbohydrates in leaves and roots of cork oak plants (means ± SE). *M* ECM treatment, *NM* non-ECM treatment, *WW* well water treatment, *WS* water stress treatment. Bars with different letters are significantly different according to Tukey's HSD test ($n = 6–7$). Significance levels of the effect of ECM treatment (M), water stress treatment (W) and their interaction (M × W) are indicated: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n.s. no significant effect

2000). However, symbiosis with *P. tinctorius* was not effective in increasing the water status of cork oak plants subjected to drought. Our data showed that inoculation did not increase any of the plant water status parameters studied, even though inoculated plants had a higher root biomass under WS, which would potentially increase water uptake. Our results are in accordance with several studies that found little or no effect of ECM on root water transport and/or plant water relations, such as root hydraulic conductance, transpiration rate, or leaf water potential (Sands et al. 1982; Coleman et al. 1990; Dominguez Nunez et al. 2009). Although trees from the genera *Quercus* and *Pinus* include ECM-forming species that grow seasonally on very dry sites, including cork oak, their survival during the driest periods has been suggested to be due to their coarse root systems that can reach several meters deep (Nadezhdina et al. 2008). Cork oak is a deep-rooting species which develops a long taproot shortly after germination that can reach deep soil layers where water is available.

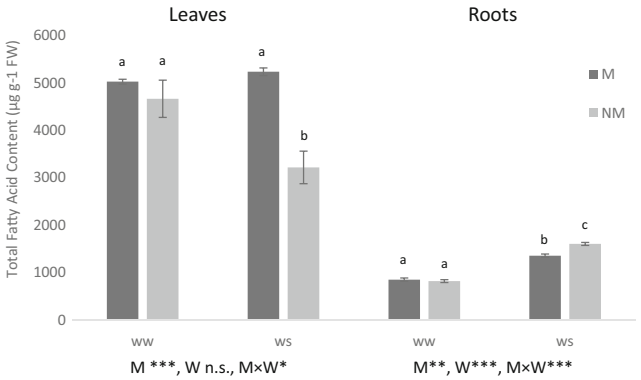

Fig. 4 Influence of ECM symbiosis and drought stress on total fatty acid content (μg g^{-1} FW) in leaves and roots of cork oak plants (means ± SE). *M* ECM treatment, *NM* non-ECM treatment, *WW* well water treatment, *WS* water stress treatment. Bars with different letters are significantly different according to Tukey's HSD test (n = 6–7). Significance levels of the effect of ECM treatment (M), water stress treatment (W) and their interaction (M × W) are indicated: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n.s. no significant effect

Inoculation with *P. tinctorius* enabled cork oak plants to acquire more nitrogen, which is in accordance with studies reporting that root colonization by *P. tinctorius* increased nutrient acquisition by the host plants, including nitrogen, phosphorus, and potassium (Alvarez et al. 2009b; Navarro-Garcia

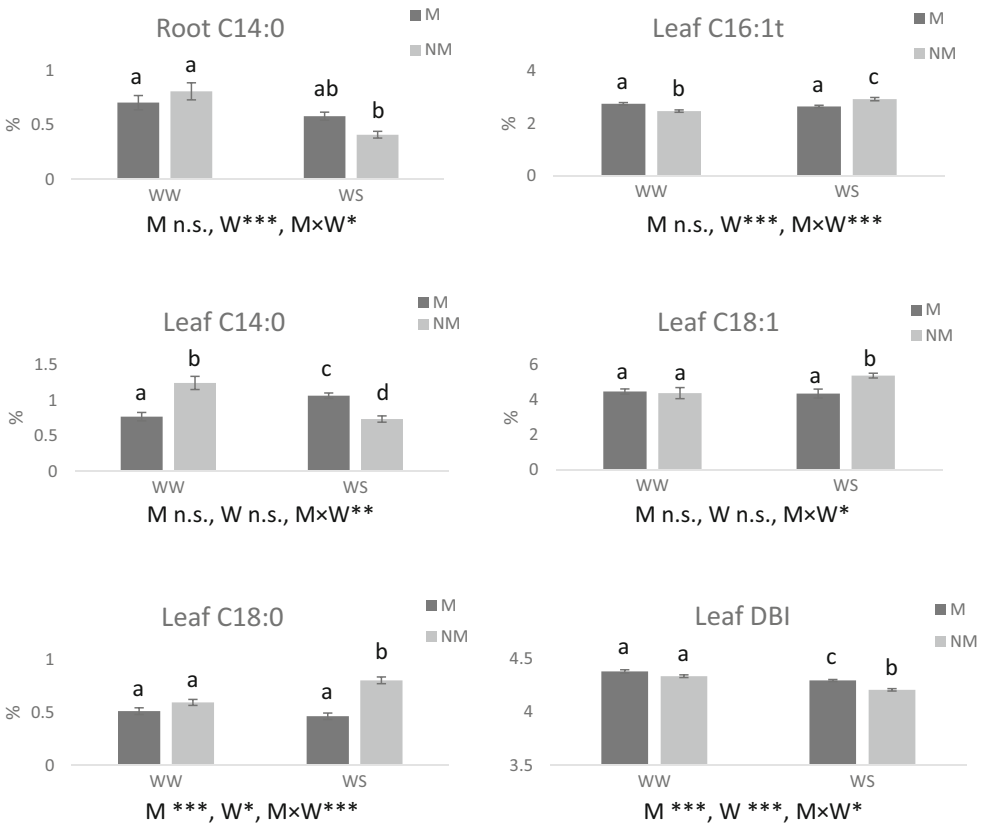

Fig. 5 Influence of ECM symbiosis and drought stress on fatty acid composition (%) and Double Bond Index (DBI) of leaves and roots of cork oak plants (means ± SE). *M* ECM treatment, *NM* non-ECM treatment, *WW* well water treatment, *WS* water stress treatment. Bars with different letters are significantly different according to Tukey's HSD test ($n = 6–7$). Significance levels of the effect of ECM treatment (M), water stress treatment (W) and their interaction (M × W) are indicated: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s. no significant effect

et al. 2011). In the temperate and boreal regions, dominant ECM fungal species, such as *P. tinctorius*, are involved in the uptake of N, the most growth-limiting nutrient in soils of these ecosystems. However, in our experiment, increased nitrogen acquisition in ECM plants was independent of the water stress treatment indicating that symbiosis with *P. tinctorius* did not increase N nutrition when plants were subjected to drought. Nutrient uptake by ECM fungi during drought conditions has been studied surprisingly little (Lehto and Zwiazek 2011). While some studies have shown that ECM fungi were able to take up N during drought and thereby promote plant resistance to water stress (Wu et al. 1999), others found no effect of ECM fungi in the sense that, although inoculation improved significantly plant N concentration, there was no added benefit on N uptake during drought acclimation (Núñez et al. 2009; Garcia et al. 2011). Our results agree with the latter reports and point to the need to increase the number of studies regarding the effect of ECM symbiosis on plant's nutrient uptake during drought conditions.

Drought has been associated to excessive production of ROS which may cause lipid peroxidation, protein carbonylation, membrane injury, and nucleic acid degradation, ultimately leading to cell death (Apel and Hirt 2004). In our experiment, the WS treatment did not cause a significant increase in leaf ROS production since no increase in lipid peroxidation, protein carbonylation, or leaf electrolyte leakage were detected during the WS treatment. Cork oak is a very drought-tolerant species which can efficiently activate the antioxidant system that protects cells from ROS that arise under stressful conditions (Faria et al. 1996). In roots, drought increased electrolyte leakage indicating a negative effect on root cell membrane stability. However, no interaction between drought and *P. tinctorius* inoculation was detected for any of the ROS-related parameters, showing that this fungus was not effective in protecting the plant from drought-induced membrane injury, at least in roots where we detected a negative ROS-related effect during water stress. Our results do not agree with the ones reported by Alvarez et al. (2009b) who found a positive effect of ECM fungi colonization on mitigating the negative effects of ROS during drought, related to the activation of antioxidant enzymes in the host plant.

The improved nutrition that results from the fungus nutrient delivery to the ECM plant comes with the price of carbohydrate transfer to the fungal partner. This flux results in a reduction of carbon in root systems of ECM plants which can receive about half of the photosynthetically fixed carbon (Nehls et al. 2010). The lower amounts of soluble sugars, specially sucrose, detected in the roots of *P. tinctorius*-inoculated cork oak plants under WW conditions, also detected earlier in this same symbiotic system (Sebastiana et al. 2017), are thus related to the increased carbon sink promoted by the ECM fungus in the symbiotic roots. In ectomycorrhizas

established with basidiomycetes such as *P. tinctorius*, plant-derived sucrose in the plant-fungus interface is hydrolysed by plant cell wall invertases into hexoses, from which glucose seems to be preferred by the mycobiont (Nehls et al. 2010). This C sink apparently did not deplete carbon content in the aerial part of the plants since % C was increased by ECM inoculation and concentration of soluble sugars was the same in ECM and non-ECM under WW conditions. It also did not affect sugar root reserves since ECM plants had increased root starch content when compared with non-ECM plants, an indication that the plant is not mobilizing its carbon reserves for transfer to the symbiotic fungus, underlining the improved performance of the ECM plants.

In our experiment, an increase in the accumulation of soluble sugars was detected in leaves and roots of cork oak plants under WS conditions. However, non-ECM plants accumulate higher amounts of soluble sugars when compared to ECM plants, which is not consistent with an increased ability of ECM plants to osmotically adjust by sugar accumulation during drought. Osmotic adjustment by sugar accumulation lowers the water potential (more negative), promoting turgor maintenance and increasing water uptake from dryer soils. However, we also did not find a correlation between leaf water potential and leaf soluble sugar content, since ECM plants had lower amounts of soluble sugars relative to non-ECM plants, and leaf water potential was not affected by *P. tinctorius* inoculation.

Increased concentrations of soluble sugars and starch upon drought have been observed in tissues of forest trees species and seems to constitute a strategy for carbon reserve accumulation intended to be used once the stress has passed, for example during resprouting after a severe disturbance, a typical characteristic of widely distributed genera like *Quercus, Populus*, or *Eucalyptus* (Zeppel et al. 2015). In this process, which has a parallel with our results, soil desiccation limits transpiration and photosynthesis, which in turn restrict growth, re-directing photoassimilates to accumulate in the stem and root tissues (Galvez et al. 2011). However, our results show that ECM plants accumulated less starch and soluble sugars under WS which is consistent with a better performance in limited water conditions. In fact, WS had no effect on ECM plants' starch concentration, whereas in non-ECM plants, WS increased significantly starch content in roots and leaves. From these observations, we conclude that under WS conditions, when compared with the non-symbiotic plants, ECM cork oak plants had a weaker environmental cue to switch from growth to accumulation of storage carbon reserves. Our results agree with a report on ECM roots of *Populus*, where drought stress promoted the accumulation of soluble sugars, which were found to be highest in WS non-ECM plants, that displayed the strongest symptoms of drought stress (Beniwal et al. 2010).

Studies suggest that cell membrane stability during drought depends largely on the content and composition of fatty acids, determining plant tolerance to dehydration (Yordanov et al. 2000; Gigon et al. 2004). The increase in fatty acid content, observed in our study in the roots of cork oak plants when subjected to drought is in accordance with the increased accumulation of carbohydrates, related with the drought stress-induced C reserve accumulation mentioned above. In the leaves, the opposite trend was detected with drought decreasing total fatty acid content, which could be due to degradative processes such as the inhibition of lipid biosynthesis, and stimulation of lipolytic and peroxidative activities that are associated with decreased membrane lipid content during drought (De Paula et al. 1990; Matos et al. 2001; Ferrari-Iliou et al. 1994). Interestingly, when subjected to drought, *P. tinctorius*-inoculated cork oak plants could maintain the same level of leaf total fatty acid content, which also suggests a beneficial effect of *P. tinctorius* symbiosis on plant drought tolerance.

Most of the changes in fatty acid composition detected in cork oak plants upon drought were detected in the leaves of non-inoculated plants indicating that *P. tinctorius* inoculated plants could maintain their fatty acid composition despite the water shortage. Drought-tolerant plants are better in maintaining their membrane composition unaltered during drought stress (Gigon et al. 2004). Furthermore, the higher double bond index (DBI) detected in the leaves of *P. tinctorius* inoculated plants under drought suggests an increased membrane fluidity. Tolerance of plants to drought depends on their ability to maintain fatty acid unsaturation (Losa and Muratab 2004; Duarte et al. 2017).

Our study is limited in the number of species tested and it would be desirable to test if our findings also hold true for other ECM fungal and plant species. In addition, different degrees of water stress and additional time-points would also have been valuable.

Concerning the mechanisms by which ECM fungi could improve the drought tolerance of host plants, our findings represent an advance in the field of ECM symbiosis water relations by showing that (1) *P. tinctorius* does not have a direct positive effect on cork oak water status that would increase photosynthetic activity during drought, (2) does not seem to contribute to increase N nutrition under drought, (3) does not promote an osmotic adjustment by sugar accumulation, (4) or protect roots from drought-induced increases in ROS. Nevertheless, our results show that symbiosis with *P. tinctorius* was beneficial to cork oak plants growing under drought conditions. Though we did not observe a significant effect of *P. tinctorius* in the growth of the aerial part of cork oak plants upon water stress, the roots of mycorrhizal plants had increased biomass under drought when compared with non-mycorrhizal plants. Furthermore, mycorrhizal plants showed less stress symptoms, such as less accumulation of soluble sugars and starch, and increased ability to maintain leaf fatty acid content and composition. Also, an increased

unsaturation level of membrane lipids, detected in mycorrhizal plants could be linked to a higher membrane fluidity and, hence, increased drought tolerance.

Whether our findings can be generalized for other ECM trees is currently hard to say as our study was restricted to a single plant-fungus combination and a single treatment. However, the better plant performance as a consequence of the ECM symbiosis is probably a good insurance for a higher drought stress tolerance in many tree species adapted to seasonally dry environments.

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