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Research article

The leaf lipid composition of ectomycorrhizal oak plants shows a drought-tolerance signature

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ABSTRACT

Ectomycorrhizas have been reported to increase plant tolerance to drought. However, the mechanisms involved are not yet fully understood. Membranes are the first targets of degradation during drought, and growing evidences support a role for membrane lipids in plant tolerance and adaptation to drought. We have previously shown that improved tolerance of ectomycorrhizal oak plants to drought could be related to leaf membrane lipid metabolism, namely through an increased ability to sustain fatty acid content and composition, indicative of a higher membrane stability under stress. Here, we analysed in deeper detail the modulation of leaf lipid metabolism in oak plants mycorrhized with *Pisolithus tinctorius* and subjected to drought stress. Results show that mycorrhizal plants show patterns associated with water deficit tolerance, like a higher content of chloroplast lipids, whose levels are maintained upon drought stress. Likewise, mycorrhizal plants show increased levels of unsaturated fatty acids in the chloroplast phosphatidylglycerol lipid fraction. As a common response to drought, the digalactosyldiacyloglycerol/monogalactosyldiacyloglycerol ratio increased in the non-mycorrhizal plants, but not in the mycorrhizal plants, associated to smaller alterations in the expression of galactolipid metabolism genes, indicative of a higher drought tolerance. Under drought, inoculated plants showed increased expression of genes involved in neutral lipids biosynthesis, which could be related to an increased ability to tolerate drought stress. Overall, results from this study provide evidences of the involvement of lipid metabolism in the response of ectomycorrhizal plants to water deficit and point to an increased ability to maintain a stable chloroplast membrane functional integrity under stress.

1. Introduction

Nowadays, due to climate change, drought represents one of the major threats to agricultural practices. Therefore, increasing knowledge on plant's response to drought is key to develop new strategies to mitigate the expected negative effects of drought on plant yield and survival. One of the first effects of drought in plants is stomatal closure, which contributes to prevent or delay tissue dehydration. This negatively affects plant biomass production and development due to decreased photosynthetic C gain. Other effects include impaired cell elongation due to loss of turgor pressure, increased oxidative stress that damages membrane components such as those from the photosynthetic machinery, nutritional deficiencies due to limited nutrient up-take and reduced energy production (Farooq et al., 2009). At the cellular level, drought affects membranes by interfering with membrane proteins and lipids. Drought can cause increased viscosity of cytoplasmic contents, leading to fusion of apposed membranes and membrane protein denaturation (Hoekstra et al., 2001). Upon drought, membrane lipid composition changes in order to contribute to membrane bilayer stabilization under stress (Gigon et al., 2004; Torres-Franklin et al., 2007; Gasulla et al., 2013). Alterations in lipid content and composition, and in lipid metabolic genes are a hallmark of plant stress response, including drought, salt, nutrient deficiency and heat stress (Okazaki and Saito, 2014). Drought tolerant plants are able to maintain the integrity and stability of cellular membranes under stress, while susceptible plants suffer membrane degradation which can lead to severe cell

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damage, hampering recovery after stress cessation (Yu and Li, 2014).

Lipids can be classified as neutral lipids (mainly storage lipids abundant in oleaginous seeds) or polar lipids, which account for nearly 90% of total lipids in plant leaves (Pham Thi et al., 1990). The main lipids constituting cellular membranes are the glycerolipids, which comprise the glycerogalactolipids from chloroplast membranes, and the phosphoglycerolipids, present in all extra-plastidial membranes. Galactolipids such as mono- and digalactosyldiacyloglycerol (MGDG, DGDG) are the most abundant lipids in chloroplast membranes, and they constitute most of the membrane lipids in plants (Shimojima and Ohta, 2011). Chloroplasts also contain minor amounts of phospholipids, mostly phosphatidylglycerol (PG). The balance between the bilayer forming DGDG and the non-bilayer forming MGDG determines membrane stability and the activity of key enzymes involved in photosynthesis (Hölzl and Dörmann, 2007). Extra-chloroplast membranes are mainly composed of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), together with minor amounts of other phospholipids such as phosphatidylinositols (PIs) which are involved in signalling (Thole and Nielsen, 2008). Fatty acids (FA), the constituents of lipids, are synthesized in plastids and consist of acyl chains growing attached to the acyl carrier protein and forming mainly C16:0- and C18:1-ACP for lipid synthesis (Li-Beisson et al., 2013). Some of these fatty acids are integrated into lipids inside plastids (the 'prokaryotic' pathway), or exported to the ER where further elongation, acyl editing, and lipid assembly occurs (the 'eukaryotic' pathway). 16:3 plants (e.g. Arabidopsis) mainly use the prokaryotic pathway for lipid synthesis, where the plastid enzyme *sn2*-acyltransferase uses exclusively C16-FA, whereas 18:3 plants (e.g pea and rice) use the eukaryotic pathway, where the ER sn2-acyltransferase has a high specificity for C18–FAs.

The role of lipids in plant water stress tolerance has been demonstrated in several studies, which have shown that adaptation to dehydration involves major remodelling of membrane lipid and fatty acid composition (Okazaki and Saito, 2014). One of the most widely reported responses is the increase in the DGDG/MGDG ratio which most often results from a decrease in MGDG content upon water stress (Gigon et al., 2004; Gasulla et al., 2013; Chen et al., 2018). In dehydration sensitive plants alterations in lipids occur at mild water stress but in tolerant plants only severe dehydration causes alterations in lipid metabolism (Sahsah et al., 1998). Another common response to drought is the decrease in fatty acid unsaturation level, mainly due to the reduction in the amount of linolenic acid (C18:3), which is often less pronounced in plants with higher tolerance to drought, indicating higher membrane fluidity and stability (Monteiro de Paula et al., 1990; Gigon et al., 2004). The presence of double bonds reduces fatty acid ability to pack together in membranes, allowing for increased membrane fluidity under stress (Vrablik and Watts, 2012).

Root symbiosis, such as mycorrhizas, are known to improve the nutritional status of plants, increasing growth and performance. In these mutualistic associations the host plant receives soil mineral nutrients, such as P and N, from the fungus in exchange for sugars and lipids which are transferred from the host plant to the fungus. In addition to the improvement in nutrition, mycorrhizas also increase resistance of plants to biotic and abiotic stress, such as pathogens, salt stress, heavy metals and drought (Smith and Read, 2008). Ectomycorrhizas (ECM) are formed between tree species such as pines and oaks, and mushroom-forming soil fungus including boletes, amanitas and truffles. In boreal and temperate forest ecosystems ECMs play a crucial role in nutrient cycling and carbon sequestration, 95% of the short roots of trees in these ecosystems being colonized by ECM fungi (Smith and Read, 2008). Several studies have shown that ECM symbiosis can improve plant performance under water stress (Davies et al., 1996; Morte et al., 2001; Alvarez et al., 2009a, 2009b; Dominguez Nunez et al., 2009, García et al., 2011). Mechanisms proposed to explain amelioration of drought stress in ECM symbiotic plants include increased water uptake by the induction of secondary root formation and fungal hyphae water transport that would induce an higher root conductance and consequently stomatal opening during water stress, enabling increased C assimilation (Duddridge et al., 1980; Plamboeck et al., 2007), a nutritional effect due to increased nutrient acquisition during drought that would increase photosynthesis efficiency (Wu et al., 1999; Alvarez et al., 2009b), or activation of the antioxidant system protecting roots from ROS produced during drought (Alvarez et al., 2009a). However, the role of ECMs in plant drought tolerance is no yet clear, with different fungal species inducing positive or negative effect on host plant responses to drought (Dosskey et al., 1991; Lamhamedi et al., 1992). Besides, in cases of positive responses, no consensual mechanism responsible for the increased drought tolerance has been found yet.

In a previous study using ECMs formed between cork oak (*Quercus suber*) and *Pisolithus tinctorius*, two drought tolerant species, we tested several mechanisms which have been suggested to confer drought tolerance to mycorrhizal plants (Sebastiana et al., 2018). Results showed a positive effect of *P. tinctorius* inoculation on cork oak growth under water limitation, mycorrhizal plants also showing less stress symptoms induced by drought. However, none of the mechanisms tested, such as ability to maintain photosynthesis under drought, increase in plant water status, increase in nutrient acquisition, effect on drought-induced ROS production or osmotic adjustment, was found to contribute to the better performance of mycorrhizal plants. Notably, we detected an increased ability of mycorrhizal plants to maintain leaf fatty acid content and composition under water stress, mycorrhizal plants also showing a higher fatty acid unsaturation level, which could improve drought tolerance. Therefore, in the present study we addressed in deeper detail leaf lipid metabolism in cork oak plants mycorrhized with *P. tinctorius* and subject to drought stress. We analysed leaf lipid and fatty acid composition, and the expression of genes involved in lipid metabolism, to examine the hypothesis that symbiosis with ECM fungi affects lipid metabolism which contributes to enhance tolerance of mycorrhizal plants against drought stress conditions.

2. Material and methods

2.1. Biological material and experimental setup

Mycorrhization of *Q. suber* with *P. tinctorius* was performed according to Sebastiana et al. (2018). Briefly, *Q. suber* acorns were germinated in 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%) in a greenhouse. Three-month old plantlets were then transferred to 10L pots containing the same soil as for seed germination. Mycorrhization with *P. tinctorius* was performed at transplanting by inoculating roots with a *P. tinctorius* inoculum produced *in vitro* using a peat-vermiculite based substrate. Non-mycorrhizal controls 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 with no fertilization applied. Sixteen months after *P. tinctorius* inoculation, plants were subjected to drought by withholding irrigation during 6 weeks in the summer. Four treatments were considered: (1) well-watered mycorrhizal plants (WW + Myc), (2) water-stressed mycorrhizal plants (WS + Myc), (3) well-watered non-mycorrhizal plants (WW-Myc) and (4) water-stressed non-mycorrhizal plants (WS-Myc). The experimental design was a randomized complete block design with pots from the different treatments being periodically rotated to ensure that all the plants received equal environmental conditions. At the end of the water stress treatment, leaves were collected, frozen in liquid nitrogen and pulverized. Six plants from each treatment, combined into 3 replicas (2 plants per replica), were used for lipid and gene expression analysis. Only plants (mycorrhizal and non-mycorrhizal) with similar size were considered for the water stress assay.

2.2. Lipid extraction and analysis

Lipids were extracted from frozen leaf tissue, after boiling for 5 min to stop lipolytic activities, using a mixture of chloroform/methanol/water (1:1:1). After being vortexed, samples were centrifuged at 4000g for 5 min. The chloroformic phase was evaporated under a stream of N_2 gas at 37 °C. Lipids were resuspended in an ethanol/toluene (1:1) solution and stored at −20 °C under N_2 atmosphere until analysis. Lipid classes were separated by thin layer chromatography (TLC) as described in Esquível et al. (2017) on silica gel plates (G-60, Merck), using the following solvent mixture: chloroform/methanol/acetone/acetic acid/water (100/20/40/20/8) for separation of the different polar lipid classes from neutral lipids. After staining with primuline (0.01% w/v in 80% acetone v/v), lipid bands were scraped off and methylated to fatty acids methyl esters (FAMEs) in a methanol/sulfuric acid (97.5:2.5) solution for 1 h at 70 °C. FAMEs were recovered by adding petroleum ether/ultrapure water (3:2) and the organic phase was collected after centrifugation. FAMEs were separated by gas chromatography (430 Gas Chromatograph, Varian) at 210 °C, equipped with a hydrogen flame ionization detector, according to Feijão et al. (2018). Heptadecanoic acid (C17:0) was used as an internal standard for quantification. Three replicates were analysed per treatment.

The double bond index (DBI) was calculated as:

DBI = 2[(% monoenoic acids) + 2 (% dienoic acids) + 3 (% trienoic acids)]/100.

2.3. Gene expression analysis

Total RNA was isolated from frozen leaf powder from each treatment (6 plants combined in 3 replicas with 2 plants per replica). RNA preparations were treated with DNase (Turbo DNA-free kit, Invitrogen) for genomic DNA removal before cDNA synthesis with Reverse Transcriptase (RevertAid™ H Minus M-MuLV Reverse Transcriptase, Fermentas) and Oligo dT primer. qPCR was preformed using a Maxima SYBR Green qPCR Master Mix (2X; Thermo Scientific), following the manufacturer's instructions and using a StepOne™ Real-Time PCR system (Applied Biosystems). Thermal cycling conditions were: 95 °C denaturation step for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at gene specific temperature (Supplementary Table 1) for 30 s. Three replicates were analysed per treatment. The following *Arabidopsis thaliana* protein sequences encoding lipid biosynthesis enzymes, were used for searching the NCBI database with TBLASTN restricted to *Q. suber* (taxid: 58331): MGDG synthase1 MGD1 (AT4G31780), DGDG synthase1 DGD1 (AT3G11670), PI synthase1 PIS1 (AT1G68000), DAG O-acyltransferases DGAT1 (AT2G19450) and DGAT2 (AT3G51520), phospholipase C PLC2 (AT3G08510), phospholipase D PLDδ1 (AT4G11850) and patatin-like phospholipase A PAT (AT2G26560). Sequences of primers used for qPCR are shown in Supplementary Table 1. Relative transcript abundance was calculated using the comparative C_T method (Schmittgen and Livak, 2008). The Elongation Factor 1-alpha (EF1α) coding gene, previously reported as a reference gene in *Q. suber-P. tinctorius* ectomycorrhizas (Sebastiana et al., 2017) and in plants subjected to drought (Jazi et al., 2016) was used for the normalization of expression data.

2.4. Data analysis

Statistical analysis was performed using the SPSS statistics software (Version 22.0 for Windows, SPSS, Chicago, USA). Two-way ANOVA was used to assess the main effects and interactions of *P. tinctorius* inoculation and water stress treatment for the lipid classes and gene expression analysis. Differences between mean values were compared using the least significant differences (LSD) post hoc test, and a P value < 0.05 was considered significant.

Since fatty acids are part of a metabolic chain, the changes are sometimes too subtle to be analysed by univariate statistical analysis being more clearly analysed using all fatty acids as a profile in a multivariate approach. Therefore, the fatty acid composition was analysed using a statistical multivariate approach that has proven to be efficient for the evaluation of the fatty acid profile of different plant ecotypes (Duarte et al., 2018). Statistical analysis was conducted using Primer 6 software. Data from the total fatty acid relative composition in each treatment was used to construct a resemblance matrix based in Euclidean distances. Canonical analysis of principal coordinates (CAP) was used to generate a statistical multivariate model based on fatty acid relative composition having this profile as modelling vectors, for each treatment. CAP analysis also allowed performing classification tests regarding the efficiency of the FA-based models to classify and separate the different treatment groups. The differences among CAP groups were evaluated using the Similarity Percentage Analysis (SIMPER) test with Primer 6 software (Clarke and Gorley, 2006). Samples were grouped according to their degree of similarity as provided by SIMPER.

Euclidean distances provided by ANOSIM from fatty acid relative quantities (Clarke and Gorley, 2006) were grouped using the clustering analysis method (UPGMA) conducted in MEGA 7 (Kumar et al., 2016). The heat map was constructed using Heatmapper (http://www.heatmapper.ca) (Babicki et al., 2016).

3. Results and discussion

3.1. The lipid composition of cork oak leaves is altered by mycorrhizal inoculation and drought

To our knowledge this is the first report describing lipid composition of cork oak leaves. TLC fractionation of total lipid extracts revealed that under control conditions, 33% of cork oak leaf fatty acids are present as neutral lipids (NL) (Supplementary Fig. 1). Regarding membrane lipids, the plastidial lipid MGDG accounted for 31% of the total, followed by DGDG, which corresponded to nearly 18% of total lipids. The third more abundant polar lipid detected was the extra-chloroplast phosphoglycerolipid PC (~8%). The plastidial phospholipid PG corresponded to 4% and PE was detected at even lower amounts (3%). Both PI and PA (phosphatidic acid), extra-plastidial phospholipids known to be involved in signalling processes, were also detected, but in very low quantities (1–2%), as expected.

Although there are several reports on the lipid composition of plant leaves, little is known regarding trees. Interestingly, the high content of NL found in cork oak leaves under control conditions, was also observed in the leaves of the Mediterranean olive tree (Guerfel et al., 2008) and contrasts with the small amounts present in the leaves of other plants such as Arabidopsis (Gigon et al., 2004) coconut (Repellin et al., 1997) or *Parkinsonia aculeata* (Benadjaoud et al., 2013). However, as in most plants, in cork oak MGDG is the most abundant polar lipid, comparing to olive tree which contains more DGDG than MGDG. The relative amounts of the different polar lipid classes detected in cork oak leaves resemble those detected in grapevine (Laureano et al., 2018), except for the NL fraction which is higher in cork oak, probably due to the nature of cork oak leaves, which accumulate large amounts of waxy materials to protect leaves from dehydration, common during the summer in the Mediterranean region where cork oak forests are located.

The CAP graphical projection clearly shows that *P. tinctorius* inoculation and water stress both affected lipid composition of cork oak leaves, resulting in specific rearrangements of the lipid profile, with samples forming 4 well defined groups corresponding to each treatment (Fig. 1A).

Comparison of our results under drought with other Mediterranean species, such as olive tree, shows that cork oak can keep a more steady state level of most lipid classes, including chloroplast lipids (MGDG, DGDG and PG), extraplastidial lipids (PC and PE) and neutral lipids (Fig. 1B), which in olive tree decrease significantly under water deficit

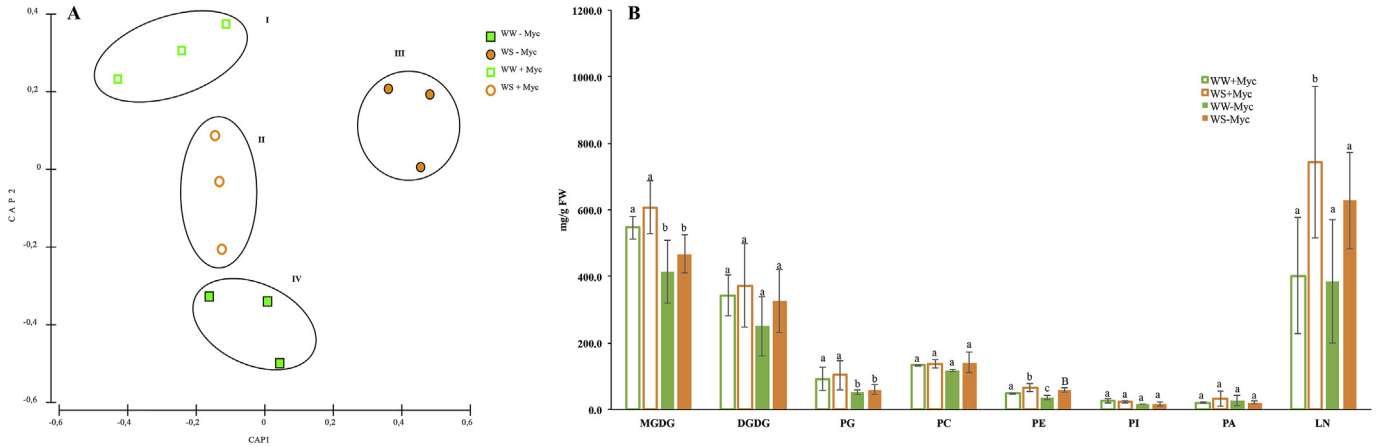

Fig. 1. Effect of water stress and *P. tinctorius* inoculation on lipid class distribution of cork oak leaves determined by TLC analysis. (A) Canonical analysis of principal coordinates (CAP) based on the lipid class relative composition. (B) Lipid profile of cork oak leaves. Bars represent averages ± SD of 3 biological replicates. Different letters represent statistical significances by two-way ANOVA ($p > 0.05$). MGDG (monogalactosyldiacylglycerol), DGDG (digalactosyldiacylglycerol), PG (phosphatidylglycerol), PC (phosphatidylcholine), PE (phosphatidylethanolamine), PI (phosphatidylinositol), PA (phosphatidic acid), NL (neutral lipids). WW + Myc (well-watered mycorrhizal plants), WS + Myc (water-stressed mycorrhizal plants), WW-Myc (well-watered non-mycorrhizal plants), WS-Myc (water-stressed non-mycorrhizal plants).

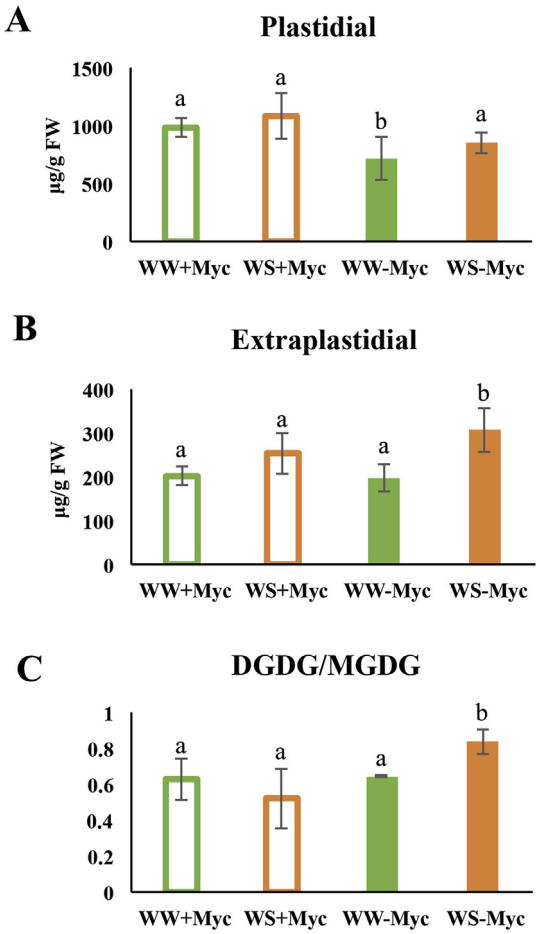

Fig. 2. Contents of (A) plastidial-, (B) extraplastidial lipids and (C) DGDG/MGDG ratio determined by TLC analysis in cork oak leaves. Treatments and lipid classes as in Fig. 1. Bars represent averages ± SD of 3 biological replicates. Different letters represent statistical significances by two-way ANOVA ($p > 0.05$).

(Guerfel et al., 2008). In fact, upon water stress these lipids are even slightly increased in cork oak leaves (Fig. 1 B). Significant increases in the DGDG content, which have been reported in several plants subjected to drought, such as maize and Arabidopsis (Gigon et al., 2004; Chen et al., 2018), were not detected in cork oak leaves (Fig. 1 B). However, the DGDG/MGDG ratio was significantly increased by the water shortage (Fig. 2C), a response that has been related to an increased conversion of MGDG into DGDG, suggested to contribute to keep chloroplast membrane structural and functional stability during dehydration (Gigon et al., 2004; Chen et al., 2018). Also, high DGDG content and DGDG/MGDG ratio have been associated with alleviation of photoinhibition through reducing of ROS produced during stress, thus improving the PSII activity under drought and alleviating drought-induced leaf senescence (Chen et al., 2018). These results are in agreement with the high tolerance of cork oak trees to water shortage.

The detailed lipid profile showed that, regarding the effect of *P. tinctorius* inoculation on cork oak lipid composition, mycorrhizal plants showed significantly higher basal levels of the chloroplast lipids MGDG and PG (Fig. 1B), and when analysed as a whole, plastidial lipids were increased in mycorrhizal plants when compared to non-mycorrhizal plants (Fig. 2A). The same trend was also observed for the extraplastidial lipids PC and PI, but to a lower extent (Fig. 1B).

Besides differences between mycorrhizal and non-mycorrhizal plants under well-water conditions, drought induced general changes, common to mycorrhizal and non-mycorrhizal plants, and others that seem to be associated to fungal inoculation. Suppression of irrigation caused an increase in the amount of total extra-plastidial membrane lipids (Fig. 2B), namely PE for both groups (Fig. 1B), whereas the increases in NL was only significant in mycorrhizal plants (Fig. 1B). The tendency for the increase in both galactolipids likely reflects the drought tolerance of cork oak plants, as also detected in several studies comparing drought-tolerant and susceptible plant species and cultivars. For example, comparison between two *Vigna unguiculata* cultivars shown that in the susceptible one there was a loss of both MGDG and DGDG, while a stability of these lipids content or even an increase was observed in the tolerant cultivar (Torres-Franklin et al., 2007). Also, in wheat, barley and coconut, a decrease in leaf galactolipid content has been described to be more pronounced in varieties requiring more water (Chetal et al., 1982; Repellin et al., 1997). High decreases in galactolipids and phospholipids were also recently observed in drought-sensitive Thyme species (*Thymus serpyllum*), compared to more tolerant ones (*Thymus vulgaris*) (Moradi et al., 2017). In drought-resistant plants, such as the desert plant *Calotropis procera*, an increase in the levels of structural lipids of photosynthetic membranes was observed (Ramadan et al., 2014) and in *Parkinsonia aculeata* an increase in membrane and

neutral lipids was detected upon dehydration (Benadjaoud et al., 2013). In our experiment we also detected a significant increase in neutral lipids upon water stress in mycorrhizal plants (Fig. 1B), indicating a higher accumulation of storage lipid species, such as triacylglycerol (TAG) in these plants. TAG accumulation is a common response to water stress in plant leaves (Gasulla et al., 2013).

Alterations in the proportion between the chloroplast galactolipids DGDG and MGDG have been observed in several plants subjected to drought, such as increases in the DGDG/MGDG ratio detected in Arabidopsis, the legume *Vigna unguiculata*, the resurrection plant *Craterostigma plantagineum* and in drought-tolerant grapevine varieties (Gigon et al., 2004; Torres-Franklin et al., 2007; Toumi et al., 2008; Gasulla et al., 2013), whereas in Thymus a decrease in this ratio was observed (Moradi et al., 2017). In cork oak, the DGDG/MGDG ratio increased with water-deficit only in the non-mycorrhizal plant group (Fig. 2C). In contrast, the mycorrhizal plants did not show significant alterations in DGDG/MGDG ratio with drought, which may result from the higher tolerance of these plants to water stress, since a more stable ratio between the two galactolipids has been reported under favourable growth conditions (Boudière et al., 2014).

3.2. Fatty acid composition alterations associated to drought and P. tinctorius inoculation

Besides quantitative changes, also qualitative alterations regarding fatty acid composition of some lipid classes could be observed. In order to determine the most important changes discriminating mycorrhizal and non-mycorrhizal plants subjected to drought stress we performed a canonical analysis of principal coordinates using the fatty acid profile of each lipid class identified (Fig. 3A).

Compared with the 4 groups from the CAP analysis of the lipid classes, showing that drought and mycorrhization both affected lipid composition (Fig. 1A), the CAP analysis of the fatty acid composition shows the formation of only 3 groups, with Myc and non-Myc plants grouping together under WW conditions, but not under WS. This means that under WW conditions the fatty acid composition is not altered by the symbiosis, but under drought mycorrhization seems to induce alterations, with mycorrhizal and non-mycorrhizal plants showing a different fatty acid profile. MGDG and PG were the lipid classes that contributed most for the observed groups in the CAP analysis. When plotting the fatty acid percentage of each lipid class, a global overview of the changes in fatty acid composition in response to each treatment could be observed (Fig. 3B).

Regarding MDGD fatty acid composition, CAP analysis and UPGMA identified 2 main clusters discriminating irrigated cork oak plants from plants subjected to water stress (Fig. 4A and B).

The fatty acid that most contributed to the observed clustering was linolenic acid (C18:3), both in water stress (63%, cluster II) and in irrigated conditions (47%, cluster I). The fatty acid composition of the chloroplast galactolipids MGDG and DGDG of cork oak plants was found to be very rich in C18:3, as is the case of other “18:3” plants, and remained relatively stable upon water deficit, although a tendency to increase in linoleic acid (C18:2) and decrease C18:3 was observed for both galactolipids (Fig. 3B, Supplementary Figs. 2 and 3). This effect appears to be more obvious when the ratios C18:2/C18:3 are calculated, showing an increase in response to drought, in both MGDG and DGDG (Fig. 5). Increases in the C18:2/C18:3 ratio have been previously observed in response to water deficit in several plants (Gigon et al., 2004; Olsson, 1995) and are likely related to the increase in membrane rigidity in response to the osmotic stress imposed by drought (Zhang et al., 2005). However, although both groups presented the same tendency with drought, the increase in C18:2/C18:3 ratio was higher for the non-mycorrhizal plants since in well irrigated mycorrhizal plants this ratio was already higher compared to non-mycorrhizal plants (Fig. 5).

The fatty acid composition of the PG fraction discriminated

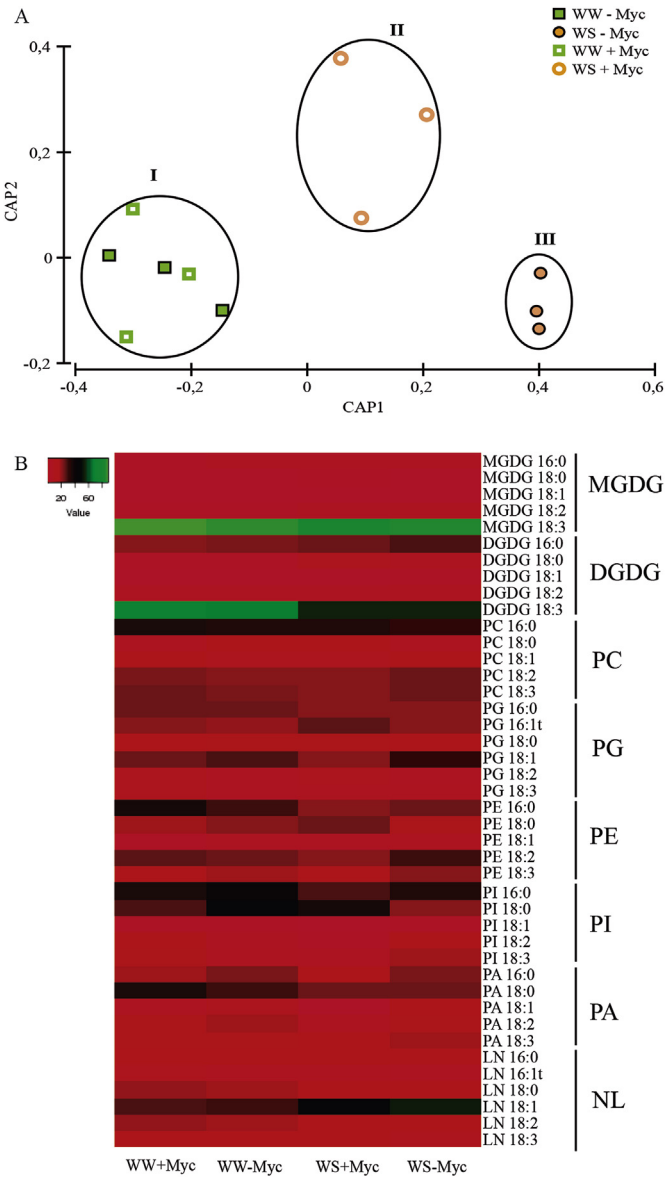

Fig. 3. Fatty acid composition of polar lipids of cork oak leaves determined by GC analysis. (A) Canonical analysis of principal coordinates (CAP) analysis based on fatty acid relative composition; (B) Heat map representing fatty acid relative composition in each lipid class. Treatments and lipid classes denoted as in Fig. 1. N = 3.

mycorrhizal and non-mycorrhizal plants independently of the irrigation treatment, forming 2 clusters of mycorrhizal and non-mycorrhizal plants (Fig. 4C and D). The main differentiating fatty acid was stearic acid (C18:0) with 63% contribution to the non-mycorrhizal cluster (cluster I), and 34% contribution to the mycorrhizal cluster (cluster II). Mycorrhizal plants PG had less C18:0 than non-mycorrhizal plants under irrigation, and also trans-hexadecenoic acid (C16:1*t*), but in mycorrhizal plants these fatty acids increased under drought (Fig. 3B, Supplementary Fig. 4). Also, these plants had increased levels of the polyunsaturated fatty acid C18:3 and the same tendency was also detected for C18:2. The percentage of C16:0 in PG tended to decrease under water stress for both mycorrhizal and non-mycorrhizal of plants. These differences in FA composition between mycorrhizal and non-mycorrhizal plants are well reflected in the higher DBI detected for PG in mycorrhizal plants, even under drought stress (Fig. 5). In a drought susceptible *Vigna unguiculata* cultivar, the PG molecular species containing C16:1*t* were severely reduced by drought (Monteiro de Paula

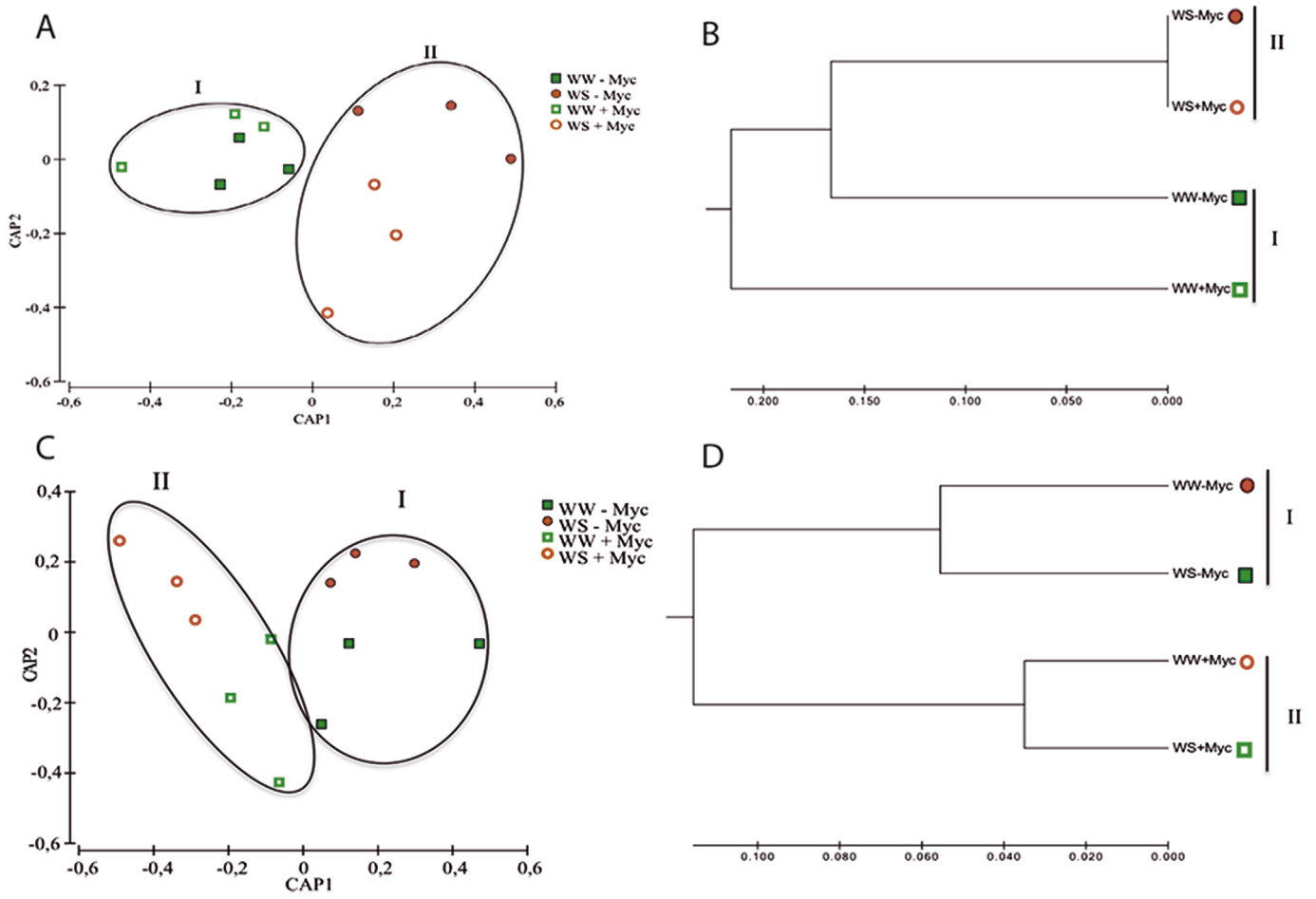

Fig. 4. Fatty acid composition of MGDG and PG determined by GC analysis. Canonical analysis of principal coordinates (CAP) based on MGDG (A) and PG (C) fatty acid relative composition; UPGMA clustering of MGDG (B) and PG (D) fatty acid relative composition based on Euclidean distance matrix generated by ANOSIM. Treatments and lipid classes denoted as in Fig. 1. N = 3.

et al., 1990). Interestingly, the opposite trend is here observed in cork oak mycorrhizal plants, suggesting that an improved ability to cope with drought is associated to mycorrhization. Since PG is the only phospholipid present in chloroplast thylakoid membranes, and is the only lipid containing C16:1*t*, differences in its fatty acid composition can be of great importance and impact on photosynthesis. In fact, in our previous work we have observed that concerning photochemical efficiency, a significant increase in the maximum quantum efficiency of the photosystem II (Fv/Fm) occurred in *P. tinctorius* mycorrhizal cork oak plants, revealing an increased photochemical potential of mycorrhizal plants (Sebastiana et al., 2018).

No mycorrhization or drought-induced changes were detected in the fatty acid composition of the major extraplastidial phospholipid PC (Fig. 3B). These results indicate that cork oak plants seem to differ from other plants where an increase in the unsaturation level of PC is observed under water deficit, such as soybean (Martin et al., 1986) and *Parkinsonia aculeata* (Benadjaoud et al., 2013). The NL fraction also showed a fairly stable fatty acid composition between treatments. The only significant difference observed in this class was in oleic acid (C18:1), which increased with drought in mycorrhizal and non-mycorrhizal plants. Interestingly, the NL fraction in cork oak leaves also contains C16:1*t*, a fatty acid which is exclusively synthetized in the chloroplast lipid PG. The existence of a specific lipase, releasing fatty acids from PG, which could be further exported from the chloroplast, reincorporated into PC and ultimately into NL, has been recently described in Arabidopsis seeds (Wang et al., 2017). Our results suggest that a similar mechanism could operate in cork oak leaves. Although the location of these PG-derived storage lipids inside the cell is out of the scope of the present work, it is possible that a fraction could remain inside chloroplasts, in the form of *plastoglobuli*, structures rich in neutral lipids which are known to contribute to stress recovery (Van Wijk and Kessler, 2017).

3.3. Gene expression profile of cork oak lipid metabolism genes upon drought and P. tinctorius inoculation

In order to get insights on the transcriptional regulation related to the changes detected in leaf lipid and fatty acid composition caused by ECM fungal inoculation and drought, the expression of key lipid metabolism genes was analysed by real-time PCR.

Regarding galactolipid biosynthetic genes, the expression of MGD1 remained stable upon water stress in the mycorrhizal cork oak plants, while in the non-mycorrhizal group there was a significant decrease (Fig. 6). In this respect, cork oak seems to differ from other plants previously analysed where a drought-induced up-regulation of MGD1 is often observed, even though the relative amounts of MGDG often decrease (Gigon et al., 2004; Torres-Franklin et al., 2007; Gasulla et al., 2013). Taking into account that additional MGD isoforms are likely to be present in cork oak, as is the case of other plant species, and that MGDG is used for DGDG and oligogalactolipid synthesis, and can also be the source of free fatty acids for the synthesis of other metabolites, a direct correlation between MGD1 expression and MDGD contents is not always found, as previously noted (Torres-Franklin et al., 2007; Gasulla et al., 2013). DGD1, the other galactolipid biosynthetic gene, was up-

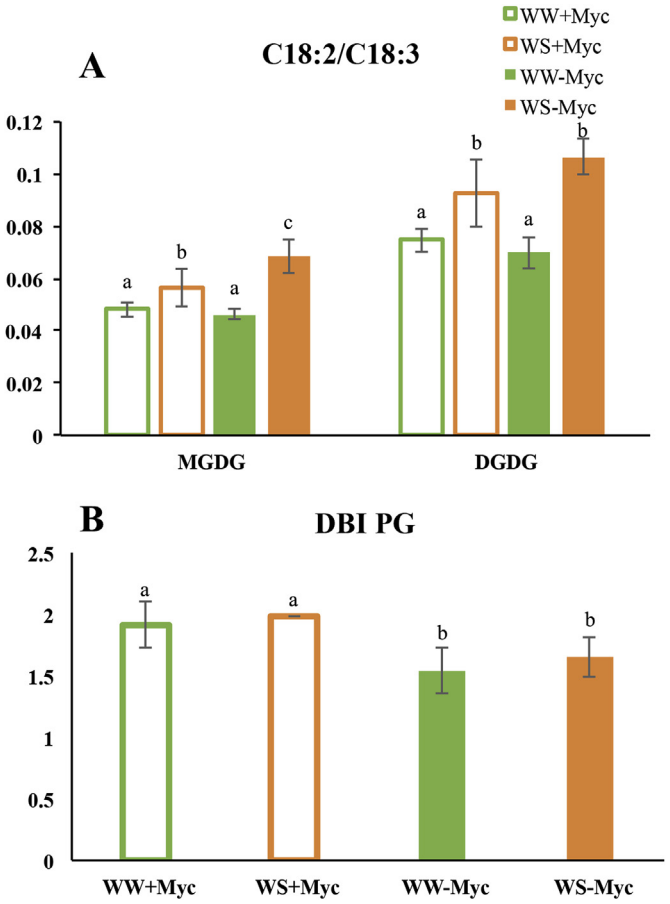

Fig. 5. MGDG and DGDG linoleic/linolenic acids ratios (C18:2/C18:3) (A) and PG double bond index (DBI) (B) determined by GC analysis. Bars represent (averages ± SD) of 3 biological replicates. Treatments and lipid classes denoted as in Fig. 1. Different letters represent statistical significances by two-way ANOVA ($p > 0.05$).

regulated by drought in both mycorrhizal and non-mycorrhizal plants (Fig. 6), in accordance with the reported induction of this gene by stress conditions, such as drought and nutrient limitation (Kelly et al., 2003; Gigon et al., 2004; Gaude et al., 2007). The higher drought-induction of DGD1, together with the down-regulation of MGD1 detected for the non-mycorrhizal plants, agree with the significant increase in DGDG/MGDG ratio detected under water stress for these plants (Fig. 2C). These results suggest that non-mycorrhizal plants have lower tolerance to drought conditions when compared with the mycorrhizal plants, since upon water stress increases in DGDG/MGDG ratio and DGD1 gene expression are more pronounced in drought-sensitive cultivars (Torres-Franklin et al., 2007; Gasulla et al., 2013). Likewise, the lower induction in DGD1 expression and the maintenance in the DGDG/MGDG ratio in mycorrhizal plants subjected to drought, when compared to non-mycorrhizal plants, suggest a higher drought tolerance in these plants. Regarding the expression of PAT, which catalyses the hydrolysis of galacto- and phospholipids, in our experiment the transcript was up-regulated in mycorrhizal and non-mycorrhizal plants upon drought (Fig. 6), as also observed in Arabidopsis and cowpea (Matos et al., 2001, 2008). Arabidopsis PAT-deficient mutant plants loose water more quickly compared to wild-type plants, indicating that this protein modulates plant response to water deficit, probably by removing oxidatively modified fatty acids from membranes (Yang et al., 2012). The lower induction of PAT in the mycorrhizal plants, seems to agree with the notion that ECM fungal inoculation contributes to increase drought tolerance since a higher up-regulation was observed in a drought-susceptible cowpea cultivar in comparison to a drought-tolerant one (Matos et al., 2001). Overall, our results on chloroplast galactolipid metabolism indicate that non-mycorrhizal plants suffer more pronounced alterations upon drought stress, consistent with the use of MGDG to synthesise DGDG or generate free fatty acids (Matos et al., 2008), commonly observed in plant response to dehydration, while mycorrhizal plants keep those alterations to a lower level.

Accumulation of neutral lipids and DGAT expression have been reported to be higher in dehydration-tolerant plants compared to sensitive ones under waters stress (Gasulla et al., 2013). The drought-induced increase in NL in cork oak was only significant in mycorrhizal plants, in agreement with the higher drought-tolerance of these plants. The NL fraction is usually rich in triacylglycerols (TAG), which are the main storage lipids in seeds, but can also serve as transient storage of acyl chains in leaves, specially under stress conditions. DGAT are key enzymes in TAG biosynthesis, but other enzymes can also contribute to the synthesis of these storage molecules. In our experiment, DGAT1 was clearly up-regulated by drought in both mycorrhizal and non-mycorrhizal plants, whereas up-regulation of DGAT2 was only detected in mycorrhizal plants (Fig. 6). Although in well-watered plants both DGAT transcripts are more expressed in the non-mycorrhizal plants than in the mycorrhizal ones, the amounts of NL are similar in both plant groups (Fig. 1B). However, since we didn't quantify TAG within the NL fraction

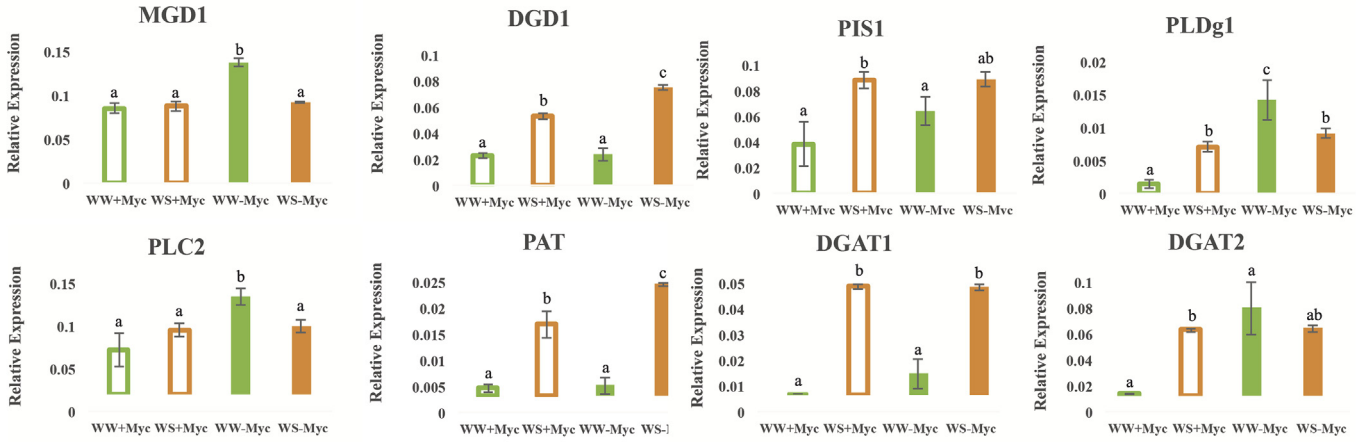

Fig. 6. Gene expression profile of lipid metabolism genes determined by real-time PCR. MGD1 (MGDG synthase1), DGD1 (DGDG synthase1), PAT (patatin-like phospholipase A), DGAT1 (DAG O-acyltransferase 1), DGAT2 (DAG O-acyltransferase 2), PIS1 (PI synthase1), PLC2 (phospholipase C2), PLDδ1 (phospholipase D1). Bars represent averages ± SD of 3 biological replicates. Treatments denoted as in Fig. 1. Different letters represent statistical significances by two-way ANOVA ($p > 0.05$).

in our samples, we cannot exclude the possibility of a different amount of this lipid between mycorrhizal and non-mycorrhizal plants under well-water conditions.

The expression of 3 genes encoding enzymes involved in the metabolism of the signalling lipids PA and PI was also analysed. Results show that there was a clear drought-induced increase in a PI synthase (PIS1) gene in mycorrhizal and non-mycorrhizal plants (Fig. 6). In this aspect, cork oak seems to differ from the desiccation-tolerant plant *C. plantagineum*, where the expression of two PI synthase genes did not change during desiccation, although an accumulation of PI was observed (Gasulla et al., 2013). Although our lipid analysis didn't show any changes in PI content with drought or mycorrhization, PI molecules are known to be involved in stress signalling, generating IP3 and diacylglycerol by the action of phospholipases C (PLC). Besides post-transcriptional regulation (Hong et al., 2016), PLC expression has also been shown to be modulated by stress conditions. For instance, in a comparison between cowpea cultivars with contrasting drought tolerance, the expression of PLC genes was only up-regulated in the tolerant cultivar (El-Maarouf et al., 2001). The fact that in non-mycorrhizal drought-treated cork oak leaves a decrease in PLC expression is observed (Fig. 6), while no change is observed in the mycorrhizal plants illustrates further the differences in lipid metabolism between mycorrhizal plants and non-mycorrhizal plants. Moreover, since it has been recently shown in rice that a PLC isoform plays a positive role in osmotic stress tolerance (Deng et al., 2019), the ability to maintain PLC transcripts levels upon water deficit could have contributed to the increase drought-tolerance displayed by mycorrhizal plants. Interestingly, a similar expression profile was observed for one of the PLD isoforms that generates the second messenger PA, (Fig. 6). These results also seem to confirm the increased tolerance of mycorrhizal plants, although a more comprehensive analysis of the many isoforms of these phospholipases is still required.

4. Conclusions

In conclusion, mycorrhizal plants show lipid metabolic patterns commonly found in drought-tolerant plants, that could be associated with their increased ability to cope with drought stress. The higher contents in chloroplast lipids, such as MGDG and PG, along with a higher unsaturation level of PG, and the ability to keep a more stable DGDG/MGDG ratio as well as C18:2/C18:3 proportions in both galactolipids, suggests an increased ability of mycorrhizal plants to keep chloroplast integrity during drought. In angiosperms, a stable DGDG/MGDG ratio is apparently found when plants are grown under favourable controlled conditions, i.e. when fed with enough nutrient sources (Boudière et al., 2014). Previously, we reported a higher N content in leaves of cork oak plants mycorrhizal with *P. tinctorius* and subjected to drought (Sebastiana et al., 2018). In Arabidopsis, drought and nutrient deficiency, such as N and P deficiency, were shown to increase DGDG/MGDG ratio, mostly by decreasing MGDG and increasing DGDG in leaves (Gigon et al., 2004; Gaude et al., 2007). Therefore, the opposite effect here observed for the DGDG/MGDG ratio and MGDG content in mycorrhizal cork oak plants subjected to drought, suggests that increased nutrient content, commonly found in leaves of plants living in symbiosis with mycorrhizal fungi, could contribute to keep chloroplast membrane integrity and stability, even under drought stress conditions, when non-mycorrhizal plants are suffering from nutrient limitation. The expression profile of several genes involved in galactolipid metabolism, such as MGD1, DGD1 and PAT, may play an important role in conferring alleviated drought-induced lipid alterations in mycorrhizal plants. Mycorrhizal plants also showed increased expression of DGAT transcripts under drought which could be related to increased ability to cope with drought stress. Likewise, the different basal and drought-responsive expression of genes encoding key signalling enzymes such as PLD and PLC also highlight the contribution of fungal inoculation to stress tolerance.

Author contributions

MS has conceived, and AM has supervised the study. MS and AM analysed the plant material. MS, BD, IC and FM performed the statistical analysis. MS, AM and FM wrote the manuscript. RM contributed to the interpretation of results and writing of the manuscript. All authors discussed the results, read and approved the manuscript.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.032.

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