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Oak protein profile alterations upon root colonization by an ectomycorrhizal fungus

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Abstract

An increased knowledge on the real impacts of ectomycorrhizal symbiosis in forest species is needed to optimize forest sustainable productivity and thus to improve forests services and their capacity to act as carbon sinks. In this study we investigated the response of an oak species to ectomycorrhizae formation using a proteomics approach complemented by biochemical analysis of carbohydrates levels. Comparative proteome analysis between mycorrhizal and non-mycorrhizal cork oak plants revealed no differences at the foliar level. However, the protein profile of 34 unique oak proteins was altered in the roots. Consistent with the results of the biochemical analysis, the proteome analysis of the mycorrhizal roots suggests a decreasing utilization of sucrose for the metabolic activity of mycorrhizal roots which is consistent with an increased allocation of carbohydrates from the plant to the fungus in order to sustain the symbiosis. In addition, a promotion of protein unfolding mechanisms, attenuation of defense reactions, increased nutrient mobilization from the plant-fungus interface (N and P), as well as cytoskeleton rearrangements and induction of plant cell wall loosening for fungal root accommodation in colonized roots, are also suggested by the results. The suggested improvement in root capacity to take up nutrients accompanied by an increase of root biomass without apparent changes in aboveground biomass strongly re-enforce the potential of mycorrizal inoculation to improve cork oak forest resistance capacity to cope with coming climate change.

Keywords: cork oak; ectomycorrhizae; symbiosis; proteome; mass spectrometry; differential in gel electrophoresis (DIGE)

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1. Introduction

The ectomycorrhizal (ECM) symbiosis is a mutualistic association between the fine roots of trees and soil inhabiting fungi, typically found in temperate and boreal forests. The intimate contact between the two partners that occurs in ectomycorrhizae results from a synchronized plant-fungus development with the final goal of nutrient transfer: the fungus provides the plant with mineral nutrients, which in turn, supplies the fungus with photosynthetically derived carbohydrates. In ectomycorrhizae, the fine hyphae of the symbiotic fungi can explore soil niches that are inaccessible to plant roots and absorb nutrients, significantly contributing to host nutrition, in particular under conditions of abiotic stress (Smith and Read 1997). Besides increasing plant growth, ectomycorrhizae also seem to bring other benefits to the plant, like a more efficient uptake of water and higher resistance to pathogens and environmental stresses (Smith and Read 1997). The use of mycorrhizal fungi in plant production systems constitutes a promising strategy to enhance plant productivity with low impact on the environment. More detailed information on the molecular processes in ECM host trees is relevant owing to their ecological significance, the economic importance of the tree species involved and the interest in exploiting this symbiosis to maximize tree productivity and sustainability. Molecular studies, including large scale gene profiling experiments have shown that the morphological and physiological changes associated with ectomycorrhizae development are accompanied by changes in gene expression in both partners (Johansson et al. 2004; Heller et al. 2008; Flores-Monterroso et al. 2013). However, considering only genes showing differential RNA accumulation will not detect all the important functions in ectomycorrhizae biology. Proteomics has the ability to complement transcriptomics by characterizing gene products (proteins) and their response to a variety of changing biological and environmental factors. Two-dimensional gel electrophoresis (2-DE) is a powerful technique which enables the separation of complex mixtures of proteins according to their isoelectric point (*pI*) and molecular mass (*Mr*). Several 2-DE pioneering studies were performed to analyze ECM symbiosis in the early 1990's (Hilbert et al. 1991; Burgess and Dell 1996) allowing researchers to detect several fungal symbiosis related (SR) proteins, up-accumulated or newly induced in ECM roots, as well as down-accumulated plant polypeptides, by comparison to control roots and mycelium. However, very few proteins were identified due to the limitation of both electrophoretic and identification methods. Improvements

in 2-DE and mass spectrometry, and the development of genomic sequence databases for peptide mass matches made it possible to achieve a high throughput of plant protein identification (Bestel-Corre et al. 2004). Recent advances in sequencing technologies and the subsequent implementation of genomic and transcriptomic databases of an increasing number of organisms, some of them establishing mycorrizal symbiosis, such as *Populus*, *Quercus*, *Pisolithus*, *Laccaria* or *Tuber*, have opened new opportunities for identifying proteins with confidence by using mass spectrometry. Differential in gel electrophoresis (DIGE) is a method that can be used to accurately quantify protein accumulation differences under various conditions. Using the DIGE technology, proteome analysis can be carried out similarly to a microarray experiment in that two samples are compared on one gel by analyzing the ratio of two fluorescent labels between two samples for each protein (Unlu et al. 1997). In this work we investigated the differences in the protein profiles between mycorrhizal and non-mycorrhizal cork oak (*Quercus suber*) plants upon inoculation with the ectomycorrhizal fungus *Pisolithus tinctorius*. Our aim was to identify plant proteins differentially regulated by the interaction with the fungal symbiont, analyze their function, and contribute to gain insights into the molecular events occurring in the plant during ECM colonization. In order to fulfill this goal we compared mycorrhizal and non-symbiotic root and foliar tissues using the 2D-DIGE technique to quantify differences in protein abundance. The differentially accumulated proteins were excised from 2D-gels and subjected to mass spectrometry and database searches for protein identification. Since proteins involved in carbon metabolic pathways were one of the most affected protein groups following inoculation with *P. tinctorius* we also analyzed soluble sugars, starch and % C in mycorrhizal and non-mycorrhizal roots.

2. Materials and Methods

2.1. Plant and fungal material

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. 2013a). *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, three months old plantlets

were transferred to 1,5 L pots containing soil and simultaneously inoculated with *P. tinctorius* peat-vermiculite inoculum (3 months old), according to Sebastiana et al. (2013a). Control plants were treated with a non-inoculated peat-vermiculite mixture. Plants were grown in pots in a greenhouse and watered once a week with 500 mL of tap water. No fertilization was applied.

2.2 Plant harvest and biomass determination

Two months after inoculation roots and leaves from inoculated and non-inoculated plants were collected. Visual inspection for the presence of mycorrhizal root tips enabled to detect five plants in the non-inoculated treatment presenting mycorrhizal roots from an unknown morphotype. These plants were discarded. In total, 25 mycorrhizal and 20 non-mycorrhizal plants were sampled. Roots were rinsed to eliminate soil particles, first with running tap water, and then with deionized water. Excess water was removed with filter paper. For each plant, the weight of the root and leaves was recorded for biomass determination. In order to account for the dilution effect in mycorrhizal plants, due to fact that only a limited number of roots in a root system are in fact colonized, only secondary roots presenting ECM root tips were sampled for protein extraction and biochemical analysis. Roots and leaves were frozen in liquid nitrogen and stored at -80 ºC for further analysis.

2.2. Protein extraction

4-5 biological replicates of mycorrhizal and non-mycorrhizal, each consisting of a pool of roots or leaves from 5 replicated plants from each group, were subjected to protein extraction and separation by 2-DE DIGE. Frozen material was grounded to a fine powder in a mortar using liquid nitrogen. Protein extraction (1 g roots/leaves) was performed according to the phenol protocol, as described previously (Sebastiana et al. 2013b). Protein quantity was measured with the 2-DE Quant Kit (GE Healthcare) using BSA as a standard. Protein extracts were concentrated using the 2-DE Clean-Up kit (GE Healthcare) and used for the 2-DE DIGE analysis.

2.3. 2-DE DIGE

The experiment included two different comparisons: (1) mycorhizal roots versus non-mycorrhizal roots and (2) “mycorrhizal” leaves versus “non-mycorrhizal” leaves. Before electrophoresis protein samples were labelled with the CyDye DIGE Fluors

(Cy5, Cy3 and Cy2; GE Healthcare). Before the labelling reaction, the pH of the extracted protein solution was adjusted to 8.5 with 100 mM NaOH solution. Each protein sample, consisting of 30 µg of root tissue / 50 µg of leaf tissue, was labelled with Cy3 or Cy5, using the CyDye DIGE Fluor minimal dyes (GE Healthcare), according to manufactures' instructions. The internal control was prepared by mixing equal quantities of protein extract from each biological replicate, and labelling with Cy2 dye. The two samples plus the internal control were combined and mixed with sample buffer [8M urea, 4% (w/v) CHAPS, 130 mM DTE, 1.5% (v/v) pharmalytes pH 4-6.5] in 1:1 proportion. Rehydration buffer [8M urea, 4% (w/v) CHAPS, 13 mM DTE, 0.75% (v/v) pharmalytes pH 4-6,5] was then added to a final volume of 400 µl. Isoelectric focusing of the combined protein samples was performed using 24 cm immobilized 4-7 pH gradient IPG strips (GE healthcare). Briefly, IPG strips were rehydrated by active rehydration for 18 h at 30 V. Isoelectric focusing (IEF) was carried out with an IPGphor system (GE Healthcare), at 20 ºC, maximum current of 50 µA/strip, and according to the following program: 1 h 150 V, 2 h 250 V, 2 h gradient from 250 V to 1000 V, 2 h 1000 V, 2 h gradient from 1000 V to 4000 V, 3 h 4000 V, 3 h gradient from 4000 V to 8000 V, 8 h 8000 V; complete run total voltage of 101 kVh. The samples were reduced at room temperature by gentle agitation for 15 minutes in equilibration buffer [6M Urea, 2% (w/v) SDS, 50mM Tris pH 8.8, 0.02% (w/v) bromophenol blue, 30% (v/v) glycerol] with 2% (w/v) DTE, followed by alkylation with 3% (w/v) iodoacetamide in the same buffer. Next, SDS-PAGE was performed using 12.5% polyacrylamide gels using the EttanDALTtwelve system (GE Healthcare). Separation was performed overnight at 20°C with 1st step at 80 V, 10 mA/gel and 1 W/gel, and 2nd step at 100 V, 17mA/gel and 1.5 W/gel. 2D-DIGE gels were scanned using low-fluorescence glass plates at a resolution of 100 µm. Images of the Cy3, Cy2 and Cy5-labeled samples were acquired in the Laser-based scanner FLA-5100 (FujiFilm) using 532 and 635 nm excitation lasers (DGR1double filter) for Cy3 and Cy5 respectively, and 473 nm excitation laser (LPB filter) for Cy2 under Image Reader FLA 500 version 1.0 (FujiFilm). All combinations of pairwise comparisons between the samples were included, as recommended in the GE Healthcare user manual. A dye swap between Cy3 and Cy5was used to avoid problems associated with preferential labelling.

2.4. Quantitative analysis of protein spots

Gel images were exported into the Progenesis SameSpot V3.31 image analysis system (Nonlinear Dynamics), where quantitative analysis of protein spots was performed. Automatic and subsequent manual editing, aligning, matching procedures and spot volume normalization were done as part of the Progenesis SameSpots workflow. Spot volume were normalized to the total spots volume. A spot was considered to be significantly differentiated between mycorrhizal and non-symbiotic tissues when one-way ANOVA $P \leq 0.05$ and power value ≥ 0.7. Normalized volumes of significant spots of the ECM root samples were corrected to account for the 0.93: 0.07 plant-fungus relative biomass in the ECM tissues analysed (determined by the ergosterol assay; see below). By this procedure we normalized the Cy dye intensity values to account for the different root protein quantity present in mycorrhizal tissue (93% root protein and 7% *P. tinctorius* protein) and non-symbiotic tissue (100% root protein). Fold change of significant spots was calculated as the ratio between ECM corrected spot volumes and non-symbiotic spot volumes (Online resource 1).

2.5. MS analysis and protein identification

Preparative Comassie 2-DE gels loaded with 400 µg of protein were used for spot picking. Differentially accumulated protein spots were excised from the gel and washed by shaking (150 rpm) first in mili-Q water for 15 minutes, and then in 50% (v/v) acetonitrile for 15 minutes at 56ºC, until complete removal of the Comassie Brilliant Blue. Gel pieces were than dehydrated by treatment with 100% acetonitrile (ACN) during 15 minutes at 37ºC, and vacuum-dried. Proteins were *in gel* digested using trypsin. Briefly, gel pieces were incubated in digestion solution containing 6.7 ng/µL of trypsin (Promega) in 50 mM ammonium bicarbonate pH 8.0, for 15 minutes at RT and then for 45 minutes at 4ºC. After removal of excess digestion solution, gel pieces were incubated with 50 mM ammonium bicarbonate pH 8.0, overnight at 37ºC. Finally the digestion was stopped by addition of formic acid to a final concentration of 5% (v/v). The tryptic peptides were concentrated and prepared for MS analysis according to Gobom et al. (1999) using homemade reverse phase micro columns. Briefly, a GEloader tip (Eppendorf) was packed with Poros R2 media (Applied Biosystems) and after an equilibration with 20 µl 5% (v/v) formic acid, the peptide solution (10-15 µl) was loaded on the column and washed with 20µl 5% (v/v) formic acid, 50% (v/v) ACN. The bounded peptides were eluted with 0.7 μL of α-cyano- 4-hydroxycinnamic acid (CHCA, Sigma) matrix [5 mg/mL in 50% ACN (v/v), 5% formic acid (v/v)] and

dropped onto the MALDI plate. Peptide mass spectra were acquired using a MALDI-TOF/TOF 4800 plus MS/MS (Applied Biosystems). Data was acquired in positive MS reflector using a *PepMix* (LaserBio Labs) to calibrate the instrument. Each reflector MS spectrum was collected in a result-independent acquisition mode, using 750 shots per spectra in 800-4000 *m/z* range and fixed laser intensity to 3200 V. Fifteen of the strongest precursors were selected for MS/MS and the analyses were performed using CID (Collision Induced Dissociation) assisted with a collision energy of 1 kV and a gas pressure of 1×10^{-6} torr. For each MS/MS spectrum, 1400 laser shots were collected, using a fixed laser intensity of 4300 V. Processing and interpretation of the MS and MS/MS spectra were performed with 4000 Series Explored™ Software (Applied Biosystem). The mass spectrometry proteomics data has been deposited in the ProteomeXchange Consortium (Vizcaino et al. 2014) via the PRIDE partner repository with the dataset identifier PXD003009.

2.6. Protein identification and annotation

Tandem mass spectral data were submitted to database searching using Mascot (Matrix Science, version 2.2.07) and ProteinPilot (Applied Biosystems, version 3.0, rev. 114732) with the following parameter settings: trypsin cleavage; one missed cleavage allowed; peptide mass tolerance of 50 ppm; fragment mass tolerance of 0.5 Da; oxidation, carbamidomethyl and deamidated as variable amino acid modifications. The following databases were used: cork oak EST consortium database available at the CorkOakDB portal (www.corkoakdb.org; 159290 EST deduced peptide sequences), oak gene index EST database (OGI_release_2.0) available at the Gene Index Project portal (http://compbio.dfci.harvard.edu/tgi/; 42144 EST sequences), red oak and white oak 454 and Sanger ESTs (Oall unigene v2) from the Fagaceae Genomics Web portal (http://www.fagaceae.org/; 480360 EST sequences), and the predicted peptide sequences of *P. tinctorius* genome available at the JGI fungi portal (http://genome.jgi.doe.gov/programs/fungi/index.jsf; 223134 sequences). Protein score confidence interval percentage and total ion score confidence interval percentage were both set above 95%. Proteins were considered if having a MASCOT protein score above 65 ($p < 0.05$) and at least one peptide with MS/MS identification. If only one peptide matched MS/MS data it was verified manually. Quality criteria for manual confirmation of MS/MS spectra were the assignment of major peaks, occurrence of uninterrupted y- or b-ion series at least with 3 consecutive amino acids and the presence

of a2/b2 ion pairs. In case of successful matching with the database, sequence annotation was verified by performing Blastp in the NCBInr (http://blast.ncbi.nlm.nih.gov). The Expasy translate tool (http://web.expasy.org/translate/) was used to translate nucleotide sequences into amino acid sequences, when needed. Gene ontology (GO) annotation was obtained using the QuickGO annotation tool (https://www.ebi.ac.uk/QuickGO/GAnnotation) with the Plant GO slim (goslim_plant) to map annotations. The UniProtKB (http://www.uniprot.org/) accession number of each differentially accumulated protein, retrieved by Blast p search against the UniProtKB database, was used for this analysis. Interpro (http://www.ebi.ac.uk/interpro/) was used for classification of proteins into families and prediction of domains and important sites. Proteins were assigned to functional categories using GO annotation, UniProtKB functional information and literature references on similar proteins. Subcellular localization of proteins was predicted using LocTree3 (https://rostlab.org/services/loctree3/) and MemPype (http://mu2py.biocomp.unibo.it/mempype). The presence of N-terminal signal peptide sequences that targets proteins for translocation across the secretory pathway was predicted using ProP 1.0 (http://www.cbs.dtu.dk/services/ProP/) and Phobius (http://phobius.binf.ku.dk/). Integral membrane proteins (containing transmembrane spans) were predicted using the MemPype sever. ER-membrane retention signal presence was predicted using the Wolf Psort program (http://www.genscript.com/psort/wolf_psort.html).

2.7. Ergosterol assay

Alterations in the relative accumulation of proteins in ECM roots were estimated by comparison with non-mycorrhizal roots. However, since protein extracts from colonized roots consist of a mixture of proteins from both symbiotic partners, an adjustment is necessary considering the proportion of plant and fungal biomass in ECM root tissue (Simoneau et al. 1993). The proportion of fungal biomass in the symbiotic roots was estimated by measuring fungal ergosterol (Martin et al. 1990). Since ergosterol is found mainly in the membranes of fungi and is rarely present in vascular plants it is commonly used for measurement of fungal biomass in ECM roots. 1-month-old aseptically grown *P. tinctorius* mycelia, mycorrhizal and non-mycorrhizal roots from the same set of plants used for protein extraction, were pulverized in liquid nitrogen and stored at -80

ºC. The ergosterol content of each sample was measured according to (Grant and West 1996). For the measurement, 2 g of freeze dried sample were extracted with 16 mL methanol, vortexed and then ultrasonicated (bath) for 30 min and centrifuged for 10 min at 1600 g. The supernatant was removed and the remaining pellet washed twice with the same volume of methanol supernatants, (16 + 16 mL). To the combined methanol supernatants, 8 mL 4% KOH (in 96% ethanol) was added and the mixture was reacted for 30 min at 80 ºC. Distilled water and hexane phase (16 mL each) were then added and the hexane phase was separated. After a repeated hexane extraction the combined hexane phases were dried in a water rotatory vacuum pump or by lyophilisation. The extracted material was dissolved in 2 mL methanol, vortexed and filtered through a 45 µm filter, and analyzed by HPLC (WATERS 2965 Separations Module, Milford, MA, USA) with a PDA detector (WATERS 2996). Briefly, the extract was separated on a 30 x 5 mm Nova Pak C18 (WAT052834) reverse-phase column packed with ODS 4 µm preceded by a Nova Pak C18 15220 guard column (WATERS), eluded by using a pure methanol (HPLC grade) mobile phase, with a flow rate of 2 mL min^{-1} and measured at 282 nm. The retention time of ergosterol was 1.9-2.0 min. Ergosterol content was determined by comparing sample peak areas with those of external standards (Fluka). Ergosterol was confirmed by comparing retention times and absorption spectrum with external standard and by co-injection of samples plus standard ergosterol. The amount of ergosterol in non-mycorrhizal roots (0.037 µg/mg root DW), probably due to microorganisms present in the roots of nursery potted plants, was subtracted from the amount in *P. tinctorius* inoculated roots (0.070 µg/mg root DW) to calculate ergosterol content of mycorrhizal roots used for the experiment (0.033 µg/mg root DW). Estimation of the fungal biomass in inoculated roots was determined based on the ergosterol content from the free living *P. tinctorius* mycelium growing in petri dish under optimal conditions (0.470 µg/mg DW), which was considered 100%. From the 0.033 µg/mg root DW ergosterol value obtained for the mycorrhizal roots we estimated a fungal biomass of 7%. Therefore a correction factor was applied in the fold change calculation for the differentially accumulated proteins and transcripts, considering a plant:fungal biomass proportion of 0.93:0.07.

2.8. Real-time PCR analysis

The following transcripts corresponding to differentially accumulated proteins were used for real-time PCR analysis: PDI, CPN60, RAD23c-like, SUMO, TIL and proteasome subunit alpha type-5-like (Online resource 2). Total RNA was extracted from the same samples used for proteome analysis according to (Wan and Wilkins 1994). mRNA purification was performed with the Dynabeads mRNA purification kit (Ambion). cDNA synthesis was done according to (Monteiro et al. 2013). Specific primers for the selected transcripts were designed with Primer Express software version 3. 0 (Applied Biosystems, Sourceforge, USA). Quantitative real-time PCR experiments were carried out using Maxima™ SYBR Green qPCR Master Mix (2×) kit (Fermentas, Ontario, Canada) in a StepOne™ Real-Time PCR system (Applied Biosystems, Sourceforge, USA) as described in (Monteiro et al. 2013). To normalize expression data the elongation factor 1α (EF1α) was used (Online resource 2). Gene expression level was calculated by the ΔΔCt method (Schmittgen and Livak 2008).

2.9. Determination of starch, soluble sugars and carbon concentration

Three biological replicates (5 plants each) of N_2 frozen grounded roots (0.1 g FW) from mycorrhizal and non-mycorrhizal roots used for protein extraction, were also used for soluble sugars, starch and carbon concentration determination. Soluble sugars were extracted according to Guy et al. (1992) and their content 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 90ºC for 20 min, followed by measurement of released D-glucose at 340nm 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) and starch content. For the carbon elemental analysis, frozen root material was dried at 70º C for 72 h and ground in a mill (Retsch Germany) to a homogenous fine powder for isotopic analysis. After grinding, samples were used for carbon (C) percentage calculation, according to Rodrigues et al. (2010), on a EuroEA 3000 Elemental Analyzer (EuroVector, Milano), with a TDC detector, at the Stable Isotopes and Instrumental Analysis Facility, Faculty of Sciences, Lisbon University. C concentration was defined as % of dry weight.

2.10. Statistical analysis

Statistical analysis of biomass, starch, soluble sugars and % C was performed using the SPSS 20.0 software package. Data were analysed for normality by the Shapiro-Wilk test. A t-test for 2 independent samples was applied to analyse results from root and leaf biomass, fructose, starch and carbon concentrations. The Mann-Whitney test for non-parametric data was used to analyse the results from the sucrose and glucose concentration. For the analysis of the 2DE-DIGE results, means of protein spot-normalized volumes were compared between mycorrhizal and non-mycorrhizal samples using one-way ANOVA test included in Progenesis SameSpots statistical package. The accepted significance level for all the tests was $p < 0.05$.

3. Results and Discussion

3.1. Ectomycorrhizae establishment and plant biomass

Two months after inoculation, inoculated plants showed distinct ectomycorrhizal root tips presenting the typical *P. tinctorius* ectomycorrhizae root morphotype (e.g. bright yellow with a thick fungal mantle) (Cairney and Chambers 1997), showing that this isolate was efficient in establishing symbiosis with cork oak (Online resource 3 c). Microscopic observations of inoculated roots showed a thick fungal mantle and a developed Hartig net surrounding epidermal root cells, indicative of a fully developed symbiosis (Online resource 3 d). In accordance, inoculated plants showed a significantly higher root biomass relative to the control non-inoculated plants (Table 1). Increases in root biomass following inoculation is a well-known effect of ECM symbiosis and has been related to an increased root branching promoted by a fungus-induced accumulation of auxin at the root apex (Felten et al. 2009). In contrast, foliar biomass was not altered by the inoculation with *P. tinctorius* (Table 1). This is probably related to the early interaction time-point used in our experiment, since plants were harvested 2 months after inoculation. Increases in leaf biomass and leaf area following mycorrhizal inoculation of oak have been reported to occur 2 years after inoculation (Dickie et al. 1997, Fini et al. 2011; Sebastiana et al. 2013a), when higher degrees of root colonization increase nutrient transfer that results in increased photosynthetic capacity (Carney and Chambers, 1997).

3.2. Accumulation profile of root and leaf proteins in response to ECM symbiosis

The accumulation profile of cork oak root and leaf proteins in response to symbiosis establishment with *P. tinctorius* was analyzed by comparing symbiotic and non-symbiotic tissues using 2D-DIGE analysis (Fig. 1 and Online Resource 4). 2D gels of proteins labeled with Cy dyes showed 378 spots across root gels and 1171 spots across leaf gels in the 4-7 pI range. Protein spot volume were statistically compared between symbiotic and non-symbiotic conditions and, in the leaves no significant alterations in protein accumulation were detected, suggesting that ECM symbiosis does not induce alteration in protein accumulation in the above-ground parts of the host plant. This contrasts with data from roots colonized with arbuscular mycorrhizae where transcriptomic analysis detected a systemic effect on gene expression which was detected in shoots, leaves and fruits (Fiorilli et al. 2009; Zouari et al. 2014). At the metabolite level, studies have revealed that leaves from ECM plants display changes in some metabolites accumulation, including amino acids and fatty acids (Luo et al. 2011). However, our results suggest that these alterations do not result from differential protein accumulation in leaves. However, we cannot exclude the possibility of an ECM effect in leaves of older plants since our plants were harvested 2 months after inoculation. In contrast, the root gels showed 66 protein spots that altered their abundance after ectomycorrhizae establishment (Fig. 1). A total of 58 differentially expressed protein spots were successfully excised from the root DIGE gels and subjected to mass spectrometry for protein identification.

3.3. Identification of differentially accumulated proteins

Using MALDI-TOF/TOF tandem mass spectrometry we successfully identified 50 differentially expressed protein spots, which corresponded to 41 unique proteins. Among the differentially expressed protein spots detected in ECM roots, 8 matched to *P. tinctorius* proteins, corresponding to 6 unique proteins, with 2 different proteins found twice (Online resource 5). Some of the identified *P. tinctorius* proteins (spot 330, 331, 585) showed a significant degree of similarity (NCBI blast p E value < 1 e^{-120}) to proteins which are known to be involved in ECM symbiosis development, such as the 32 kDa-cell wall symbiosis regulated acidic polypeptide (SRAP 32) and its precursor protein (Hilbert et al. 1991). Transcripts encoding SRAPs are strongly accumulated during *P. tinctorius* ectomycorrhizae formation, when fungal hyphae form the mantle

around root tips (Burgess and Dell 1996). There is no evidence of sequence homologies between SRAPs and previously identified proteins but the presence of an Arg-Gly-Asp (RGD) motif found in cell adhesion-proteins, suggests a role in cell-cell adhesion needed for aggregation of hyphae in ECM roots (Laurent et al. 1999). We also identified two *P. tinctorius* proteins with some sequence homology (NCBI blast p E value < 1 e^{-6}) to serine protease inhibitors (spot 480, 582, and 593). Fungal protease inhibitor proteins are highly expressed during ectomycorrhizae formation by *Laccaria bicolor* suggesting that these proteins may play a role in counteracting plant secreted proteases expressed during fungal apoplastic growth (Vincent et al. 2012), partially explaining the low level of plant defense reactions observed in the colonized host roots (Martin and Nehls 2009). However, we didn't identify any plant proteases in our 2-DE gels.

Since our main interest was the alteration of the host plant proteome in response to ectomycorrhizae establishment, only spots identified as plant proteins were analyzed further. Although the genomic sequence of *Q. suber* has not yet been characterized, we were still able to identify 42 cork oak protein spots via homology with translated protein sequences from 454 sequence databases from cork oak and other oak species, such as *Q. petraea*, *Q. rubor*, *Q. alba* and *Q. rubra*. The 42 oak protein spots identified in our experiment matched to 34 unique proteins, with 5 proteins found twice or more (Table 2). Most of these multiple spots differed from each other in their pI values forming a line of spots with the same molecular weight. This pattern on the gel indicates multiple protein species and/or post translational modifications (PTMs), such as changes in charge state caused by phosphorylation. In this group we identified several proteins that have been reported to exist in multiple forms, such as protein disulphide-isomerase (spot 630, 634, 635, and 636) (Selles et al. 2011), purple acid phosphatase (spot 81 and 82) (Wang et al. 2011), UDP-glucose pyrophosphorylase (spot 149 and 653) (Chen et al. 2007), and actin (spot 202 and 205) (Slajcherova et al. 2012). The existence of multiple forms of these proteins in our experiment suggests an important role in plant responses to ectomycorrhiza establishment.

Gene ontology (GO) annotation of the biological processes affected by the interaction of cork oak roots with *P. tinctorius* is shown in Figure 2. As expected, "metabolic process" and "cellular process" were the most abundant categories. More than 65% of the proteins were assigned with the GO annotations "carbohydrate metabolic process",

448 "protein metabolic process" and "transport". Another relevant category was "response
449 to stress".

450 Functional analysis and database searches revealed a putative involvement of the
451 identified proteins in several cellular pathways such as, carbon and energy metabolism,
452 protein folding, stability and degradation, stress and defense, nutrient acquisition, lipid
453 transport/metabolism, cell wall remodelling and cytoskeleton.

454 In order to better characterize the mycorrhizal responsive proteins identified in our
455 experiment we analyzed their sequences for sub-cellular location prediction (Table 2).
456 Proteins predicted to be cytoplasm-located were mostly found to be involved in carbon
457 metabolism, such as glycolysis, stress and defense response, cell wall organization and
458 the cytoskeleton. Secretory pathway proteins included endoplasmic reticulum located
459 proteins involved in protein processing, such as folding and degradation (e.g. protein
460 disulfide-isomerase, ubiquitin receptor RAD23d-like), extracellular proteins putatively
461 involved in nutrient transfer (e.g. purple acid phosphatase, acid phosphatase 1-like), and
462 proteins related to lipid transport/metabolism (e.g. membrane steroid-binding protein,
463 phosphatidylglycerol/phosphatidylinositol transfer protein).

464 When analyzing the differentially accumulated proteins for their association to
465 membranes using MemPype (Table 2), 4 proteins were predicted to be integral
466 membrane proteins containing transmembrane helices for anchoring to membranes of
467 the endomembrane system (membrane steroid-binding protein and
468 phosphatidylglycerol/phosphatidylinositol transfer protein), mitochondria
469 (mitochondrial malate dehydrogenase) and the cell membrane (sinapyl alcohol
470 dehydrogenase-like).

471 In the next sections we will discuss the possible role of the identified proteins in the
472 context of ECM symbiosis.

473 **3.4. Carbon/energy metabolism and cell wall remodeling**

474 A decreased accumulation was detected for several proteins involved in carbon flux
475 through oxidative degradation pathways, like the glycolysis and the TCA cycle [e.g.
476 fructokinase (spot 268), enolase (spot 133, 137, 656), fructose bisphosphate aldolase
477 (spot 210), triosephosphate isomerase (spot 366) and malate dehydrogenase (spot 286)].
478 We also observed lower levels for several proteins involved in generation of energy,

such as homologues to mitochondrial ATP synthase subunits (spot 145, 414, 655), which are part of the F1F0 ATPase enzymatic complex that catalyzes the final step of ATP synthesis in the mitochondrial respiratory chain. Previous microarray studies on ECM roots have shown that the transcript levels of genes encoding enzymes in the TCA cycle and the respiratory chain were decreased in the plant partner (Johansson et al. 2004; Frettinger et al. 2007; Flores-Monterroso et al. 2013). Furthermore, studies have reported that ECM roots have lower respiratory rates when compared with non-symbiotic roots (Martins et al. 1997), a phenomenon that could involve a lower carbon flow through glycolysis and the TCA cycle in colonized roots. Also in arbuscular mycorrhizae (AM) symbiosis, proteomic and transcriptomic studies have revealed a decreased expression of genes and proteins involved in glucose breakdown pathways (Cangahuala-Inocente et al. 2011; Abdallah et al. 2014; Xu et al. 2015). Reduced abundance of C assimilative enzymes and electron transport chain proteins is suggestive of a lower sugar availability in colonized roots which agrees with the increased carbon sink promoted by the ECM fungus in symbiotic roots. In ECM root systems, up to 50% of the carbon fixed during photosynthesis can be "lost" to feed the fungal partner that, unlike wood decomposers, has a limited capacity to use carbohydrates present in the humus and litter layers of forest soils and, as so depends on the host fixed C (Nehls et al. 2010). This is consistent with the lower levels of soluble sugars, especially sucrose, found in cork oak mycorrhizal roots, compared with the non-symbiotic roots (Table 1). It is commonly accepted that in ectomycorrhizae established with basidiomycotic fungi like *P. tinctorius*, plant derived sucrose in the plant-fungus interface is hydrolyzed by plant cell wall invertases into hexoses, from which glucose seems to be preferred by the mycobiont (Nehls et al. 2010). This carbon drain to the fungal partner, suggested by our results, does not seem to result in biomass loss since foliar biomass was unaltered and root biomass was even increased by the inoculation with *P. tinctorius* (Table 1). This is suggestive of a fully functional symbiotic relationship between *Q. suber* and *P. tinctorius* under our experimental conditions. Together with the apparent decreased activity of glycolysis and TCA cycle related proteins, we also notice decreased levels for UDP-glucose pyrophosphorylase (UGPase) (spot 138, 149, 653) an enzyme which in roots is involved in sucrose breakdown for starch biosynthesis using the UDPG produced by Suc synthase (SuSy) (Kleczkowski et al. 2004). Down-accumulation of this protein in our experiment could suggest a carbon decreased flow towards starch

accumulation in ECM roots. However, our results do not support this assumption since starch levels were not decreased in mycorrizal roots compared with non-inoculated roots (Table 1). In fact, starch levels were identical in mycorrhizal and non-mycorrhizal roots, suggesting that the plant is not mobilizing stored sugar pools for transfer to the symbiotic fungus. Besides its role in sucrose metabolism, this enzyme is also involved in the synthesis of UDPG for cell wall polysaccharide synthesis (Kleczkowski et al. 2004). Interestingly, we also detected decreased levels of sinapyl alcohol dehydrogenase (spot 240) and isoflavone reductase (spot 281), both involved in the phenylpropanoid pathway that leads to the production of lignin and lignan, cell wall phenolic compounds that confer structural support and vascular integrity (Dixon et al. 1995; Li et al. 2001). Production of phenolic compounds and cell wall lignification are found in interactions between plant pathogens and resistant hosts (Miedes et al. 2014). Evidence suggests that ECM colonization down-regulates the phenylpropanoid pathway in roots, since decreased deposition of cell wall phenolics and inhibition of genes encoding enzymes of the phenylpropanoid pathway have been reported in pine and poplar ECM roots (Heller et al. 2008; Luo et al. 2009). The decreased levels of these proteins observed in our experiment and the molecular data referred above are perfectly in agreement with ultrastructural data of Duddridge (1986) showing that, in incompatible ECM interactions both lignification and cell wall appositions are induced in the "host" plant. This strongly suggests an induced "softening" of the plant cell wall in ECM roots, probably in order to facilitate the progression of the fungal hyphae in the root apoplast and the establishment of the plant-fungus interface for nutrient transport and exchange (compatible interaction). This is in line with the lower % C observed for the *P. tinctorius* colonized roots compared to the non-colonized roots (Table 1), since the majority of the cellular carbon pool is associated with the cell wall material, such as cellulose, hemicellulose and lignin. Since the amount of non-structural carbohydrates does not vary between mycorrhizal and non-mycorrhizal cork oak roots (Table 1), the % C difference between inoculation treatments probably reflects different contents of structural carbohydrates related to the cell wall. It can be hypothesized that suppression of pathways that lead to cell wall re-enforcement could be adopted by the plant as a means to facilitate symbiosis since ECM fungi apparently have lost the enzymatic capacity to degrade plant cell walls (Martin et al. 2008, 2010). In addition, the decreased abundance of cell wall phenolic-related enzymes can be interpreted also as an

attenuation of a defensive reaction in cork oak allowing *P. tinctorius* hyphae to accommodate between root cells.

3.5. Protein folding, stability and degradation

Among the down-accumulated spots, two proteins with chaperone activity, namely a protein disulfide isomerase (PDI) (spot 630, 634, 635, 636) and a protein showing sequence homology to the Rubisco large subunit-binding protein (spot 118) were identified. PDIs are involved in the oxidative-folding of nascent proteins in the endoplasmic reticulum catalysing the post-translational redox formation of disulfide bonds, acting also as chaperones by inhibiting the aggregation of misfolded proteins (Wilkinson and Gilbert 2004). Generally, they function with other chaperones to form functional proteins. The Rubisco large subunit-binding protein belongs to the chaperonin Cpn60/TCP-1 family which comprises proteins essential for the correct folding and assembly of polypeptides into oligomeric structures, acting also to stabilize or protect disassembled proteins under stress conditions (Hemmingsen et al.1988; Prasad and Stewart, 1992). There are evidences supporting a role of molecular chaperones in the interaction of plants and microorganisms. For example, PDIs are strongly up-regulated during pathogen attack (Ray et al. 2003, Caplan et al. 2009) and studies suggest a role of ER chaperones in the secretion of pathogenesis-related proteins (Wang et al. 2005) and in the folding of membrane receptor-like kinases required for innate immunity (Caplan et al. 2009). Interestingly, molecular chaperones also seem to be involved in mutualistic interactions such as the one between *Arabidopsis* and the endophytic fungus *Piriformospora indica,* which colonizes roots conferring beneficial effects to host plants (Qiang et al. 2012). Studies have shown that *Arabidopsis* proteins with chaperone activity, such as PDIs, are supressed by *P. indica* resulting in an impairment of root ER function suggested to disturb the secretion of antimicrobial proteins as a means of supressing plant defences (Qiang et al. 2012). It is well known that mycorrhizal fungi are able to supress the host plant defense system, by a yet unknown mechanism. Although our experimental system is very different from the *Arabidopsis-P. indica* interaction since *Arabidopsis* is not a mycorrhizal plant, an ectomycorrhizal fungus-induced suppression of protein folding/chaperone activity in the host cell could contribute to inhibit the secretion of proteins participating in defense reactions. Another protein identified in our study, which is also involved in post-translational redox-based modifications was identified as a thioredoxin-like protein

578 CXXS1 (spot 579) and on the contrary showed increased accumulation in colonized
579 roots. Thioredoxins are disulfide reductases that modulate the catalytic activity of their
580 target proteins by reducing disulfide bonds (conversion of S-S to –SH) (Meyer et al.
581 1999). The thioredoxin identified in our work shows sequence homology with the
582 cytosolic monocysteinic (WCXXS) thioredoxins from the subgroup H, present in all
583 higher plants (Serrato et al. 2008). A thioredoxin H from *Arabidopsis* plays an
584 important role in plant defense by catalyzing the oligomer-to-monomer switch of the
585 protein NPR1 involved in salicylic acid signaling (Tada et al. 2008). The down-
586 accumulation of PDI and up-accumulation of TRX in our study suggests that in cork
587 oak roots, a fungus-induced pathway counteracting protein oligomer formation (folding)
588 and promoting the formation of protein monomers (unfolding) is active.

589 Two spots corresponding to proteins involved in the ubiquitin/proteasome system were
590 identified, and included an up-accumulated subunit from the 26S proteasome (spot 381)
591 and a down-accumulated ubiquitin receptor RAD23 related protein (spot 160). The
592 ubiquitin/proteasome system is responsible for the selective degradation of proteins in
593 which substrates, marked by the covalent attachment of Ub, are degraded by the 26 S
594 proteasome (Book et al. 2010). Proteins from the RAD23 family are known to regulate
595 the degradation of ubiquinated proteins by a mutually contradictory mechanism in
596 which their UBA domains are reported to suppress the degradation of ubiquitinated
597 proteins by the proteasome, and their Ubl domains are otherwise enhancing proteasome
598 degradation (Lambertson et al. 2003; Raasi and Picard 2003). Decreased levels of
599 proteins with chaperone activity, such as Cpn60, and increased accumulation of proteins
600 involved in the proteasome complex were also reported to occur in AM symbiosis
601 between grapevine and two *Glomus* species (Cangahuala-Inocente et al. 2011). Our
602 results agree with these observations and suggest an impairment in protein folding
603 activity accompanied by an activation of protein degradation pathways to eliminate
604 unfolded proteins in colonized roots.

605 **3.6. Stress and defense**

606 Within the stress and defense category we observed alterations in spot abundance for
607 five different proteins. ROS (Reactive Oxygen Species) scavenging enzymes, such as
608 superoxide dismutase (spot 548, 549) showed increased levels in colonized roots.
609 Superoxide dismutase acts against ROS by converting highly reactive superoxide anion

radicals to H_2O_2 and O_2. Our results are in agreement with the Baptista et al. (2007) and Alvarez et al. (2009), who reported increased levels of antioxidant enzymes in roots colonized by ECM fungi. Increased levels of these proteins in ECM roots would improve their capacity to cope with oxidative stress and agree with the reported lower accumulation of ROS in mycorrhizal roots when compared with roots infected by pathogenic fungi (Espinoza et al. 2014). Several studies concluded that contact of roots with mycorrhizal fungi can evoke an unspecific redox defense reaction (Garcia-Garrido and Ocampo 2002; Baptista et al. (2007), although attenuated when compared with the oxidative burst elicited by pathogenic fungi (Espinoza et al. 2014). Also up-accumulated in roots under our experimental conditions was a small ubiquitin-like modifier (SUMO) protein (spot 594). SUMOs are regulatory proteins that can be covalently attached in a reversible manner to target proteins as a post-translational modification (SUMOylation) and thereby modify protein function. In plants, SUMO has been shown to be involved in stress responses, pathogen defense, absisic acid signaling and flower induction (Novatchkova et al. 2004). Nothing is known about the role of SUMOylation in symbiotic interactions but a SUMO protein was transcriptionally induced in legume symbiotic nodules (Rose et al. 2012). This type of modification could be regulating the activity, stability or sub-cellular localization of proteins involved in the response of plants to ECM fungi (Miura et al. 2007). Among the down-accumulated proteins in the stress and defense category, a protein spot (416) corresponding to a putative temperature induced lipocalin (TIL) was identified in our study. TILs belong to a poorly understood family of proteins predicted to act in the protection of cells against membrane lipid peroxidation during oxidative stress conditions in plants and animals (Charron et al. 2008; Boca et al. 2014). Also down-accumulated were 3 spots corresponding to a major latex-like protein (MLP) (spots 437, 646 and 647). MLPs belong to the Bet v I family and constitute a poorly known protein family, found only in plants that have been associated with pathogen defense responses, response to wounding or abiotic stress (Liu et al. 2006). The biological function of these proteins is unknown but an involvement in lipid binding, such as trafficking of membrane components was suggested (Radauer et al. 2008). This agrees with the predicted ER membrane retention signals KKXX-like motifs (IAKA and HITK; Online resource 5) detected in the C-terminus of the MLPs identified as differentially accumulated in colonized corks roots. Proteins with homology to the Bet v I family

have been detected in other ECM systems, and just like in our study they have been reported to be down-regulated in ECM roots (Heller et al. 2008). The decreased accumulation of proteins with a suggested role in pathogen defense is in good agreement with the reported inhibition of plant defense genes in mycorrhizae (Le Quéré et al. 2005; Xu et al. 2015) as a way to supress defence reactions against symbiotic fungi and facilitate its establishment in the roots, although the mechanism remains unknown.

3.8. Lipid metabolism/transport

Increased levels of two proteins putatively involved in lipid metabolism and transport were recorded. One example was a membrane steroid-binding protein 2-like (spot 311). Noteworthy, a *Medicago truncatula* membrane steroid-binding protein MtMSBP1, was reported to be critical for AM arbuscular mycorrhiza establishment (Kuhn et al. 2010). Like the MSBP1 from *M. truncatula*, the protein identified in our experiment was also predicted to contain a single N-terminal transmembrane region (amino acids 18-42) for membrane anchoring, a conserved cytochrome b5-like heme/steroid binding domain (Pfam motif PF00173) for steroid binding and a conserved tryptophan residue for progesterone binding (Trp 150) (Online resource 5). In addition, a C-terminal ER-membrane retention signal KKXX-like motif (DVAK; Online resource 5) was also detected, suggesting that, like MSBP1 from *M. truncatula*, the protein identified in cork oak could also be an ER integral membrane protein. These data suggest that these two proteins could be functional orthologous necessary for the response of plants to both ecto- and endo-fungal symbionts. Membrane steroid-binding proteins are presumed to have a conserved role in the control of sterol biosynthesis by binding and regulating cytochrome P450 enzymes in the ER membrane (Hughes et al. 2007). The increased abundance of this protein upon ECM symbiosis may contribute to sustain the delivery of new membrane material to the plant-fungus interface region, or proteins and metabolites in transport vesicles to the plant-fungus interface. Another putative lipid-interacting protein, which was the most strongly accumulated protein in cork oak roots after *P. tinctorius* inoculation, and was also predicted to be an internal membrane protein, showed high sequence similarity to posphatidylglycerol/phosphatidylinositol transfer proteins (PG/PI-TP) (spot 583) from several plants. This protein contains the MD-2 related lipid-recognition domain (Pfam domain PF02221) (Online resource 5),

675 from the ML family of proteins which are involved in the interaction with specific lipids
676 and lipid recognition (Inohara and Nuñez 2002). The protein identified in our
677 experiment contains a predicted transmembrane helix for anchoring to internal
678 membranes (amino acids 37-54; Online resource 5) which is suggestive of a protein
679 localization at the secretory pathway. The ML family contains multiple members of
680 unknown function in animals and plants. In animals they have been implicated in
681 regulation of lipid metabolism, response to pathogen components such as
682 lipopolysaccharides, and other cellular functions involving lipid recognition (Inohara
683 and Nuñez 2002). These results suggest that ECM fungal colonization induces major
684 alterations in internal membranes of the host root cells. The establishment of the plant-
685 fungus interface for nutrient exchange is probably accompanied by a reorganization of
686 the plasma membrane in both partners, implying an increased formation of transport
687 vesicles for sustaining the delivery of new membrane material and/or
688 extracellular/plasma membrane proteins.

689 **3.9. Nutrient exchange**

690 A glycine cleavage system H protein (spot 439), which is part of the mitochondrial
691 glycine decarboxylase complex (GDC), was accumulated in our experiment. GDC is an
692 essential component of glycine catabolism in non-photosynthetic tissues, where it plays
693 a role in organic nitrogen assimilation in root tissues (Hartung and Ratcliffe 2002).
694 Ectomycorrhizal fungi are very important for their host N nutrition, since in temperate
695 and boreal forest ecosystems concentration of mineral N forms in the soil is often very
696 low due to the reduced levels of N mineralization. N translocation from the soil through
697 the fungus and to the plant is a defining characteristic of this symbiosis (Müller et al.
698 2007). The process involved in the transference of N from the fungus to the symbiotic
699 tissues is still poorly understood but there is evidence that amino acids are released from
700 the fungal cells to the plant apoplast, where specific transporters translocate them to the
701 symbiotic roots for assimilation (Müller et al. 2007). The increased levels of this protein
702 in *P. tinctorius* colonized roots supports a role of organic N (amino acids) as a major N
703 form for translocation into the host roots, also suggested from transcriptomic studies in
704 *Q. suber-P. tinctorius* mycorrhizae (Sebastiana et al. 2014).

705 A protein showing homology to purple acid phosphatases (spot 81, 82) from several
706 plants was down accumulated, in agreement with previous results from EST sequencing

and microarray analysis on ECM roots showing a repression of genes involved in soil phosphorus transport and acquisition (Heller et al. 2008; Luo et al. 2009; Flores-Monterroso et al. 2013; Sebastiana et al. 2014). Sequence analysis revealed a signal peptide motif (1-16 amino acids) and a secretory pathway location, which is in accordance with the notion that purple acid phosphatases (PAPs) are secreted outside the root cells to the extracellular environment, where they hydrolyze various phosphates, including inorganic pyrophosphate (Tran et al. 2010). Decreased levels of these proteins in roots after mycorrhiza establishment with *P. tinctorius* suggests a disinvestment in soil phosphorus up-take pathways by the colonized roots since phosphorus can be supplied directly by the fungus, evidenced from phosphorus increases in roots after long-term ECM colonization (Luo et al. 2009). In accordance, we detected increased volumes for two protein spots corresponding to a plant acid phosphatase (spot 308, 310), which are proteins reported to be involved in P transfer from the fungus to the host plant (Alvarez et al. 2012). The majority of soil P is not available to plants because it is sequestered in organic forms (Hinsinger 2001). In ectomycorrhizae, phosphatase enzymes produced by the fungal symbiont play a role in the conversion of soil organic P compounds into plant accessible forms such as polyphosphate-P (poly-P), which concentrate into fungal tissues (Cairney 2011). The limited substrate specificity of acid phosphatases allows these enzymes to target the poly-P in the Hartig Net region for P transfer from the fungus to the host plant (Alvarez et al. 2012). Increased accumulation of acid phosphatases was also found to occur in *Medicago truncatula* in response to AM symbiosis (Valot et al. 2005) and during symbiotic nodule development in soybean (Penheiter et al. 1998), suggesting that these proteins are common to the different symbiotic programs in plants. We also detected increased levels of a protein showing sequence similarity to the copper transport protein ATOX1 (spot 589). In *Arabidopsis*, the copper chaperone ATX1 (the homolog of the mammalian ATOX1 and yeast ATX1) is involved in copper homeostasis conferring tolerance to both excess and deficiency of copper (Shin et al. 2012). Little is known about the physiological significance of copper chaperones in plants, besides their putative dual involvement in copper trafficking and detoxification (Harrison et al. 1999). The *P. tinctorius* strain used in our study was isolated in a copper mine area, where very high levels of copper metal are present in the soil (Sebastiana et al. 2013a). Adaptation to this condition probably results in an increased capacity to absorb and

accumulate copper into fungal tissues. The *P. tinctorius* mycelium colonizing cork oak roots could be actively involved in translocating copper from the surrounding soil to the vicinity of the roots which could result in the increased accumulation of a copper chaperone in the colonized roots.

3.7. Cytoskeleton

Cytoskeletal proteins were mainly down-accumulated in cork oak ECM roots, including two spots corresponding to actin (spot 202, 205), and one spot corresponding to the actin-binding protein, profilin (spot 580), which regulates actin polymerization. Ultrastuctural studies using immunological methods have revealed that in heavily colonized portions of ECM roots, cytoskeleton actin filaments disappear (Timonen and Peterson 1993), which agrees with the down accumulation of actin protein observed in our experiment and suggests a cytoskeleton rearrangement in ECM colonized root cells. Down accumulation of actin was also reported in grapevine roots heavily colonized by an endomycorrhizal fungus (Cangahuala-Inocente et al. 2011). Microorganisms can interact with the plant cytoskeleton, as observed for an effector molecule from a plant pathogen (*Pseudomonas syringae*), shown to disrupt the actin cytoskeleton of *Arabidopsis* cells and resulting in inhibition of endocytosis and trafficking to the vacuoles as a way to elude plant defense responses (Kang et al. 2014). Remarkably, this pathogen seems to target actin 7 (ACT7) (Jelenska et al. 2014), the same which we found to be down accumulated upon *P. tinctorius* colonization of ECM cork oak roots. By negatively interfering with the actin cytoskeleton, *P. tinctorius* could disturb vesicular protein secretion of antimicrobial proteins.

4. Correlation between protein and mRNA transcription levels

Several transcripts encoding differentially accumulated proteins identified in our 2D experiment (PDI, CPN60, TIL, RAD23-like, proteasome subunit alpha type-5-like and SUMO) were analyzed by real-time PCR in order to evaluate the correlation with the results from the proteomics analysis (Fig. 3). Most of them showed the same accumulation profile as the one detected for the proteins in the 2D electrophoresis analysis, showing that our results are consistent.

5. Conclusions

Using 2D-DIGE and MS technologies we investigated the differences in the protein profiles between *P. tinctorius* mycorrhizal and non-mycorrhizal cork oak plants. The detection of differentially accumulated proteins only at the root level and not at the foliar level suggests that the response of plants to ectomycorrhizal inoculation is mainly a local effect, as opposed to a systemic effect from the roots to the aerial parts, contrary to what generally occurs in arbuscular mycorrhizae. At the root level, the results allowed us to gain novel insights into the molecular events involved on host plant response to ECM fungal colonization. Most of the proteins detected in ECM roots had lower accumulation levels when compared with the non-symbiotic roots. This strongly suggests a decreased metabolic activity in mature ECM roots. Studies on mycorrhizal herbaceous plants, like *Medicago*, have reported a major up-accumulation of root proteins, compared with down-accumulated proteins, following AM colonization (Bestel-Corre et al. 2002; Aloui et al. 2008). However, on grapevine, a woody species like cork oak, a proteomic study revealed a generalized down-accumulation of root proteins following AM colonization (Cangahuala-Inocente et al. 2011). This, and the fact that many of the proteins identified in our study were also detected in AM grapevine, suggests that in woody plants mycorrhization results in a different reprograming of the host genes in order to accommodate the fungal symbiont (Cangahuala-Inocente et al. 2011). Proteins that showed decreased levels following mycorrhiza formation were mainly implicated in carbohydrate and energy metabolism, protein folding/assembling, cell wall re-enforcement, defense, cytoskeleton biogenesis and soil Pi acquisition. Conversely, proteins related to the antioxidant defense system, N assimilation, membrane lipid transport/metabolism and P transfer from the symbiotic fungus had their levels increased. A schematic overview of the major findings from our proteomics study is shown in Fig. 4. Our results suggest a decreased activity of metabolic pathways, like glycolysis, the TCA cycle and the respiratory chain, which could be related to the transfer of carbon to "feed" the fungal symbiont that occurs in ectomycorrhizae. This suggestion is supported by a decrease in soluble sugar content in mycorrhizal roots when compared with non-mycorrhizal roots. In the context of mineral nutrient exchange, a nitrogen assimilation pathway involving the transfer of amino acids into the host root is suggested by the results. In addition, a disinvestment in soil P assimilation and activation of enzymes related to the transfer of P from the ECM fungus to the host plant was detected. A cell wall softening of the colonized roots is evidenced

from the proteomics and analysis of total carbon concentration, which could facilitate the progression of the fungal mycelium in the apoplast during the formation of the Hartig net for mutual nutrient exchange. Furthermore, a previously unreported mechanisms promoting the unfolding of proteins in colonized roots is suggested, which could be related to the known fungal-induced inhibition of defense responses in ECM symbiotic plants. These findings represent a step forward towards a better understanding of ECM symbiosis on forest trees and constitute an indication of the benefits of promoting mycorrhization of cork oak forests, especially in the context of climate change. According to our results mycorrhizal colonization increases root biomass which could have a positive impact on the global capacity to up-take soil nutrients. Taken together, results from this study further suggest that ectomycorrhizal symbiosis can have very positive-role effects in coming scenarios of increasing aridity and extreme climatic events.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1 2D-gel electrophoresis analysis of proteins extracted from cork oak roots colonized by *P. tinctorius* (A) and from non-inoculated roots (B) visualized by Comassie blue staining. Proteins differentially accumulated in colonized roots are marked by arrows and are described in Table 2 and online resource 5. A merged Cy5/Cy3 2D-gel representative image from the two conditions (mycorrhizal root and non-mycorrhizal root) (C)

Fig. 2 Gene ontology (GO) annotation (biological process) of the identified cork oak root proteins differentially accumulated upon mycorrhiza formation with *P. tinctorius*. On the X axis is represented the proportion of proteins assigned to each GO functional category

Fig. 3 Gene expression levels of selected candidates in roots inoculated with *P. tinctorius* (Inoc) and in non-inoculated roots (non-Inoc). Bars represent standard deviation. TIL: temperature-induced lipocalin; RAD23: Ubiquitin receptor RAD23c; PDI: Protein disulfide-isomerase; Cpn60: Chaperonin 60; PROTα5: Proteasome subunit alpha type-5; SUMO: Small ubiquitin-related modifier 1

Fig. 4 Summary of the major molecular events occurring in mature ectomycorrhizal cork oak root cells colonized by *P. tinctorius*. Proteins identified in this study (Table 2)

are represented by boxes. Red boxes denote up-accumulated proteins. Green boxes denote down-accumulated proteins. Dashed arrows denote pathways down-regulated by ECM inoculation, whereas complete arrows refer to up-accumulated pathways. PDI: protein disulfide-isomerase; TIL: temperature-induced lipocalin; RAD23: Ubiquitin receptor RAD23c; PROTα5: Proteasome subunit alpha type-5; MLP: MLP-like protein 328; PG/PI TP: phosphatidylglycerol/phosphatidylinositol transfer protein; MSBP: Membrane steroid-binding protein 2-like; SOD: Superoxide dismutase; TRX H: Thioredoxin-like protein CXXS1; GDC: Glycine cleavage system H protein 2; ATPase: ATPase subunit 1; MDH: Malate dehydrogenase 2; TPI: Triosephosphate isomerase family protein; FBPA: Fructose-bisphosphate aldolase; ENO: Enolase; FK: Fructokinase-1; UGPase: UDP-glucose pyrophosphorylase; SAD: Sinapyl alcohol dehydrogenase-like; IR: allergenic isoflavone reductase-like Bet v 6.0102; PAP: Purple acid phosphatase; AP: Acid phosphatase

Table captions

Table 1 – Root and leaf biomass, concentration of soluble sugars (glucose, sucrose, and fructose), starch, non-structural carbohydrates and carbon in *Q. suber* roots inoculated with *P. tinctorius* and in non-inoculated roots. Data indicate means ± standard deviation (n= 20-25 for biomass; n=3 for soluble sugars, starch and % C). Different letters in the same column indicate significant differences between the treatments at $p < 0.05$.

Table 2 - List of differentially accumulated proteins when comparing *P. tinctorius* inoculated and non-inoculated roots identified by 2D-electrophoresis and mass spectrometry (MALDI-TOF/TOF).

Electronic supplementary material

Online Resource 1 (Supplementary Table 1) Quantitative analysis of the protein spots. Column A: spot number; Column B: normalised spot volume in mycorrhizal roots according to Progenesis SameSpot; Column C: spot volume upon correction for the 0.93:0.07 plant-fungal proportion in mycorrhizal roots; Column D: normalised spot volume in non-mycorrhizal roots according to Progenesis SameSpot; Column E: Fold Change (FC) between mycorrhizal and non-mycorrhizal roots calculated as C/D;

Column F: for representation proposes, a -1/FC transformation was applied to FC values between 0 and 1 (down-accumulated spots).

Online Resource 2 (Supplementary Table 2) Target genes for real-time PCR analysis: accession in cork oak transcriptome database (www.corkoakdb.org), primers sequences, annealing (Ta) and melting (Tm) temperature.

Online Resource 3 (Supplementary Fig. 1) The interaction between *P. tinctorius* and the roots of cork oak. (a) Example of a colonized (right) and a non-colonized (left) plant. (b) Non-inoculated roots. (c) Colonized root, 2 months after inoculation with *P. tinctorius*. (d) Transverse section of a colonized root (2 months after inoculation) showing the fungal mantle (m) surrounding the root and the hartig net (hn) on root epidermal cells; scale 50 µm (Sebastiana et al. 2014)

Online Resource 4 (Supplementary Fig. 2) Representative images of Cy labelled 2-DE gels for mycorrhizal leaves (A) and non-mycorrhizal leaves (B)

Online Resource 5 (Supplementary Table 3) The identity of differentially expressed protein spots as determined by tandem mass spectrometry