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Title:

In-depth analysis of the *Quercus suber* metabolome under drought stress and recovery reveals potential key metabolic players

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Highlights

- Cork oak metabolome significantly changes between drought stress and rehydration
- sPLS analysis allowed for a comprehensive study of the two tested water conditions, revealling potential metabolic biomarkers research.
- Secondary metabolism has a crucial role during both drought stress and rehydration.
- Specific flavonoid and terpenoid compounds migth be key players in drought stress response and recovery.

Running title: *Quercus suber* drought and rehydration metabolome

Abstract

Cork oak (*Quercus suber* L.) is a species of ecological, social and economic importance in the Mediterranean region. Given its xerophytic adaptability, the study of cork oak's response to drought stress conditions may provide important data in the global scenario of climate change. The mechanisms behind cork oak's adaptation to drought conditions can inform the design and development of tools to better manage this species under the changing climate patterns. Metabolomics is one of the most promising omics layers to capture a snapshot of a particular physiological state and to identify putative biomarkers of stress tolerance. Drastic changes were observed in the leaf metabolome of *Q. suber* between the different experimental conditions, namely at the beginning of the drought stress treatment, after one month under drought and post rehydration. All experimental treatments were analyzed through sPLS to inspect for global changes and stress and rehydration responses were analyzed independently for specific alterations. This allowed a more in-depth study and a search for biomarkers specific to a given hydric treatment. The metabolome analyses showed changes in both primary and secondary metabolism, but highlighted the role of secondary metabolism. In addition, a compound-specific response was observed in stress and rehydration. Key compounds such as L-phenylalanine and epigallocatechin 3-gallate were identified in relation to early drought response, terpenoid leonuridine and the flavonoid glycoside (-)-epicatechin-3'-O-glucuronide in long-term drought response, and flavone isoscoparine was identified in relation to the recovery process. The results here obtained provide novel insights into the biology of cork oak, highlighting pathways and metabolites potentially involved in the response of this species during drought and recovery that may be essential for its adaptation to long periods of drought. It is expected that this knowledge can encourage further functional studies in order to validate potential biomarkers of drought and recovery that maybe used to support decision-making in cork oak breeding programs.

Keywords: *Quercus suber*, drought, metabolome, rehydration, secondary metabolism, integrated analysis.

Introduction

Climate change presents a major challenge to the sustainable management of forests in Europe. In this climate change scenario, extreme weather conditions such as heat waves and long periods of drought are expected to be more frequent and particularly severe in some regions, such as the Mediterranean [1]. *Quercus suber* forms part of the characteristic flora of the Mediterranean region, but unlike other species (e.g. *Quercus ilex)*, its distribution is quite narrow [1] presenting greater vulnerability to environmental changes. In addition to its ecological and social importance, this species has also an important economic value due to cork production, which can also be compromised under the foreseen extreme climatic conditions.

Drought is one of the main environmental constraints affecting plant yield and survival, especially in Mediterranean ecosystems [2]. Although *Quercus suber* shows a great tolerance to drought, being considered a xerophytic species [3], a greater comprehension of the adaptation strategies developed by this species under unfavorable climate conditions is still required. This knowledge will contribute for the design of sustainable breeding programmes to ensure the survival of this species under extreme abiotic stress conditions [4]. Although less studied, recovery ability is a pivotal trait in plant´s drought response and may dictate the difference between a susceptible or a resistant phenotype [5]. The high resilience of the photosynthetic apparatus to drought as well as good recovery in cork oak was reported for trees under field conditions [6]

The metabolome provides a snapshot of the physiological state of the plant in a particular condition and can help to understand specific response mechanisms [7]. Metabolomics is one of the omics layers closest to the phenotype, and highly environmentally-sensitive [8–11] representing very promising for the search and discovery of new candidate biomarkers [9,12]. Furthermore, the integration of metabolome data with other omics or physiological data is expected to greatly increase the method robustness and confidence in meaningful knowledge discovery.

Plant responses to low water availability can affect the concentration, composition, and distribution of both primary (maintaining life processes and facilitate growth) and secondary metabolites [13]. However, the role of secondary or specialized metabolism in drought defense and plant adaptation mechanisms has been

emphasized [14,15]. These specialized metabolites can be divided into three main distinct groups: terpenes, phenolics, and nitrogen-containing compounds. Water limitation induces different patterns of secondary metabolites accumulation (e.g. terpenoids and phenolics) including increase, reduction or no alteration [16–18]. This has been attributed to the concentrations of secondary metabolites being specific to the species, the experimental conditions, the class of secondary metabolite and even compound-specific [16,19,20]. Metabolomics research applied to forest trees has advanced slowly due to the long-life cycles of trees, the lack of genomic tools and limited databases [21,22], but a few studies have highlighted the potential of this tool. López-Hidalgo *et al.* [23] reported the impact of drought in *Q. ilex* primary metabolism in which the biosynthesis of secondary metabolites was less evident. Interestingly, in a different study, the total phenol and specialized metabolites such as flavonoids increased in *Q. brantii* under drought particularly in the susceptible provenances [24].

The aim of this study was to develop a novel integrative and explorative approach to discover potential key metabolic biomarkers and pathways related to drought tolerance of *Quercus suber*. To that end, the drought and recovery of 1-year-old cork oak plants was analyzed using an untargeted metabolomic analysis. An integrative bioinformatics analysis was carried out, combining metabolomic data with physiological data (physiological dataset previously published by this team [25]). The analysis was performed at the different water availability conditions (control, drought and recovery) and stress periods (short term and long term). This strategy allowed to gain in resolution over the metabolic status in each of the different stress and recovery phases considered in the experiment, resulting in the identification of metabolic signatures associated with each one of them. The physiological dataset included several parameters used to monitor the cork oak plants physiological performance over the assay, namely plant water status (relative water content and water potential), proline and malondialdehyde contents [25]. By using this novel integrative analysis, the present work aimed to identify potential key metabolic markers (stress and rehydration), pathways and add new fundamental knowledge that will drive useful data to scientific-based decision-making for *Q. suber* management programs. By identifying these biomarkers of drought tolerance in seedlings we seek to aid programs of early selection and field establishment of plants genetic material more

adapted to the foreseen climate change scenarios, crucial to minimize cork oak decline.

Material and Methods

Plant material and experimental design

One-year-old cork oak plants grown from seeds from provenance region II, Grandola (South of Portugal) were acquired from a forest plant producer (Anadiplanta, Portugal). Plants were transplanted to 1L containers with a peat:perlite mixtures (1:1) and acclimated for 1 month in a climatic chamber at 25 day/ 20 °C night, 60-70% relative humidity, 16 h of light photoperiod and 250 mmol m^{-2} s^{-1} of photosynthetic photonic flux. During this period, plants were fertilized twice with a solution of Nutriquisa 5-8-10® (5 ml/L) until runoff. All plants were watered to 60% field capacity (FC) and the stress was initiated by withholding water until soil moisture reached 18% FC (water stress, WS) which took five days. A group of well-watered (WW, control group) plants was held at 60% FC during the entire assay. The percentages of FC were maintained by adding the amount of water lost by evapotranspiration, which was monitored by weighing the containers every day. The drought stress was imposed for 30 days under same conditions during acclimatization; following that, stressed plants were re-watered until well-watered conditions during one week. The experiment was designed to replicate a scenario of water limitation period, followed by a period of rewatering, which may be experience by seedlings in the field after plantation. For analysis, three pools (6 plants each) of fully expanded leaves were collected from WS plants at four points: (T1) the first day of WS when soil moisture reached 18% FC; (T2) 30 days after T1; (R1) one day after re-watering; and (R2) one week after rewatering. The experimental design was as in Almeida et al. [25] with a few adaptations. For metabolomics studies, control plants were collected at the beginning (T1WW) and at the end of the experiment (R2WW). These two controls were selected for metabolomics and integrative analysis based in the results of physiological data assessed in Almeida *et al.* [25]. In this previous article all controls did not show significant differences in the physiological parameters analyzed over time, except for MDA that increase and stabilized one month later (T2WW, R1WW, R2WW) comparing to the first control (T1WW). Based on these results change of this single parameter, we decided to include two controls,

T1WW and only one control after one month, the final time R2WW. A diagram of the experiment and sample collection is available in Figure 1. For metabolite analysis, leaf samples were immediately frozen in liquid nitrogen and kept at 80 °C until further use.

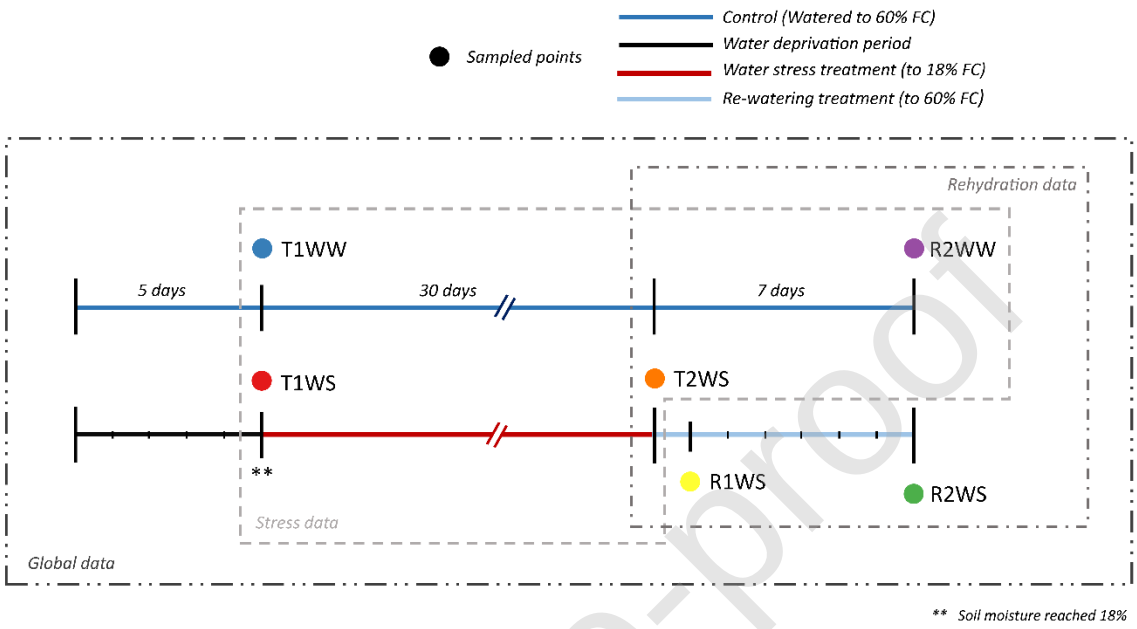

Figure 1. Time diagram of the experiment, sample collection points and datasets considered for different analysis are depicted. The different dotted lines separate the data used for the analyses (global, stress and rehydration). Small separations on the trial are equivalent to one day. Collection points: T1WW, control plants well-watered at day 1; R2WW, control plants well-watered the whole experiment; T1WS, water-stress plants at first day of water stress; T2WS, water-stress plants 30 days after T1WS; R1WS, water-stress plants one day after rehydration; and R2WS, water-stress plants one week of rehydration. ** Indicated when soil moisture reached 18%.

Briefly Plant Physiological parameters

Stem water potential (WatPot) was measured on the main stem after a clean cut near the base using a Scholander-type pressure chamber (PMS Instrument Co). To determine relative water content (RWC), four leaf discs (diameter = 11 mm) were used per plant. Tissue fresh weight (FW), turgid weight (TW) and dry weight (DW) were recorded and RWC was calculated using the following equation: RWC (%) = (FW-DW)/(TW-DW)*100. Lipid peroxidation was estimated by determining the MDA content in leaves according to Elkahoui *et al.* [26] using 100 mg frozen leaves.

Proline content was assessed as described by Khedr *et al.* [27] using 100 mg of frozen leaves.

The physiological data of lipid peroxidation (MDA), proline content, water potential (WatPot) and relative water content (RWC) from Almeida *et al.*[25] were used in this study and combined with metabolomics data to construct the integrated networks.

Extraction of metabolites

Polar metabolites were extracted as described by Valledor *et al.* [28] using 100 mg of leaf fresh weight from T1WW, T1WS, T2WS, R1WS, R2WS and R2WW treatments. Briefly, samples were ground in liquid nitrogen and 600 µl of cold (4°C) metabolite extraction solution – Methanol:Chloroform:H2O (2.5:1:0.5) – was immediately added to each tube. Samples were then centrifuged at 20,000 g for 4 min at 4°C and the supernatant transferred to new tubes. Finally, 800 µl of Chloroform:water (1:1) were added, the tubes vortexed and again centrifuged at 20,000 g for 4 min at 4°C. Following the formation of two layers, the upper aqueous layer, containing the polar metabolites, was transferred to new tubes and dried in a speed vac.

Polar metabolite identification and quantification using LC-Orbitrap-MS analysis

The polar fraction of each sample was analyzed twice on a LC-Orbitrap-MS, using both positive and negative ion modes. To check and evaluate the results, the recordings of the high-resolution mass spectrometer and the DAD detector were monitored and stored. Dionex Ultimate 3000 (ThermoFisher Scientific, USA) was used as the high-performance liquid chromatography component. The LC-Orbitrap-MS system (controlled by Xcalibur version 2.2, Thermo Fisher Corporation) was run according to the procedure of Escandón et al. [29]. Mass spectrometry (MS) was performed using an LTQ Orbitrap XL- high resolution mass spectrometer (ThermoFisher Scientific, USA) equipped with a HESI II (Heated electrospray ionization) source. After the analysis of each batch (30 samples), resolution and sensitivity of the Orbitrap were controlled by the injection of a mixed standard (caffeic acid, proline and sucrose) and with the aid of lock masses (phthalates). Blanks were also analyzed during the sequence.

MZmine software [30] was used to identify and analyze raw data of LC-Orbitrap-MS. MS1 spectra were filtered establishing a noise threshold at $4.5E^{03}$ and minimum peak height at $7E^{03}$ with a minimum time peak of 0.15 min. Peaks were smoothed and deconvoluted by using a local minimum search algorithm (98% chromatographic threshold, minimum retention range 5 min, minimum relative height of 90%, and minimum ratio top/edge of 1.2). Chromatograms were aligned using the RANSAC algorithm with a tolerance of 5 ppm of m/z and one-minute retention time. Normalized peak areas were used for quantification. The individual peaks were identified following different approaches; the first step was performed against an in-house library (> 100 compounds) and manual annotation considering m/z and retention times. In a second step, masses were assigned using the KEGG, MassBank, HMDB and Plantcyc databases with a built-in MZmine utility with a 5 ppm threshold. Metabolites were considered as undoubtedly "identified" if identified after comparison with the in-house standard compound library, or as "tentatively assigned" for those with molecular ions with exact masses corresponding to the identified metabolites in databases (Supplementary Table S1). Metabolite identification against in-house library was confirmed by retention time (RT), mass, isotopic pattern and ring double bound parameters. Metabolomics pathways of each metabolite (Supplementary Table S2a) were searched against KEGG pathway maps (http://www.genome.jp/kegg/tool/map_pathway1.html) and p-values of each metabolomics pathways (Supplementary Table S2b) in MBROLE 2.0 [31].

Statistical and bioinformatics analysis

Three biological replicates were used for metabolites and physiological parameters analysis. Statistical procedures were carried out using R programming language running under the open-source computer software R v2.15.2 (R Development Core Team, 2015) and RStudio [32]. Recommendations from Valledor *et al.* [33] were followed to pre-processed metabolome (missing value imputation, abundance balancing and filtering datasets) using pRocessomic v.1. (available on web direction: http://github.com/Valledor/pRocessomics). Sample-centric approach was used to transform the data. Scaled and centered values (z-scores) were subjected to multivariate analysis and Heatmap clustering. Heat mapping was performed using the Manhattan distance method to grouping metabolites in different KEGG pathways.

Multivariate analysis (Sparse Partial Least Squares (sPLS), network analyses and Principal Component Analysis (PCA)) was performed. The sPLS algorithm was used to find correlations between predictor (metabolites matrix) and response variables (physiological parameters) in the following three different datasets: 1) Global analysis: Metabolites and physiological parameters data of all treatments (T1WW, T1WS, T2WS, R1WS, R2WS, R2WW) (Supplementary Dataset 1). This global approach also supported the decision of separating the analysis in two different blocks; 2) Drought stress: Metabolites and physiological parameters data of water limitations (T1WW, T1WS, T2WS, R2WW) (Supplementary Dataset 2) for analyzing short- and long-term water stress treatments (5 and 30 days respectively); 3) Recovery: Metabolites and physiological parameters data after rehydration (T2WS, R1WS, R2WS, R2WW) (Supplementary Dataset 3) for analyzing changes after rehydration. Datasets were combined following a normalization for each dataset. sPLS and PCA analysis were conducted with mixOmics [34] and pRocessomic v.1. Network topology was defined after applying sPLS regression using the function "network()" of the mixOmics package and filtered (only edges equal or higher than |0.85| were maintained) in Cytoscape v.3.3.0 [35] as described in Escandón *et al.* [36]. Univariate analyses one-way ANOVA and Student's t-test were conducted for metabolites and physiological parameters with $P \leq 0.05$ and FDR (5%). Graphics were plotted using the libraries ggplot2 [37] and pheatmap [38].

Results and Discussion

No morphological signs of stress were observed one-month after water deficit imposition. However physiological changes occurred during this period confirming the physiological stress response already reported by Almeida et al. [25]. Leaf water status measured by RWC decrease at T1WS and reached 71.41% at T2WS in comparison with 85.82% determined in T1WW plants (Table 1). The water potential (WatPot), in turn, showed the lowest value at T1WS (-1.69 MPa) while at T2WS this value increased for -0.78 MPa, being still lower than T1WW value (-0.32 MPa) (Table 1). This pinpoint a first quick response and then a great plasticity to cope with 18% of FC for a longer period. Following re-watering, the water stressed plants rapidly recovered and displayed values of control plants (T1WW and R2WW) for both parameters. Proline content was maintained constant in WW and WS plants during drought and recovery (Table 1). On the other hand, MDA content was affected by water deficit, showing an increase at T2WS (60.74 nmol g^{-1} FW) compared to T1WW (21.75 nmol g^{-1} FW) (Table 1). After one day of recovery (R1WS), MDA levels remained higher (50.59 nmol g^{-1} FW) compared to R2WW plants (36.17 nmol g^{-1} FW); however, these differences disappeared after one week of re-watering. These results confirm the great adaptability of this species to drought stress imposition [39]. The low impact on survival of juvenile cork oak plants under dry conditions is well recognized [3,4], however the knowledge of the mechanisms underlying this notable stress tolerance is still scarce. Here we seek to integrate these physiological traits with the metabolomic data to identify key drought response mechanisms and potential key markers that later may help to explore the existent intraspecific variation to drought response [3,4].

Exploring metabolome of Q. suber related to drought stress and rehydration

LC-Orbitrap-MS allowed the reliable quantification of 3,112 metabolites (Supplementary Table S1) in six sampling points analyzed (T1WW, T1WS, T2WS, R1WS, R2WS, R2WW), with more than 40% of the metabolites (1252) differentially accumulated (FDR 5%, Supplementary Dataset 1). A customized algorithm combining custom in-house and public databases resulted in the unequivocal identification of 16 ions (identical matches to in-house compound library) and 869

ions that were tentatively assigned after comparing their accurate mass against reference compound databases (Supplementary Table S1). The use of this technology yielded a wide spectrum of primary and secondary metabolites, yet key changes in the secondary metabolism behind drought and recovery in *Q. sube*r were highlighted.

An initial analysis of the obtained metabolites revealed a high specificity with more than 100 unique metabolites associated with each individual treatment (Table 2 and Supplementary Table S3). The treatment exhibiting the highest number of specific metabolites was T1WS followed by the two rehydration points (R1WS and R2WS) and the treatment after one month under stress (T2WS). The classes of metabolites found in all treatments were highly diverse, including compounds related to phenylpropanoids biosynthesis pathways (e.g. sinapyl alcohol) and flavonoids (e.g. quercetin 3-(4"-acetylrhamnoside) 7-rhamnoside) present in T2WS. Furthermore, stress treatments (TIWS and T2WS) shared 20 unique metabolites only, including flavonoids such as epigallocatechin 3-gallate and 3,5-digalloylepicatechin. This indicates the high variability and specificity of the metabolome during the different water status imposed, establishing a good baseline for biomarkers research to predictor of plant drought performance.

A global multivariable analysis (sPLS and PCA) was used to glimpse the different processes throughout the assay. The sPLS analysis (Fig. 2, Supplementary Table S5 and Supplementary Fig. S1 for the blocks plots (metabolites and physiological parameters separately)) and the analysis of most changeable KEGG pathways (Fig. 3) showed the relevance of time course in the metabolome. Component 1 in the sPLS clustered the plants at the beginning of the assay (T1WW, T1WS) from around one month older plants (T2WS, R1WS, R2WS, R2WW). Similar results were shown in component 1 of PCA (Dataset 1, Supplementary Fig. S2 and Table S4). This time course effect was considered during the experimental design, and justify the existence of two controls for the block “stress” (T1WW and R2WW) and only the second control (R2WW) for the block “recovery”. sPLS Component 2 established the stress variables better than component 3 of the PCA, showing a lower stress effect in R2WS than in R1WS. In this sPLS component flavonoids, terpenes (such as leonuridine) and hormone (trans-zeatin riboside monophosphate) showed high loadings (Supplementary Table S6).

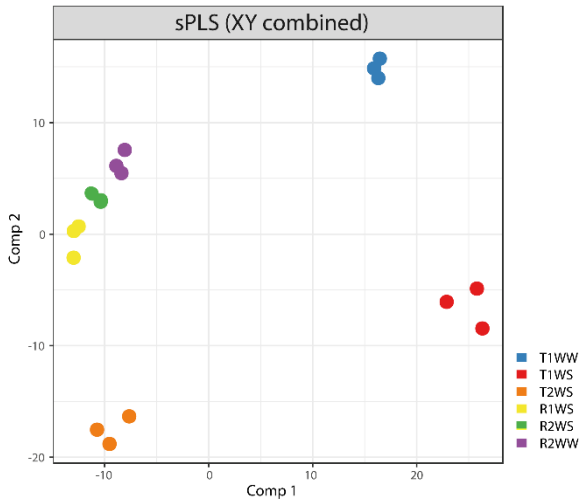

Fig. 2: Multivariate analysis sPLS of metabolome and physiological data, using metabolites as the predictor matrix and physiological measurements as the response matrix. Abbreviations: T1WW, control plants well-watered at day 1; R2WW, control plants well-watered the whole experiment; T1WS, water-stress plants at first day of water stress; T2WS, water-stress plants 30 days after T1WS; R1WS, water-stress plants one day after rehydration; and R2WS, water-stress plants one week of rehydration.

Analysis of the most changeable KEGG pathways (Figure 3 with the average for treatment and supplementary; Fig. S3 for each replicate), showed the pathways *Aminoacyl-tRNA biosynthesis, Pentose phosphate pathway, ABC transporters and alanine, Aspartate and glutamate metabolism* were more active on the first week of the experiment (T1WS and T1WW) but activity was drastically slowed down one month after (T2WS, R1WS, R2WS and R2WW). Nevertheless, there were key pathways at different times of the experiment. On the one hand, *Phenylalanine metabolism* was the most active pathway in control (T1WW) and curiously, the ones that most intensely recovered after the first day of rehydration (R1WS). Regarding the intensity of the stress, *Phenylalanine, tyrosine and tryptophan biosynthesis* was a very active pathway only in the first week of stress (T1WS), while *Phenylpropanoid biosynthesis* was very active during all stress treatments (T1WS and T2WS). These pathways are related, finding common metabolites such as L-phenylalanine a precursor for several of the pathways. The last pathway to highlight is *Flavone and flavonol biosynthesis* that was active in the first week of stress and with a reactivation in R1WS. This pathway is responsible for the synthesis of flavones such as isoscoparine; these compounds could act as antioxidants under stress in terms of inhibiting the formation of malonaldehyde in lipids oxidized by UV [40].

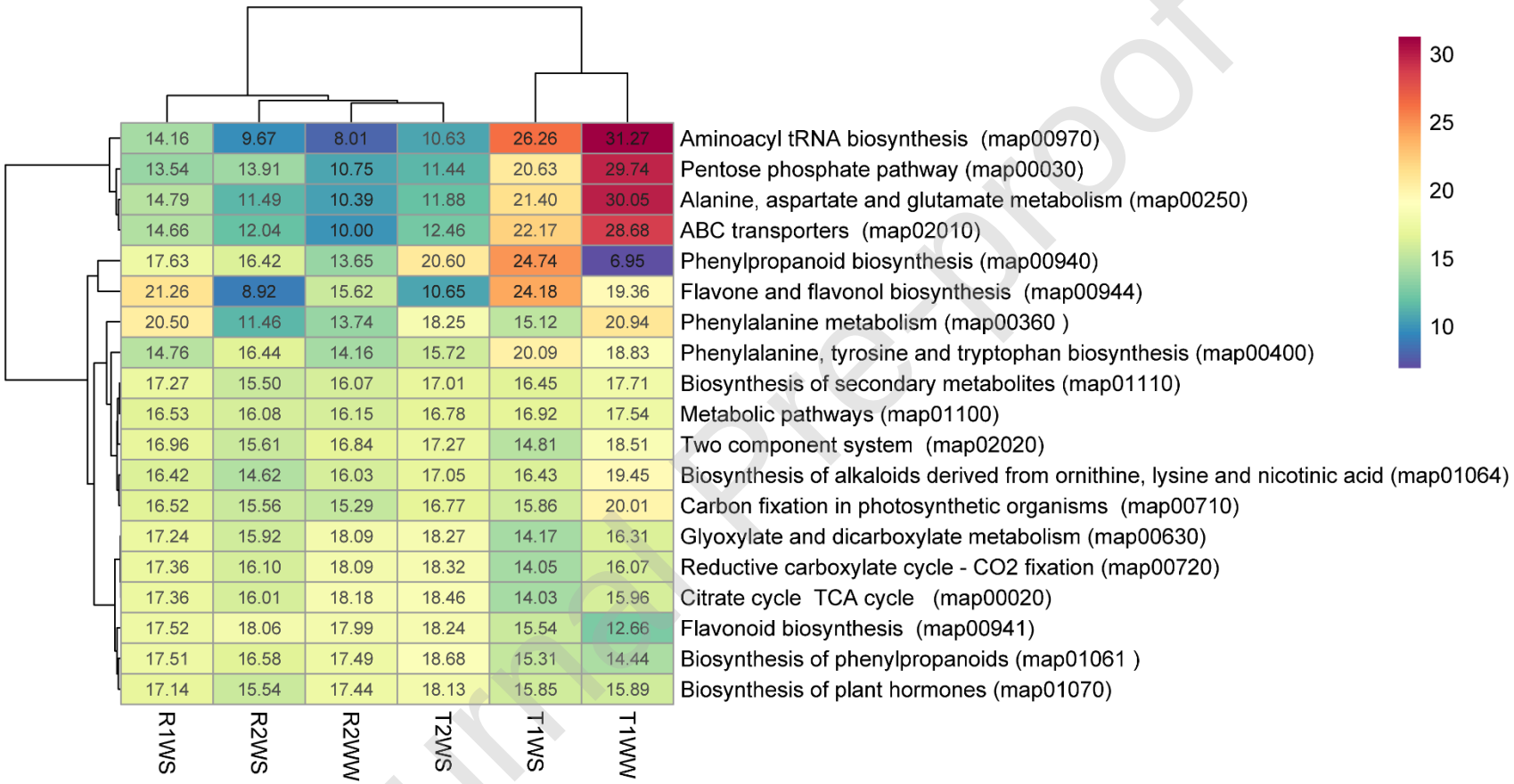

Fig. 3: Heatmap-clustering analyses of KEGG pathways considering only the pathways that exceed MBROLE FDR-correction (p < 0.001). Represented is the average of the three replicates, see supplementary Fig. S3 for KEGG pathways of the separate replicates. Numbers inside boxes indicate normalized abundance of each pathway (as a percentage) calculated as the sum of all identified/assigned metabolites within each pathway according to KEGG pathway. Abbreviations: T1WW, control plants well-watered at day 1; R2WW, control plants well-watered the whole experiment; T1WS, water-stress plants at first day of water stress; T2WS, water-stress plants 30 days after T1WS; R1WS, water-stress plants one day after rehydration; and R2WS, water-stress plants one week of rehydration.

Through the use of KEGG pathways and sPLS analyses it was possible to simplify the dimensionality of the assay, as well the implementation of a scientific base-decision to support the separation of the assay in two key stages: drought (T1WW, T1WS, T2WS, R2WW) and recovery (T2WS, R1WS, R2WS, R2WW) (Figure 1, stress data and rehydration data respectively).

Integrative analysis of metabolome revealed short-term and long-term drought stress response

The experimental design carried out allowed a more in-depth analysis of a short and long-term water stress treatments (5 and 30 days) and their respective controls (T1WW and R2WW, Supplementary dataset 2). sPLS and PCA plot (Fig. 4A and Fig. S4) revealed a clear clustering of treatments, in which the water stress treatments were separated from control treatments by component 2 (sPLS) or PC3 (PCA). These results clearly confirmed that the stronger component (comp 1), both metabolites and physiological traits, differentiated the different points of the time course (T1WW, T1WS) and (T2WW, R2WW). The sPLS interaction networks built using comp1 and comp2 (Fig. 4B and Supplementary Table S7) showed 3 insolated nodes (RWC, WatPot and MDA) which binds numerous metabolites interrelated with each other in a positive or negative way, highlighting the high abundance of flavonoids and terpenes. MDA and WatPot parameters showed the highest number of metabolites associations. In the case of MDA, most of the metabolite correlations were positive, while WatPot accumulated negative ones. So, MDA node was positively linked to metabolites included in the *Phenylpropanoid biosynthesis pathway* (coniferyl alcohol and 1-O-sinapoyl-beta-D-glucose), precursor pathway to the production of flavonoids. Moreover, terpenes (as rutaevin and mascaroside) and flavonoids (as isoscoparine), were positively correlated with MDA, recalling the importance of these compounds in the drought stress response. In other stresses has been observed that some flavonoids act as antioxidants, contributing to the adaptation to environmental changes such as cold, high temperatures or irradiation [7,9,41,42] and it is feasible a similar function for these compounds under drought conditions. Furthermore, flavonoids appeared in MDA negative correlations (quercetin-

3-O-glucoside and gallocatechin-(4alpha->8)-epigallocatechin)) indicating that flavonoids showed different accumulation patterns depending on the compound studied.

On the other hand, WatPot presented also numerous correlations (although most of them negative) with different metabolites (as L-isoleucine amino acid), emphasizing again the flavonoids, among others: epigallocatechin 3-gallate and 3,5-digalloylepicatechin negatively correlated, or (-)-epicatechin-3'-O-glucuronide, positively correlated. Both flavonoid accumulation patterns may be possible; while flavonoids often emphasize their antioxidant role, glycosylated flavonoids are often associated with transport and storage function [43].

The last node RWC, showed few connections but with high loading in component 2 (Supplementary Table S5): Terpenes with positive (curcubitacin P) and negative correlation (leonuridine) and the flavonoid (sophoraflavanone G) with positive correlation. Again, compounds belonging to the same class (in this case terpenes) show different patterns of accumulation depending on the compound, supporting the compound-specific effect of severe drought on terpenoid fluxes in Mediterranean ecosystems [2].

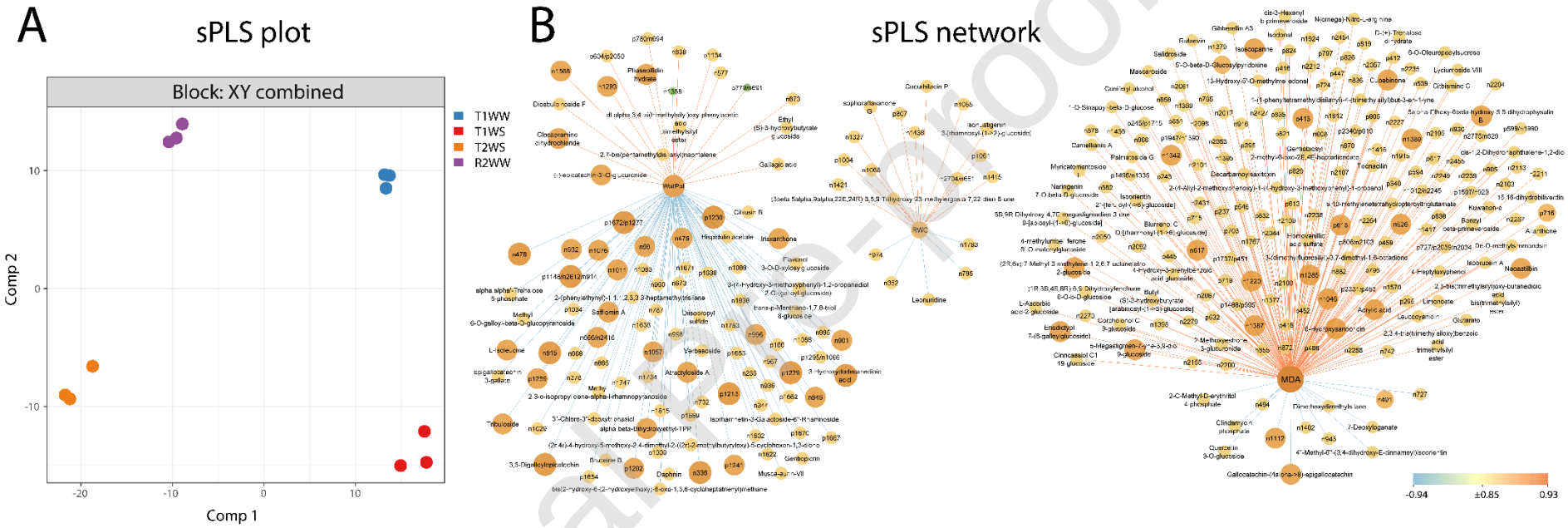

Fig. 4: Multivariate and integrative analysis of metabolome, and physiological parameters during drought stress. (A) Classification of the different samples according to sPLS. Components 1 and 2 allowed the clustering of control treatments versus drought stress treatments. **(B)** Interaction networks constructed after sPLS analysis using metabolites as the predictor matrix and physiological measurements as the response matrix. Edge color represents the correlation value. Only those correlations equal or higher, in absolute value, than 0.85 are shown. Color nodes reflects the amount of control that this node exerts over the interactions of other nodes in the network (higher control = darker color). Abbreviations: T1WW, control plants well-watered at day 1; R2WW, control plants well-watered the whole experiment; T1WS, water-stress plants at first day of water stress; T2WS, water-stress plants 30 days after T1WS.

Rehydration metabolome analysis showed the importance of flavonoids and terpenoids but with distinct specific players

To study the performance of recovery from stress a sPLS and a PCA analysis including the T2WS, R1WS, R2WS and R2WW treatments were performed (Supplementary dataset 3). sPLS and PCA plot (Fig. 5A and Fig. S5) revealed the rehydration phase in component 1 from one month of drought stress (T2WS), to the rehydration treatments (R1WS and R2WS) and finally with the control (R2WW). On the other hand, component 2 highlighted the differential metabolites induced on the first day of rehydration (R1WS) as opposed to the remaining treatments.

The sPLS interaction networks related to rehydration analysis (Fig. 5B and Supplementary Table S6) showed the same three central nodes (RWC, WatPot and MDA) already evident in drought conditions (Fig. 4B); however, in this case, using the same threshold in the pruning of the network (0.85), the three nodes were interconnected by other metabolites. This indicates that these interconnections exists or are more stronger in this response than in the drought response.

In the first main group, the identified metabolites connected with the three nodes (negatively with WatPot and RWC, positively with MDA) were the diterpenoid mascaroside and some amino acids such as N-acetyl-D-tryptophan and indole-3-acetyl-L-aspartic acid. In the case of acetylated amino acids at the N-terminal end, these have been shown to have a significant impact on protein activity, stability, folding patterns and other processes [44].

The second main group was connected with two nodes (negatively with WatPot and RWC). Despite the high number of metabolites, this group presented few identifications and most of them with unknown functions. Among the identified metabolites, the flavonoid (subclass flavans) epigallocatechin 3-gallate was highlighted, already found in the stress network.

The third main group was negatively correlated with the WatPot and positively correlated with MDA. In this group, several classes of metabolites were identified: some terpenes glycoside (leonuridine and tarennoside), the hormone trans-zeatin riboside

monophosphate, the amino acid L-phenylalanine and the suberic acid related to cell wall biosynthesis. Within the terpenes, in general, their antioxidant role under drought stress has been reported. Some diterpenes (e.g. carnosic acid) display high antioxidant activity which has been associated with protection of biological membranes from lipid peroxidation [45] and, monoterpenes have also shown an antioxidant role against high temperatures, but are less effective than isoprenoids [46].

Finally, there are numerous metabolites only connected with one of the nodes. Among these, there are metabolites included in the phenylpropanoid biosynthesis such as the 1-O-sinapoyl-beta-D-glucose (negatively connected with WatPot), the sinapyl alcohol or the phenylpyruvate (both negatively connected with RWC). Moreover, the analysis reveals once again the relevance of flavonoids with different trends like quercetin 3-(4"-acetylrhamnoside) 7-rhamnoside or kaempferol 7-(6”-galloylglucoside) (both negatively connected with RWC) and quercetin-3-arabinoside (positively connected with RWC).

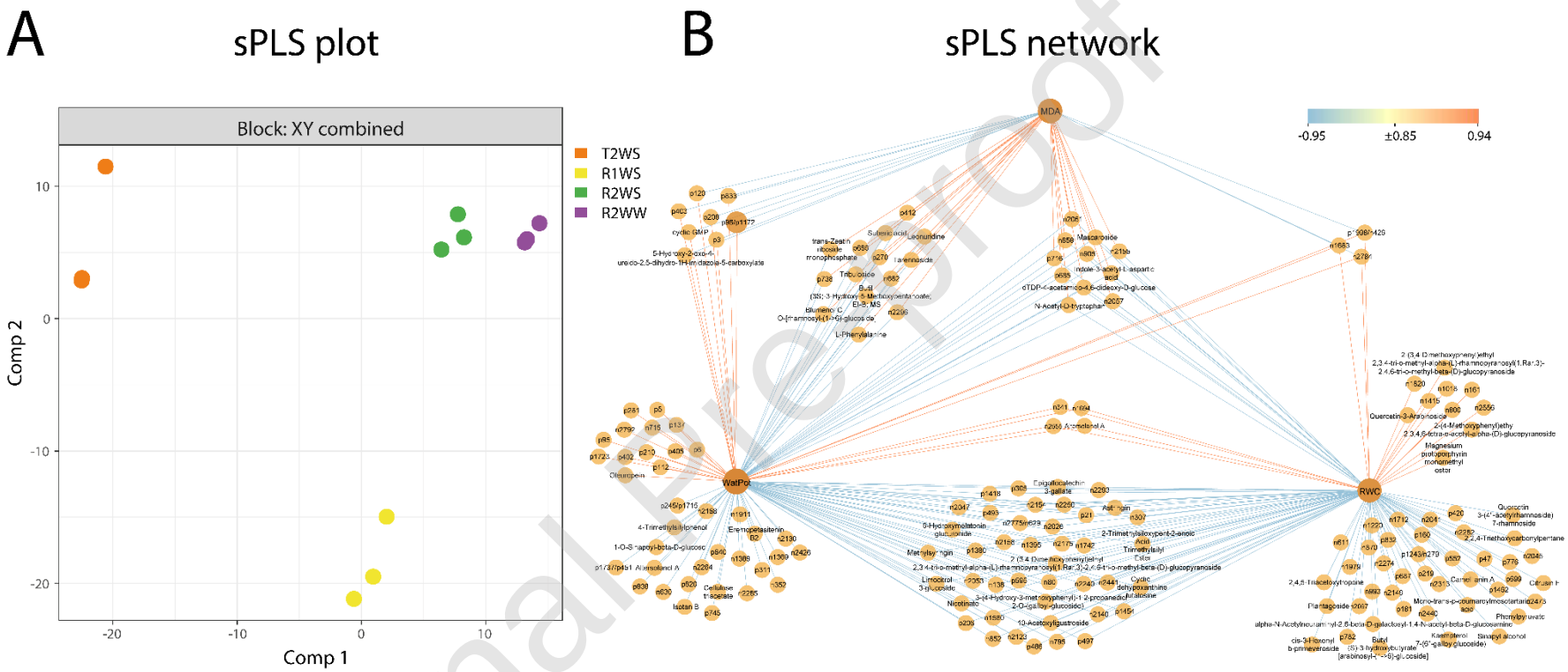

Fig. 5: Multivariate analysis of metabolome and physiological parameters during rehydration. (A) Classification of the different samples according to sPLS. Components 1 and 2 allowed the visualization of 3 groups: rehydration (R1WS, R2WS), control (R2WW) and long-term water stress treatment (T2WS). **(B)** Interaction networks constructed after sPLS analysis using metabolites as the predictor matrix and physiological measurements as the response matrix. Edge color represents the correlation value. Only correlations equal or higher, in absolute value, than 0.85 are shown. Color nodes reflects the amount of control that this node exerts over the interactions of other nodes in the network (higher control = darker color). Abbreviations:R2WW, well-watered control group at the end of experiment; T2WS, water-stress group 30 days after day one of water stress; R1WS, water-stress group one day after rehydration; and R2WS, water stress group one week after rehydration.

Unveiling possible candidate markers for drought tolerance and recovery plasticity from Q. suber

The global analysis of KEGG pathways, as well as the study of stress and rehydration separately has shown the key pathways involved in both responses. Flavonoids and terpenoids seemed to play a key role both during water deficit and recovery. Within these broad groups, it appeared that different compounds are specifically associated with each process [15,16]. In fact, in this work, only 24 common metabolites (10 identified and 14 unidentified) were found both in stress and rehydration networks.

Secondary metabolism appears to play a strategic role in drought responses [14], which was confirmed by the results in this work. Even so, metabolites belonging to primary metabolism (e.g. amino acids and fatty acids) were also found in the candidate networks. The amino acids L-isoleucine and L-phenylalanine (Fig. 6A and B) were largely accumulated in the short response to stress, while acetyl amino acids such as tryptophan and L-aspartic acid (Fig. 6C and D) were largely accumulated after a month post drought. In studies with other species, the accumulation of L-isoleucine has been reported as being associates with drought response [47–49], as well as the accumulation of L-phenylalanine [48,50,51]. The accumulation of amino acids during different stresses (including drought) is related to the synthesis of their activation/inhibition, and degradation and/or decomposition of proteins [52–54]. Currently, it is not clear whether isoleucine accumulation (a branched-chain amino acids, BCAAs) can act as a compatible solute, as BCAAs are known to accumulate in very high amounts under stress [55], or may act as signalling molecules in response to drought stress [56]. Proline did not show differences during this experiment (Table 1) although its role as an osmoprotectant is already recognised [57–59], while the role of other amino acids requires an exhaustive study [60].

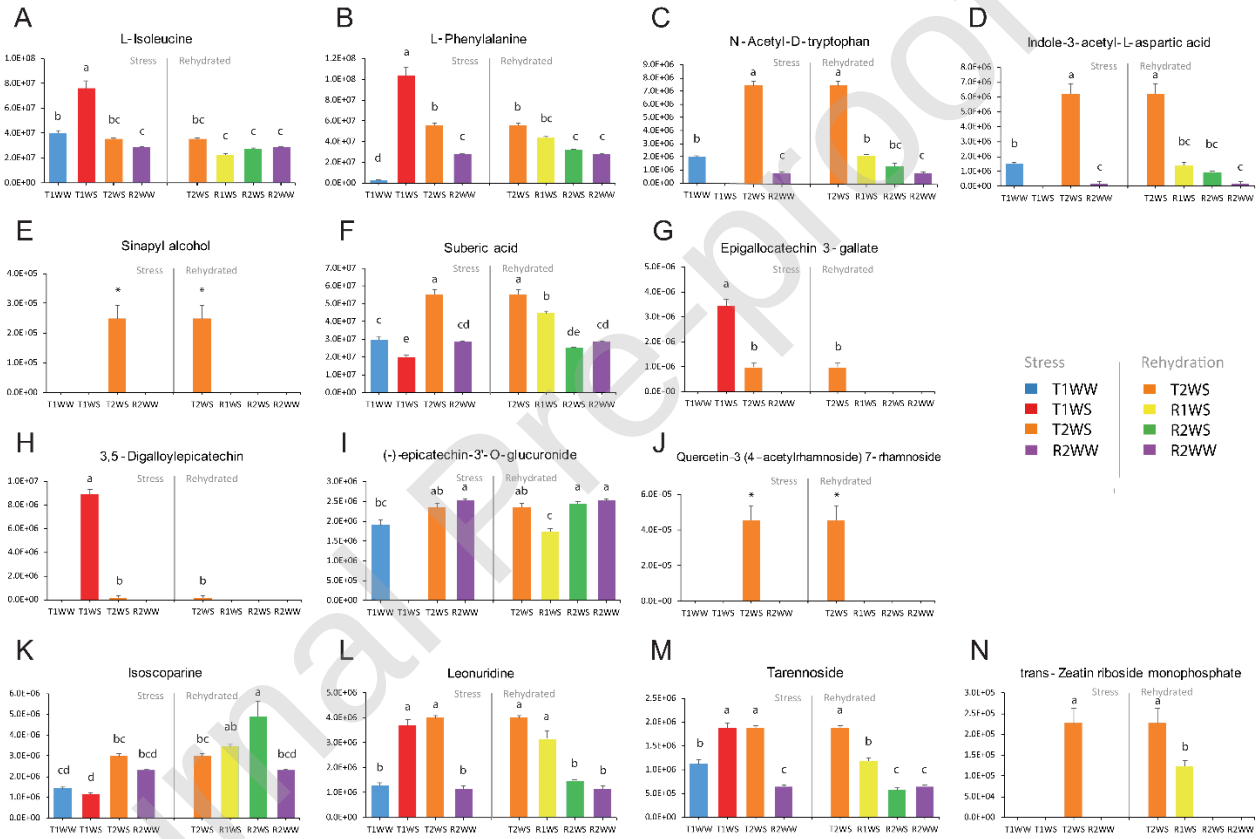

Fig. 6: Levels of key metabolites associated to stress and rehydration process selected based on the presence in the sPLS analysis networks, as well as in key metabolic pathways in both processes. The metabolite levels are shown as mean ± standard deviation**.** Means followed by the same letter were not significantly different ($P \leq 0.05$) according to a Tukey test and asterisk denote significant differences attested by FDR (5%). Abbreviations: T1WW, control plants well-watered at day 1; R2WW, control plants well-watered the whole experiment; T1WS, water-stress plants at first day of water stress; T2WS, water-stress plants 30 days after T1WS; R1WS, water-stress plants one day after rehydration; and R2WS, water-stress plants one week of rehydration.

The accumulation of L-phenylalanine was particularly important, as it was closely related to the increase of secondary metabolism. Phenylalanine and tryptophan, both aromatic amino acids, were the most accumulated primary metabolites in maize leaves under drought [61] and are known to be precursors from a large number of secondary metabolites [62]. L-phenylalanine was involved in numerous pathways, being a precursor in the *Phenylpropanoid biosynthesis pathway* (Map 00940) [63,64], which was a pathway over-expressed under the drought treatment (Fig. 3). Regarding this pathway, another candidate was also identified, the monolignol sinapyl alcohol (only presented in the long-term stress) (Fig. 6E). This metabolite is a precursor of lignins, and could play a role in the acclimation response of *Q. suber* after one month under drought stress. Increased lignification is a common response to biotic and abiotic stress [65], observed in the response to drought by different herbaceous species [66–68] and trees [69]. In addition, suberic acid (a dicarboxylic acid part of suberin) showed its highest accumulation after one month under drought stress (Fig. 6F); to our knowledge it is the first time this compound is associated with drought stress. This metabolite was associated to cell wall biosynthesis, which seems to be in agreement with Geng *et al.* [70], reporting that modification of cell wall architecture could improve plant growth under drought conditions.

The role of secondary metabolites in plants-environment interaction and adaptation is well documented [14,71,72]. Here, the integrative analysis of metabolome and physiological data reveals the importance of secondary metabolism during drought and recovery in *Q. suber* showing that terpenes and phenolic compounds are highly represented, with flavonoids standing out from phenolics.

Flavonoids such as epigallocatechin 3-gallate and 3,5-digalloylepicatechin (Fig. 6G and H), both flava-3-ols of the subclass flavans, were only present under cork oak drought conditions. Their higher short-term accumulation suggests its involvement in the early defense mechanism against drought response. When a plant is subjected to drought, an accumulation of reactive species of oxygen (ROS) takes place, because there is an imbalance between ROS production and ROS scavenging, which triggers an oxidative stress in the plant [73]. Epigallocatechin 3-gallate and the flavanols epicatechin gallate have shown to have an excellent antioxidant capacity *in vitro*, showing inclusive an activity up to five times higher

than α-tocopherol and ascorbic acid [74]. Therefore, these metabolites could play an important role in detoxifying ROS, in particular, in the short-term drought response. Some studies showed that epigallocatechin gallate, epicatechin and epicatechin gallate concentrations increased progressively during drought in tea plants and *Crataegus* ssp. [75,76], reaching maximum values after 30 days under stress in the case of *Crataegus clusii.* However, in other drought studies, these compounds have also remained unchanged in tea plants [77,78] or decreased in drought-stressed plants exposed to short water deficit [79]. This is the first time that 3,5-digalloylepicatechin is associated to drought stress which corroborates the specificity of the flavonoids between experimental conditions [16].

This metabolome specialization was also seen as other flavonoids seem to be crucial for *Q. suber* acclimation to stress condition one month under water deficit. This was the case of the flavonoids glycoside (-)-epicatechin-3'-O-glucuronide and quercetin 3-(4''-acetylrhamnoside) 7-rhamnoside) (Fig. 6 I and J), both present in long-term water stress treatment, but not detected in the short-term water stress treatment. Both metabolites are for the first time associated with drought stress. Although, it is common to find glycosylated flavonoids in plant leaves, as it increases their solubility in the aqueous cell medium and preserves the most reactive functional groups of auto oxidation [80]. However, few flavonoids with this modification are effective antioxidants [74] being more associated to its transport and storage in the vacuole [43]. Accumulated flavonoid glycosides have a role in the osmoregulation of the plant against drought, probably as soluble sugars as seen under cold stress [81,82]. By accumulating these solutes in the vacuoles plant are able to decrease their osmotic potential, maintain turgor and reduce water potential, which may explain the increase of WatPot one month after water limitation in our experiment. In addition, (-)-epicatechin-3'-O-glucuronide reduced its presence with the onset of re-watering but returned to control levels one week after plants were rehydrated, potentially as an adaptation mechanism to an eventually future recurrence of a drought event.

The isoscoparine (Fig. 6K) was the last selected flavonoid belonging to the flavone subclass, specifically a 6-C-glucosides part of the glycosides of flavones. Contrary to the above metabolites, isoscoparine seems to play an important role in the recovery process, being for the first time described in connection with this process. The metabolite was accumulated

during the rehydration phase, reaching its maximum accumulation one week after rehydration. Glycosides of flavones displayed different patterns of accumulation under drought [83]. Few examples of flavones induced by pathogens or plant stress have been described [84,85]. Generally, in these studies, various flavone C glycosides were accumulated in cereal crops against pathogens, including isoscoparine [86]. However, other studies have shown no effect [87]. Further studies are needed to clarify the role of glycosylated flavones in the recovery process, being isoscoparine an interesting candidate to study this response in *Q. suber*.

Finally, to be noted are the compounds leonuridine and tarennoside (Fig. 6L and 6M) which are significantly accumulated during the stress stage and then decreased noticeably once the rehydration process begins. These compounds belong to another major class of secondary metabolites, the terpenes, specifically, terpene glycosides (non-volatile compounds).This is the first time that leonuridine is associated with drought stress response and tarennoside related to *Q. suber*. Tarennoside has been previously reported as being associated with arid provenances of *Pinus pinaster* [7]. . Terpenes concentration has been generally found to increase in drought conditions [88–90] while terpenes emission may be reduced when the stress is severe; Haberstroh et al. [2] reported a reduction in monoterpenes emission by *Q. suber* under summer drought stress. With terpenoids also showing a high compound specificity, as seen in flavonoids, we could point out that leonuridine and tarennoside could have an important role in this species' response to drought.

Many terpenoids are involved in fundamental plant processes such as growth regulation by phytohormones (gibberellins, abscisic acid or brassinosteroids) [91]. There is also a relationship between terpenoids and cytokinins. Terpenoid backbone biosynthesis pathway is precursor of both the terpenoid biosynthesis pathway and the zeatin biosynthesis pathway. Trans-zeatin riboside monophosphate (Fig. 6N) was one of the metabolites accumulated at the end of the drought period, remaining present only on the first day of rehydration. Nucleosides were considered the main form of translocation of cytokinins [92]. This could explain a movement of these metabolites to other parts of the plant after rehydratation; here, they would become active for growth restart. In the current study, this was apparent, since one week after rehydration trans-zeatin riboside monophosphate is not present in leaves.

Conclusions

Increased drought stress tolerance of forest trees will be a key feature to maintain forest sustainability in climate-threatened regions. The presented integrative approach to study the leaf metabolome of *Quercus suber* has highlighted the role of drought stress response and subsequent recovery in metabolism modulation. Metabolome profile is significantly correlated with water availability and intimately associated with specific metabolites to both, drought and recovery. Both primary and secondary metabolism are significant altered in both phases yet highlighting the role of the secondary metabolism. The most promising compounds identified in short-term drought response were, L-phenylalanine, which is essential precursor of numerous secondary metabolites, and epigallocatechin 3-gallate, which present high antioxidant capacity necessary to deal with the oxidative damage in the first impact of stress. In the long-term drought response that lead to an acclimation response, the terpenoid leonuridine (first time associated with drought stress) and the flavonoid glycoside (-)-epicatequine-3'-O-glucuronide are featured, and might be key players. Finally, during recovery, and first time reported, the most relevant candidate is the isoscoparine flavone, although it is role in drought stress requires further research. Unraveling the key processes that underlie drought stress tolerance will not just contribute to fill the scientific knowledge gap on tree adaptation mechanisms but, for cork oak in particular, also has a significant economic and ecologic importance. The development of markers linked to drought tolerance and recovery ability are of particular importance, as they offer the potential to accelerate breeding programs by exploring the existent natural variation of cork oak provenances through marker-assisted selection.

Conflicts of interest: *The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest*.

Authors and Contributors

TA, GP, BC and SG designed and performed the experimental work. TA, GP and BC processed the samples. ME integrated the datasets, and completed the metabolome analyses.

MM performed the mass spectrometry and helped with statistical analyses. TA and ME wrote the manuscript while GP, BC, SG, and MM supervised the manuscript. All authors read and approved the final manuscript.

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References

[1] H.N. Kim, H.Y. Jin, M.J. Kwak, I. Khaine, H.N. You, T.Y. Lee, T.H. Ahn, S.Y. Woo, Why does Quercus suber species decline in Mediterranean areas?, J. Asia-Pacific Biodivers. 10 (2017) 337–341. https://doi.org/https://doi.org/10.1016/j.japb.2017.05.004.

[2] S. Haberstroh, J. Kreuzwieser, R. Lobo-do-Vale, M.C. Caldeira, M. Dubbert, C. Werner, Terpenoid Emissions of Two Mediterranean Woody Species in Response to Drought Stress., Front. Plant Sci. 9 (2018) 1071. https://doi.org/10.3389/fpls.2018.01071.

[3] J.A. Ramírez-Valiente, F. Valladares, D. Sánchez-Gómez, A. Delgado, I. Aranda, Population variation and natural selection on leaf traits in cork oak throughout its distribution range, Acta Oecologica. 58 (2014) 49–56. https://doi.org/https://doi.org/10.1016/j.actao.2014.04.004.

[4] L. Matías, I.M. Pérez-Ramos, L. Gómez-Aparicio, Are northern-edge populations of cork oak more sensitive to drought than those of the southern edge?, Environ. Exp. Bot. 163 (2019) 78–85. https://doi.org/https://doi.org/10.1016/j.envexpbot.2019.04.011.

[5] M.M. Chaves, J. Flexas, C. Pinheiro, Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell, Ann. Bot. 103 (2009) 551–560. https://doi.org/10.1093/aob/mcn125.

[6] M. Vaz, J.S. Pereira, L.C. Gazarini, T.S. David, J.S. David, A. Rodrigues, J. Maroco, M.M. Chaves, Drought-induced photosynthetic inhibition and autumn recovery in two Mediterranean oak species (Quercus ilex and Quercus suber)., Tree Physiol. 30 (2010) 946–

956. https://doi.org/10.1093/treephys/tpq044.
[7] M. Meijón, I. Feito, M. Oravec, C. Delatorre, W. Weckwerth, J. Majada, L. Valledor, Exploring natural variation of Pinus pinaster Aiton using metabolomics: Is it possible to identify the region of origin of a pine from its metabolites?, Mol. Ecol. 25 (2016) 959–976. https://doi.org/10.1111/mec.13525.
[8] A.M. de Leonardis, M. Fragasso, R. Beleggia, D.B.M. Ficco, P. de Vita, A.M. Mastrangelo, Effects of Heat Stress on Metabolite Accumulation and Composition, and Nutritional Properties of Durum Wheat Grain, Int. J. Mol. Sci. 16 (2015) 30382–30404. https://doi.org/10.3390/ijms161226241.
[9] M. Escandón, M. Meijón, L. Valledor, J. Pascual, G. Pinto, M.J. Cañal, Metabolome Integrated Analysis of High-Temperature Response in Pinus radiata, Front. Plant Sci. 9 (2018) 485. https://doi.org/10.3389/fpls.2018.00485.
[10] J. Pascual, M.J. Cañal, M. Escandón, M. Meijón, W. Weckwerth, L. Valledor, Integrated Physiological , Proteomic , and Metabolomic Analysis of Ultra Violet (UV) Stress Responses and Adaptation Mechanisms in Pinus radiata, MCP. 16 (2017) 485–501. https://doi.org/10.1074/mcp.M116.059436.
[11] V. Shulaev, D. Cortes, G. Miller, R. Mittler, Metabolomics for plant stress response, Physiol. Plant. 132 (2008) 199–208. https://doi.org/10.1111/j.1399-3054.2007.01025.x.
[12] O. Fernandez, M. Urrutia, S. Bernillon, C. Giauffret, F. Tardieu, J. Le Gouis, N. Langlade, A. Charcosset, A. Moing, Y. Gibon, Fortune telling: metabolic markers of plant performance, Metabolomics. 12 (2016) 158. https://doi.org/10.1007/s11306-016-1099-1.
[13] F.M. Mundim, E.G. Pringle, Whole-Plant Metabolic Allocation Under Water Stress, Front. Plant Sci. 9 (2018) 852. https://doi.org/10.3389/fpls.2018.00852.
[14] Ü. Niinemets, Uncovering the hidden facets of drought stress: secondary metabolites make the difference, Tree Physiol. 36 (2015) 129–132. https://doi.org/10.1093/treephys/tpv128.
[15] A.B. McKiernan, B.M. Potts, T.J. Brodribb, M.J. Hovenden, N.W. Davies, S.A.M. McAdam, J.J. Ross, T. Rodemann, J.M. O'Reilly-Wapstra, Responses to mild water deficit and rewatering differ among secondary metabolites but are similar among provenances within Eucalyptus species., Tree Physiol. 36 (2016) 133–147. https://doi.org/10.1093/treephys/tpv106.
[16] A.B. McKiernan, M.J. Hovenden, T.J. Brodribb, B.M. Potts, N.W. Davies, J.M. O'Reilly-Wapstra, Effect of limited water availability on foliar plant secondary metabolites of two Eucalyptus species, Environ. Exp. Bot. 105 (2014) 55–64. https://doi.org/https://doi.org/10.1016/j.envexpbot.2014.04.008.
[17] J.-S. Blanch, J. Peñuelas, J. Sardans, J. Llusià, Drought, warming and soil fertilization effects on leaf volatile terpene concentrations in Pinus halepensis and Quercus ilex, Acta Physiol. Plant. 31 (2009) 207. https://doi.org/10.1007/s11738-008-0221-z.
[18] J.A. Marchese, J.F.S. Ferreira, V.L.G. Rehder, O. Rodrigues, Water deficit effect on the accumulation of biomass and artemisinin in annual wormwood (Artemisia annua L., Asteraceae), Brazilian J. Plant Physiol. 22 (2010) 1–9.
[19] L.C. Pizarro, A.J. Bisigato, Allocation of biomass and photoassimilates in juvenile plants of six Patagonian species in response to five water supply regimes, Ann. Bot. 106 (2010) 297–307. https://doi.org/10.1093/aob/mcq109.
[20] Q. Zhang, L.-J. Su, J.-W. Chen, X.-Q. Zeng, B.-Y. Sun, C.-L. Peng, The antioxidative role of anthocyanins in Arabidopsis under high-irradiance, Biol. Plant. 56 (2012) 97–104. https://doi.org/10.1007/s10535-012-0022-5.
[21] D.B. Neale, A. Kremer, Forest tree genomics: growing resources and applications, Nat. Rev. Genet. 12 (2011) 111–122.
[22] A.M. Rodrigues, C. Miguel, I. Chaves, C. Antonio, Mass spectrometry-based forest tree metabolomics., Mass Spectrom. Rev. (2019). https://doi.org/10.1002/mas.21603.
[23] C. López-Hidalgo, V.M. Guerrero-Sánchez, I. Gómez-Gálvez, R. Sánchez-Lucas, M.A.

Castillejo-Sánchez, A.M. Maldonado-Alconada, L. Valledor, J. V Jorrín-Novo, A Multi-Omics Analysis Pipeline for the Metabolic Pathway Reconstruction in the Orphan Species Quercus ilex, Front. Plant Sci. 9 (2018) 935. https://doi.org/10.3389/fpls.2018.00935.

[24] S. Jafarnia, M. Akbarinia, B. Hosseinpour, S.A.M.M. Sanavi, S.A. Salami, Effect of drought stress on some growth, morphological, physiological, and biochemical parameters of two different populations of Quercus brantii, iForest - Biogeosciences For. (2018) 212–220. https://doi.org/10.3832/ifor2496-010.

[25] T. Almeida, G. Pinto, B. Correia, C. Santos, S. Gonçalves, Plant Physiology and Biochemistry QsMYB1 expression is modulated in response to heat and drought stresses and during plant recovery in Quercus suber, Plant Physiol. Biochem. 73 (2013) 274–281. https://doi.org/10.1016/j.plaphy.2013.10.007.

[26] S. Elkahoui, J.A. Hernández, C. Abdelly, R. Ghrir, F. Limam, Effects of salt on lipid peroxidation and antioxidant enzyme activities of Catharanthus roseus suspension cells, Plant Sci. 168 (2005) 607–613. https://doi.org/https://doi.org/10.1016/j.plantsci.2004.09.006.

[27] A.H.A. Khedr, M.A. Abbas, A.A.A. Wahid, W.P. Quick, G.M. Abogadallah, Proline induces the expression of salt- stress- responsive proteins and may improve the adaptation of Pancratium maritimum L. to salt- stress, J. Exp. Bot. 54 (2003) 2553–2562. https://doi.org/10.1093/jxb/erg277.

[28] L. Valledor, M. Escandón, M. Meijón, E. Nukarinen, M.J. Cañal, W. Weckwerth, A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms, Plant J. 79 (2014) 173–180. https://doi.org/10.1111/tpj.12546.

[29] M. Escandón, L. Valledor, J. Pascual, G. Pinto, M. Cañal, M. Meijón, System-wide analysis of short-term response to high temperature in Pinus radiata, 2017. https://doi.org/10.1093/jxb/erx198.

[30] T. Pluskal, S. Castillo, A. Villar-briones, M. Orešič, MZmine 2 : Modular framework for processing , visualizing , and analyzing mass spectrometry- based molecular profile data, BMC Bioinformatics. 11 (2010) 395.

[31] J. López-Ibáñez, F. Pazos, M. Chagoyen, MBROLE 2.0—functional enrichment of chemical compounds, Nucleic Acids Res. . (2016). https://doi.org/10.1093/nar/gkw253 .

[32] RStudio Team, RStudio: Integrated Development Environment for R, (2015).

[33] L. Valledor, M.C. Romero-Rodriguez, J. V Jorrin-Novo, Standardization of data processing and statistical analysis in comparative plant proteomics experiment., Methods Mol. Biol. 1072 (2014) 51–60. https://doi.org/10.1007/978-1-62703-631-3_5.

[34] F. Rohart, B. Gautier, A. Singh, K.-A. Lê Cao, mixOmics: An R package for 'omics feature selection and multiple data integration, PLOS Comput. Biol. 13 (2017) e1005752. https://doi.org/10.1371/journal.pcbi.1005752.

[35] M.S. Cline, M. Smoot, E. Cerami, A. Kuchinsky, N. Landys, C. Workman, R. Christmas, I. Avila-Campilo, M. Creech, B. Gross, K. Hanspers, R. Isserlin, R. Kelley, S. Killcoyne, S. Lotia, S. Maere, J. Morris, K. Ono, V. Pavlovic, A.R. Pico, A. Vailaya, P.-L. Wang, A. Adler, B.R. Conklin, L. Hood, M. Kuiper, C. Sander, I. Schmulevich, B. Schwikowski, G.J. Warner, T. Ideker, G.D. Bader, Integration of biological networks and gene expression data using Cytoscape., Nat. Protoc. 2 (2007) 2366–2382. https://doi.org/10.1038/nprot.2007.324.

[36] M. Escandón, L. Lamelas, V. Roces, V.M. Guerrero-Sanchez, M. Meijón, L. Valledor, Protein Interaction Networks: Functional and Statistical Approaches, in: J. Jorrin-Novo, L. Valledor, M. Castillejo, M. Rey (Eds.), Plant Proteomics. Methods Mol. Biol. Vol 2139, New York, NY, 2020: pp. 21–56. https://doi.org/10.1007/978-1-0716-0528-8_3.

[37] H. Wickham, ggplot2: Elegant Graphics for Data Analysis., Springer-Verlag New York, New York, 2009. https://doi.org/10.1007/978-0-387-98141-3.

[38] R. Kolde, pheatmap: Pretty Heatmaps. R package version 0.7.7., (2015).

[39] O.M. Grant, Ł. Tronina, J.C. Ramalho, C. Kurz Besson, R. Lobo-do-Vale, J. Santos Pereira,

H.G. Jones, M.M. Chaves, The impact of drought on leaf physiology of Quercus suber L. trees: comparison of an extreme drought event with chronic rainfall reduction, J. Exp. Bot. 61 (2010) 4361–4371. https://doi.org/10.1093/jxb/erq239.

[40] T. Nishiyama, Y. Hagiwara, H. Hagiwara, T. Shibamoto, Inhibitory effect of 2"-O-glycosyl isovitexin and alpha-tocopherol on genotoxic glyoxal formation in a lipid peroxidation system., Food Chem. Toxicol. 32 (1994) 1047–1051. https://doi.org/10.1016/0278-6915(94)90145-7.

[41] H. Doerfler, D. Lyon, T. Nagele, X. Sun, L. Fragner, F. Hadacek, V. Egelhofer, W. Weckwerth, Granger causality in integrated GC-MS and LC-MS metabolomics data reveals the interface of primary and secondary metabolism., Metabolomics. 9 (2013) 564–574. https://doi.org/10.1007/s11306-012-0470-0.

[42] H. Doerfler, X. Sun, L. Wang, D. Engelmeier, D. Lyon, W. Weckwerth, mzGroupAnalyzer--predicting pathways and novel chemical structures from untargeted high-throughput metabolomics data., PLoS One. 9 (2014) e96188. https://doi.org/10.1371/journal.pone.0096188.

[43] J. Zhao, R.A. Dixon, The "ins" and "outs" of flavonoid transport., Trends Plant Sci. 15 (2010) 72–80. https://doi.org/10.1016/j.tplants.2009.11.006.

[44] K.T. Nguyen, S.-H. Mun, C.-S. Lee, C.-S. Hwang, Control of protein degradation by N-terminal acetylation and the N-end rule pathway, Exp. Mol. Med. 50 (2018) 91. https://doi.org/10.1038/s12276-018-0097-y.

[45] S. Munné-Bosch, M. Mueller, K. Schwarz, L. Alegre, Diterpenes and antioxidative protection in drought-stressed Salvia officinalis plants, J. Plant Physiol. 158 (2001) 1431–1437. https://doi.org/https://doi.org/10.1078/0176-1617-00578.

[46] J. Peñuelas, J. Llusià, D. Asensio, S. Munné-Bosch, Linking isoprene with plant thermotolerance, antioxidants and monoterpene emissions, Plant. Cell Environ. 28 (2005) 278–286. https://doi.org/10.1111/j.1365-3040.2004.01250.x.

[47] D. Todaka, Y. Zhao, T. Yoshida, M. Kudo, S. Kidokoro, J. Mizoi, K.-S. Kodaira, Y. Takebayashi, M. Kojima, H. Sakakibara, K. Toyooka, M. Sato, A.R. Fernie, K. Shinozaki, K. Yamaguchi-Shinozaki, Temporal and spatial changes in gene expression, metabolite accumulation and phytohormone content in rice seedlings grown under drought stress conditions, Plant J. 90 (2017) 61–78. https://doi.org/10.1111/tpj.13468.

[48] J.A. Hein, M.E. Sherrard, K.P. Manfredi, T. Abebe, The fifth leaf and spike organs of barley (Hordeum vulgare L.) display different physiological and metabolic responses to drought stress., BMC Plant Biol. 16 (2016) 248. https://doi.org/10.1186/s12870-016-0922-1.

[49] E.T. Hamanishi, G.L.H. Barchet, R. Dauwe, S.D. Mansfield, M.M. Campbell, Poplar trees reconfigure the transcriptome and metabolome in response to drought in a genotype- and time-of-day-dependent manner, BMC Genomics. 16 (2015) 329. https://doi.org/10.1186/s12864-015-1535-z.

[50] F. Gagné-Bourque, A. Bertrand, A. Claessens, K.A. Aliferis, S. Jabaji, Alleviation of Drought Stress and Metabolic Changes in Timothy (Phleum pratense L.) Colonized with Bacillus subtilis B26, Front. Plant Sci. 7 (2016) 584. https://doi.org/10.3389/fpls.2016.00584.

[51] G.L.H. Barchet, R. Dauwe, R.D. Guy, W.R. Schroeder, R.Y. Soolanayakanahally, M.M. Campbell, S.D. Mansfield, Investigating the drought-stress response of hybrid poplar genotypes by metabolite profiling., Tree Physiol. 34 (2014) 1203–1219. https://doi.org/10.1093/treephys/tpt080.

[52] C. Di Martino, S. Delfine, R. Pizzuto, F. Loreto, A. Fuggi, Free amino acids and glycine betaine in leaf osmoregulation of spinach responding to increasing salt stress, New Phytol. 158 (2003) 455–463. https://doi.org/10.1046/j.1469-8137.2003.00770.x.

[53] F. Kaplan, J. Kopka, D.W. Haskell, W. Zhao, K.C. Schiller, N. Gatzke, D.Y. Sung, C.L. Guy, Exploring the Temperature-Stress Metabolome of Arabidopsis, Plant Physiol. 136 (2004) 4159 LP-4168. https://doi.org/10.1104/pp.104.052142.

[54] K.L. Ford, A. Cassin, A. Bacic, Quantitative proteomic analysis of wheat cultivars with differing drought stress tolerance., Front. Plant Sci. 2 (2011) 44. https://doi.org/10.3389/fpls.2011.00044.

[55] V. Joshi, J.-G. Joung, Z. Fei, G. Jander, Interdependence of threonine, methionine and isoleucine metabolism in plants: accumulation and transcriptional regulation under abiotic stress., Amino Acids. 39 (2010) 933–947. https://doi.org/10.1007/s00726-010-0505-7.

[56] E. Nambara, H. Kawaide, Y. Kamiya, S. Naito, Characterization of an Arabidopsis thaliana mutant that has a defect in ABA accumulation: ABA-dependent and ABA-independent accumulation of free amino acids during dehydration., Plant Cell Physiol. 39 (1998) 853–858. https://doi.org/10.1093/oxfordjournals.pcp.a029444.

[57] S. Hayat, Q. Hayat, M.N. Alyemeni, A.S. Wani, J. Pichtel, A. Ahmad, Role of proline under changing environments: a review, Plant Signal. Behav. 7 (2012) 1456–1466. https://doi.org/10.4161/psb.21949.

[58] J.-Y. Zhang, M.H. Cruz DE Carvalho, I. Torres-Jerez, Y. Kang, S.N. Allen, D. V Huhman, Y. Tang, J. Murray, L.W. Sumner, M.K. Udvardi, Global reprogramming of transcription and metabolism in Medicago truncatula during progressive drought and after rewatering., Plant. Cell Environ. 37 (2014) 2553–2576. https://doi.org/10.1111/pce.12328.

[59] M. Singh, J. Kumar, S. Singh, V.P. Singh, S.M. Prasad, Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review, Rev. Environ. Sci. Bio/Technology. 14 (2015) 407–426. https://doi.org/10.1007/s11157-015-9372-8.

[60] V.K. Rai, Role of Amino Acids in Plant Responses to Stresses, Biol. Plant. 45 (2002) 481–487. https://doi.org/10.1023/A:1022308229759.

[61] T. Obata, S. Witt, J. Lisec, N. Palacios-Rojas, I. Florez-Sarasa, S. Yousfi, J.L. Araus, J.E. Cairns, A.R. Fernie, Metabolite Profiles of Maize Leaves in Drought, Heat, and Combined Stress Field Trials Reveal the Relationship between Metabolism and Grain Yield, Plant Physiol. 169 (2015) 2665–2683. https://doi.org/10.1104/pp.15.01164.

[62] T. Vogt, Phenylpropanoid Biosynthesis, Mol. Plant. 3 (2010) 2–20. https://doi.org/10.1093/mp/ssp106.

[63] V. Tzin, G. Galili, The Biosynthetic Pathways for Shikimate and Aromatic Amino Acids in Arabidopsis thaliana, Arabidopsis Book. 8 (2010) e0132. https://doi.org/10.1199/tab.0132.

[64] H. Yoo, J.R. Widhalm, Y. Qian, H. Maeda, B.R. Cooper, A.S. Jannasch, I. Gonda, E. Lewinsohn, D. Rhodes, N. Dudareva, An alternative pathway contributes to phenylalanine biosynthesis in plants via a cytosolic tyrosine:phenylpyruvate aminotransferase, Nat. Commun. 4 (2013) 2833.

[65] J.C.M.S. Moura, C.A.V. Bonine, J. de Oliveira Fernandes Viana, M.C. Dornelas, P. Mazzafera, Abiotic and biotic stresses and changes in the lignin content and composition in plants., J. Integr. Plant Biol. 52 (2010) 360–376. https://doi.org/10.1111/j.1744-7909.2010.00892.x.

[66] B.-R. Lee, S. Muneer, W.-J. Jung, J.-C. Avice, A. Ourry, T.-H. Kim, Mycorrhizal colonization alleviates drought-induced oxidative damage and lignification in the leaves of drought-stressed perennial ryegrass (Lolium perenne)., Physiol. Plant. 145 (2012) 440–449. https://doi.org/10.1111/j.1399-3054.2012.01586.x.

[67] K. Yoshimura, A. Masuda, M. Kuwano, A. Yokota, K. Akashi, Programmed proteome response for drought avoidance/tolerance in the root of a C(3) xerophyte (wild watermelon) under water deficits., Plant Cell Physiol. 49 (2008) 226–241. https://doi.org/10.1093/pcp/pcm180.

[68] B.-R. Lee, K.-Y. Kim, W.-J. Jung, J.-C. Avice, A. Ourry, T.-H. Kim, Peroxidases and lignification in relation to the intensity of water-deficit stress in white clover (Trifolium repens L.)., J. Exp. Bot. 58 (2007) 1271–1279. https://doi.org/10.1093/jxb/erl280.

[69] S. Srivastava, R.K. Vishwakarma, Y.A. Arafat, S.K. Gupta, B.M. Khan, Abiotic stress induces change in Cinnamoyl CoA Reductase (CCR) protein abundance and lignin deposition in

developing seedlings of Leucaena leucocephala., Physiol. Mol. Biol. Plants. 21 (2015) 197–205. https://doi.org/10.1007/s12298-015-0289-z.

[70] D. Geng, P. Chen, X. Shen, Y. Zhang, X. Li, L. Jiang, Y. Xie, C. Niu, J. Zhang, X. Huang, F. Ma, Q. Guan, MdMYB88 and MdMYB124 Enhance Drought Tolerance by Modulating Root Vessels and Cell Walls in Apple, Plant Physiol. 178 (2018) 1296 LP-1309. https://doi.org/10.1104/pp.18.00502.

[71] M. Wink, Evolution of Secondary Plant Metabolism, eLS. (2008). https://doi.org/doi:10.1002/9780470015902.a0001922.pub2.

[72] L. Yang, K.-S. Wen, X. Ruan, Y.-X. Zhao, F. Wei, Q. Wang, Response of Plant Secondary Metabolites to Environmental Factors., Molecules. 23 (2018). https://doi.org/10.3390/molecules23040762.

[73] Z. Xu, G. Zhou, H. Shimizu, Plant responses to drought and rewatering, Plant Signal. Behav. 5 (2010) 649–654. https://doi.org/10.4161/psb.5.6.11398.

[74] C. Rice-Evans, N. Miller, G. Paganga, Antioxidant properties of phenolic compounds, Trends Plant Sci. 2 (1997) 152–159. https://doi.org/https://doi.org/10.1016/S1360-1385(97)01018-2.

[75] I. Hernandez, L. Alegre, S. Munne-Bosch, Drought-induced changes in flavonoids and other low molecular weight antioxidants in Cistus clusii grown under Mediterranean field conditions., Tree Physiol. 24 (2004) 1303–1311. https://doi.org/10.1093/treephys/24.11.1303.

[76] A. Kirakosyan, E. Seymour, P.B. Kaufman, S. Warber, S. Bolling, S.C. Chang, Antioxidant capacity of polyphenolic extracts from leaves of Crataegus laevigata and Crataegus monogyna (Hawthorn) subjected to drought and cold stress., J. Agric. Food Chem. 51 (2003) 3973–3976. https://doi.org/10.1021/jf030096r.

[77] I.C. Arts, B. van De Putte, P.C. Hollman, Catechin contents of foods commonly consumed in The Netherlands. 2. Tea, wine, fruit juices, and chocolate milk., J. Agric. Food Chem. 48 (2000) 1752–1757. https://doi.org/10.1021/jf000026+.

[78] I. Hernandez, L. Alegre, S. Munne-Bosch, Enhanced oxidation of flavan-3-ols and proanthocyanidin accumulation in water-stressed tea plants., Phytochemistry. 67 (2006) 1120–1126. https://doi.org/10.1016/j.phytochem.2006.04.002.

[79] W. Wang, H. Xin, M. Wang, Q. Ma, L. Wang, N.A. Kaleri, Y. Wang, X. Li, Transcriptomic Analysis Reveals the Molecular Mechanisms of Drought-Stress-Induced Decreases in Camellia sinensis Leaf Quality, Front. Plant Sci. 7 (2016) 385. https://doi.org/10.3389/fpls.2016.00385.

[80] I.S. Pearse, K.D. Heath, J.M. Cheeseman, Biochemical and ecological characterization of two peroxidase isoenzymes from the mangrove, Rhizophora mangle, Plant. Cell Environ. 28 (2005) 612–622. https://doi.org/10.1111/j.1365-3040.2005.01307.x.

[81] M. Groenbaek, E. Tybirk, S. Neugart, U.K. Sundekilde, M. Schreiner, H.L. Kristensen, Flavonoid Glycosides and Hydroxycinnamic Acid Derivatives in Baby Leaf Rapeseed From White and Yellow Flowering Cultivars With Repeated Harvest in a 2-Years Field Study, Front. Plant Sci. 10 (2019) 355. https://doi.org/10.3389/fpls.2019.00355.

[82] M. Korn, S. Peterek, H.-P. Mock, A.G. Heyer, D.K. Hincha, Heterosis in the freezing tolerance, and sugar and flavonoid contents of crosses between Arabidopsis thaliana accessions of widely varying freezing tolerance, Plant. Cell Environ. 31 (2008) 813–827. https://doi.org/10.1111/j.1365-3040.2008.01800.x.

[83] A. Piasecka, A. Sawikowska, A. Kuczynska, P. Ogrodowicz, K. Mikolajczak, K. Krystkowiak, K. Gudys, J. Guzy-Wrobelska, P. Krajewski, P. Kachlicki, Drought-related secondary metabolites of barley (Hordeum vulgare L.) leaves and their metabolomic quantitative trait loci., Plant J. 89 (2017) 898–913. https://doi.org/10.1111/tpj.13430.

[84] L.V. Modolo, F.Q. Cunha, M.R. Braga, I. Salgado, Nitric oxide synthase-mediated phytoalexin accumulation in soybean cotyledons in response to the Diaporthe phaseolorum f. sp. meridionalis elicitor., Plant Physiol. 130 (2002) 1288–1297. https://doi.org/10.1104/pp.005850.

[85] D.J. McNally, K. V Wurms, C. Labbé, S. Quideau, R.R. Bélanger, Complex C-Glycosyl Flavonoid Phytoalexins from Cucumis sativus, J. Nat. Prod. 66 (2003) 1280–1283. https://doi.org/10.1021/np030150y.

[86] E. Grotewold, M. Chamberlin, M. Snook, B. Siame, L. Butler, J. Swenson, S. Maddock, G. St Clair, B. Bowen, Engineering secondary metabolism in maize cells by ectopic expression of transcription factors., Plant Cell. 10 (1998) 721–740.

[87] Y. Du, H. Chu, M. Wang, I.K. Chu, C. Lo, Identification of flavone phytoalexins and a pathogen-inducible flavone synthase II gene (SbFNSII) in sorghum, J. Exp. Bot. 61 (2010) 983–994. https://doi.org/10.1093/jxb/erp364.

[88] J. Llusià, J. Peñuelas, Changes in terpene content and emission in potted Mediterranean woody plants under severe drought, Can. J. Bot. 76 (1998) 1366–1373. https://doi.org/10.1139/b98-141.

[89] S. Turtola, A.-M. Manninen, R. Rikala, P. Kainulainen, Drought Stress Alters the Concentration of Wood Terpenoids in Scots Pine and Norway Spruce Seedlings, J. Chem. Ecol. 29 (2003) 1981–1995. https://doi.org/10.1023/A:1025674116183.

[90] J. Llusià, J. Peñuelas, G.A. Alessio, M. Estiarte, Seasonal contrasting changes of foliar concentrations of terpenes and other volatile organic compound in four dominant species of a Mediterranean shrubland submitted to a field experimental drought and warming, Physiol. Plant. 127 (2006) 632–649. https://doi.org/10.1111/j.1399-3054.2006.00693.x.

[91] J. Gershenzon, N. Dudareva, The function of terpene natural products in the natural world., Nat. Chem. Biol. 3 (2007) 408–414. https://doi.org/10.1038/nchembio.2007.5.

[92] H. Sakakibara, Cytokinins: activity, biosynthesis, and translocation., Annu. Rev. Plant Biol. 57 (2006) 431–449. https://doi.org/10.1146/annurev.arplant.57.032905.105231.

Table 1: Mean, standard deviation and FDR analysis of physiological parameters included. Asterisks (*) indicate significant differences at 5% of FDR and different lowercase letters indicate significant differences between treatments ($P \leq 0.05$). Abbreviations: T, treatment; T1WW, control plants well-watered at day 1; R2WW, control plants well-watered the whole experiment; T1WS, water-stress plants at first day of water stress; T2WS, water-stress plants 30 days after T1WS; R1WS, water-stress plants one day after rehydration; and R2WS, water-stress plants one week of rehydration.

T	T1WW	T1WS	T2WS	R1WS	R2WS	R2WW	FDR (5%)
MDA (mg g^{-1}FW)	21.75 ± 1.21 d	19.07 ± 0.49 d	60.74 ± 3.57 a	50.59 ± 1.67 b	40.89 ± 2.23 bc	36.17 ± 2.11 c	*
Proline (mg g^{-1}FW)	0.0267 ± 0.0006 a	0.0294 ± 0.002 a	0.0296 ± 0.0015 a	0.0314 ± 0.011 a	0.0291 ± 0.0006 a	0.0291 ± 0.0004 a	
RWC (%)	85.82 ± 1.55 a	73.85 ± 0.83 b	71.41 ± 1.13 b	89.64 ± 1.91 a	87.69 ± 0.76 a	88.58 ± 2.28 a	*
WatPot (MPa)	-0.45 ± 0.04 a	-1.69 ± 0.29 b	-0.78 ± 0.05 a	-0.45 ± 0.05 a	-0.35 ± 0.03 a	-0.30 ± 0.05 a	*

Table 2. Total number of specific metabolites present and number of identified in each treatment (sorted by highest to lowest). Abbreviations: T1WW, control plants well-watered at day 1; R2WW, control plants well-watered the whole experiment; T1WS, water-stress plants at first day of water stress; T2WS, water-stress plants 30 days after T1WS; R1WS, water-stress plants one day after rehydration; and R2WS, water-stress plants one week of rehydration.

Treatments	Treatment specific metabolites	(tentatively identified)
T1WS	186	(43)
R1WS	160	(22)
R2WS	145	(32)
T2WS	141	(30)
R2WW	127	(32)
T1WW	124	(20)