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Untargeted MS-Based Metabolomics Analysis of the Responses to Drought Stress in *Quercus ilex* L. Leaf Seedlings and the Identification of Putative Compounds Related to Tolerance

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Abstract: The effect and responses to drought stress were analyzed in *Quercus ilex* L. seedlings using a nontargeted metabolomic approach, implementing the approaches of previous studies in which other -omics platforms, transcriptomics, and proteomics were employed. This work aimed to characterize the *Q. ilex* leaf metabolome, determining possible mechanisms and molecular markers of drought tolerance and identifying putative bioactive compounds. Six-month-old seedling leaves subjected to drought stress imposed by water withholding under high-temperature and irradiance conditions were collected when leaf fluorescence decreased by 20% (day 17) and 45% (day 24) relative to irrigated seedlings. A total of 3934 compounds were resolved, with 616 being variable and 342 identified, which belonged to five chemical families. Out of the identified compounds, 33 were variable, mostly corresponding to amino acids, carboxylic acids, benzenoids, flavonoids and isoprenoids. Epigallocatechin, ellagic acid, pulegone, indole-3-acrylic acid and dihydrozeatin-O-glucoside were up-accumulated under drought conditions at both sampling times. An integrated multi-omics analysis of phenolic compounds and related enzymes was performed, revealing that some enzymes involved in the flavonoid pathways (chalcone synthase, anthocyanidin synthase and anthocyanidin reductase) were up-accumulated at day 24 in non-irrigated seedlings. Some putative markers of tolerance to drought in *Q. ilex* are proposed for assisting breeding programs based on the selection of elite genotypes.

Keywords: *Quercus ilex*; drought; metabolome; climate change; molecular markers; secondary metabolites; integrated omics analysis

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1. Introduction

According to climate data and derived predictive models, longer and more intense episodes of drought, accompanied by high temperatures and irradiance, are expected to occur throughout the 21st century [1]. These will be more drastic in specific areas such as the Mediterranean Basin [2,3], the natural habitat of the forest tree species under study, Holm oak (*Quercus ilex* L.), with defined zones of low survival probability [4]. It has been argued that tree mortality and dieback are caused by drought and high-temperature conditions,

accompanied by biotic stress (insects, fungi), which for some species is becoming a serious threat [5,6]. Even though it is considered the most drought-tolerant species within the European *Quercus* genus [7], episodes of tree damage and mortality have been observed in the last 50 years in natural stands and reforested areas [8]. Known as the decline syndrome, the causal agents are not well defined, with a combination of biotic (e.g., *Phytophthora cinnamomii*) and abiotic (drought) agents proposed as possible drivers [9,10].

Quercus ilex is a nondomesticated species, with biological characteristics (e.g., allogamy, wind pollen and animal seed dispersal) that make the exploitation of natural variability and the selection of elite genotypes the only plausible approach in plant breeding programs, which serve as the basis of sustainable management, conservation and reforestation programs [11–13]. For such a purpose, the characterization of the variability and the mechanisms of tolerance to stresses at the molecular level and the search for gene, gene products and metabolites to be used as markers should be a priority for selecting resilient genotypes for ulterior propagation and introduction in the dehesa and natural forests [14,15]. This requires the use of different techniques of morphometry, cell biology, physiology and molecular biology because tree mortality is not easy to predict [16].

We have previously investigated the responses to drought in *Q. ilex* individuals from different Andalusian populations to confirm differences in response and characterize the mechanisms of tolerance and of the genes implicated [17,18]. Thus, studies have been carried out on morphometry, physiology, classic biochemistry, transcriptomics and proteomics approaches [7,19–24]. In order to provide a more complete and realistic view of the processes and mechanisms, we should implement our previous work with the third gene expression level, that of metabolites, by using a nontargeted metabolomics approach. For that, we used a holistic, nontargeted, MS-based metabolomics approach. Such an approach has been recently used in the phytochemical analysis and variability between *Q. ilex* acorn morphotypes [25]. It has been reported that specific metabolites such as osmolytes, phenolics, and other secondary compounds play a key role in the response and tolerance to drought in particular and to abiotic stresses in general [26–30].

It was in the early 2000s that MS-based metabolomics started to be used in plant biology research [31], with pioneer papers dealing with stress response and water deficits appearing by the end of the decade [32,33]. Most of the work published focused on model systems and crops [34], with much less research carried out on trees [35–37]. Some of the published work used the genus *Quercus* and *Q. ilex* species as the experimental system [3,38–40].

Here, we characterized the leaf metabolome of *Q. ilex* in six-month-old seedlings and the changes that take place in response to drought stress by water withholding under high-temperature and irradiance conditions. Drought was imposed by water withholding in seedlings grown in perlite for 28 days, as previously described in San-Eufrasio et al. [7]. The analysis included two time points corresponding to a leaf fluorescence decrease of 20% and 45% relative to irrigated seedlings. The final goal was the characterization of the *Q. ilex* leaf metabolome, the identification of novel metabolites with an emphasis on those with bioactivity previously reported, the characterization of tolerance to drought from a metabolomics point of view, the identification of possible markers of tolerance and the integratation of the metabolite data with those obtained by us and other groups using other -omics or classic approaches. The knowledge generated can be translated to ecological studies and to breeding programs based on the molecular-assisted selection of elite resilient genotypes to be used in restoration and reforestation projects.

2. Materials and Methods

2.1. Plant Material, Treatment and Experimental Design

Mature and healthy acorns were collected from trees located in Almaden de la Plata (Seville, Andalusia, Spain; 37°52′ N, 6°28′ W) and germinated as previously described in San-Eufrasio et al. [7]. Six-month-old seedlings grown in 3 L black plastic pots containing perlite were subjected to drought conditions by water withholding for 28 days. The

irrigated, control, and seedling samples were maintained at 100% moisture. The experiment was performed by July 2018 in Cordoba, Andalusia, Southern Spain (37°54′ N, 4°43′ O), where extreme dry conditions prevail throughout the whole month (under a mean 37 °C temperature, 28 W m^{-2} solar irradiance, and 41% humidity).

An experiment based on a completely randomized design was developed by using ten biological replicates per treatment [7]. Out of the biological replicates, three asymptomatic (nondamaged) non-irrigated seedlings were randomly selected for metabolomics. All leaves were collected when the leaf chlorophyll fluorescence dropped by 20% and 45% in the non-irrigated seedlings compared to the irrigated ones (at days 17 and 24, respectively) [7]. After collection, leaves from the three biological replicates per treatment and sampling time were washed with tap water, blot dried with filter paper, shock-frozen in liquid nitrogen, and stored at −80 °C until metabolite extraction.

2.2. Extraction of Metabolites

Metabolites were extracted from leaves as described by Valledor et al. [41], with minor modifications. An extraction solution containing 600 µL of ice-cold methanol–chloroform–water (5:2:2) was added to 50 mg dry weight leaf tissue and vortexed for 10 s. The mixture was sonicated (ultrasonic bath, 40 kHZ for 10 min) and after centrifugation at 20,000× *g* at 4 °C for 4 min, the supernatant was transferred to a new tube. Then, 200 µL of cold methanol–chloroform–water (5:2:2) was added to the pellets and the process was repeated once. After combining both supernatants, they were vacuum dried at 30 °C (Speedvac, Eppendorf Vacuum Concentrator Plus/5301, Eppendorf, Leicestershire, UK). Dried extracts were reconstituted in methanol, centrifuged at 20,000× *g* for 10 min, and filtered through 0.22 µm PTPE membranes (Thermo Fisher Scientific, Courtaboeuf, France) and the filtrate was collected in 1.5 mL LC/MS certified sample vials.

2.3. Metabolite Identification and Quantification Using LC–Orbitrap MS Analysis

Dried extracts were re-dissolved in 1 mL of 50% methanol and 5 µL of sample were subjected to chromatographic separation with a Dionex Ultimate 3000 RS UHPLC system (Thermo Fisher Scientific, Bremen, Germany) equipped with an Acquity UPLC BEH (bridged ethyl hybrid) C18 column (1.7 µm, 100 × 2.1 mm, Waters Corporation, Manchester, UK) at 40 °C. A fifteen-minute mobile phase gradient was employed. A gradient elution chromatography was performed with solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol), as follows: (i) 95% A for 0.5 min; (ii) a linear increase from 5% to 100% in solvent B for 10 min; and (iii) return to 95% A for 2.9 min. A flow rate of 0.5 mL/min was used.

Column eluent was analyzed using a quadrupole Orbitrap Q Exactive hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source (HESI) operating in positive and negative polarities. The operating parameters, in positive ion mode, were a sheath gas flow rate at 60 kV, an auxiliary gas flow rate at 25 kV, a sweep gas flow rate at 2 kV, a spray voltage at 3.50 kV, a capillary temperature at 320 °C, an S-lens RF level at 50 kV, and an auxiliary gas heater temperature at 400 °C. For negative ion mode, all parameters remained the same except that the spray voltage was set to 3.00 kV. The Xcalibur v3.1 software was used for instrument control and data acquisition. Spectra data were acquired in full scan (FS) mode at a resolution of 70,000 (full-width half-maximum, FWHM at m/z 200) for MS1, and in a data-dependent (dd-MS2/dd-SIM) manner for MS2, fragmenting the five most abundant precursor ions per MS1 scan (TopN, 5), acquiring MS/MS data between 200 and 2000 m/z at a resolution of 17.500.

Three biological replicates of each treatment and quality control mix (QC) were analyzed. The QC samples were prepared using equal volumes of all samples and were injected after every six samples for continuous quality assurance and to promote confidence in the data. Moreover, the QC samples were analyzed in a data-dependent (dd-MS2/dd-SIM) manner for feature annotation. All acquired data were exported by Xcal-

ibur software to be analyzed by the Compound Discoverer v3.1 software (Thermo Fisher Scientific, Bremen, Germany). Raw data were deposited in the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org, accessed on 13 September 2020. The data can be accessed directly via DataTrack ID:3106.

2.4. Data Processing

UPLC-MS/MS data treatment, alignment, peak selection, deconvolution, normalization, and annotation were performed using the Compound Discoverer v3.1 software (Thermo Fisher Scientific, Bremen, Germany). The alignment was performed with a retention time with a maximum shift of 0.1 min and a mass tolerance of 5 ppm. Then, peak selection and deconvolution allowed the detection of feature groups across all samples. Moreover, elemental compositions (chemical formula hypothesis) for all compounds were predicted and the chemical background was hidden using blank samples. The metabolites were annotated using a ddMS2 similarity search (sustained by the agreement between theoretical and experimental isotopic patterns) in mzCloud (https://www.mzcloud.org/, accessed on 13 September 2020), and the formula or exact mass (mass error $\leq$ 5 ppm) were searched in ChemSpider (https://www.chem-spider.com/, accessed on 13 September 2020), which was also used for the literature references. Finally, metabolites were classified and mapped to biological pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/, accessed on 13 September 2020).

Considering the areas of the corresponding chromatographic peaks of the MS1 precursor, a relative comparison among the different samples was performed. Neutral masses obtained in the positive and negative modes were evaluated to avoid duplicities (neutral mass in different modes and similar retention time) while retaining the most intense peaks. A table (Table S1) was generated that included the following information: mass-to-charge ratio (m/z), retention time, and the abundance of the metabolites in the samples.

2.5. Statistical Analysis

All the statistical analyses were performed in the environment RStudio v1.2.158 running under R v3.6.1. Three biological replicates were used for all statistical procedures. The metabolomic data were pre-processed using missing value imputation (Random Forest algorithm with a threshold of 0.35), abundance balancing (values were sample-centric and then each value was multiplied by the average intensity (sum of the peak area of all variables within a sample) of all samples) and filtering (consistency-based criteria with a threshold of 0.5) using pRocessomics (available on web direction: http://github.com/Valledor/pRocessomics, accessed on 27 August 2021).

A multivariate analysis (principal component analysis, PCA) with all samples, including QC samples, was performed. With this analysis, the robustness of the analytical procedure was demonstrated by the tight clustering of the QC samples. In addition, QCs located in the center of the PCA plot ensure that the separation between the groups is not random but is due to a real variability. In addition, to determine which metabolites were different between conditions, a one-way Kruskal–Wallis test per single metabolite was carried out on area values. A PCA was carried out with these metabolites (cut-off of the p-value < 0.05).

2.6. Muti-Omics Integrated Analysis

Datasets of transcriptomics and proteomics, previously reported by Guerrero-Sánchez et al. [24], and the dataset of metabolomics generated in this work were used to perform a multi-omics integration by using the KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathway database. The presence of transcripts, proteins and metabolites related to the following pathways were manually selected: phenylpropanoids; lignins; coumarins; stilbenoids; flavonoids; flavones and flavonols; anthocyanins; and isoflavonoids. The resulting molecular profile obtained for the different pathways was

followed by a manual integration of *Q. ilex* phenolic metabolism. Once the integration was carried out, the levels of transcripts, proteins and metabolites were statistically analyzed (p-value < 0.05 in the Student t-test) under drought conditions.

3. Results and Discussion

3.1. Drought Treatment in Q. ilex Seedlings

Studies on drought stress in *Q. ilex* seedlings are justified considering that this is the main cause of plant mortality upon transplanting from the nursery [42,43], and hence, the main cause of the failure of restoration and reforestation programs. Because there is great variability in the response to drought stress between individuals and populations [7,21], the final goal of the investigation is to select highly tolerant genotypes, characterize the mechanisms of tolerance and identify key molecular markers associated with resilience to be used in breeding programs. The latter should be sustained by in vitro holistic -omics strategies, covering the different levels of the central dogma of molecular biology and the omics cascade, which connect genotype and phenotype from genomics, transcriptomics, proteomics and metabolomics.

The current research was intended to implement previous studies on the effect and responses to drought in *Q. ilex* seedlings, in which morphometric (growth, damage symptoms, mortality), physiological (water content, photosynthesis), classic biochemistry (pigments, sugar, amino acids, phenolics), and omics (transcriptomics and proteomics) approaches were used [7,24]. Six-month-old seedlings from highly drought-tolerant, asymptomatic individuals located in the Almaden de la Plata (Sevilla, Andalusia, Spain), grown on perlite, were subjected to drought stress imposed by water withholding under high-temperature and irradiance conditions [7]. Leaf samples were collected at day 17 and 24, when leaf fluorescence dropped to values of 20 and 45% with respect to the irrigated seedlings. The soil matric potential was −15 (day 17) and −26 (day 24) kPa in the non-irrigated seedlings, the relative leaf water content at day 24 was 85% (irrigated seedlings) and 54% (non-irrigated ones), and the quantum-yield (Qy) was 0.73 (irrigated seedlings) and 0.60 (day 17) and 0.40 (day 24) (non-irrigated ones), indicating severe stress conditions. There were no differences in photosynthetic rate and stomatal conductance between treatments at day 9; on the contrary, they were very much impaired under drought conditions at day 24, with values of 11 (irrigated seedlings) and 2 (non-irrigated ones) μmol CO_2 m^{-2} s^{-1} (photosynthetic efficiency), and 0.18 (irrigated seedlings) and 0.05 (non-irrigated ones) mol H_2O m^{-2} s^{-1} (stomatal conductance) [7]. At day 24, there were no significant differences between irrigated and non-irrigated seedlings in terms of the content of photosynthetic pigments, and there was a small reduction in the content of anthocyanins [7], proving the integrity of the photosynthetic machinery. Leaves from non-irrigated seedlings had higher levels in total sugars, amino acids and phenolics [7], which is quite a common response to drought, as these compounds enhance osmoprotection and prevent water loss [26,38].

3.2. Untargeted Metabolome Profiling in Q. ilex Leaves

The leaf metabolome from irrigated and non-irrigated *Q. ilex* seedling leaves at the two sampling times (days 17 and 24) was analyzed by LC-Q-Orbitrap-MS of water-methanolic extracts, operating in the positive and negative ion modes (Table 1).

Table 1. LC-MS metabolic profile of leaf tissue extracts from irrigated and non-irrigated seedlings. The number of resolved positive and negative ion features are shown, corresponding to the mean of three biological replicates.

Treatment	Day	Total		Variable	
		Positive	Negative	Up	Down
Control	17	1369	1152		
	24	1318	1184		
Drought	17	1366	1182	250 [a] (127 [b]/123 [c])	219 [a] (117 [b]/102 [c])
	24	1523	1312	305 [a] (87 [b]/218 [c])	233 [a] (128 [b]/105 [c])

[a] Total, [b] quantitative (p-value < 0.05 in the Kruskal–Wallis test) and [c] qualitative (absence of a variable ion in the three biological replicates of a treatment) variables. Up- and down-accumulated ions at the two sampling times (days 17 and 24) are included.

In total, 1152–1312 (negative) and 1318–1523 (positive) ion features were resolved at both treatments and sampling times (Table 1). Qualitative differences (absence of a variable ion in the three biological replicates of a treatment) allowed 127 up- and 117 down-accumulated and 87 up- and 128 down-accumulated ions to be identified at days 17 and 24, respectively (Table 1). Quantitative differences (p-value < 0.05 in the Kruskal–Wallis test) identified 123 up- and 102 down-accumulated, and 218 up- and 105 down-accumulated ions at days 17 and 24, respectively (Table 1). These values are lower than those previously reported for *Q. ilex* acorns by using Q-TOF [25], *Quercus suber* leaves by using a double extraction system [44], and *Nicotiana tabacum* leaves by using IT-TOF [45]. The original dataset was filtered for consistency (present in the three replicates) and differences between samples (p-value < 0.05 in the Kruskal–Wallis test) (Table 1). Around 20% of the whole metabolome visualized changed in abundance, with a similar number of compounds being more/less abundant, and showing quantitative/qualitative changes in non-irrigated leaves, except for those more abundant at day 24. Venn diagrams were performed with the total (3934), variable (616) and annotated (342) features identified in irrigated and non-irrigated *Q. ilex* leaf seedlings at days 17 and 24 (Figure S1). Out of 616 variable metabolites, 54 were present at both treatments and both sampling times, representing a small percentage of the total (8.77%). Regarding variable compounds, 137 variable metabolites were commonly identified at both treatments and both sampling times. On the other hand, 15 metabolites were commonly observed at both sampling times in the non-irrigated seedlings, whereas 10 and 41 were detected at days 17 and 24, respectively, which indicates the existence of permanent and transitory changes.

A PCA analysis was performed to reduce the dimensionality of the data and visualize the relationship among samples. The PCA of the total dataset (Figure S2) showed that the different replicates within samples, including the QC ones, were grouped, demonstrating the high quality of the data. PC1 and PC2 accounted for a low percentage of the variance, at 15.10% and 12.84%, respectively. This is quite common for *Q. ilex* -omics analysis [24]. The PCA test of the variable features dataset (616) discriminated treatments and sampling times (Figure 1); thus, PC1 (34.09% of the variance) separated non-irrigated seedlings at day 24 for the rest of the treatments, whereas PC2 (23.62% of the variance) separated both sampling times. A similar PCA analysis has been previously performed with the variable transcripts and proteins at the two treatments and sampling times [24]. Higher variability was found among replicates in transcriptomics than proteomics or metabolomics data. A different distribution of the samples was obtained with the different -omics datasets. From the protein and transcript PCA, PC1 clearly separated treatments and PC2 sampling times [24]. This indicates that the transcript and protein profiles were more variable at the assayed times than the metabolite one, and that changes in transcripts and proteins occurred earlier than in metabolites.

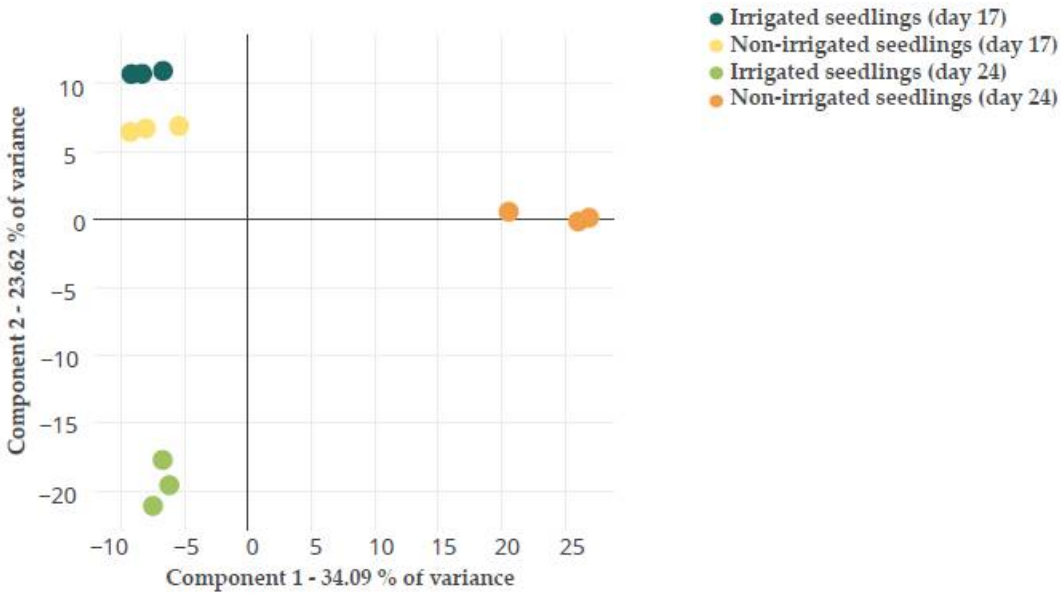

Figure 1. PCA analysis of the variable features dataset identified in *Q. ilex* leaf seedlings during both treatments (irrigated and non-irrigated seedlings) and both sampling times (days 17 and 24).

From the m/z of the ion precursor and derived fragments, 342 compounds were annotated (Table 2 and Table S1), with redundancy for some of them. They belonged to different chemical families (Table 2), with 22 having three or more identified compounds. The secondary metabolism group, and concretely, that of phenolic compounds, at the number of 131, was the most represented. Out of 342 annotated compounds, 28 were included in very heterogeneous classes (Table 2 and Table S1). These data show, as previously observed in *Q. ilex* acorns [25], the complexity and richness of the *Q. ilex* secondary metabolites.

Table 2. Total number of annotated compounds classified by chemical families.

Chemical Family	Number of Annotated Compounds
Carbohydrate conjugates and sugar derivates	28
Amino acids, peptides and analogues	35
Lipids and fatty acid-related	19
Carboxylic acids and derivates	33
Amines	4
Benzenoids	51
Coumarins	15
Flavonoids	37
Aurones	2
Stilbenes	6
Monolignols	9
Tannins (galloyl derivatives)	8
Lignans	5
Isoprenoids/Terpenoids (mono, di and sesqui)	36
Steroids	6
Indoles	4
Plant growth regulators (jasmonic acid, cytokinins)	3
Vitamins (Vit B5)	3
Pyridines	4
Quinones	3
Pyrones	3
Others	28

3.3. Differential Metabolite Abundance Analysis

Next, data on annotated variable compounds are presented and discussed. Out of the 342 annotated compounds, 33 were variable (p-value < 0.05 in the Kruskal–Wallis test) (Table 3), belonging to the following groups: amino acids (9), carbohydrates and carbohydrate conjugates (3), phenolics (9), terpenoids (6), phytohormones (2), and others (4). The number of identified variable compounds is lower than that reported for *Q. suber* [44], but it is in the range of that reported in other *Quercus* spp., or even plant species [46,47]. From our previous proteomics and transcriptomics analysis [24], the number of expected variable metabolites, according to the enzyme protein and transcript profiles, should be much higher, which clearly indicates the limitation of the metabolomics strategy.

As previously shown in previous studies on metabolome changes under drought conditions, and in agreement with data presented here, amino acids, carbohydrates, organic acids, and the group of secondary metabolites (phenolics, and terpenoids) are the most affected [44,61,62]. Some preliminary studies on changes in the metabolome profile have been caried out in *Quercus* spp., with agreements on changes in chemical groups, but differences in individual components can be explained by the different species or genotype, experimental design or metabolomics platform. As an example, Aranda et al. [47] reported an experiment with older seedlings using GC-MS. Some annotated variable compounds have not been previously identified in *Quercus* spp. (e.g., aesculin, dihydrophaseic acid), others are of very limited distribution or not reported in the plant kingdom (e.g., 7-oxoheptanoate and 10-hydroxy-2decenoic-acid), whereas others are ubiquitous (e.g., aspartate, L-tyrosine) (Table 3). In addition, for some of them, bioactivities and health benefits have been claimed (e.g., emodin, miglitol, 7-deoxyloganin). As some of the compounds have been described in animal systems or microorganisms, its identification should be considered questionable, and it should be validated by NMR and other approaches. Those that were not previously described in nonplant systems are kept out of the discussion. The pattern observed depended on the compound, with qualitative and quantitative differences among treatments, and up- or down-accumulation tendencies at both or individual sampling times.

Regarding the phenolics compounds, they are the major secondary metabolites in the genus *Quercus*, with some of them showing bioactivity and having interesting pharmacological properties (epigallocatechin, ellagic acid) [53]. The clearest metabolome response of *Q. ilex* to drought stress was the changes in the phenolic profile with variable phenolic compounds of the different chemical groups, from simple benzenoids to more complex flavonoids and tannins through coumarins and monolignols (Table 3), which agrees with Almeida et al. [44]. Benzenoids (C_6–C_1, C_6–C_2, C_6–C_3 and C_6–C_7), either increased (3,4-Dihydroxymandelaldehyde, L-dopa) or decreased (Isovanillic acid) under drought conditions. The implication of L-dopa and other catecholamines in the responses to biotic and abiotic stresses has been suggested [63] and associated with its allelochemical potential [64]. Coumarins have been previously reported in the genus *Quercus*, being present in bark, contributing to the taste of wines produced and stored in wood barrels [65]. Three compounds belonging to this group were identified as variable ones: the simplest coumarin, aesculin, and scoparone. They were down-accumulated in the response to drought in *Q. ilex*, except for aesculin at day 24. Both epigallocatechin and ellagic acid were up-accumulated under drought conditions at both sampling times [66,67].

Table 3. List of variable annotated metabolites identified in *Q. ilex* seedlings subjected to drought conditions.

Group	Compound	Kegg ID/ PubChem ID [a]	FC (Day 17/Day 24) [b]	Previously Detected in *Quercus* spp. (If Not Ubiquitous)
Amino acids and derivates	4-Oxoproline	C01877	Down/Down	-
	Aspartate	C00049	Down/Down	*Q. ilex* [25,48]
	Gamma-aminobutyric acid (GABA)	C00334	Down/Up	*Q. robur*, *Q. pubescens and Q. petraea* [49]
	Homoarginine	C01924	ND/Up	-
	L (+)-citrulline	C00327	Up/Up	-
	L-methionine sulfoxide	C02989	ND/Up	-
	L-tyrosine	C00082	Up/Up	*Q. rubra* [50], *Q. ilex* [38]
	Proline	C00148	Down/Down	*Q. ilex* [48]
	L-3,4-dihydroxyphenylalanine (L-DOPA)	C00355	ND/Up	-
Carbohydrates and carbohydrate conjugates	(−)-Quebrachitol	C08257	Down/Down	-
	N-acetyl-beta-D-galactosamine	C05021	Down/Down	-
	Miglitol	D00625	Up/Up	-
Phenolic compounds	Isovanillic acid	C05582	ND/Up	*Quercus robur*, *Quercus salicina*, *Quercus glauca*, *Quercus acuta*, *Quercus phillyraeoides*, *Quercus myrsinaefolia* [51]
	3,4-Dihydroxymandelaldehyde	C05577	Up/Down	-
	(−)-Epigallocatechin	C12136	Up/Up	*Q. ilex* [25] *Q. resinosa*, *Q. grisea*, *Q. arizonica and Q. covallata* [52], *Q. macrocarpa* [53], *Q. suber* [44]
	Trans-cinnamaldehyde	C00903	Down/ND	-
	Aesculin (Esculin)	C09264	Down/Up	-
	Coumarin	C05851	Down/Down	*Quercus canariensis* [54]
	Scoparone	C09311	Down/Down	-
	4-Coumaryl alcohol	C02646	ND/Up	-
	Ellagic acid	C10788	Up/Up	*Q. ilex* [25], *Quercus infectoria* [55], *Q. pyrenaica* [56], *Quercus petraea*, *Q. robur* [57], *Q. suber* [58]
Terpenoids	(+)-exo-5-hydroxycamphor	C03448	Down/Down	-
	Dihydrophaseic acid	C15971	Down/Down	-
	7-deoxyloganin	C01433	ND/Up	*Q. ilex* [25]
	(−)-trans-carveol	C11409	ND/Up	-
	Arjunic acid	15385516	Up/Down	*Quercus faginea* [59]
	Pulegone	C09893	Up/Up	-

Table 3. *Cont.*

Group	Compound	Kegg ID/ PubChem ID [a]	FC (Day 17/Day 24) [b]	Previously Detected in *Quercus* spp. (If Not Ubiquitous)
Phytohormones	Indole-3-acrylic acid	5375048	Up/Up	-
	Dihydrozeatin-O-glucoside	C16448	Up/Up	-
Others	2-Furoic acid	C01546	Up/Up	*Q. petrea*, *Q. robur* [60]
	3-Ureidoisobutyrate	C05100	Down/Down	-
	Emodin	C10343	ND/Up	-
	Pantothenic acid	C00864	Up/Up	-

[a] The identification of metabolites has been prioritized according to the KEGG database, otherwise PubChem identification has been indicated. [b] Fold change (FC) of each metabolite at both sampling times (days 17 and 24) indicating the response of the metabolite to drought (up: up-accumulated; down: down-accumulated; ND: not detected).

Quercus spp., and concretely *Q. ilex*, are rich in terpenoids, mostly mono- and sesquiterpenes [44]. They may contribute to the drought-tolerant phenotype as, for example, they are components of the cuticular waxes [68]. In *Q. ilex* under drought conditions, the amount of terpenoids in root exudates and terpene emissions increased [69,70]; additionally, in *Q. suber*, an increase in the terpenoid leonuridine was a characteristic late response to drought [44]. Among the annotated variable terpenoids in this work, the following compounds are included: the triterpenoid saponin arjunic acid (down-accumulated in the non-irrigated seedlings at day 24), the sesquiterpenoid dihydrophaseic acid (detected at days 17, in the irrigated seedlings, and 24 in the non-irrigated ones), and the monoterpenes pulegone and (+)-exo-5-hydroxycamphor (detected in the irrigated seedlings at day 17). A clear tendency, up- or down-accumulated, for this group has not been found, which is not surprising, as it has been previously discussed in [71].

As for the amino acids annotated in the *Q. ilex* leaf metabolome, L-tyrosine was observed in the non-irrigated seedlings, being ten times more accumulated at day 24 than at day 17, which agrees with Fabregas and Fernie [72]. Tyrosine has proven to be accumulated in drought-stressed chickpea plants and is also more abundant in drought-tolerant varieties [73]. Other annotated variable amino acids were also up-accumulated at day 24 (homoarginine, GABA, L-citrulline, L-methionine sulfoxide, L-tyrosine and L-dopa), even though they were not detected, or even at lower abundance in the non-irrigated seedlings at day 17. Amino acid accumulation under stress conditions has been reported in several plants species, including the genus *Quercus* [44,47,72], with GABA and proline being two clear examples linked to a stress-tolerant character [74,75]. In *Quercus* spp., the up-accumulation of GABA and glutamic acid was one of the clearest responses to drought in *Quercus* spp. [47].

Among the changes in the group of carbohydrates in the metabolic profile of seedlings subjected to drought, the increase in the levels of glucose, fructose, oligosaccharides or derivatives (polyols and other osmotic active compounds) seems to be a general tendency. In *Q. ilex*, a slight increase in glucose, fructose, and galactose, but not sucrose or raffinose, has been previously reported [47]. These sugars were not identified in the present work, which can be due to the employed extraction and MS analysis. Out of the 28 annotated carbohydrates, only three were variable in response to drought, namely N-acetyl-beta-D-galactosamine (decreased under drought conditions at both sampling times), (−)-quebrachitol (less abundant in the non-irrigated seedlings at both sampling times) and miglitol (increased under drought conditions at both sampling times). To our knowledge, not much information has been reported about the presence of N-acetyl-beta-D-galactosamine and miglitol in plant tissues [76]. Quebrachitol is one of the major methylated cyclitols in some plant species but has not been previously reported in the genus *Quercus*. With no clear biological roles assigned, its chemical nature suggests its participation in the response to osmotic stress [77].

Indole-3-acrylic acid, generated from tryptophan and reported as an auxin growth regulator, was identified in this study (Table 3). As most of the plant hormones, auxins seem to play a role in the responses to abiotic stresses, either directly or through hormone signaling pathway crosstalk [46,78,79]. Another variable growth regulator annotated was the cytokinin dihydrozeatin-O-glucoside that increased in abundance at both sampling times. In this direction, a few published works have reported an increase in cytokinin levels in plants under drought conditions [80,81].

Abscisic acid (ABA) has been reported as the key player in plant responses to drought. ABA has been identified in this metabolomic study, but statistically significant differences between samples have not been found. The most typical effect of ABA is stomata closing, which reduces water loss and gas exchange. In our system, the photosynthetic rate and stomatal conductance were only significantly reduced at late times of the experiment, even though no changes in ABA were observed. For some of the up-accumulated compounds, some previous evidence may support a possible role in drought tolerance. This is the case of the riboflavin precursor 6,7-dimethyl-8-(1-D-ribityl)lumazine. The riboflavin treatment

of tobacco plants enhanced drought tolerance [82]. Other metabolites have been shown to be changed in response to drought in *Q. ilex* and other *Quercus* spp., and these changes are either general or species-specific. Such changes have not been observed with the employed individual or under our experimental conditions. It is the case of some organic acids such as succinic and malic acids.

Because the search of possible molecular markers is related to *Q. ilex* drought tolerance response, we propose those metabolites that were up-accumulated in non-irrigated seedlings (Table 3). This list includes L-citrulline, L-tyrosine, epigallocatechin, ellagic acid, pulegone, indole-3-acrylic acid, dihydrozeatin-O-glucoside, 2-furoic acid and pantothenic acid.

3.4. Integrated Multi-Omics Data

A total number of 25,169 transcripts, 3312 proteins that were previously reported in Guerrero-Sánchez et al. [24], and 342 metabolites reported in this work, were annotated in *Q. ilex* seedlings. As expected, the transcriptomic dataset is the most comprehensive and informative and includes more than 10,000 transcripts, followed by the proteomics dataset with about 3000 proteins, and, finally, the metabolomics dataset containing a few hundred meabolites [83]. An integrated multi-omics analysis of phenolic compounds' andrelated enzymes was performed, considering that the phenolic metabolism pathway is the most represented in the metabolome of *Q. ilex*. All the transcripts, proteins and metabolites related to the biosynthesis of phenylpropanoids and derivates, lignins, coumarins, stilbenoids, diarylheptanoids and gingerols, flavonoids, flavones and flavonols, anthocyanins, and isoflavonoids were selected (Table S2). A total of 38 enzymes involved in phenolics metabolism were identified in the pathways analyzed, with the biosynthesis of phenylpropanoid and flavonoids being the most represented (13 and 16, respectively). The number of enzymes found on the three omics levels was quite low (leucoanthocyanidin reductase, cinnamoyl-CoA reductase and chalcone synthase), which indicates that the data integration is challenging when a combined analysis of metabolomics data is integrated with other omics data [84]. However, when the presence of enzymes is identified at two omics levels, the number of enzymes is higher, at 18 out of 38. The rest of the enzymes were detected only at the transcript level (Table S2).

The enzymes involved in the pathways of lignins, stilbenoids and isoflavonoids were not affected by drought conditions (Figure 2). In contrast, some enzymes involved in the pathways of phenylpropanoids and derivates, coumarins and flavonoids were altered in the *Q. ilex* seedlings subjected to drought. Most of the enzymes involved in the pathways of phenylpropanoids and derivates and coumarins were down-accumulated in response to drought (Figure 2). It is remarkable the significant late response to drought observed in the flavonoid pathways in some enzymes (chalcone synthase, anthocyanidin synthase and anthocyanidin reductase) that were up-accumulated at day 24 (Figure 2). Flavonoids have a high antioxidant activity against reactive oxygen species (ROS), which are enhanced under drought stress [72,85]. Chalcone synthase is a key enzyme in the flavonoid biosynthesis pathway that monitors the changes in response to drought in many plants. In *Nicotiana bentamiana* and *Populus*, the expression of the chalcone synthase gene increased under drought conditions [86,87]. Recently, chalcone synthase has been proposed as a putative marker of drought tolerance, which was up-accumulated in *Q. ilex* seedlings from several populations subjected to drought [88]. Li et al. [89] reported that anthocyanidin synthase from *Morus alba* contributes to the protection of plants against abiotic stress such as drought by improving the ROS-scavenging ability. Kubra et al. [90] also showed that the expression of the anthocyanidin synthase gene in *Arachis hypogaea* was significantly increased in drought. In anthocyanidin reductase, although it was described as a down-accumulated gene in response to drought in *Camellia sinensis* [91], other studies reported that its expression is increased (e.g., *Vitis vinifera*) under this abiotic stress [92]. Wang et al. [93] described that, in *C. sinensis*, this enzyme was first decreased and then increased in response to drought stress. Considering the role of these enzymes in the

response to drought as well as their identification in at least two omics approaches, they could be proposed as markers of resilience to drought stress to be used in breeding and reforestation programs. Therefore, they deserve further attention and in-depth functional study and validation as potential molecular markers.

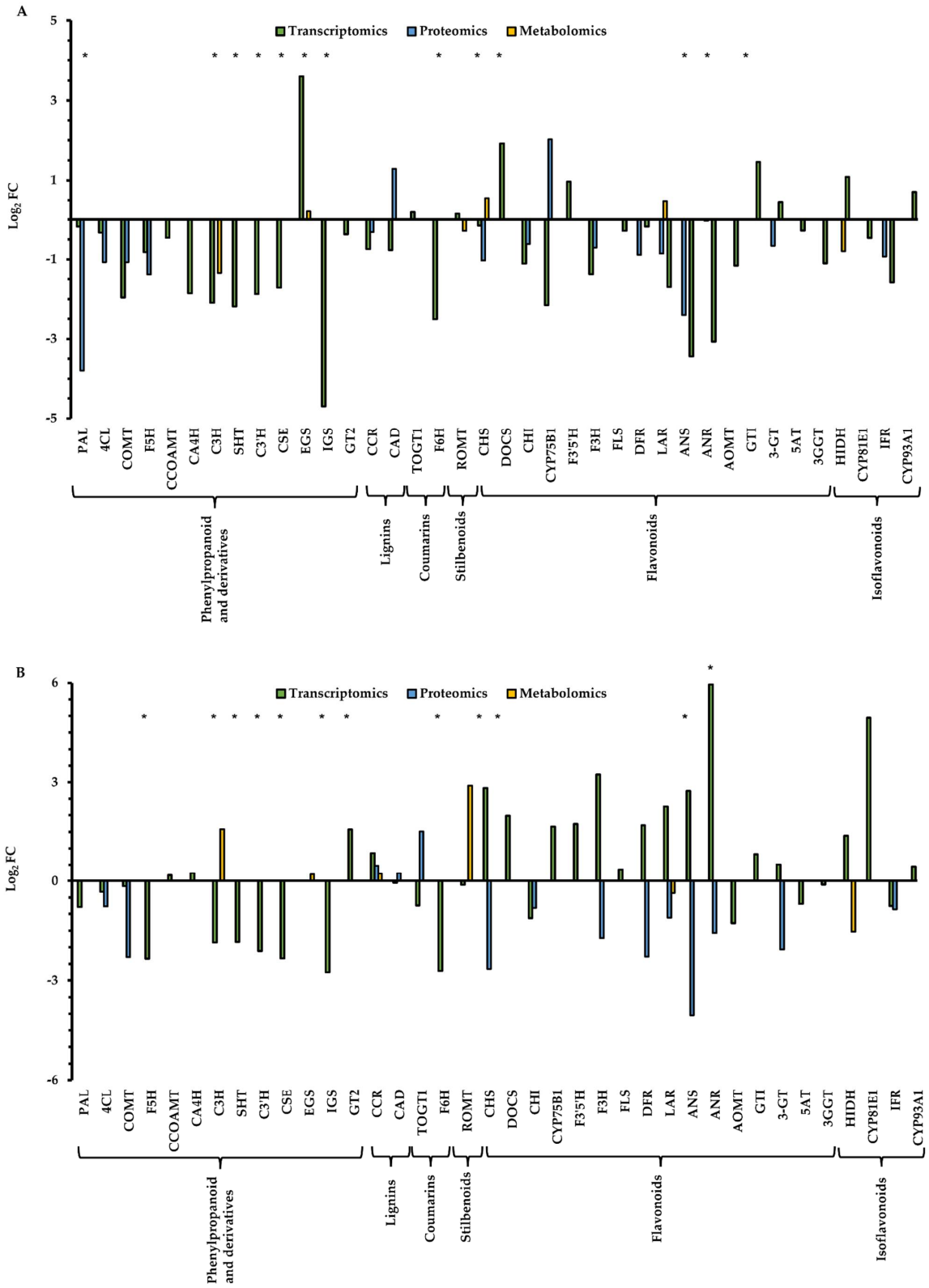

Figure 2. Integrated multi-omics analysis of phenolic compound-related enzymes in *Q. ilex*. The

asterisk indicates significant differences in the differential expression of the enzymes related to phenolic compounds in response to drought at days 17 (**A**) and 24 (**B**) at the transcriptomic, proteomic and metabolomic levels. Expression levels are represented as $\log_2$ of fold change (FC). PAL: phenylalanine ammonia-lyase; 4CL: 4-coumarate CoA ligase; COMT: caffeic acid 3-O-methyltransferase; F5H: ferulate 5-hydroxylase; CCOAMT: caffeoyl-CoA O-methyltransferase; CA4H: cinnamate 4-hydroxylase; C3H: coumarate 3-hydroxylase; SHT: shikimate O-hydroxycinnamoyltransferase; C3′H: p-coumaroyl ester 3′-hydroxylase; CSE: caffeoylshikimate esterase; EGS: eugenol synthase; IGS: isoeugenol synthase; GT2: cinnamate beta-D-glucosyltransferase; CCR: cinnamoyl-CoA reductase; CAD: cinnamyl alcohol dehydrogenase; TOGT1: scopoletin glucosyltransferase; F6H: feruloyl CoA ortho-hydroxylase; ROMT: trans-resveratrol di-O-methyltransferase; CHS: chalcone synthase; DOCS: NAD(P)H-dependent 6′-deoxychalcone synthase; CHI: chalcone isomerase; CYP75B1: flavonoid 3′-hydroxylase; F3′5′H: flavonoid 3′,5′-hydroxylase; F3H: flavanone-3-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol reductase; LAR: leucoanthocyanidin reductase; ANS: anthocyanidin synthase; ANR: anthocyanidin reductase; AOMT: flavonoid 3′,5′-methyltransferase; GTI: flavonol 3-O-glucosyltransferase; HIDH: 2-hydroxyisoflavanone dehydratase; CYP81E1: isoflavone 2′-hydroxylase; IFR: isoflavone reductase; CYP93A1: 3,9-dihydroxypterocarpan 6A-monooxygenase; 3-GT: anthocyanidin 3-O-glucosyltransferase; 5AT: anthocyanin 5-aromatic acyltransferase; 3GGT: anthocyanidin 3-O-glucoside 2″-O-glucosyltransferase. Asterisk indicates significant differences between irrigated and non-irrigated seedlings (* $p < 0.05$).

4. Conclusions

The molecular study of the effect and responses to drought stress in *Q. ilex* is a key feature in assisting breeding programs based on the selection of elite genotypes and hence, maintaining the sustainability of Mediterranean ecosystems and the agrosilvopastoral system "*dehesa*" under stress and climate change conditions. For this, two omics approaches, transcriptomics and proteomics, were employed with this species to decipher molecular mechanisms involved in the response to drought as well as to identify gene, gene products and molecular markers associated with drought tolerance [24,93]. As a complement analysis, in this work, changes in the metabolome profile of *Q. ilex* seedlings subjected to drought conditions by water withholding was performed. The metabolomic analysis, at two sampling times (days 17 and 24) under drought conditions, revealed important changes in the metabolome (3934 features resolved, with 616 variable and 342 annotated compounds). The chemical groups more affected by the stress were amino acids, carbohydrates, organic acids, and the group of secondary metabolites, including phenolics and terpenoids. The list of annotated variable compounds included some that were not previously identified in *Quercus* spp. (e.g., aesculin, dihydrophaseic acid), that were not previously reported in the plant kingdom (e.g., 7-oxoheptanoate and 10-hydroxy-2decenoic-acid), or that were ubiquitous (e.g., aspartate, L-tyrosine). It is remarkable that some of them have been previously described in other plant species as being bioactive or having health benefits (e.g., epigallocatechin, ellagic acid, emodin, 7-deoxyloganin). Among the variable compounds, such as L-citrulline, L-tyrosine, epigallocatechin, ellagic acid, pulegone, indole-3-acrylic acid, dihydrozeatin-O-glucoside, 2-furoic acid and pantothenic acid, some could be considered putative markers of tolerance as they are accumulated in non-irrigated seedlings at the two sampling times. The integrated multi-omics analysis was performed with transcripts and proteins of phenolic pathway enzymes that were previously reported [24] and the metabolome analysis presented here. The flavonoid biosynthesis pathway was the most represented in *Q. ilex*, with a total of 16 transcripts, 10 proteins and 2 metabolites identified. Three enzymes (chalcone synthase, anthocyanidin synthase and anthocyanidin reductase) were up-accumulated at day 24 in response to drought and identified in at least two omics approaches. These enzymes, together with the up-accumulated metabolites at both sampling times, could be proposed as markers of drought tolerance to be used in breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13040551/s1, Table S1. (A) List of the total metabolites obtained in three biological replicates of Q. ilex leaf seedlings under drought conditions at two sampling times (days 17 and 24), showing the mass-to-charge ratio (m/z), retention time, and abundance. (B) List of annotated variable metabolites (p-value < 0.05 in the Kruskal–Wallis test) showing the ID, neutral mass (Da), retention time (min) and description. Table S2. List of enzymes involved in the phenolics metabolism identified at the transcriptomic, proteomic and metabolomic levels in *Q. ilex*. The presence of the enzyme is shown with an "x". Figure S1. Venn diagrams of the total 3934 (A), and 616 variable (B) features, and 342 annotated compounds (C) in irrigated and non-irrigated Q. ilex leaf seedlings at both sampling times (days 17 and 24). Figure S2. PCA analysis of the total dataset of metabolites including the quality control mix (QC).

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