Topsoil depth substantially influences the responses to drought of the foliar metabolomes of Mediterranean forests

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Abbreviations

H-Forest: Forest with high canopies

L-Forest: Forest with low canopies

VS: Vegetation structure

DUSL: Depth of upper soil layers

D50: Diameter at 50 cm from soil

SEM: Structural Equation Model

LC-MS: Liquid chromatography coupled to mass spectrometry

Abstract

The upper soil provides support, water, and nutrients to terrestrial plants and is therefore crucial for forest dynamics. We hypothesised that a tree's metabolic activity (and therefore its metabolome; the total set of metabolites) would be affected by both the depth of upper soil layers and water availability. We sampled leaves for stoichiometric and metabolomic analyses once per season from differently sized *Quercus ilex* trees under natural and experimental drought conditions representing the likely conditions in the coming decades). Although the metabolomes varied according to tree size, smaller trees did not show higher concentrations of biomarker metabolites related to drought stress. However, the effect of the drought treatment on the metabolomes was greatest for small trees growing in shallow soils. Our results suggest that tree size is more dependent on the depth of the upper soil, which indirectly affects a tree's metabolome, rather than on the moisture content in the upper soil. Metabolomic profiling of *Q. ilex* supports our finding that water availability in the upper soil is not necessarily correlated with tree size. The higher impact of drought on trees growing in shallower soils nevertheless indicates that any increase in the frequency, intensity, and duration of drought - as has been projected for the Mediterranean Basin and other areas - would affect small trees most. Metabolomics has proved to be a useful means for investigating the links between plant metabolism and environmental conditions.

Keywords: Vegetation structure, soil depth, soil moisture, metabolomics, *Quercus ilex*

Introduction

Soil provides a physical support system and a reservoir of water for terrestrial primary producers (Montheith, 1981). A scarcity of soil resources, particularly water, is often associated with restricted development of plant-soil systems and reduced biomass (Huxman et al., 2004; Knapp and Smith, 2001; Orwig and Abrams, 1997). Soil biological activity and tree growth can be limited by several factors such as nutrients (Bowman et al., 1993; Sardans and

Peñuelas, 2015; Sardans et al., 2012a, 2012b), light (Poorter, 1999), temperature (Epstein et al., 1997), or water (Huxman et al., 2004; Rosenzweig, 1968; Sala et al., 1988). Topographic factors such as slope variation and/or soil texture also play important roles in the retention and storage of soil water (Farahani et al., 1998; Fernandez-Illescas et al., 2001) and can influence soil enzymatic activity (Bastida et al., 2008) and erosion (Kinnell and Cummings, 1993). Soil depth is tightly linked with the physiology and respiration rates of tree roots (Pregitzer et al., 1998), the composition of microbial communities (Fierer et al., 2003), and even plant biodiversity (Fuhlendorf and Smeins, 1998). Most of the biological activity and nutrient recycling in soil occurs in the upper topsoil layers, so water availability in these layers is crucial to forests (Jobbagy and Jackson, 2000; Wardle et al., 2004). Hydraulic lift, mainly the transport of water from deep to shallower soil layers through roots to maintain physiological activity (Canadell et al., 1996; Nepstad et al., 1994; Schulze et al., 1996), is common in several plant species (Caldwell and Richards, 1989; Caldwell et al., 1998; Peñuelas and Filella, 2003; Prieto et al., 2012; Wan et al., 2000). Plants also intensely influence soil; interdependence between changes in plant communities and in soil properties, such as fertility, has been observed in the Mediterranean Basin, especially in dry areas (Ruiz-Sinoga et al., 2011). For example, increased plant cover had a direct effect on soil porosity by increasing water infiltration and decreasing runoff (Garcia-Estringana et al., 2010; Goberna et al., 2007; Johnson-Maynard et al., 2002). The enhancement of soil quality by plant cover thus improves fertility (Gallardo et al., 2000) and enzymatic activity (Garcia et al., 2002), which in turn have positive effects on plant metabolism and growth (Ruiz-Sinoga et al., 2011; Sadaka and Ponge, 2003). The depth and texture of the topsoil, amongst other topographic factors, and the link between vegetation and soil are thus fundamental to the availability of water and nutrients for plants and may further play crucial roles in determining plant cover, habitat fragmentation, landscape patchiness, and changes in biodiversity (Fahrig, 2003; Sardans and Peñuelas, 2014; Allen and Breshears, 1998).

Mediterranean and other arid and semi-arid ecosystems have marked seasonality, with hot and dry summers (Aschmann 1973; Schwinning et al., 2004). Soil depth can play a critical role in these ecosystems, because summer drought is an important factor limiting the growth of plants (Ogaya et al., 2003) and determining tree mortality (Barbeta et al., 2013). High temperatures and the absence of precipitation during summer deplete moisture in the upper soil layers, so plant activity can only be sustained where enough moisture is available in deep soil layers (Barbeta et al., 2015). Mortality and crown damage caused by extreme droughts are usually minimised where soil is deep (Lloret et al., 2012). The most recent climatic models project more frequent and severe droughts in Mediterranean ecosystems (IPCC, 2007). The

effects of the increased drought conditions have already been observed during the last two decades affecting forest communities by increasing crown defoliation and tree mortality (Allen et al., 2010; Bigler et al., 2006; Carnicer et al., 2011; Galiano et al., 2011, 2010; McDowell et al., 2008; Poyatos et al., 2013; Rebetez and Dobbertin, 2004). The effects of drought on plant fitness and performance may also trigger important cascade effects through various trophic levels (Harrison, 2001; Kuske et al., 2003), thus producing important changes to entire ecosystems. A future exacerbation of drought in Mediterranean and other arid and semi-arid ecosystems may thus potentially affect, in a more intense or different way, the metabolism of trees growing in shallow soils with low capacities to store water, thereby leading to landscape patchiness and desertification (Sardans and Peñuelas, 2014). Forest decline and/or large changes in the structure of the vegetation and habitat are thus more likely in areas with shallower soil that are more susceptible to extreme drought (Galiano et al., 2012; Lloret et al., 2004).

Plants can adjust their metabolisms to maintain homeostasis under marked seasonality (Bertram et al., 2010; Falasca et al., 2013; Rivas-Ubach et al., 2014, 2012). Extreme droughts can cause drastic vegetation shifts, especially in Mediterranean, semi-arid, and arid ecosystems (Allen and Breshears, 1998; Hanson and Weltzin, 2000; Mueller et al., 2005), so the study of plant metabolomes can contribute to our understanding of how plants can metabolically cope with intense drought stress. Plants under drought conditions can adjust their chemistry to maintain physiological functions by, for example, increasing foliar concentrations of K (Cakmak and Engels, 1999; Sardans and Peñuelas, 2015), proline (Tymms and Gaff, 1979; Yamada et al., 2005), antioxidants (Rivas-Ubach et al., 2014), sugars (Ingram and Bartels, 1996; Porcel and Ruiz-Lozano, 2004; Rivas-Ubach et al., 2014), and/or other species-specific compounds (Sardans et al., 2011).

The metabolome, the chemical phenotype of an organism, is the total set of low molecular weight metabolites (typically <1200 Da) present in an organism at a particular moment (Fiehn, 2002). The metabolome, includes thus amino acids, sugars, and nucleotides from primary plant metabolism and many secondary metabolites such as phenolics and terpenes representing the diverse physiological processes in an organism for maintaining internal homeostasis and function. The first functional responses of an organism facing abiotic and biotic stressors are typically at the metabolomic level (Peñuelas and Sardans, 2009). Metabolomics represents thus a powerful tool for ecological studies (ecometabolomics) to identify the main changes in organisms directly associated with metabolism and performance (Sardans et al., 2011). Metabolomics allows us to understand the metabolic variation of organisms under stressful environmental conditions, including the complete set of metabolites

and not just single compounds or families of metabolites (Fiehn, 2002; Bundy et al., 2008; Sardans et al., 2011). The study of the metabolomic changes of wild plant species helps to comprehend the mechanisms behind plant physiological responses to natural or experimental stressors. Metabolomic techniques can also assess the plasticity of specific metabolomes and detect and quantify the metabolic biomarkers linked with specific environmental stressors (Bundy et al., 2008; Sardans et al., 2011; Rivas-Ubach et al., 2016a). Ecometabolomics has advanced our understanding of the natural variability and flexibility of the metabolomes of wild organisms under climatic stressors (Gargallo-Garriga et al., 2014; Rivas-Ubach et al., 2014), amongst seasons (Rivas-Ubach et al., 2012), and under attack from folivorous insects (Rivas-Ubach et al., 2016b). Ecometabolomics is thus valuable for exploring the organism-environment interaction by detecting and quantifying the final phenotypic response of an organism to environmental changes.

The physiological response to drought of *Quercus ilex* L., an evergreen sclerophyllous tree species widely distributed in the Mediterranean Basin (Barbero et al., 1992), has been extensively studied (Filella et al., 1998; Nardini et al., 2000; Ogaya and Peñuelas, 2003; Peñuelas et al., 2000; Sala and Tenhunen, 1996). *Q. ilex* is a keystone species in many Mediterranean ecosystems and is currently expanding its dominance by recolonising abandoned cropland and pastures and by out-competing Mediterranean conifers that are more sensitive to rising temperatures (Carnicer et al., 2013). Drought-induced declines in *Q. ilex* forests have been reported (Galiano et al., 2012; Camarero et al., 2015), but this tree possesses an array of functional and morphological traits (such as an extensive root system) for surviving periods of drought. Ecometabolomic studies of *Q. ilex* could thus identify the key metabolites involved in drought tolerance and resistance as well as measure how flexible are the individual metabolomes under stress conditions. We sampled once per season the leaves of differently sized mature *Q. ilex* trees of the same age from a forest exposed to a moderate experimental drought and analysed the elemental stoichiometries and metabolomes. We discuss three important issues of *Q. ilex* metabolic responses to environmental variables or factors by multivariate approximations: (i) we hypothesise that the depth of the upper soil layers (DUSL, typically the A + B horizons), where most of the biological activity and water uptake occur, may determine vegetation structure (VS) and overall metabolomic composition; (ii) we evaluate how different VSs, with special attention to trees growing in shallower soils, respond to the marked seasonality of the Mediterranean Basin and to experimental drought stress; and (iii) we apply the results of this study to illustrate the crucial necessity in ecometabolomic studies of controlling the factors potentially able to produce large metabolomic shifts in plants.

Material & Methods

Study site

This study was carried out in a natural *Q. ilex* forest in the Prades Mountains in southern Catalonia, Spain (41°21'N, 1°2'E). The climate is mesic-Mediterranean, with a marked three-month summer drought. The average annual rainfall is 658 mm, and the average temperature is 12 °C. The forest canopy is dominated by *Q. ilex*, followed by *Phillyrea latifolia* and *Arbutus unedo*, with average trunk basal areas at 50 cm of 20.8, 7.7, and 6.9 m² ha⁻¹, respectively. Other species adapted to drought conditions are *Juniperus oxycedruys*, *Erica arborea*, and *Cistus albidus*, with sporadic individuals of deciduous species such as *Acer monspesulanum* and *Sorbus torminalis*.

Tree size in the forest is naturally variable, so VS is also variable. We categorised the VSs by designating the areas dominated by taller trees and higher canopies as H-Forest and the areas dominated by smaller trees and lower canopies as L-Forest. The average trunk diameters at 50 cm above the soil (D50s) of the trees of H- and L-Forests were 41.2 and 27.8 cm, respectively.

Experimental design

Eight plots (15 × 10 m) were established in March 1999 at the same altitude (930 m a.s.l.) on a 25% mountain slope facing south-southeast (Ogaya et al., 2003). Four plots were established in each of H- and L-Forest areas to represent the natural variation of the VS of the *Q. ilex* forest. Two randomly chosen plots for each VS received a drought treatment, and the other two served as control plots. The plots were separated by a minimum of 15 m and were located along the same slope position. The drought treatment consisted of covering approximately 30% of the soil surface with 14 × 1 m PVC strips from the top to the bottom edges of the plots at 0.5-0.8 m above the soil, which partially excluded throughfall. Upslope runoff was intercepted by ditches 0.8-1 m deep dug along the entire top edges of the treatment plots. All water intercepted by the strips and ditches was channelled to the bottom edges of the drought plots.

Measurement of plot parameters

Soil depth was measured by inserting a 1.5-m metallic corer with a pneumatic hammer 0.7-1 m from the trunk of each sampled tree. The depths of the A and B horizons (upper soil layers; DUSL) were measured *in situ*, and soil samples were collected for calculating the proportion of fine soil (% <2mm). The samples were dried for 72 h at 60 °C and were then sieved through a 2-mm mesh, and the fine-soil and stony fractions were weighed. The density of each horizon

was calculated. Stone density was considered to be 2.65 g cm^{-3} (Chepil, 1950). The proportions of fine soil and stones were thus calculated at the tree level. The moisture content of the upper soil was measured during each campaign by time-domain reflectometry (Tektronix 1502C, Beaverton, USA) (Gray and Spies, 1995; Zegelin et al., 1989). Three stainless-steel cylindrical rods 25 cm long were vertically installed in the upper 25 cm of the soil at four randomly selected locations in each plot. The time-domain reflectometer was manually attached to the ends of the rods for each measurement.

The aboveground biomass (AB) in the plots was estimated by allometric relationships between tree AB and D50. Twelve *Q. ilex* trees were harvested outside the plots, their circumferences at a height of 50 cm were measured, and their ABs were weighed after drying in an oven to constant weight. We used the calculated allometric relationships to estimate the AB ($\ln AB = 4.9 + 2.3 \ln D50$; $R^2 = 0.98$; $n = 12$; $P < 0.001$).

Moisture content of the upper soil layers and precipitation were monitored every 30 min in all plots (see Ogaya and Peñuelas (2006) for details). Fig. 1a shows the seasonal accumulated annual precipitation in the study area for 1999-2009. Fig. 1b shows the differences of soil moisture amongst the plots and VSs for each season for 2004-2009 (5 years prior to sampling).

Sampling and processing of leaves

We randomly selected five *Q. ilex* individuals from each plot as study cases. Leaves were sampled once per season February in winter, May in spring, August in summer, and November in autumn. A small sunlit branch was cut from each tree with a pole, and a fraction of the youngest leaves was immediately frozen in liquid nitrogen for the stoichiometric and metabolomic analyses. The youngest leaves were always sampled to standardise the cohort and control any ontogenic shift with season (see Rivas-Ubach et al. (2014) for details). The leaves were collected within a short period between 11:00 and 14:00 to avoid large temporal metabolomic variation (Rivas-Ubach et al., 2013).

The leaves were processed as described in detail by Rivas-Ubach et al. (2013). Briefly, the frozen leaves were lyophilised, placed in plastic cans, and stored at -20°C until ground with a ball mill at 1600 rpm for 6 min (Mikrodismembrator-U, B. Braun Biotech International, Melsungen, Germany) to produce a fine powder for each sample. All samples were then stored in hermetic cans at -80°C until preparation for metabolite extraction.

Metabolite extraction for LC-MS

Metabolites were extracted as described by t'Kindt et al. (2008) with some modifications. Two sets of 2-mL tubes were labelled (A and B). Set A was used for the extractions, and the extracts

were transferred to set B. One hundred milligrams of powder of each foliar sample were introduced into a tube of set A. One millilitre of MeOH/H₂O (80:20) was added to each tube. The tubes were vortexed for 15 min and sonicated for 5 min at room temperature (22 °C) and were then centrifuged at 23000 × g for 5 min, and 0.6 mL of supernatant was collected from each tube and transferred to the corresponding tube of set B. This procedure was repeated to perform two extractions of each sample. The tubes of set B were then centrifuged at 23000 × g for 5 min. The supernatants were collected by crystal syringes, filtered with 0.22-µm pore microfilters, and transferred to a labelled set of HPLC vials. The vials were stored at -80 °C until LC-MS analysis.

Elemental analysis

For the C and N analyses, 1.4 mg of powder were transferred to a tin microcapsule. C and N concentrations were determined by elemental analysis using combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).

P and K concentrations were determined using acid digestion in a microwave at high pressure and temperature (Sardans et al., 2010); 250 mg of powder were weighed in a Teflon tube, and 5 mL of 65% nitric acid and 2 mL of H₂O₂ were added. The sample was then digested in a MARSXpress microwave (CEM, Mattheus, USA). All digested contents were added to a 50-mL flask and dissolved with Milli-Q water to 50 mL. P and K concentrations were determined by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (The Perkin-Elmer Corporation, Norwalk, USA).

LC-MS analyses

LC-MS chromatograms were obtained using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC, Dionex, Waltham, USA) coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with an HESI II (heated electrospray ionisation) source. Chromatography was performed on a reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3 µm particle size; Thermo Scientific, Waltham, USA) at 30 °C. The mobile phases consisted of acetonitrile (A) and 0.1% acetic acid (B). Both mobile phases were filtered and degassed for 10 min in an ultrasonic bath prior to use. The elution gradient began at 10% A (90% B) at a flow rate of 0.3 mL min⁻¹ and was maintained for 5 min, then changed linearly to 10% B (90% A) for the next 15 min. The initial proportions (10% A, 90% B) were gradually recovered over the next 5 min, and the column was then washed and stabilised for 5 min before injection of the next sample. The injection volume of the samples was 5 µL. All samples were injected twice, once with the HESI operating in

negative ionisation mode (-H) and once in positive ionisation mode (+H). The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60000). The resolution and sensitivity of the spectrometer were monitored by injecting a caffeine standard after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analysed during the sequence (see Rivas-Ubach et al., 2016b for more details of the instrumentation parameters).

Processing of LC-MS chromatograms

The raw data files from the Orbitrap were processed by MZmine 2.12 (Pluskal et al., 2010). Chromatograms were baseline corrected, deconvoluted, aligned, and autoassigned (see Table A.1 for details). Metabolomic variables were assigned by the exact mass and retention time of our metabolite library performed by the injection of over 200 standards representing several common metabolites of both primary and secondary metabolism (see Table A.2 for assignments). Numerical data sets were exported to CSV and posteriorly filtered; outliers and variables present in fewer than six individuals were removed from the data set. Outlier variables were defined as measurements 3-fold higher than the 3rd quartile or 3-fold lower than the 1st quartile of each cell factor. The areas under the peaks of the deconvoluted chromatograms do not reflect the real concentration as weight of metabolite per weight of the sample but are directly correlated with the concentration of the corresponding variable. The areas are thus suitable for metabolomic comparative analyses, as demonstrated in several metabolomic studies (Gargallo-Garriga et al., 2014; Lee and Fiehn, 2013; Leiss et al., 2013; Mari et al., 2013; Rivas-Ubach et al., 2014, 2016a, 2016b). We use the term *concentration* to refer to the relative concentration of a metabolite.

Statistical analyses

The data set consisted of three categorical independent variables, season (winter, spring, summer, and autumn), VS (H-Forest and L-Forest), and treatment (control and drought), and 2784 dependent continuous variables, ten of which were elemental concentrations and stoichiometric variables (C, N, P, and K concentrations and C/N, N/P, C/P, C/K, N/K, and K/P ratios) and 2774 of which were metabolomic variables, including 35 identified by our plant library.

The D50 and DUSL of all trees and the AB of the plots were subjected to one-way ANOVAs with VS as a factor to test for differences between H- and L-Forests (Fig. A.1). Shapiro and Levene's tests were used on all metabolomic and stoichiometric variables to assess the

normality and homogeneity of the variances, respectively. All known variables were normally distributed, and the variances of the groups were homogeneous. Any unidentified metabolomic variable that was not normally distributed or for which the variance of the group was not homogeneous was removed from the data set before statistical analysis to comply with the assumptions of the tests (0.64% of the variables were removed from the data set).

The entire data set, including all stoichiometric and metabolomic variables of the *Q. ilex* leaves (2784 variables), were first subjected to a PERMANOVA analysis using the *Bray Curtis* distance to test for differences in elemental stoichiometries and metabolomes between seasons, in the experimental drought treatment, and in VS (L-Forest/H-Forest). The number of permutations was set at 10000 (Table 1). The same data set was also subjected to a principal component analysis (PCA) (Fig. 2) to determine the natural variability amongst samples. The coordinates of cases of the PCA plot were subjected to a one-way ANOVA to identify statistical differences between the analysed groups (See Rivas-Ubach et al. (2013) for details).

The Euclidian distances for each *Q. ilex* individual between control and droughted trees of both H-Forest and L-Forest (HFC vs HFD and LFC vs. LFD, respectively) and between H-Forest and L-Forest controls (HFC. vs. LFC) were calculated using the coordinates of the first 15 PCs of the PCA, which explained the 54.7% of the total variance. The explained variation of each PC was used for the calculation of the Euclidian distance (PC1 (9.7%); PC2 (6.5%); PC3 (6.1%); PC4 (5.0%); PC5 (4.5%); PC6 (3.8%); PC7 (3.4%); PC8 (2.8%); PC9 (2.2%); PC10 (2.1%); PC11 (2.0%); PC12 (1.8%); PC13 (1.7%); PC14 (1.6%); PC15 (1.5%)). The distances between groups were then submitted to one-way ANOVAs (Fig. 3).

PLS-DAs (partial least squares discriminant analyses) and PCAs were also performed for each season for the elemental and stoichiometric data and the foliar metabolomic fingerprints of the *Q. ilex* (Figs. 4 and A.2, respectively). The PLS-DAs were performed to clearly identify the variables that could discriminate the various factors studied. The PLS-DAs were represented in bi-plots, and case distribution in multidimensional space was represented by different colours. Individual cases of the PLS-DAs are presented in Fig. A.3.

PC2 of the PCA clearly separated the metabolomes of H- and L-Forests (Fig. 2), so we used a structural equation model (SEM) to detect the overall relationships (total, direct, and indirect) of the soil data (DUSL, texture, and moisture) and the experimental drought on D50 and the metabolomic variation along PC2 of the PCA of the stoichiometric and metabolomic data from all seasons (Fig. 5; Table 2). We selected the variation along PC2 of the seasonal PCA (Fig. 2) because it clearly separated H- and L-Forests. The data for soil humidity of the SEM corresponded to the seasonal average for 2004-2009. All cases for each season were included in the model, so each *Q. ilex* tree in each plot and season had a corresponding measure of soil

humidity. We incorporated the experimental treatment (categorical variable) into the model by transforming it into a *dummy* variable (control = 1, drought = 0). Standard errors and the significance levels (*P* values) of the direct, indirect, and total effects were calculated with bootstrapping (with 1200 repetitions) (Davison et al., 1986). We ran the model with various stipulated relationships and chose the simplest model explaining the most variance of PC2 (metabolomic variance) (Fig. 5). The initial model is shown in Fig. A.4, and the total, direct, and indirect effects are shown in Table A.6. Plant metabolomes can vary considerably in response to small environmental shifts, so the SEM model was also performed using the soil humidity of the sampled year (2009-2010), but the overall results were consistent with the seasonal averaged data for 2004-2009. We finally used the 5 year averaged data (2004-2009) of soil moisture for the SEM, because it is a better proxy of tree size than the soil moisture of the sampling year. A heat map of all assigned variables was plotted for all control trees in all seasons to identify the main metabolite shifts amongst seasons and VSs. The relative concentrations for each variable were scaled to the same level before plotting the heat map (Fig. 6).

All statistical analyses were performed with R (R Core Team, 2013). The Shapiro tests and ANOVAs were performed with the *shapiro.test* and *aov* functions, respectively, in the “R stats” package (R Core Team, 2013). Levene’s test was performed with the *levene Test* function in the “car” package (Fox and Weisberg, 2011). The PERMANOVA analysis was conducted with the *adonis* function in the “vegan” package (Oksanen et al., 2013). The PCAs and PLS-DAs were performed by the *pca* and *plsda* functions, respectively, of the R “mixOmics” package (Le Cao et al., 2015). The SEM was performed with the *sem* function of the “sem” package (Fox et al., 2015). The heat map was constructed with the *heatmap.2* function in “gplots” package (Warnes et al., 2015). Post-hoc tests were conducted with the *HSD.test* function in “agricolae” package (de Mendiburu 2015).

Results

D50, DUSL, and AB were higher in H-Forest than in L-Forest ($F = 17.7$, $P < 0.001$; $F = 11.1$, $P < 0.01$; and $F = 60.1$, $P < 0.0001$; respectively) (Fig. A.1). Spring was the wettest season, as expected, followed by winter, autumn, and summer ($F = 10.45$; $P < 0.0001$) (Fig. 1a). Autumn was drier than winter from 1999 to 2009, but the difference was not statistically significant ($P > 0.05$). A factorial ANOVA of soil moisture, including all categorical factors, was significant for season ($F = 108.2$; $P < 0.0001$), VS ($F = 6.4$; $P < 0.05$), and treatment ($F = 13.1$; $P < 0.001$), but none of the interactions were significant. Soil moisture was significantly higher in winter and spring than in summer and autumn (Fig. 1b). The PERMANOVA clearly identified significant

differences in the elemental concentrations, stoichiometric ratios, and metabolomes for the *Q. ilex* leaves between seasons (Pseudo- $F = 31.04$, $P < 0.0001$), between VS types (Pseudo- $F = 3.31$, $P < 0.05$), between experimental treatments (Pseudo- $F = 3.98$, $P < 0.05$), and in the VS×treatment interaction (Pseudo- $F = 9.07$, $P < 0.001$). The season×VS and season×treatment interactions were not significant ($P > 0.05$) (Table 1).

The one-way ANOVA of case coordinates of the seasonal PCA identified significant differences amongst seasons along PC1 ($F = 10.9$, $P < 0.0001$) and marginal differences along PC2 ($F = 2.27$, $P = 0.08$) (marginal differences are not indicated in the graph), but the case plot did not clearly cluster cases for each season (Fig. 2b). The cases, however, were better separated between the two VSs in the annual PCA in both PCs, but especially in PC2 ($F = 7.48$, $P < 0.01$ for PC1 and $F = 351$, $P < 0.0001$ for PC2) (Fig. 2c). The experimental drought treatment produced significant separation along PC1 when excluding VS ($F = 15.5$, $P < 0.001$). The significance of the drought treatment, however, was extended to PC2 within each VS (H-Forest: $F = 7.75$, $P < 0.01$ for PC1 and $F = 5.89$, $P < 0.05$ for PC2; L-Forest: $F = 58.9$, $P < 0.0001$ for PC1 and $F = 6.71$, $P < 0.05$ for PC2) (Fig. 2c). The metabolomes of the leaves shifted most strongly across seasons, but VS and the experimental drought also had significant effects (Table 1).

One-way ANOVAs of the metabolomic distances (Euclidian distances) calculated with the first 15 PCs of the PCA showed that the HFC vs. HFD distance in each of the seasons was smaller than the HFC vs. LFC distance. The LFC vs. LFD and HFC vs. LFC distances, however, did not differ for summer, autumn, or winter, and the LFC vs. LFD distance was even larger in spring (Fig. 3). The LFC vs. LFD distance was largest in winter and spring, the wetter seasons.

The factorial ANOVAs of all known variables, excluding the effects of seasonality (seasons taken together because the treatment×season and VS×season interactions were not significant in the PERMANOVA, see Table 1), identified several variables with a significant VS×treatment effect, suggesting that the drought treatment affected each VS differently (Table A.3). All elemental and stoichiometric variables, except C concentrations, and metabolites such as pentoses, valine, arginine, malic acid, epigallocatechin, homoorientin, chlorogenic acid, α -humulene, caryophyllene, and pyridoxine responded significantly to the VS×treatment interaction ($P < 0.05$). The factorial ANOVAs of all individual variables for the VS×treatment interaction for each season separately did not have many common responses with VS and treatment for specific variables amongst the seasons (Table A.4). The PLS-DA, however, clearly indicated some common responses of VS and treatment with the seasons (Fig. 4). The changes amongst VS and the treatments were not significant for all seasons, but L-Forest under the

drought treatment generally had higher concentrations of P, N, α -humulene, caryophyllene, disaccharides, pentoses, hexoses, leucine, succinic acid, lactic acid, chlorogenic acid, and catechin and higher N/K and C/K ratios (Fig. 4). The PLS-DAs, as expected, identified larger metabolomic differences along components 1 and 2 of the case plot between the control and droughted plants in L-Forest than in H-Forest in all seasons (Figs 4 and A.2).

The SEM indicated that the proportion of fine soil was not correlated with the moisture content of the upper soil, and DUSL did not influence the soil texture; soil texture was thus not included in the final model. All included variables (drought treatment, upper soil moisture, DUSL, and D50) had significant direct or indirect effects on the metabolomes of the leaves along PC2, explaining 38.4% of its total variation. D50 explained most of the variation, followed by the indirect effect of DUSL (Fig. 5; Table 2). DUSL and the effect of the experimental drought explained 48.8% of the variance of the D50 of the *Q. ilex* trees, with DUSL the most significant factor ($R^2 = 0.59$, $P < 0.00001$). Of the variables included in the model, only the drought treatment had a significant effect on upper soil moisture, explaining 3.5% of the total variance (Fig. 5; Table 2).

The factorial ANOVAs of all known variables for the control trees, with season and VS as categorical factors, indicated that several of the variables shifted significantly amongst seasons and VSs (Fig. 6). Biomarkers of drought and oxidative stress, i.e. epigallocatechin, epicatechin, catechin, gallic acid, quinic acid, kaempferol, rhamnetin, proline, and quercetin, did not differ significantly between VSs. Concentrations of quinic acid were higher in summer. The N:P ratio was generally lowest and the concentrations of some amino acids were generally higher in trees in spring, although not significantly (Fig. 6).

Discussion

Metabolomic variation, VS, and drought.

The drought treatment significantly affected the metabolomes and stoichiometries of the natural populations of *Q. ilex* (Table 1), as also reported for other plant species (Gargallo-Garriga et al., 2014; Rivas-Ubach et al., 2012; J. Sardans et al., 2012c; Sardans et al., 2011 and citations therein; Urano et al., 2009). The effects of the drought treatment on the foliar metabolomes and stoichiometries differed in the L- and H-Forests, as shown by the PERMANOVA (Table 1) and the case plot of the seasonal PCA, with the two VSs clearly separated along PC2 (Fig. 2c). Interestingly, the metabolomic distance in all seasons was smaller between the control and droughted trees in H-Forest (HFC vs HFD) than between the control trees of H-Forest and L-Forest (HFC vs LFC) (Fig. 3), indicating that tree metabolism was affected more by VS than by the experimental drought treatment. The distance between the

control and droughted trees, however, was significantly larger in all seasons in L-Forest than in H-Forest and this distance was larger in winter and spring, the wetter seasons, than in summer and autumn (Fig. 3). Moreover, case clustering in the PLS-DAs identified larger differences between the control and droughted trees in L-Forest than in H-Forest (Fig. 4). These results demonstrated a higher metabolomic sensitivity to drought of trees in shallower soils (L-Forest), supporting the importance of VS to the response to drought. In fact, shallower soil should be an important factor in VS evolution from continuous to patchy vegetation cover under increasingly arid conditions in desertified areas of the Mediterranean (Sardans and Peñuelas, 2014). In this scenario, areas with deeper soils would be more likely to remain covered. The large difference in the tree metabolomes between the two VSs exposed to the same experimental stress demonstrated the necessity of including VS as an important factor when designing experiments, especially metabolomic studies, in natural ecosystems. Most plants require well-balanced soil humidity to maintain physiological homeostasis, fitness, and growth. The SEM, however, indicated that the moisture content of the upper soil did not explain the variation in D50 of the plots (Fig. 5), in contrast to the results of previous studies (Huxman et al., 2004; Knapp and Smith, 2001; Orwig and Abrams, 1997). Other Mediterranean trees also depend on water stored in deep soil layers or in rock fractures to cope with summer drought (Barbeta et al., 2015; Voltas et al., 2015). The upper soil layers are typically very dry during summer (Fig. 1), so growth is determined more by the availability of deep water pools that may allow trees to reduce the period of growth cessation than by the amount of moisture in the upper layers (Lempereur et al., 2015). The effect of the drought treatment on soil moisture was noticeable in all seasons, including winter, when deep-water pools are replenished (Brooks et al., 2010). The effect the treatment on growth and mortality rates has been dampened over time by the larger reductions in density in the drought plots (Barbeta et al., 2013), but extremely dry summers still affect the drought plots more negatively (Barbeta et al., 2015). The negative effect of the drought treatment on D50 may thus be mediated by the depletion of deep-water pools caused by a chronically lower replenishment during cold seasons (Barbeta et al., 2015).

Interestingly, the moisture content of the upper soil was nevertheless significantly correlated with the metabolomic variation of *Q. ilex* L-Forest along PC2 (Fig. 5), which separated not only the VSs but also the experimental drought treatment within each VS (Fig. 2). The treatment, however, was not correlated with PC2, probably due the larger separation of VSs than the drought treatment along PC2. The significant relationship between soil moisture and PC2 potentially illustrates the effects of water availability on the metabolomes of plants, as has been reported in other metabolomic studies (Gargallo-Garriga et al., 2014;

Griesser et al., 2015; Rivas-Ubach et al., 2012, 2014; Sardans et al., 2011 and citations therein; Sun et al., 2014; Zhang et al., 2014). DUSL was not correlated with the moisture content of the upper soil, as expected, but was highly correlated with D50, which together with the effects of the experimental drought explained 48.6% of the D50 variance (Fig. 5). Hydraulic lift has been described in *Q. ilex* (David et al., 2007), but the SEM suggested that tree size (D50) and therefore VS were more dependent on the changes in DUSL than on upper soil moisture, indicating that upper soil moisture alone could not exclusively account for the VSs. The large metabolomic distance between the control and droughted trees (Figs. 2 and 3) and the significant VS×treatment interaction (Table 1), however, suggest that moisture content of the upper soil still played an important role in determining the physiology and homeostasis of the trees (Huxman et al., 2004; Rosenzweig, 1968; Sala et al., 1988), especially those under the effects of drought and in shallower soils (L-Forest).

Seasonality had a large effect on the *Q. ilex* metabolomes, in accordance with previous metabolomic studies (Rivas-Ubach et al., 2012, 2014), but the effect varied significantly between VSs (Fig. 6). The smaller distance between the control and droughted trees of L-Forest in summer and autumn, the driest seasons (Fig. 1), suggest that the control trees experienced some level of natural drought stress in those seasons (Figs. 1 and 3). This seasonal trend, however, was not significant in H-Forest. The significant shift with season in metabolomic distance between the control and droughted trees in L-Forest, largest in winter and spring and smallest in summer and autumn (Fig. 3), indicated that the responses of *Q. ilex* to drought in shallower soils were tightly linked with seasonality. This variable response of VS to seasonality also suggests that *Q. ilex* trees in shallower soils are more sensitive to the typically large natural environmental changes in Mediterranean ecosystems. Drivers of global change such as drought can lead to long-term changes in VS by reducing the growth of trees (Barber et al., 2000; Bréda et al., 2006), which may consequently negatively impact soil fertility (Gallardo et al., 2000) and enzymatic activity (Garcia et al., 2002) and may increase runoff (Garcia-Estringana et al., 2010; Goberna et al., 2007; Johnson-Maynard et al., 2002), thereby reducing soil depth. Shallower soils can generally have negative feedbacks on plants (Ruiz-Sinoga et al., 2011; Sadaka and Ponge, 2003), which may result in a higher vulnerability to increases in the intensity, frequency, and duration of drought forecasted for the coming decades, especially in the Mediterranean Basin (IPCC, 2007).

Foliar chemical changes, drought, and VS.

Foliar K concentrations were similar in the two VSs and in the droughted and control trees (Fig. 4; Table A.4). K concentrations, however, interestingly varied significantly amongst seasons, with the highest concentrations in summer, the driest season (Figs. 2 and 3). Several plant species allocate large amounts of K to leaves during the driest season, especially in Mediterranean evergreen and dry tropical forests (Milla et al., 2005; Rivas-Ubach et al., 2012; Sardans and Peñuelas 2015). K is involved in several plant mechanisms that mitigate drought stress (Sardans and Peñuelas, 2015), for example the maintenance of cellular turgor and osmotic pressure (Ashraf et al., 2002; Levi et al., 2011), the control of transpiration and water conductance (Arquero et al., 2006; Benlloch-González et al., 2010; Harvey and van den Driessche, 1999), the control of stomata (Benlloch-González et al., 2010; Talbott and Zeiger, 1996), and the maintenance of transmembrane potentials (Su et al., 2001; Waraich et al., 2011). K can even act as an osmolyte (Babita et al., 2010; Levi et al., 2011) and can thus improve the capacity to retain water (Nandwal et al., 1998). K concentrations were not higher in the droughted trees but were higher in all trees in the driest season, as reported in other studies (Sardans and Peñuelas 2015 and citations therein), suggesting that increases in foliar K concentration are a programmed phenological response of plants to cope with the driest season.

Oxidative stress is expected to increase in plants under conditions of drought, especially in water-limited ecosystems such as those in the Mediterranean Basin (Price et al., 1989; Dat et al., 2000; Munné-Bosch & Peñuelas, 2004; Peñuelas et al., 2004). The concentrations of antioxidants has been reported to increase in water-stressed plants for coping with oxidative stress, so these compounds are excellent biomarkers of drought stress (Farooq et al., 2009; Reddy et al., 2004; Wang et al., 2003). Their role as electron donors and the ability to alter peroxidation kinetics are the main properties that provide their strong antioxidant activity (Rice-Evans et al., 1997). The concentrations of most of the antioxidants identified in our study (epigallocatechin, epicatechin, catechin, gallic acid, quinic acid, kaempferol, rhamnetin, and quercetin) did not differ significantly between VSs (Figs. 4 and 6). Additionally, the concentration of proline, a well-known multifunctional amino acid that typically acts as osmoregulator under drought conditions (Szabados and Saviouré, 2010), was not significantly higher in L-Forest than in H-Forest (Figs. 4 and 6; Tables A.3 and A.4), suggesting that L-Forest trees, even growing in shallower soils, were not necessarily more water-stressed than H-Forest trees. The levels of most antioxidant compounds were not significantly higher in summer (Fig. 6), but flavonoid concentrations have been reported to be generally higher in summer in H-Forest trees (Rivas-Ubach et al., 2014). The concentration of quinic acid was higher in summer, in accordance with previous studies (Rivas-Ubach et al., 2012, 2014), especially in L-Forest

trees (Fig. 6). Several flavonoids are synthesised from quinic acid, tyrosine, and phenylalanine by the shikimic acid pathway (Draths et al., 1999; Harborne 1988), suggesting a potential activation of this metabolic route in summer to cope with oxidative stress. The metabolism of amino acids is complex; each amino acid is involved in several metabolic pathways, which complicates the interpretation of changes in their concentrations. The concentrations of several amino acids, however, were higher in spring, especially when compared to summer (Fig. 6).

Spring is the principal growing season in Mediterranean ecosystems, and the higher concentrations of several amino acids in spring may have been associated with higher growth rates (Rivas-Ubach et al., 2012). N:P ratios were also generally lowest in spring, and the tendency of lower N:P ratios in H-Forest trees than L-Forest trees potentially indicates the higher growth rate in spring in H-Forest than L-Forest according to the growth rate hypothesis (Elser et al., 1996). The concentrations of malic, pyruvic, and citric acids also tended to be higher in H-Forest trees, which may also account for the higher growth than in L-Forest trees (Fig. 6). The increase in carbohydrates in droughted plants is a common strategy to cope with drought stress. Carbohydrates can act as osmolytes, improving the hydric potential and thus maintaining cellular turgor (Ingram and Bartels, 1996; Leprince et al., 1993; Porcel and Ruiz-Lozano, 2004; Rivas-Ubach et al., 2014). The concentrations of carbohydrates were higher in our droughted L-Forest trees than in the other plots, including the H-Forest droughted plants (Figs. 2 and 4), which potentially indicates a higher drought stress in the droughted L-Forest trees.

We have reported some clues indicating drought stress in droughted *Q. ilex* trees, but plants can use various metabolic pathways to cope with drought depending on the intensity of the stress. Such responses are also typically species-specific, so some plant species may use proportionally higher concentrations of metabolites than others for osmoprotection (Leprince et al., 1993; Ingram and Bartels, 1996). The accumulation of carbohydrates in leaves, however, may also be due to the impairment of growth as drought progresses. The C-loading of trees is higher during periods of zero growth, such as the summer cessation of growth in Holm oak forests (Lemepreur et al., 2015), because source activity (photosynthesis) exceeds sink activity (growth) (Estiarte and Peñuelas 1999; Körner, 2003; Peñuelas and Estiarte 1998). The accumulation of carbohydrates can consequently be an acclimative mechanism, but it may also be a passive process indicating the impairment of sink activities at the individual level (Estiarte and Peñuelas 1999; Peñuelas and Estiarte 1998).

The synthesis of terpenes is typically associated with attacks by folivores and pathogens and is one of the main induced defensive chemical mechanisms in plants (Achotegui-Castells et

al., 2013; Huang et al., 2012; Köpke et al., 2010; Mumm and Hilker, 2006; Pare and Tumlinson, 1997). L-Forest droughted trees tended to have higher foliar concentrations of caryophyllene and α -humulene (Fig. 4), suggesting a higher herbivory pressure in L- than H-Forest trees during drought. The synthesis of caryophyllene, an indirect defensive compound that attracts predators and parasitoids of the herbivore (Köllner et al., 2008; Rasmann et al., 2005), is increased under folivory (Gouinguéné et al., 2001; Rivas-Ubach et al., 2016b). Rivas-Ubach et al. (2014) suggested that droughted H-Forest *Q. ilex* trees had significantly higher levels of folivory associated with the foliar concentrations of carbohydrates and antioxidants due drought stress. The higher foliar terpene concentrations in the droughted L-Forest trees found in our study, in addition to the increased drought stress, provide clues for the potentially higher susceptibility of these trees to herbivory and/or pathogenic stress (Gaylord et al., 2013; Rivas-Ubach et al., 2014; Rouault et al., 2006; Desprez-Loustau et al., 2006).

Metabolomics in natural ecosystems.

This study has demonstrated that metabolomic techniques are excellent tools for identifying metabolic shifts in plants and other organisms under conditions of stress (Sardans et al., 2011; Shulaev et al., 2008). These techniques are sufficiently sensitive to detect small shifts in the metabolomes of plants over seasons (Rivas-Ubach et al., 2012); under experimental drought treatments (Urano et al., 2009) and herbivorous attack (Rivas-Ubach et al., 2016a, 2016b); with salinity (Sanchez et al., 2008), warming (Gargallo-Garriga et al., 2015; Rivas-Ubach et al., 2012), nutrient toxicity (Navascués et al., 2012), and ultraviolet light (Broeckling et al., 2005); and amongst other stressors or environmental gradients (Sardans et al., 2011 and citations therein). Plant metabolomes can vary widely amongst individuals of the same species, even under the same environmental conditions (Gargallo-Garriga et al., 2015, 2014; Rivas-Ubach et al., 2014, 2012), which is indicative of their high plasticity. The metabolomic shifts detected in response to each of the factors in this study (season, treatment, and VS) are likely to have different directions, which prevents a clear clustering of cases from the same groups in the PCA and complicates the interpretation of the results for each of the factors (Figs. 2 and 6). It is also clear in the PERMANOVA showing the significance of the VS×treatment interaction suggesting that the effects of the drought treatment vary between VSs (Table 1). The large metabolomic variation between H- and L-Forests in our analyses (see Figs. 2, 3, 4 and 6) compelled us to include VS as another significant independent factor, which is not often included in ecophysiological field studies. The high sensitivity of metabolomics for detecting significant differences of slight metabolic shifts requires an awareness of any environmental factor that could potentially affect the composition of the metabolomes.

Our results have thus demonstrated the necessity of identifying and including in metabolomic studies any factor that may produce large metabolomic variation amongst individuals within the same group level of the primary factors studied (seasons and drought treatment in our study). Any uncontrolled and/or unknown environmental factor causing substantial shifts in the metabolomes could lead to inconclusive or even incorrect interpretations of the results for the primary factors. All ecometabolomic studies should thus pay special attention to including any factor in the experimental design that may significantly affect the metabolomes of plants.

Conclusions

- The metabolome of *Q. ilex* trees was highly dependent on the vegetation structure defined by tree size (H-Forest, L-Forest).
- The depth rather than the moisture content of the upper soil layers was correlated with vegetation structure, suggesting that shallow soils may not be able to sustain large *Q. ilex* trees.
- The effects of drought on the metabolomes of *Q. ilex* trees were stronger in trees in shallower soils (L-Forest). The depth of the upper soil layers is thus a potential factor determining the future of tree populations under drought conditions and possible further desertification, especially in Mediterranean ecosystems.
- Potassium concentrations were higher in summer, as in other studies, but were not higher in trees growing in shallower soils nor in droughted trees, supporting our hypothesis that the increase in K concentrations is a programmed phenological response of plants to cope with dry seasons.
- Metabolomics is a very sensitive technique for detecting shifts in metabolomes amongst individuals and for improving our understanding of the relationships of metabolomes with environmental variables. This high sensitivity indicates the necessity of identifying and analysing any component able to strongly affect the metabolomes of individuals within the same group.

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Table 1. Full factorial PERMANOVA model (excluding the triple interaction) of the complete metabolomic and stoichiometric data set: season, vegetation structure (VS), drought treatment, season×VS, season×drought treatment, and VS×drought treatment. Bold type indicate significant effects.

	Degrees of freedom	Sum of squares	Mean of squares	Pseudo- <i>F</i>	<i>P</i>
Season	3	4.06	1.35	31.0	0.00001
Vegetation structure (VS)	1	0.14	0.14	3.31	0.044
Drought treatment	1	0.17	0.17	3.98	0.025
Season×VS	3	0.12	0.04	0.90	0.47
Season×Treatment	3	0.17	0.06	1.33	0.24
VS×Treatment	1	0.40	0.40	9.07	0.0005
Residuals	147	6.40	0.04	0.56	
Total	159	11.5	1.00		

Table 2. Correlation coefficients (R^2) and the corresponding P values for the total, direct, and indirect effects of the simplified structural equation model (SEM) with tree diameter (D50), moisture content of the upper soil, and metabolomic variation (PC2) as endogenous variables and drought treatment, moisture content of the upper soil, depth of the upper soil layers (DUSL), and D50 as exogenous variables. The model is described in Figure 5.

TOTAL EFFECTS				
	Drought treatment	Upper soil moisture	DUSL	D50
D50	0.39 ($P < 0.001$)		0.64 ($P < 0.001$)	
Upper soil moisture	0.19 ($P < 0.001$)			
PC2	0.25 ($P < 0.001$)	0.24 ($P < 0.001$)	0.34 ($P < 0.001$)	0.53 ($P < 0.001$)
DIRECT EFFECTS				
	Drought treatment	Upper soil moisture	DUSL	D50
D50	0.39 ($P < 0.001$)		0.64 ($P < 0.001$)	
Upper soil moisture	0.19 ($P < 0.001$)			
PC2		0.24 ($P < 0.001$)		0.53 ($P < 0.001$)
INDIRECT EFFECTS				
	Drought treatment	Upper soil moisture	DUSL	D50
D50				
Upper soil moisture				
PC2	0.25 ($P < 0.001$)		0.34 ($P < 0.001$)	

Figure Captions.

Figure 1. Box plots indicating the medians and distributions of a) seasonal precipitation (mm) for 1999-2009 and b) seasonal soil moisture (v/v.) in the upper soil layers for each control and drought plot from H-Forest and L-Forest for 2004-2009. Black and white circles indicate the medians for the control and drought plots, respectively. H- and L-Forest boxes are white and grey, respectively. Different letters denote statistical differences after Tukey's post-hoc tests ($P < 0.05$).

Figure 2. Principal component (PC) 1 vs. PC2 of the principal component analysis (PCA) of the metabolomic and stoichiometric variables over four seasons for the leaves of *Q. ilex*. **(a)** Carbon (C), nitrogen (N), phosphorus (P), and potassium (K) ratios are shown in red. Metabolomic families are indicated by different colours: blue, sugars; green, amino acids; cyan, nucleotides; orange, organic acids of the tricarboxylic acid cycle; yellow, phenolics; dark red, other metabolites; violet, terpenes. Unassigned metabolomic variables are not represented in the graph. Metabolite abbreviations: Disacch (disaccharides), Hex (hexoses), Pent (pentoses), Ala (alanine), Arg (arginine), Asp (aspartic acid), Glu (glutamic acid), Leu (leucine), Phe (phenylalanine), Pro (proline), Trp (tryptophan), Tyr (tyrosine), Val (valine), Cit.ac (citric acid), Mal.ac (malic acid), Succ.ac (succinic acid), Pyr (pyruvate), Lac.ac (lactic acid), Cat (catechin), Chlo.ac (chlorogenic acid), Hom (homoorientin), Epi (epicatechin), Epigallo (epigallocatechin), Gall.ac (gallic acid), Kaemp (kaempferol), Lut (luteolin), Rham (rhamnetin), aHum (α -humulene), Cary (caryophyllene), Quer (quercetin), Qui.ac (quinic acid), and Pyrid (pyridoxine). **(b)** Case plot representing the cases by season (blue for winter, green for spring, red for summer, and orange for autumn). The coloured arrows indicate the means for PC1 of the corresponding season. **(c)** Case plot representing the cases by vegetation structure and experimental drought treatment. Black arrows indicate the means for the PC1 and PC2 of the corresponding vegetation structure (L-Forest or H-Forest). The coloured arrows indicate the means for PC1 and PC2 of the corresponding treatment (control or drought) in each vegetation structure. Different letters by the arrows indicate significant differences between seasons, vegetation structures, or experimental treatments ($P < 0.05$).

Figure 3. Metabolomic distance (based on Euclidian distances) for each season of the paired groups of samples H-Forest-Control vs H-Forest-Drought (HFC vs HFC, squares), L-Forest-Control vs L-Forest-Drought (LFC vs LFD, circles), and H-Forest-Control vs L-Forest-Control (HFC vs LFC, triangles). Different letters denote statistical significance amongst all distances after Tukey's post-hoc tests ($P < 0.05$).

Figure 4. Bi-plots of component 2 vs. component 1 of the partial least squares discriminant analysis of the metabolomic and stoichiometric variables for the leaves of *Q. Ilex* for (a) winter, (b) spring, (c) summer, and (d) autumn. The variables in the plots are the same as in Figure 2 and have the same codes. The areas of distribution of the cases are coloured by vegetation structure and experimental treatment: light green for H-forest controls, dark green for H-Forest droughted trees, orange for L-Forest controls, and red for L-Forest droughted trees. The original representations of the cases are shown in Figure A.3.

Figure 5. Simplified structural equation model including the moisture content of the upper soil, tree diameter (D50), and metabolomic variation (PC2) as endogenous variables and depth of the upper soil layers (DUSL) and experimental drought treatment as exogenous variables. The model coefficient (R^2) and the corresponding P value for each relationship are shown between the variables. Total, direct, and indirect effects are shown in Table 2.

Figure 6. P values of factorial ANOVAs for each known variable with season and vegetation structure (VS) as categorical factors (a). Significant and marginally significant P values are shown in red and blue, respectively. Heat map (hierarchical variable relationship) of the known variables for each season (winter, spring, summer, and autumn) and vegetation structure (H-Forest (H-F) and L-Forest (L-F)) (b). The colours represent the relative metabolite concentration amongst each of the groups. Red represents the highest concentration, and greater colour differences for each variable indicate larger differences amongst the groups. Different letters indicate statistical significance after HSD post-hoc test ($P < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

R^2 for endogenous variables		
Upper soil moisture	D50	PC2 (Metabolomic variation)
0.035	0.49	0.38

Figure 6