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*

78 **Introduction**

79 Soil provides a physical support system and a reservoir of water for terrestrial primary
80 producers (Montheith, 1981). A scarcity of soil resources, particularly water, is often
81 associated with restricted development of plant-soil systems and reduced biomass (Huxman et
82 al., 2004; Knapp and Smith, 2001; Orwig and Abrams, 1997). Soil biological activity and tree
83 growth can be limited by several factors such as nutrients (Bowman et al., 1993; Sardans and
84 Peñuelas, 2015; Sardans et al., 2012a, 2012b), light (Poorter, 1999), temperature (Epstein et
85 al., 1997), or water (Huxman et al., 2004; Rosenzweig, 1968; Sala et al., 1988). Topographic
86 factors such as slope variation and/or soil texture also play important roles in the retention
87 and storage of soil water (Farahani et
88 al., 1998; Fernandez-Illescas et al., 2001) and can influence soil enzymatic activity (Bastida et
89 al., 2008) and erosion (Kinnell and Cummings, 1993). Soil depth is tightly linked with the
90 physiology and respiration rates of tree roots (Pregitzer et al., 1998), the composition of
91 microbial communities (Fierer et al., 2003), and even plant biodiversity (Fuhlendorf and
92 Smeins, 1998). Most of the biological activity and nutrient recycling in soil occurs in the upper
93 topsoil layers, so water availability in these layers is crucial to forests (Jobbagy and Jackson,
94 2000; Wardle et al., 2004). Hydraulic lift, mainly the transport of water from deep to shallower
95 soil layers through roots to maintain physiological activity (Canadell et al., 1996; Nepstad et al.,
96 1994; Schulze et al., 1996), is common in several plant species (Caldwell and Richards, 1989;
97 Caldwell et al., 1998; Peñuelas and Filella, 2003; Prieto et al., 2012; Wan et al., 2000). Plants
98 also intensely influence soil; interdependence between changes in plant communities and in
99 soil properties, such as fertility, has been observed in the Mediterranean Basin, especially in
100 dry areas (Ruiz-Sinoga et al., 2011). For example, increased plant cover had a direct effect on
101 soil porosity by increasing water infiltration and decreasing runoff (Garcia-Estringana et al.,
102 2010; Goberna et al., 2007; Johnson-Maynard et al., 2002). The enhancement of soil quality by
103 plant cover thus improves fertility (Gallardo et al., 2000) and enzymatic activity (Garcia et al.,
104 2002), which in turn have positive effects on plant metabolism and growth (Ruiz-Sinoga et al.,
105 2011; Sadaka and Ponge, 2003). The depth and texture of the topsoil, amongst other
106 topographic factors, and the link between vegetation and soil are thus fundamental to the
107 availability of water and nutrients for plants and may further play crucial roles in determining
108 plant cover, habitat fragmentation, landscape patchiness, and changes in biodiversity (Fahrig,
109 2003; Sardans and Peñuelas, 2014; Allen and Breshears, 1998).

110 Mediterranean and other arid and semi-arid ecosystems have marked seasonality, with
111 hot and dry summers (Aschmann 1973; Schwinning et al., 2004). Soil depth can play a critical
112 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 m^2 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.

218 Measurement of plot parameters

219 Soil depth was measured by inserting a 1.5-m metallic corer with a pneumatic hammer 0.7-1 m
220 from the trunk of each sampled tree. The depths of the A and B horizons (upper soil layers;
221 DUSL) were measured *in situ,* and soil samples were collected for calculating the proportion of
222 fine soil (% <2mm). The samples were dried for 72 h at 60 °C and were then sieved through a
223 2-mm mesh, and the fine-soil and stony fractions were weighed. The density of each horizon
224 was calculated. Stone density was considered to be 2.65 g cm^{-3} (Chepil, 1950). The proportions
225 of fine soil and stones were thus calculated at the tree level. The moisture content of the
226 upper soil was measured during each campaign by time-domain reflectometry (Tektronix
227 1502C, Beaverton, USA) (Gray and Spies, 1995; Zegelin et al., 1989). Three stainless-steel
228 cylindrical rods 25 cm long were vertically installed in the upper 25 cm of the soil at four
229 randomly selected locations in each plot. The time-domain reflectometer was manually
230 attached to the ends of the rods for each measurement.

231 The aboveground biomass (AB) in the plots was estimated by allometric relationships
232 between tree AB and D50. Twelve *Q. ilex* trees were harvested outside the plots, their
233 circumferences at a height of 50 cm were measured, and their ABs were weighed after drying
234 in an oven to constant weight. We used the calculated allometric relationships to estimate the
235 AB ($\ln AB = 4.9 + 2.3 \ln D50$; $R^2 = 0.98$; n = 12; $P < 0.001$).
236 Moisture content of the upper soil layers and precipitation were monitored every 30 min in all
237 plots (see Ogaya and Peñuelas (2006) for details). Fig. 1a shows the seasonal accumulated
238 annual precipitation in the study area for 1999-2009. Fig. 1b shows the differences of soil
239 moisture amongst the plots and VSs for each season for 2004-2009 (5 years prior to sampling).
240

241 Sampling and processing of leaves

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

250 The leaves were processed as described in detail by Rivas-Ubach et al. (2013). Briefly, the
251 frozen leaves were lyophilised, placed in plastic cans, and stored at -20 °C until ground with a
252 ball mill at 1600 rpm for 6 min (Mikrodismembrator-U, B. Braun Biotech International,

253 Melsungen, Germany) to produce a fine powder for each sample. All samples were then stored
254 in hermetic cans at -80 °C until preparation for metabolite extraction.
255
256 Metabolite extraction for LC-MS
257 Metabolites were extracted as described by t'Kindt et al. (2008) with some modifications. Two
258 sets of 2-mL tubes were labelled (A and B). Set A was used for the extractions, and the extracts
259 were transferred to set B. One hundred milligrams of powder of each foliar sample were
260 introduced into a tube of set A. One millilitre of MeOH/H_2O (80:20) was added to each tube.
261 The tubes were vortexed for 15 min and sonicated for 5 min at room temperature (22 °C) and
262 were then centrifuged at 23000 × g for 5 min, and 0.6 mL of supernatant was collected from
263 each tube and transferred to the corresponding tube of set B. This procedure was repeated to
264 perform two extractions of each sample. The tubes of set B were then centrifuged at 23000 × g
265 for 5 min. The supernatants were collected by crystal syringes, filtered with 0.22-µm pore
266 microfilters, and transferred to a labelled set of HPLC vials. The vials were stored at -80 °C until
267 LC-MS analysis.
268
269 Elemental analysis
270 For the C and N analyses, 1.4 mg of powder were transferred to a tin microcapsule. C and N
271 concentrations were determined by elemental analysis using combustion coupled to gas
272 chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).
273 P and K concentrations were determined using acid digestion in a microwave at high
274 pressure and temperature (Sardans et al., 2010); 250 mg of powder were weighed in a Teflon
275 tube, and 5 mL of 65% nitric acid and 2 mL of H_2O_2 were added. The sample was then digested
276 in a MARSXpress microwave (CEM, Mattheus, USA). All digested contents were added to a 50-
277 mL flask and dissolved with Milli-Q water to 50 mL. P and K concentrations were determined
278 by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (The Perkin-Elmer
279 Corporation, Norwalk, USA).
280
281 LC-MS analyses
282 LC-MS chromatograms were obtained using a Dionex Ultimate 3000 HPLC system (Thermo
283 Fisher Scientific/Dionex RSLC, Dionex, Waltham, USA) coupled to an LTQ Orbitrap XL high-
284 resolution mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with an HESI
285 II (heated electrospray ionisation) source. Chromatography was performed on a reversed-
286 phase C18 Hypersil gold column (150 × 2.1 mm, 3 µm particle size; Thermo Scientific,
287 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 assignations). 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

323 stoichiometric variables (C, N, P, and K concentrations and C/N, N/P, C/P, C/K, N/K, and K/P
324 ratios) and 2774 of which were metabolomic variables, including 35 identified by our plant
325 library.
326 The D50 and DUSL of all trees and the AB of the plots were subjected to one-way ANOVAs
327 with VS as a factor to test for differences between H- and L-Forests (Fig. A.1). Shapiro and
328 Levene's tests were used on all metabolomic and stoichiometric variables to assess the
329 normality and homogeneity of the variances, respectively. All known variables were normally
330 distributed, and the variances of the groups were homogeneous. Any unidentified
331 metabolomic variable that was not normally distributed or for which the variance of the group
332 was not homogeneous was removed from the data set before statistical analysis to comply
333 with the assumptions of the tests (0.64% of the variables were removed from the data set).
334 The entire data set, including all stoichiometric and metabolomic variables of the *Q. ilex*
335 leaves (2784 variables), were first subjected to a PERMANOVA analysis using the *Bray Curtis*
336 distance to test for differences in elemental stoichiometries and metabolomes between
337 seasons, in the experimental drought treatment, and in VS (L-Forest/H-Forest). The number of
338 permutations was set at 10000 (Table 1). The same data set was also subjected to a principal
339 component analysis (PCA) (Fig. 2) to determine the natural variability amongst samples. The
340 coordinates of cases of the PCA plot were subjected to a one-way ANOVA to identify statistical
341 differences between the analysed groups (See Rivas-Ubach et al. (2013) for details).
342 The Euclidian distances for each *Q. ilex* individual between control and droughted trees of
343 both H-Forest and L-Forest (HFC vs HFD and LFC vs. LFD, respectively) and between H-Forest
344 and L-Forest controls (HFC. vs. LFC) were calculated using the coordinates of the first 15 PCs of
345 the PCA, which explained the 54.7% of the total variance. The explained variation of each PC
346 was used for the calculation of the Euclidian distance (PC1 (9.7%); PC2 (6.5%); PC3 (6.1%); PC4
347 (5.0%); PC5 (4.5%); PC6 (3.8%); PC7 (3.4%); PC8 (2.8%); PC9 (2.2%); PC10 (2.1%); PC11 (2.0%);
348 PC12 (1.8%); PC13 (1.7%); PC14 (1.6%); PC15 (1.5%)). The distances between groups were then
349 submitted to one-way ANOVAs (Fig. 3).
350 PLS-DAs (partial least squares discriminant analyses) and PCAs were also performed for
351 each season for the elemental and stoichiometric data and the foliar metabolomic fingerprints
352 of the *Q. ilex* (Figs. 4 and A.2, respectively). The PLS-DAs were performed to clearly identify the
353 variables that could discriminate the various factors studied. The PLS-DAs were represented in
354 bi-plots, and case distribution in multidimensional space was represented by different colours.
355 Individual cases of the PLS-DAs are presented in Fig. A.3.
356 PC2 of the PCA clearly separated the metabolomes of H- and L-Forests (Fig. 2), so we used
357 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

393 expected, followed by winter, autumn, and summer (F = 10.45; P < 0.0001) (Fig. 1a). Autumn
394 was drier than winter from 1999 to 2009, but the difference was not statistically significant (P
395 > 0.05). A factorial ANOVA of soil moisture, including all categorical factors, was significant for
396 season (F = 108.2; P < 0.0001), VS (F = 6.4; P < 0.05), and treatment (F = 13.1; P < 0.001), but
397 none of the interactions were significant. Soil moisture was significantly higher in winter and
398 spring than in summer and autumn (Fig. 1b). The PERMANOVA clearly identified significant
399 differences in the elemental concentrations, stoichiometric ratios, and metabolomes for the *Q.*
400 *ilex* leaves between seasons (Pseudo-F = 31.04, P < 0.0001), between VS types (Pseudo-F =
401 3.31, P < 0.05), between experimental treatments (Pseudo-F = 3.98, P < 0.05), and in the
402 VS×treatment interaction (Pseudo-F = 9.07, P < 0.001). The season×VS and season×treatment
403 interactions were not significant (P > 0.05) (Table 1).
404 The one-way ANOVA of case coordinates of the seasonal PCA identified significant
405 differences amongst seasons along PC1 (F = 10.9, P < 0.0001) and marginal differences along
406 PC2 (F = 2.27, P = 0.08) (marginal differences are not indicated in the graph), but the case plot
407 did not clearly cluster cases for each season (Fig. 2b). The cases, however, were better
408 separated between the two VSs in the annual PCA in both PCs, but especially in PC2 (F = 7.48, P
409 < 0.01 for PC1 and F = 351, P < 0.0001 for PC2) (Fig. 2c). The experimental drought treatment
410 produced significant separation along PC1 when excluding VS (F = 15.5, P < 0.001). The
411 significance of the drought treatment, however, was extended to PC2 within each VS (H-
412 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
413 for PC1 and F = 6.71, P < 0.05 for PC2) (Fig. 2c). The metabolomes of the leaves shifted most
414 strongly across seasons, but VS and the experimental drought also had significant effects
415 (Table 1).
416 One-way ANOVAs of the metabolomic distances (Euclidian distances) calculated with the
417 first 15 PCs of the PCA showed that the HFC vs. HFD distance in each of the seasons was
418 smaller than the HFC vs. LFC distance. The LFC vs. LFD and HFC vs. LFC distances, however, did
419 not differ for summer, autumn, or winter, and the LFC vs. LFD distance was even larger in
420 spring (Fig. 3). The LFC vs. LFD distance was largest in winter and spring, the wetter seasons.
421 The factorial ANOVAs of all known variables, excluding the effects of seasonality (seasons
422 taken together because the treatment×season and VS×season interactions were not significant
423 in the PERMANOVA, see Table 1), identified several variables with a significant VS×treatment
424 effect, suggesting that the drought treatment affected each VS differently (Table A.3). All
425 elemental and stoichiometric variables, except C concentrations, and metabolites such as
426 pentoses, valine, arginine, malic acid, epigallocatechin, homoorientin, chlorogenic acid, α-

427 humulene, caryophyllene, and pyridoxine responded significantly to the VS×treatment
428 interaction ($P < 0.05$). The factorial ANOVAs of all individual variables for the VS×treatment
429 interaction for each season separately did not have many common responses with VS and
430 treatment for specific variables amongst the seasons (Table A.4). The PLS-DA, however, clearly
431 indicated some common responses of VS and treatment with the seasons (Fig. 4). The changes
432 amongst VS and the treatments were not significant for all seasons, but L-Forest under the
433 drought treatment generally had higher concentrations of P, N, α-humulene, caryophyllene,
434 disaccharides, pentoses, hexoses, leucine, succininc acid, lactic acid, chlorogenic acid, and
435 catechin and higher N/K and C/K ratios (Fig. 4). The PLS-DAs, as expected, identified larger
436 metabolomic differences along components 1 and 2 of the case plot between the control and
437 droughted plants in L-Forest than in H-Forest in all seasons (Figs 4 and A.2).
438 The SEM indicated that the proportion of fine soil was not correlated with the moisture
439 content of the upper soil, and DUSL did not influence the soil texture; soil texture was thus not
440 included in the final model. All included variables (drought treatment, upper soil moisture,
441 DUSL, and D50) had significant direct or indirect effects on the metabolomes of the leaves
442 along PC2, explaining 38.4% of its total variation. D50 explained most of the variation, followed
443 by the indirect effect of DUSL (Fig. 5; Table 2). DUSL and the effect of the experimental drought
444 explained 48.8% of the variance of the D50 of the *Q. ilex* trees, with DUSL the most significant
445 factor ($R^2 = 0.59$, $P < 0.00001$). Of the variables included in the model, only the drought
446 treatment had a significant effect on upper soil moisture, explaining 3.5% of the total variance
447 (Fig. 5; Table 2).
448 The factorial ANOVAs of all known variables for the control trees, with season and VS as
449 categorical factors, indicated that several of the variables shifted significantly amongst seasons
450 and VSs (Fig. 6). Biomarkers of drought and oxidative stress, i.e. epigallocatechin, epicatechin,
451 catechin, gallic acid, quinic acid, kaempferol, rhamnetin, proline, and quercitin, did not differ
452 significantly between VSs. Concentrations of quinic acid were higher in summer. The N:P ratio
453 was generally lowest and the concentrations of some amino acids were generally higher in
454 trees in spring, although not significantly (Fig. 6).
455
456 **Discussion**
457 **Metabolomic variation, VS, and drought.**
458 The drought treatment significantly affected the metabolomes and stoichiometries of the
459 natural populations of *Q. ilex* (Table 1), as also reported for other plant species (Gargallo-
460 Garriga et al., 2014; Rivas-Ubach et al., 2012; J. Sardans et al., 2012c; Sardans et al., 2011 and
461 citations therein; Urano et al., 2009). The effects of the drought treatment on the foliar

462 metabolomes and stoichiometries differed in the L- and H-Forests, as shown by the
463 PERMANOVA (Table 1) and the case plot of the seasonal PCA, with the two VSs clearly
464 separated along PC2 (Fig. 2c). Interestingly, the metabolomic distance in all seasons was
465 smaller between the control and droughted trees in H-Forest (HFC vs HFD) than between the
466 control trees of H-Forest and L-Forest (HFC vs LFC) (Fig. 3), indicating that tree metabolism was
467 affected more by VS than by the experimental drought treatment. The distance between the
468 control and droughted trees, however, was significantly larger in all seasons in L-Forest than in
469 H-Forest and this distance was larger in winter and spring, the wetter seasons, than in summer
470 and autumn (Fig. 3). Moreover, case clustering in the PLS-DAs identified larger differences
471 between the control and droughted trees in L-Forest than in H-Forest (Fig. 4). These results
472 demonstrated a higher metabolomic sensitivity to drought of trees in shallower soils (L-Forest),
473 supporting the importance of VS to the response to drought. In fact, shallower soil should be
474 an important factor in VS evolution from continuous to patchy vegetation cover under
475 increasingly arid conditions in desertified areas of the Mediterranean (Sardans and Peñuelas,
476 2014). In this scenario, areas with deeper soils would be more likely to remain covered. The
477 large difference in the tree metabolomes between the two VSs exposed to the same
478 experimental stress demonstrated the necessity of including VS as an important factor when
479 designing experiments, especially metabolomic studies, in natural ecosystems. Most plants
480 require well-balanced soil humidity to maintain physiological homeostasis, fitness, and growth.
481 The SEM, however, indicated that the moisture content of the upper soil did not explain the
482 variation in D50 of the plots (Fig. 5), in contrast to the results of previous studies (Huxman et
483 al., 2004; Knapp and Smith, 2001; Orwig and Abrams, 1997). Other Mediterranean trees also
484 depend on water stored in deep soil layers or in rock fractures to cope with summer drought
485 (Barbeta et al., 2015, Voltas et al., 2015). The upper soil layers are typically very dry during
486 summer (Fig. 1), so growth is determined more by the availability of deep water pools that
487 may allow trees to reduce the period of growth cessation than by the amount of moisture in
488 the upper layers (Lempereur et al., 2015). The effect of the drought treatment on soil moisture
489 was noticeable in all seasons, including winter, when deep-water pools are replenished
490 (Brooks et al., 2010). The effect the treatment on growth and mortality rates has been
491 dampened over time by the larger reductions in density in the drought plots (Barbeta et al.,
492 2013), but extremely dry summers still affect the drought plots more negatively (Barbeta et al.,
493 2015). The negative effect of the drought treatment on D50 may thus be mediated by the
494 depletion of deep-water pools caused by a chronically lower replenishment during cold
495 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 quercitin) 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 Savouré, 2010), was not significantly

566 higher in L-Forest than in H-Forest (Figs. 4 and 6; Tables A.3 and A.4), suggesting that L-Forest
567 trees, even growing in shallower soils, were not necessarily more water-stressed than H-Forest
568 trees. The levels of most antioxidant compounds were not significantly higher in summer (Fig.
569 6), but flavonoid concentrations have been reported to be generally higher in summer in H-
570 Forest trees (Rivas-Ubach et al., 2014). The concentration of quinic acid was higher in summer,
571 in accordance with previous studies (Rivas-Ubach et al., 2012, 2014), especially in L-Forest
572 trees (Fig. 6). Several flavonoids are synthesised from quinic acid, tyrosine, and phenylalanine
573 by the shikimic acid pathway (Draths et al., 1999; Harborne 1988), suggesting a potential
574 activation of this metabolic route in summer to cope with oxidative stress. The metabolism of
575 amino acids is complex; each amino acid is involved in several metabolic pathways, which
576 complicates the interpretation of changes in their concentrations. The concentrations of
577 several amino acids, however, were higher in spring, especially when compared to summer
578 (Fig. 6).
579 Spring is the principal growing season in Mediterranean ecosystems, and the higher
580 concentrations of several amino acids in spring may have been associated with higher growth
581 rates (Rivas-Ubach et al., 2012). N:P ratios were also generally lowest in spring, and the
582 tendency of lower N:P ratios in H-Forest trees than L-Forest trees potentially indicates the
583 higher growth rate in spring in H-Forest than L-Forest according to the growth rate hypothesis
584 (Elser et al., 1996). The concentrations of malic, pyruvic, and citric acids also tended to be
585 higher in H-Forest trees, which may also account for the higher growth than in L-Forest trees
586 (Fig. 6). The increase in carbohydrates in droughted plants is a common strategy to cope with
587 drought stress. Carbohydrates can act as osmolytes, improving the hydric potential and thus
588 maintaining cellular turgor (Ingram and Bartels, 1996; Leprince et al., 1993; Porcel and Ruiz-
589 Lozano, 2004; Rivas-Ubach et al., 2014). The concentrations of carbohydrates were higher in
590 our droughted L-Forest trees than in the other plots, including the H-Forest droughted plants
591 (Figs. 2 and 4), which potentially indicates a higher drought stress in the droughted L-Forest
592 trees.
593 We have reported some clues indicating drought stress in droughted *Q. ilex* trees, but
594 plants can use various metabolic pathways to cope with drought depending on the intensity of
595 the stress. Such responses are also typically species-specific, so some plant species may use
596 proportionally higher concentrations of metabolites than others for osmoprotection (Leprince
597 et al., 1993; Ingram and Bartels, 1996). The acumulation of carbohydrates in leaves, however,
598 may also be due to the impairment of growth as drought progresses. The C-loading of trees is
599 higher during periods of zero growth, such as the summer cessation of growth in Holm oak
600 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 (Gouinguené 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$\times$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-*F*	*P*
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 ($P < 0.05$).

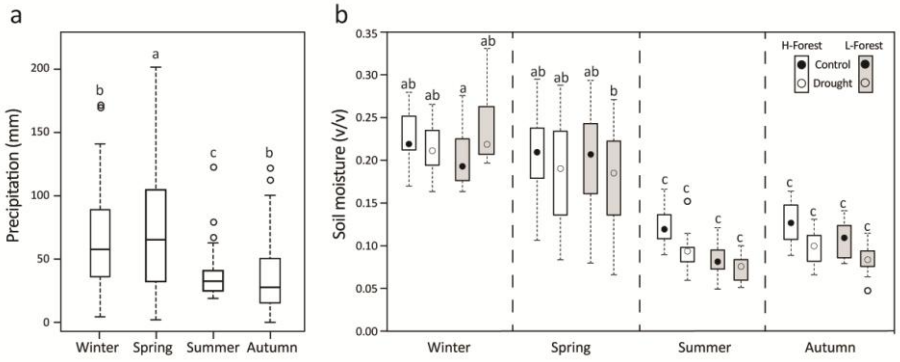

Figure 2. Principal component (PC) 1 vs. PC2 of the principal component analysis (PCA) of the metabolomic and stoichiometric variables over four seasons for the leaves of *Q. ilex.* (**a**) Carbon (C), nitrogen (N), phosphorus (P), and potassium (K) ratios are shown in red. Metabolomic families are indicated 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 ($P < 0.05$).

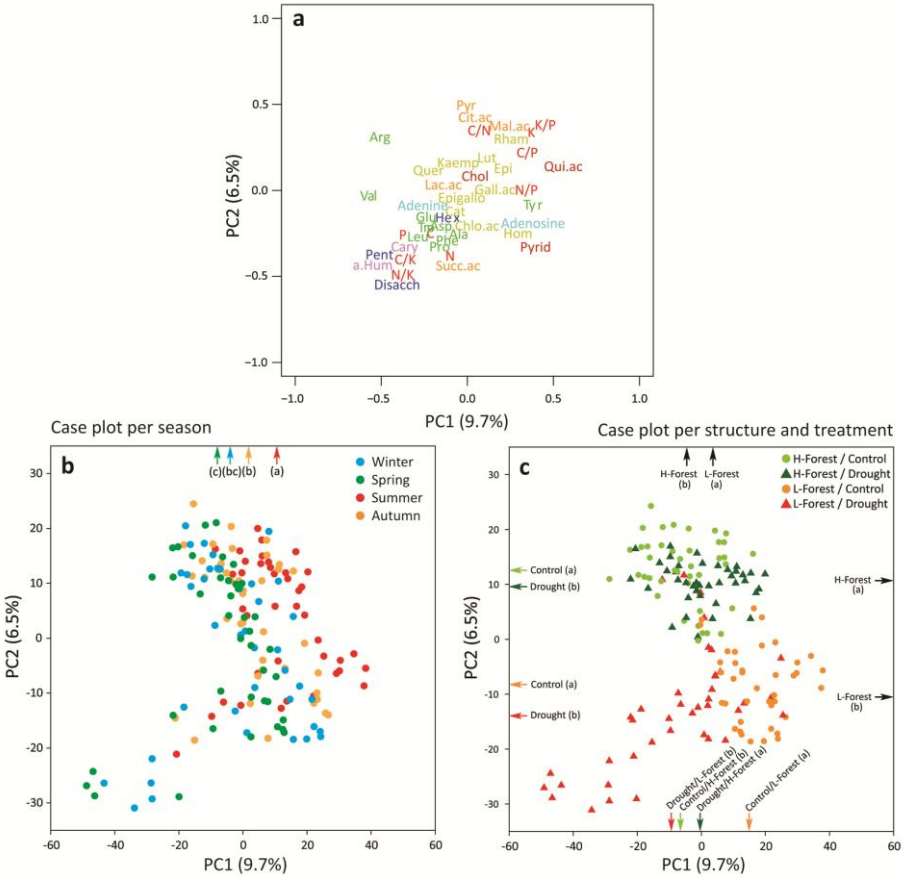
a
PC2 (6.5%)
PC1 (9.7%)
Pyr
Cit.ac
C/N
Mal.ac
K/P
K
Rham
Arg
C/P
Kaemp
Lut
Epi
Qui.ac
Quer
Chol
Lac.ac
Gall.ac
N/P
Epigallo
Val
Tyr
Adenine
Cat
Glu
Hex
Inos
Chlo.ac
Adenosine
Hom
Leu
Ala
Phe
Pyrid
Cary
Pro
Pent
C/K
N
a.Hum
Succ.ac
N/K
Disacch
Case plot per season
b
(c)(bc)(b)
(a)
Winter
Spring
Summer
Autumn
PC2 (6.5%)
PC1 (9.7%)
Case plot per structure and treatment
c
H-Forest
(b)
L-Forest
(a)
H-Forest / Control
H-Forest / Drought
L-Forest / Control
L-Forest / Drought
Control (a)
Drought (b)
H-Forest
(a)
Control (a)
Drought (b)
L-Forest
(b)
Drought/L-Forest (b)
Control/H-Forest (b)
Drought/H-Forest (a)
Control/L-Forest (a)
PC2 (6.5%)
PC1 (9.7%)

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).

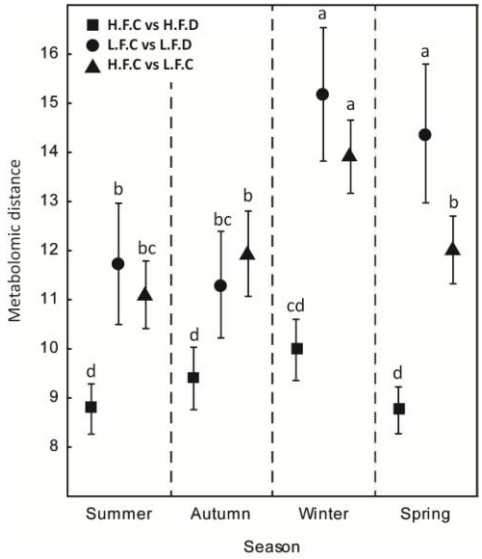

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Figure 4. Bi-plots of component 2 vs. component 1 of the partial least squares discriminant analysis of the metabolomic and stoichiometric variables for the leaves of *Q. Ilex* for (**a**) winter, (**b**) spring, (**c**) summer, and (**d**) autumn. 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.

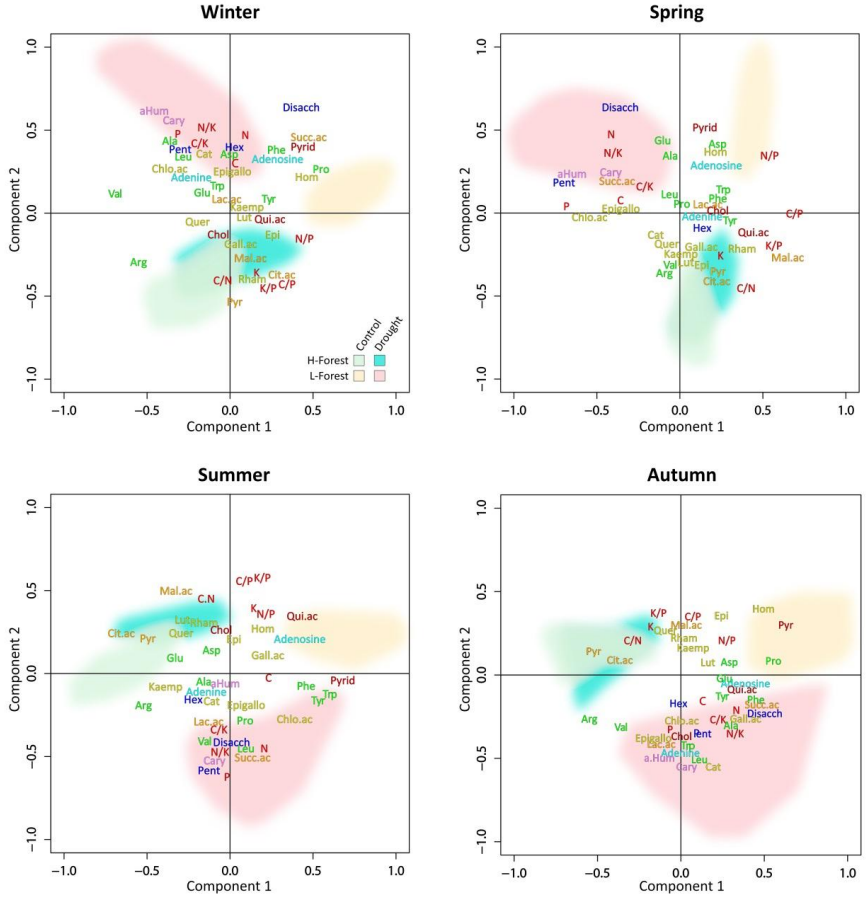

Figure 5. Simplified structural equation model including the moisture content of the upper soil, tree diameter (D50), and metabolomic variation (PC2) as endogenous variables and depth of the upper soil layers (DUSL) and experimental drought treatment as exogenous variables. The model coefficient (R^2)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

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

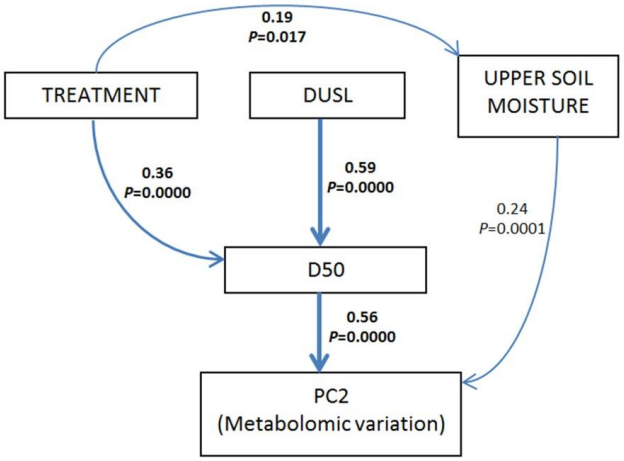

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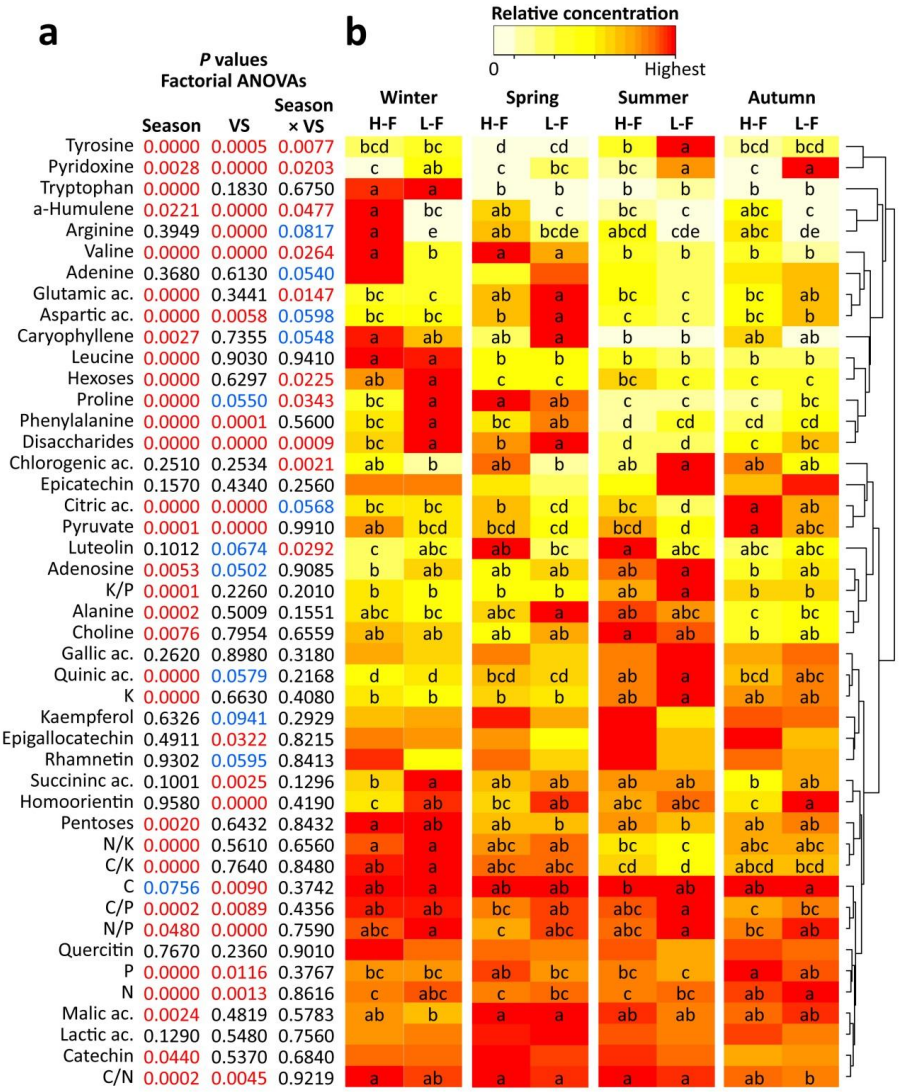
Figure 6. *P* values of factorial ANOVAs for each known variable with season and vegetation structure (VS) as categorical factors (a). Significant and marginally significant *P* values are shown in red and blue, respectively. Heat map (hierarchical variable relationship) of the known variables for each 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 statistically significance after HSD post-hoc test ($P < 0.05$).



	Season	VS	Season × VS	Winter H-F	Winter L-F	Spring H-F	Spring L-F	Summer H-F	Summer L-F	Autumn H-F	Autumn L-F
Tyrosine	0.0000	0.0005	0.0077	bcd	bc	d	cd	b	a	bcd	bcd
Pyridoxine	0.0028	0.0000	0.0203	c	ab	c	bc	bc	a	c	a
Tryptophan	0.0000	0.1830	0.6750	a	a	b	b	b	b	b	b
a-Humulene	0.0221	0.0000	0.0477	a	bc	ab	c	bc	c	abc	c
Arginine	0.3949	0.0000	0.0817	a	e	ab	bcde	abcd	cde	abc	de
Valine	0.0000	0.0000	0.0264	a	b	a	a	b	b	b	b
Adenine	0.3680	0.6130	0.0540								
Glutamic ac.	0.0000	0.3441	0.0147	bc	c	ab	a	bc	c	bc	ab
Aspartic ac.	0.0000	0.0058	0.0598	bc	bc	b	a	c	c	bc	b
Caryophyllene	0.0027	0.7355	0.0548	a	ab	ab	a	b	b	ab	ab
Leucine	0.0000	0.9030	0.9410	a	a	b	b	b	b	b	b
Hexoses	0.0000	0.6297	0.0225	ab	a	c	c	bc	c	c	c
Proline	0.0000	0.0550	0.0343	bc	a	a	ab	c	c	c	bc
Phenylalanine	0.0000	0.0001	0.5600	bc	a	bc	ab	d	cd	cd	cd
Disaccharides	0.0000	0.0000	0.0009	bc	a	b	a	d	d	c	bc
Chlorogenic ac.	0.2510	0.2534	0.0021	ab	b	ab	b	ab	a	ab	ab
Epicatechin	0.1570	0.4340	0.2560								
Citric ac.	0.0000	0.0000	0.0568	bc	bc	b	cd	bc	d	a	ab
Pyruvate	0.0001	0.0000	0.9910	ab	bcd	bcd	cd	bcd	d	a	abc
Luteolin	0.1012	0.0674	0.0292	c	abc	ab	bc	a	abc	abc	abc
Adenosine	0.0053	0.0502	0.9085	b	ab	ab	ab	ab	a	b	ab
K/P	0.0001	0.2260	0.2010	b	b	b	b	ab	a	b	b
Alanine	0.0002	0.5009	0.1551	abc	bc	abc	a	ab	abc	c	bc
Choline	0.0076	0.7954	0.6559	ab	ab	ab	ab	a	ab	b	ab
Gallic ac.	0.2620	0.8980	0.3180								
Quinic ac.	0.0000	0.0579	0.2168	d	d	bcd	cd	ab	a	bcd	abc
K	0.0000	0.6630	0.4080	b	b	b	b	ab	a	ab	ab
Kaempferol	0.6326	0.0941	0.2929								
Epigallocatechin	0.4911	0.0322	0.8215								
Rhamnetin	0.9302	0.0595	0.8413								
Succininc ac.	0.1001	0.0025	0.1296	b	a	ab	ab	ab	ab	b	ab
Homoorientin	0.9580	0.0000	0.4190	c	ab	bc	ab	abc	abc	c	a
Pentoses	0.0020	0.6432	0.8432	a	ab	ab	b	ab	b	ab	ab
N/K	0.0000	0.5610	0.6560	a	a	abc	ab	bc	c	abc	abc
C/K	0.0000	0.7640	0.8480	ab	a	abc	abc	cd	d	abcd	bcd
C	0.0756	0.0090	0.3742	ab	a	ab	ab	b	ab	ab	a
C/P	0.0002	0.0089	0.4356	ab	ab	bc	ab	abc	a	c	bc
N/P	0.0480	0.0000	0.7590	abc	a	c	abc	abc	a	bc	ab
Quercitin	0.7670	0.2360	0.9010								
P	0.0000	0.0116	0.3767	bc	bc	ab	bc	bc	c	a	ab
N	0.0000	0.0013	0.8616	c	abc	c	bc	c	bc	ab	a
Malic ac.	0.0024	0.4819	0.5783	ab	b	a	a	ab	ab	ab	ab
Lactic ac.	0.1290	0.5480	0.7560								
Catechin	0.0440	0.5370	0.6840								
C/N	0.0002	0.0045	0.9219	a	ab	a	a	a	a	ab	b

Appendix A

Topsoil depth substantially influences the tree metabolomes of Mediterranean forests.

Rivas-Ubach et al., 2015

Table A.1. LC-MS chromatogram processing. Chromatograms were obtained by LC-MS and processed by MZmine 2.12. The following table summarizes the processes and parameters applied to the *Q. ilex* foliar metabolomic chromatograms.

		(+H) Chromatograms		(-H) Chromatograms
1	**Baseline correction**			
	Chromatogram type	TIC		TIC
	MS level	1		1
	Smoothing	10E7		10E7
	Asymmetry	0.001		0.001
2	**Mass detection** (exact Mass)			
	Noise level	5×10^5		4×10^5
3	**Chromatogram builder**			
	Minimum time span	0.05		0.05
	Minimum height	25000		25000
	m/z tolerance	0.002		0.002
4	**Smoothing**			
	Filter width	5		5
5	**Chromatogram deconvolution (local minimum search)**			
	Chromatographic threshold	65%		65%
	Search minimum in RT range (min)	0.1		0.1
	Minimum relative height	5.0%		5.0%
	Minimum absolute height	30000		30000
	Minimum ratio of peak top/edge	2		2
	Peak duration range	0.0-2.0		0.0-2.0
6	**Chromatogram alignment (join alignment)**			
	m/z tolerance	0.001		0.001
	Weight for m/z	80		80
	RT tolerance	0.15		0.2
	Weight for RT	20		20
7	**Gap filling (Peak Finder)**			
	Intensity tolerance	20%		20%
	m/z tolerance	0.001		0.001
	Retention time tolerance	0.1		0.1
	RT correction	marked		marked
8	**Filtering**			
	Minimum peaks in a row	8		8
9	**Metabolite Assignation**			
	m/z tolerance	0.0005		0.0005
	RT tolerance	0.25		0.25
	Ions excluded from database	<75 83.05 102.05 114.09 227.17 607.29 Between 0.0 and 1 min Between 28.5 and 30 min		<85 119.035 223.082 391.196 159.25 186.186 Between 0.0 and 1.1 min Between 27.0 and 30 min

RT, retention time; m/z, mass to charge ratio

Table A.2. Metabolite assignment by LC-MS. The assignment of the metabolites was based on the standards. The following table summarizes the retention time (RT) and mass to charge ratio (m/z) of the assigned metabolites in both positive and negative ionization modes.

Mode	Compound	RT (min)	m/z
Negative	Catechin	3.44	289.0718
Negative	Chlorogenic acid	3.11	353.0873
Negative	Citric acid	1.77	191.0196
Negative	Deoxyhexose	1.42	163.0618
Negative	Disaccharides	1.43	341.108
Negative	Epicatechin	4.93 - 5.2	289.0713
Negative	Epigallocatechin	1.54 - 2.64	305.0667
Negative	Gallic acid	1.55 - 1.83	169.0147
Negative	Hexose	1.44	179.056
Negative	Homoorientin	9.45	447.0923
Negative	Kaempferol	14.82	285.0404
Negative	Lactic acid	1.52; 1.75	89.0245
Negative	Malic acid	1.51; 1.78	133.0143
Negative	Pentose	1.43	149.0456
Negative	Pyruvate	1.65	87.0089
Negative	Quercetin	13.72	301.0355
Negative	Quinic acid	1.47	191.056
Negative	Rhamnetin	15.98	315.0509
Negative	Sodium salicylate	10.51	137.0245
Negative	Succinic acid	1.74; 1.78	117.0194
Positive	Adenine	1.42; 1.77	136.0614
Positive	Adenosine	1.49; 1.75	268.1038
Positive	α-Humulene	20.27	205.1949
Positive	Alanine	1.43	90.054
Positive	Arginine	1.34	175.119
Positive	Aspartic acid	1.5	134.044
Positive	Caryophyllene	21.46	221.1899
Positive	Catechin	3.44	291.0863
Positive	Chlorogenic acid	3.11	355.084
Positive	Epigallocatechin	1.54 - 2.64	307.0812
Positive	Glutamic acid	1.41	148.0604
Positive	Glutamine	1.46	147.076
Positive	Kaempferol	14.82	287.0552
Positive	Leucine	1.76	132.101
Positive	Luteolin	13.68	287.0551
Positive	Phenylalanine	1.91	166.086
Positive	Proline	1.49	116.07
Positive	Pyridoxine	1.38	170.0812
Positive	Quercetin	13.72	303.0498
Positive	Rhamnetin	15.95	317.0653
Positive	Tryptophan	2.49	205.097
Positive	Tyrosine	1.54 - 1.77	182.081
Positive	Valine	1.53	118.086

Table A.3. Results of the factorial ANOVAs of all stoichiometries and assigned metabolites extracted from *Q. ilex* leaves with vegetation structure (VS) and experimental drought treatment as factors for all seasons combined. The whole factorial model and the decomposed model are shown in the table for each of the known variables. Statistical significance ($P < 0.05$) is shown in red.

	Whole model		Decomposed model			
				VS	Treatment	VS×treatment
C/N	F (*P*)	14.81 (0.00000)	F (*P*)	37.94 (0.00000)	0.56 (0.45658)	5.94 (0.01594)
N/P	F (*P*)	15.36 (0.00000)	F (*P*)	6.78 (0.01012)	28.28 (0.00000)	11.03 (0.00112)
C/P	F (*P*)	13.43 (0.00000)	F (*P*)	1.98 (0.16178)	18.55 (0.00003)	19.76 (0.00002)
C/K	F (*P*)	4.64 (0.00389)	F (*P*)	4.52 (0.03510)	3.27 (0.07269)	6.14 (0.01429)
K/P	F (*P*)	9.92 (0.00001)	F (*P*)	5.20 (0.02400)	9.25 (0.00276)	15.30 (0.00014)
N/K	F (*P*)	10.30 (0.00000)	F (*P*)	15.94 (0.00010)	3.80 (0.05300)	11.15 (0.00105)
[N]	F (*P*)	15.44 (0.00000)	F (*P*)	41.17 (0.00000)	0.02 (0.88056)	5.13 (0.02487)
[P]	F (*P*)	15.42 (0.00000)	F (*P*)	3.37 (0.06831)	24.19 (0.00000)	18.69 (0.00003)
[K]	F (*P*)	4.57 (0.00426)	F (*P*)	4.45 (0.03640)	2.18 (0.14197)	7.08 (0.00863)
[C]	F (*P*)	5.67 (0.00104)	F (*P*)	4.91 (0.02821)	10.74 (0.00129)	1.35 (0.24648)
Hexoses	F (*P*)	3.00 (0.03254)	F (*P*)	0.00 (0.96126)	8.76 (0.00357)	0.23 (0.63114)
Pentoses	F (*P*)	8.26 (0.00004)	F (*P*)	6.03 (0.01515)	10.03 (0.00185)	8.73 (0.00362)
Disaccharides	F (*P*)	6.85 (0.00023)	F (*P*)	20.10 (0.00001)	0.02 (0.88967)	0.43 (0.51054)
Alanine	F (*P*)	3.83 (0.01104)	F (*P*)	7.53 (0.00677)	0.19 (0.66749)	3.78 (0.05362)
Aspartic acid	F (*P*)	4.14 (0.00745)	F (*P*)	7.18 (0.00818)	4.98 (0.02713)	0.26 (0.61018)
Glutamic acid	F (*P*)	1.77 (0.15486)	F (*P*)	3.81 (0.05271)	0.51 (0.47805)	1.00 (0.31933)
Valine	F (*P*)	4.69 (0.00366)	F (*P*)	2.02 (0.15730)	1.83 (0.17776)	10.21 (0.00169)
Leucine	F (*P*)	2.88 (0.03801)	F (*P*)	1.30 (0.25584)	5.82 (0.01702)	1.51 (0.22115)
Phenylalanine	F (*P*)	3.22 (0.02439)	F (*P*)	6.02 (0.01528)	0.68 (0.40935)	2.96 (0.08737)
Proline	F (*P*)	1.31 (0.27473)	F (*P*)	3.43 (0.06594)	0.22 (0.64043)	0.27 (0.60572)
Arginine	F (*P*)	13.45 (0.00000)	F (*P*)	20.43 (0.00001)	0.41 (0.52359)	19.50 (0.00002)
Tryptophan	F	0.46	F	0.12	0.85	0.40

	(*P*)	(0.71267)	(*P*)	(0.72664)	(0.35927)	(0.52639)
Tyrosine	F (*P*)	2.66 (0.05040)	F (*P*)	6.50 (0.01174)	0.36 (0.54711)	1.10 (0.29533)
Adenine	F (*P*)	11.64 (0.00000)	F (*P*)	0.14 (0.70824)	34.76 (0.00000)	0.00 (0.96702)
Adenosine	F (*P*)	2.51 (0.06096)	F (*P*)	7.24 (0.00791)	0.28 (0.59435)	0.00 (0.97502)
Succinic acid	F (*P*)	9.77 (0.00001)	F (*P*)	21.51 (0.00001)	6.54 (0.01149)	1.26 (0.26316)
Citric acid	F (*P*)	6.33 (0.00045)	F (*P*)	17.32 (0.00005)	0.83 (0.36286)	0.83 (0.36427)
Malic acid	F (*P*)	7.07 (0.00017)	F (*P*)	12.41 (0.00056)	2.30 (0.13104)	6.50 (0.01175)
Pyruvate	F (*P*)	8.76 (0.00002)	F (*P*)	23.92 (0.00000)	1.57 (0.21232)	0.79 (0.37513)
Lactic acid	F (*P*)	1.66 (0.17840)	F (*P*)	0.31 (0.57576)	4.57 (0.03416)	0.09 (0.76162)
Catechin	F (*P*)	12.03 (0.00000)	F (*P*)	0.01 (0.93463)	35.30 (0.00000)	0.79 (0.37528)
Epicatechin	F (*P*)	1.49 (0.21979)	F (*P*)	0.65 (0.42025)	0.50 (0.47949)	3.31 (0.07076)
Epigallocatechin	F (*P*)	10.61 (0.00000)	F (*P*)	2.71 (0.10183)	5.61 (0.01909)	23.51 (0.00000)
Homoorientin	F (*P*)	9.80 (0.00001)	F (*P*)	10.39 (0.00154)	2.13 (0.14608)	16.88 (0.00006)
Kaempferol	F (*P*)	3.05 (0.03033)	F (*P*)	4.73 (0.03122)	4.36 (0.03839)	0.07 (0.79761)
Luteolin	F (*P*)	2.43 (0.06734)	F (*P*)	7.04 (0.00880)	0.25 (0.61678)	0.00 (0.98348)
Quercitin	F (*P*)	5.67 (0.00103)	F (*P*)	10.21 (0.00169)	3.91 (0.04968)	2.89 (0.09140)
Rhamnetin	F (*P*)	5.90 (0.00077)	F (*P*)	15.56 (0.00012)	0.00 (0.97022)	2.13 (0.14638)
Chlorogenic acid	F (*P*)	8.85 (0.00002)	F (*P*)	5.64 (0.01877)	10.18 (0.00171)	10.74 (0.00129)
Gallic acid	F (*P*)	0.46 (0.71304)	F (*P*)	0.18 (0.67443)	1.14 (0.28793)	0.06 (0.81406)
α-Humulene	F (*P*)	11.56 (0.00000)	F (*P*)	5.09 (0.02549)	18.43 (0.00003)	11.17 (0.00104)
Caryophyllene	F (*P*)	8.27 (0.00004)	F (*P*)	8.89 (0.00332)	6.25 (0.01342)	9.65 (0.00224)
Choline	F (*P*)	1.03 (0.38100)	F (*P*)	0.88 (0.34980)	0.49 (0.48699)	1.73 (0.19094)
Pyridoxine	F (*P*)	29.10 (0.00000)	F (*P*)	59.86 (0.00000)	17.87 (0.00004)	9.56 (0.00235)
Quinic acid	F (*P*)	0.99 (0.40146)	F (*P*)	0.49 (0.48713)	0.26 (0.60927)	2.21 (0.13934)

Table A.4. Results of the decomposed models of the factorial ANOVAs of all stoichiometries and assigned metabolites extracted from *Q. ilex* leaves with vegetation structure (VS) and experimental drought treatment (TRT) as factors for each season. Statistical significance ($P < 0.05$) is shown in red.

		SUMMER			AUTUMN			WINTER			SPRING		
		TRT	VS	VS×TRT	TRT	VS	VS×TRT	TRT	VS	VS×TRT	TRT	VS	VS×TRT
C/N	F (*P*)	0.02 (0.90)	5.29 (0.027)	1.18 (0.28)	1.67 (0.19968)	21.29 (0.00002)	4.42 (0.03882)	0.08 (0.7747)	12.56 (0.00111)	0.54 (0.46681)	0.06 (0.80691)	22.21 (0.00004)	7.92 (0.00786)
N/P	F (*P*)	5.37 (0.0263)	0.70 (0.40846)	3.56 (0.06719)	9.14 (0.00342)	5.23 (0.02495)	6.71 (0.01151)	20.38 (0.00007)	1.54 (0.22312)	0.94 (0.33774)	3.75 (0.06079)	1.95 (0.17120)	5.96 (0.01972)
C/P	F (*P*)	5.22 (0.02832)	0.20 (0.65922)	6.06 (0.01875)	3.32 (0.07236)	0.83 (0.36569)	14.06 (0.00034)	19.93 (0.00008)	1.77 (0.19220)	1.57 (0.21794)	3.34 (0.07582)	2.46 (0.12584)	17.08 (0.00020)
C/K	F (*P*)	0.65 (0.42694)	0.46 (0.50328)	5.52 (0.02435)	2.78 (0.09955)	2.95 (0.09006)	3.26 (0.07514)	0.90 (0.34991)	2.53 (0.12025)	1.47 (0.23280)	1.56 (0.22035)	0.72 (0.40140)	0.38 (0.54178)
K/P	F (*P*)	3.45 (0.07139)	0.37 (0.54952)	12.87 (0.00099)	4.43 (0.03852)	4.53 (0.03650)	8.40 (0.00491)	4.80 (0.03508)	3.64 (0.06436)	0.75 (0.39353)	4.14 (0.04935)	3.33 (0.07645)	5.84 (0.02082)
N/K	F (*P*)	1.41 (0.24226)	2.11 (0.15485)	7.66 (0.00885)	2.62 (0.10945)	12.90 (0.00058)	8.30 (0.00514)	1.38 (0.24788)	6.87 (0.01279)	2.23 (0.14365)	2.66 (0.11182)	6.82 (0.01306)	3.41 (0.07291)
[N]	F (*P*)	0.18 (0.67769)	5.99 (0.01935)	0.81 (0.37346)	0.79 (0.37622)	23.16 (0.00001)	4.41 (0.03912)	0.44 (0.5119)	13.86 (0.00067)	0.37 (0.54567)	0.60 (0.44463)	25.02 (0.00001)	9.81 (0.00344)
[P]	F (*P*)	6.48 (0.01534)	1.06 (0.31024)	5.62 (0.02326)	6.48 (0.0129)	0.88 (0.35117)	12.23 (0.00079)	20.80 (0.00006)	2.60 (0.11552)	1.84 (0.18338)	6.26 (0.01704)	2.86 (0.09928)	14.17 (0.00060)
[K]	F (*P*)	0.49 (0.48791)	0.39 (0.53847)	7.87 (0.00807)	1.69 (0.19754)	3.62 (0.06097)	2.53 (0.11613)	0.70 (0.40799)	2.65 (0.11229)	0.68 (0.41592)	1.51 (0.22754)	1.51 (0.22683)	0.22 (0.64182)
[C]	F (*P*)	1.81 (0.18711)	2.88 (0.09852)	2.99 (0.09238)	7.93 (0.00619)	2.00 (0.16113)	0.13 (0.71668)	2.08 (0.15787)	0.79 (0.38043)	1.34 (0.25426)	3.55 (0.06752)	0.36 (0.55210)	0.16 (0.69474)
Hexoses	F (*P*)	1.49 (0.22996)	0.99 (0.32744)	1.99 (0.16653)	7.65 (0.00714)	0.67 (0.41543)	3.25 (0.07549)	7.71 (0.00866)	2.89 (0.09769)	0.34 (0.56262)	3.20 (0.08210)	0.95 (0.33643)	2.27 (0.14057)
Pentoses	F (*P*)	1.49 (0.22947)	0.59 (0.44667)	3.75 (0.06074)	7.95 (0.00613)	4.34 (0.04070)	3.87 (0.05290)	1.96 (0.17037)	1.81 (0.18696)	2.21 (0.14572)	16.04 (0.00030)	6.02 (0.01912)	7.45 (0.00976)
Disaccharides	F (*P*)	0.00 (0.96174)	0.86 (0.36043)	4.35 (0.04408)	0.00 (0.97119)	17.95 (0.00006)	0.77 (0.38258)	0.34 (0.56569)	43.34 (0.00000)	0.89 (0.35220)	0.12 (0.73313)	26.81 (0.00001)	2.22 (0.14482)
Alanine	F (*P*)	5.33 (0.02687)	0.04 (0.83609)	0.93 (0.34045)	0.04 (0.85173)	8.81 (0.00401)	1.34 (0.25123)	11.10 (0.002)	0.80 (0.37662)	4.05 (0.05158)	0.08 (0.77374)	8.30 (0.00664)	1.16 (0.28883)
Aspartic acid	F	2.96	1.43	0.00	8.88	11.01	0.93	1.01	4.19	0.41	6.62	8.69	0.10

	(P)	(0.09395)	(0.23958)	(0.98786)	(0.00387)	(0.00139)	(0.33794)	(0.32121)	(0.04809)	(0.52525)	(0.01435)	(0.00560)	(0.75819)
Glutamic acid	F (P)	3.80 (0.05923)	3.85 (0.05757)	0.48 (0.49307)	0.36 (0.54784)	13.12 (0.00052)	0.38 (0.54198)	1.23 (0.27546)	0.35 (0.55883)	1.26 (0.26969)	0.00 (0.99999)	11.29 (0.00186)	0.66 (0.42117)
Valine	F (P)	3.19 (0.08234)	0.00 (0.96725)	3.52 (0.06886)	0.03 (0.87114)	1.86 (0.17694)	1.28 (0.26058)	7.03 (0.01186)	1.05 (0.31208)	22.92 (0.00003)	0.12 (0.72827)	1.83 (0.18435)	0.57 (0.45378)
Leucine	F (P)	10.90 (0.00218)	1.42 (0.24189)	2.24 (0.14336)	0.05 (0.81675)	2.77 (0.10020)	1.52 (0.22127)	7.01 (0.01193)	0.27 (0.60653)	0.59 (0.44925)	0.72 (0.40108)	1.47 (0.23360)	0.25 (0.62315)
Phenylalanine	F (P)	0.04 (0.85115)	5.14 (0.02950)	0.69 (0.41204)	0.02 (0.88406)	1.47 (0.22951)	2.67 (0.1066)	3.84 (0.05795)	6.43 (0.01572)	1.51 (0.22641)	0.70 (0.40810)	0.22 (0.64427)	2.35 (0.13401)
Proline	F (P)	1.77 (0.19136)	0.97 (0.33127)	0.12 (0.72589)	0.52 (0.47378)	0.87 (0.35414)	0.32 (0.5743)	0.04 (0.83840)	6.45 (0.01553)	6.57 (0.01472)	0.48 (0.49218)	0.10 (0.75109)	1.15 (0.29162)
Arginine	F (P)	3.50 (0.06937)	7.16 (0.01117)	7.13 (0.01131)	0.06 (0.80970)	7.15 (0.00915)	4.54 (0.0364)	0.23 (0.63283)	9.42 (0.00407)	13.04 (0.00092)	0.20 (0.65685)	2.34 (0.13509)	1.43 (0.23999)
Tryptophan	F (P)	0.19 (0.66927)	12.84 (0.00100)	2.16 (0.15036)	0.05 (0.82535)	0.01 (0.92317)	2.73 (0.10244)	4.35 (0.04419)	0.02 (0.87845)	0.27 (0.60591)	0.15 (0.70002)	0.03 (0.85919)	5.16 (0.02920)
Tyrosine	F (P)	0.31 (0.5792)	10.14 (0.00299)	0.24 (0.62667)	0.27 (0.60408)	1.07 (0.30513)	0.26 (0.61135)	5.71 (0.02222)	0.10 (0.74873)	2.80 (0.10286)	0.20 (0.65685)	0.02 (0.89701)	1.81 (0.18666)
Adenine	F (P)	12.11 (0.00133)	2.21 (0.14616)	0.23 (0.63599)	9.44 (0.00295)	0.25 (0.61515)	1.29 (0.25893)	25.61 (0.00001)	0.01 (0.93598)	0.84 (0.36471)	2.93 (0.09548)	0.00 (0.95977)	2.45 (0.12620)
Adenosine	F (P)	1.21 (0.27862)	0.99 (0.32701)	0.06 (0.80169)	0.06 (0.81493)	3.64 (0.06028)	0.00 (0.98862)	0.36 (0.55060)	4.13 (0.04968)	0.15 (0.70189)	0.68 (0.41657)	1.32 (0.25839)	0.00 (0.96691)
Succinic acid	F (P)	2.38 (0.13169)	4.79 (0.03514)	4.56 (0.0396)	4.55 (0.03610)	8.37 (0.00497)	0.37 (0.54305)	0.12 (0.73180)	14.06 (0.00062)	2.38 (0.13139)	8.64 (0.00572)	3.47 (0.07068)	0.68 (0.41386)
Citric acid	F (P)	0.79 (0.37998)	37.26 (0.00000)	0.67 (0.41708)	0.09 (0.77076)	5.70 (0.01947)	1.64 (0.20441)	6.07 (0.01863)	1.68 (0.20303)	0.68 (0.41353)	1.01 (0.32186)	7.40 (0.01000)	1.99 (0.16697)
Malic acid	F (P)	0.03 (0.85751)	15.92 (0.00031)	3.63 (0.06484)	1.83 (0.18051)	5.16 (0.02596)	8.41 (0.00487)	1.37 (0.24945)	1.58 (0.21718)	0.14 (0.70657)	2.06 (0.15981)	9.58 (0.00380)	6.96 (0.01226)
Pyruvate	F (P)	0.47 (0.49817)	13.26 (0.00085)	0.00 (0.97975)	0.27 (0.60510)	7.02 (0.00980)	0.94 (0.3349)	1.88 (0.17902)	9.56 (0.00383)	0.06 (0.81333)	2.07 (0.15839)	4.50 (0.04095)	0.46 (0.50403)
Lactic acid	F (P)	1.39 (0.24690)	0.11 (0.73741)	0.22 (0.64378)	0.58 (0.44887)	0.13 (0.72193)	0.22 (0.63672)	4.23 (0.04703)	0.07 (0.79515)	0.39 (0.53534)	1.37 (0.24950)	0.00 (0.99999)	0.07 (0.78705)
Catechin	F (P)	18.55 (0.00012)	1.53 (0.22408)	0.00 (0.97594)	11.15 (0.00130)	0.03 (0.87445)	0.43 (0.51597)	10.86 (0.00222)	1.45 (0.23562)	0.74 (0.39602)	4.06 (0.05152)	1.22 (0.27626)	0.00 (0.97209)
Epicatechin	F (P)	0.96 (0.33376)	0.28 (0.59968)	5.19 (0.02870)	0.75 (0.39020)	0.75 (0.38936)	0.57 (0.45355)	3.94 (0.05470)	0.01 (0.90799)	0.07 (0.79156)	0.17 (0.67973)	2.18 (0.14821)	0.19 (0.66629)
Epigallocatechin	F (P)	0.96 (0.33300)	0.43 (0.51388)	6.01 (0.01917)	5.87 (0.01783)	0.87 (0.35406)	14.02 (0.00035)	0.02 (0.88314)	1.78 (0.19046)	3.13 (0.08516)	4.46 (0.04167)	1.06 (0.31109)	13.15 (0.00088)

Homoorientin	F (P)	0.28 (0.59892)	0.45 (0.50837)	1.44 (0.23747)	1.30 (0.25754)	6.55 (0.01245)	8.99 (0.00366)	0.52 (0.47592)	4.09 (0.05062)	7.13 (0.01128)	0.44 (0.51034)	3.11 (0.08617)	3.38 (0.07408)
Kaempferol	F (P)	1.89 (0.17805)	5.23 (0.02817)	0.90 (0.34784)	0.61 (0.43879)	1.77 (0.18797)	0.03 (0.87326)	3.05 (0.08908)	0.04 (0.84802)	0.42 (0.52110)	2.60 (0.11581)	2.28 (0.13947)	0.15 (0.69812)
Luteolin	F (P)	0.01 (0.91111)	7.59 (0.00915)	0.02 (0.89501)	0.53 (0.46851)	1.92 (0.16968)	0.47 (0.49321)	5.09 (0.03031)	0.11 (0.74737)	0.98 (0.32992)	0.31 (0.58000)	5.51 (0.02455)	1.33 (0.25554)
Quercitin	F (P)	0.31 (0.58009)	5.31 (0.02711)	0.95 (0.33678)	2.21 (0.14159)	2.81 (0.09775)	1.60 (0.20917)	1.58 (0.21712)	2.58 (0.11689)	0.36 (0.55452)	2.80 (0.10322)	1.30 (0.26168)	0.77 (0.38461)
Rhamnetin	F (P)	0.28 (0.59808)	3.16 (0.08386)	0.45 (0.50820)	0.19 (0.66677)	4.63 (0.03461)	0.83 (0.36510)	0.01 (0.91982)	9.07 (0.00473)	0.93 (0.34148)	0.74 (0.39516)	3.88 (0.05647)	0.40 (0.53296)
Chlorogenic acid	F (P)	6.74 (0.01356)	7.18 (0.01104)	1.64 (0.20889)	0.34 (0.56135)	0.69 (0.40882)	18.53 (0.00005)	16.22 (0.00028)	0.05 (0.82225)	2.77 (0.10468)	3.24 (0.08044)	0.78 (0.38193)	18.13 (0.00014)
Gallic acid	F (P)	0.54 (0.46657)	0.94 (0.33823)	0.10 (0.75092)	0.82 (0.36835)	0.09 (0.77082)	0.45 (0.50444)	0.00 (0.94698)	0.62 (0.43741)	0.00 (0.99585)	0.39 (0.53444)	3.45 (0.07164)	0.17 (0.68275)
α-Humulene	F (P)	3.05 (0.0892)	0.35 (0.55577)	0.16 (0.68974)	8.00 (0.00597)	3.75 (0.05660)	9.25 (0.00323)	15.56 (0.00035)	3.42 (0.07280)	6.28 (0.01690)	6.21 (0.01742)	4.10 (0.05041)	7.54 (0.00936)
Caryophyllene	F (P)	1.55 (0.22177)	1.01 (0.32231)	10.19 (0.00293)	4.17 (0.04450)	5.20 (0.02546)	4.90 (0.0298)	3.11 (0.08641)	6.26 (0.01704)	8.39 (0.00637)	3.99 (0.05348)	5.17 (0.02911)	3.26 (0.07937)
Choline	F (P)	4.99 (0.03176)	1.08 (0.30599)	0.10 (0.74929)	7.83 (0.00650)	0.00 (0.97177)	2.38 (0.12669)	0.01 (0.93255)	0.61 (0.44102)	0.05 (0.82079)	2.85 (0.09982)	0.45 (0.50573)	4.54 (0.04001)
Pyridoxine	F (P)	7.87 (0.00806)	29.84 (0.00000)	0.40 (0.53193)	12.62 (0.00066)	21.53 (0.00001)	11.16 (0.0013)	0.79 (0.38009)	21.18 (0.00005)	0.59 (0.44933)	1.56 (0.21996)	14.37 (0.00055)	1.65 (0.20772)
Quinic acid	F (P)	0.31 (0.57817)	0.85 (0.36174)	2.47 (0.12462)	2.35 (0.12978)	0.33 (0.56506)	0.00 (0.97309)	4.06 (0.05142)	0.05 (0.82288)	4.98 (0.03195)	7.73 (0.00858)	1.22 (0.27700)	0.04 (0.83896)

Table A.5. *P* values of the one-way ANOVAs of coordinates of cases along PC1 and PC2 of the experimental drought treatment and the vegetation structure of the PCAs for each season (Fig. A.2).

Season			PC axis	*P*
Winter	Treatment (Global)		PC1	NS
			PC2	0.0097
	Structure (Global)		PC1	NS
			PC2	0.00001
	Treatment (within structure)	For H-Forest (Control vs. Drought)	PC1	0.0399
			PC2	0.0027
		For L-Forest (Control vs. Drought)	PC1	0.00001
			PC2	NS
	Structure (within treatment)	For Control (H-Forest vs. L-Forest)	PC1	0.00000
			PC2	0.00000
		For Drought (H-Forest vs. L-Forest)	PC1	0.027
			PC2	0.00000
Spring	Treatment (Global)		PC1	0.0065
			PC2	NS
	Structure (Global)		PC1	NS
			PC2	0.00000
	Treatment (within structure)	For H-Forest (Control vs. Drought)	PC1	NS
			PC2	NS
		For L-Forest (Control vs. Drought)	PC1	0.00037
			PC2	NS
	Structure (within treatment)	For Control (H-Forest vs. L-Forest)	PC1	0.00000
			PC2	0.00000
		For Drought (H-Forest vs. L-Forest)	PC1	0.00000
			PC2	0.00000
Summer	Treatment (Global)		PC1	NS
			PC2	NS
	Structure (Global)		PC1	NS
			PC2	0.00000
	Treatment (within structure)	For H-Forest (Control vs. Drought)	PC1	0.0267
			PC2	0.073 (MS)
		For L-Forest (Control vs. Drought)	PC1	0.0046
			PC2	NS
	Structure (within treatment)	For Control (H-Forest vs. L-Forest)	PC1	0.00014
			PC2	0.00000
		For Drought (H-Forest vs. L-Forest)	PC1	0.098 (MS)
			PC2	0.00005
Autumn	Treatment (Global)		PC1	NS
			PC2	NS
	Structure (Global)		PC1	0.000051
			PC2	0.0033
	Treatment	For H-Forest (Control vs. Drought)	PC1	NS

	(within structure)		PC2	0.0756 (MS)
		For L-Forest (Control vs. Drought)	PC1	0.0029
			PC2	NS
	Structure (within treatment)	For Control (H-Forest vs. L-Forest)	PC1	0.00000
			PC2	0.0886 (MS)
		For Drought (H-Forest vs. L-Forest)	PC1	NS
			PC2	0.0165

NS, not significant; MS, marginally significant

Table A.6. R^2 coefficients and the corresponding *P* values for the total, direct, and indirect effects of the structural equation model (SEM) with tree diameter (D50), upper soil moisture content, soil texture, and metabolomic variation (PC2) as endogenous variables. The model is described in Fig. A.4.

TOTAL EFFECTS					
	Drought treatment	Soil texture	Upper soil moisture	DUSL	D50
Upper soil moisture	0.2 (*P* < 0.001)	-0.015 (*P* = 0.42)		0.056 (*P* = 0.26)	
D50	0.39 (*P* < 0.0001)	-0.00074 (*P* = 0.45)	0.048 (*P* = 0.22)	0.64 (*P* < 0.0001)	
Soil texture				-0.097 (P = 0.1)	
PC2	0.26 (*P* < 0.0001)	-0.0041 (*P* = 0.42)	0.27 (*P* < 0.00001)	0.35 (*P* < 0.0001)	0.52 (*P* < 0.0001)
DIRECT EFFECTS					
	Drought treatment	Soil texture	Upper soil moisture	DUSL	D50
Upper soil moisture	0.2 (*P* < 0.001)	-0.015 (*P* = 0.42)		0.054 (*P* = 0.26)	
D50	0.38 (*P* < 0.0001)		0.048 (*P* = 0.21)	0.64 (*P* < 0.0001)	
Soil texture				-0.097 (*P* = 0.1)	
PC2	0.004 (*P* = 0.48)		0.24 (*P* < 0.001)		0.52 (*P* < 0.0001)
INDIRECT EFFECTS					
	Drought treatment	Soil texture	Upper soil moisture	DUSL	D50
Upper soil moisture				0.0015 (*P* = 0.44)	
D50	0.0098 (*P* = 0.24)	-0.00074 (*P* = 0.45)		0.0027 (*P* = 0.36)	
Soil texture					
PC2	0.26 (*P* < 0.0001)	-0.0041 (*P* = 0.42)	0.025 (*P* = 0.22)	0.35 (*P* < 0.0001)	

Fig A.1. Means ±SE for each vegetation structure for (a) trunk diameter (cm) measured at 50 cm from soil surface (D50) of the analyzed trees, (b) the depth of upper soil layers (DUSL) near the analyzed trees and, (c) the aboveground biomass (AB)(Kg of dry weight) of *Q. ilex* trees in the plots. A one way ANOVA was performed for each plot. *F* and *P* values are also shown.

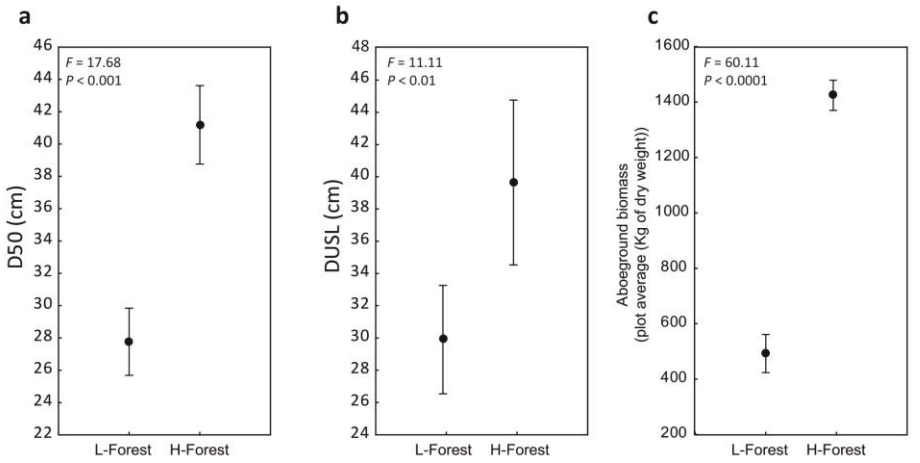


Fig A.2. Principal component (PC) 1 vs. PC2 of the principal component analysis of the metabolomic and stoichiometric variables for the *Q. Ilex* leaves for each season. Variable plots for (**a**) winter, (**b**) spring, (**c**) summer, and (**d**) autumn, and case plots for (**e**) winter, (**f**) spring, (**g**) summer, and (**h**) autumn. The variables in the variable plots are the same as in Figure 1 and have the same codes. The case plots represent the cases by vegetation structure and experimental drought treatment. Black arrows indicate the means for the PC1 and PC2 of the corresponding vegetation structure (H-Forest or L-Forest) that differed significantly. Colored arrows indicate the means for PC1 and PC2 of the corresponding treatment (control or drought) in each vegetation structure that differed significantly. Different letters beside the arrows indicate significant differences between seasons, vegetation structures, or experimental treatments ($P < 0.05$).

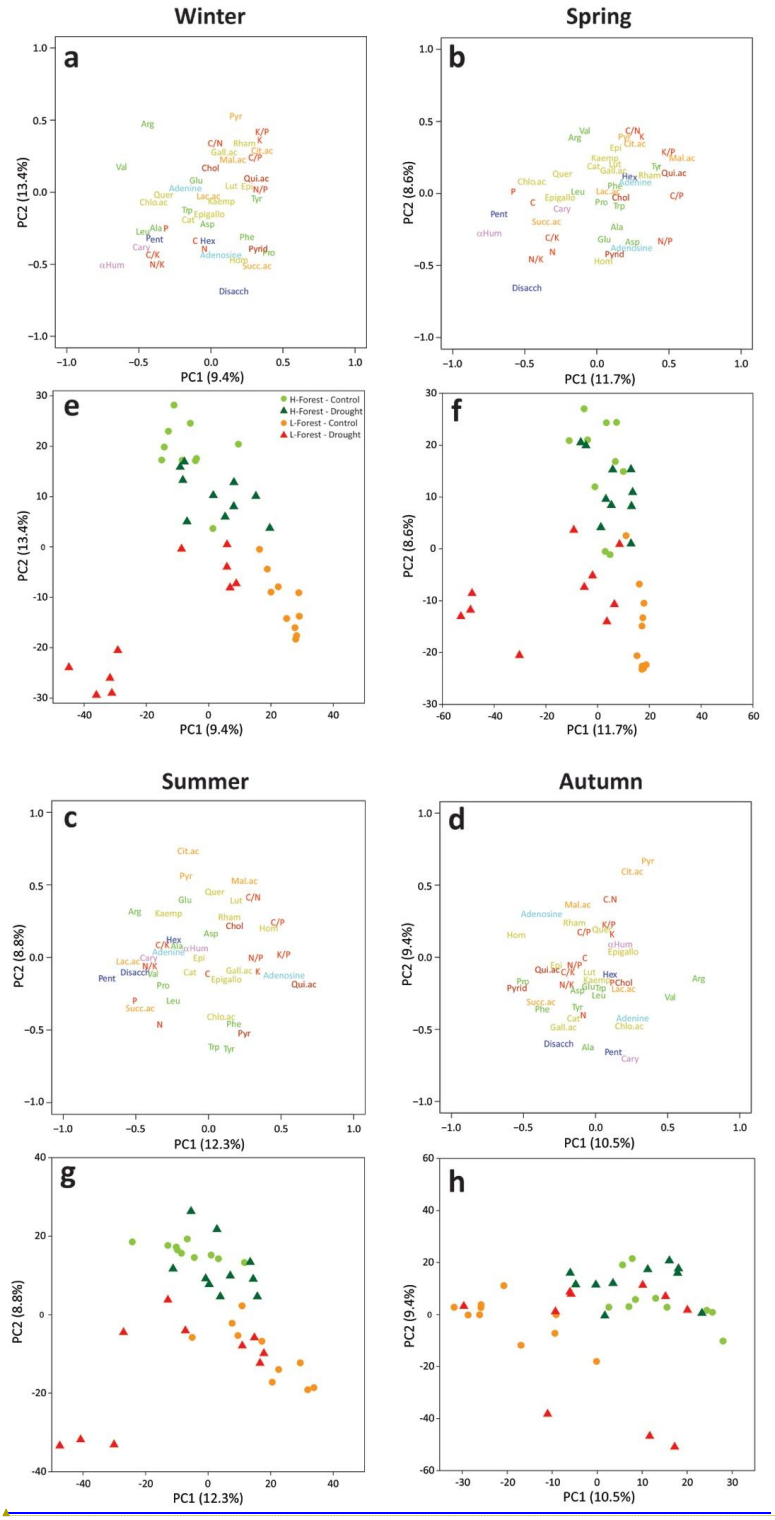


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Fig A.3. Case plots of component 1 vs. component 2 of the partial least squares discriminant analysis of the metabolomic and stoichiometric variables for the *Q. ilex* leaves for each season.

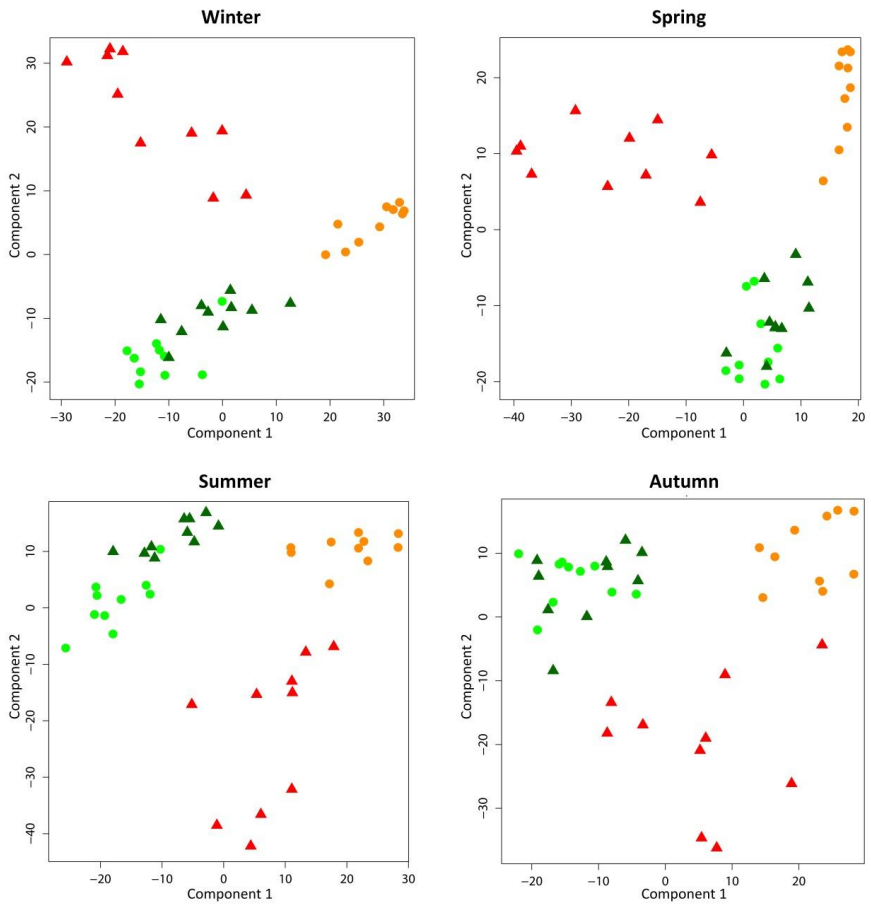

Fig A.4. Structural equation model including the soil moisture, tree diameter (D50), soil texture, and metabolomic variation (PC2) as endogenous variables and depth of upper soil layers (DUSL), and experimental drought treatment as exogenous variables. The model coefficient (R^2) and the corresponding *P* value for each relationship are shown between the two related variables. Total, direct, and indirect effects are shown in Table A.6.

R^2 for endogenous variables			
Upper soil moisture	D50	Soil Texture	PC2 (Metabolomic variation)
0.0444	0.4883	0.0094	0.3978