Drought stress enhances folivory rates by shifting foliar metabolomes.

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Abstract.

Plants respond to external environment such as drought or marked year seasons by shifting the foliar C:N:P:K stoichiometry and metabolome. Folivory rates have usually associated with the water status of plants and/or foliar nutrient concentrations, especially N, but all present studies focused mainly in foliar nutrient concentrations or in foliar specific metabolic families. The new emerging ecometabolomic techniques allow studying the metabolomes, the total set of metabolites present in an organism in a specific moment. This study tries to integrate both stoichiometric and metabolomic techniques to understand the responses of *Quercus ilex* throughout year seasons and under moderated drought experimental conditions and the further relationship with folivory rates. As expected, foliar K concentrations increased in summer, the driest Mediterranean season, since K acts as osmoprotector front water stress and consequently presented higher foliar K/P and lower C/K and N/K ratios. Moreover, trees exposed to a field moderated drought showed higher foliar concentrations of total sugars and polyphenolic compounds (flavonoids) than controls. These compounds were mainly associated to water-stress avoidance by the osmotic protection of sugars and the antioxidant function of most of the assigned flavonoids. The results of the present study suggest that the increase of sugar and flavonoids in *Q. ilex* leaves in droughted trees lead to an increase in the herbivore attack. These results suggest the evidence of the

indirect relationship between the drought increases and the folivory rates by the produced shifts in metabolomes. The present study represents a step in understanding potential cascade effects of drought at different trophic levels and the possible further implications in the ecosystem structure, function and evolution.

Introduction

Drought, one of the most important factors promoting climate change, which is predicted to increase in the following decades in several regions of the world such as in the Mediterranean basin (IPCC, 2007), is a potential driver of changes in the elemental C:N:P:K stoichiometries of different plant organs and ecosystems (Sardans et al., 2012 PEES, Rivas-Ubach et al., 2012 PNAS, Sardans et al., 2012 Functional Ecology) and this way end up affecting ecological processes and finally the ecosystem structure and function (Elser et al., 1996 BioScience; Sterner and Elser 2002; Sardans et al., 2011 Biogeochemistry). Even though N and P have been the most studied elements in the ecological stoichiometry context (Sterner and Elser 2002), several studies have demonstrated the central role that foliar K concentrations plays under drought conditions (Ingram and Bartels 1996 annual review of plant physiology and plant molecular biology; Sardans et al., 2012 Functional Ecology; Wang et al., 2013 International Journal of Molecular Sciences). For example, seasonal studies in plants in Mediterranean climate showed that foliar K concentrations tend to to increase in both summer, the driest season, and also under experimental drought conditions (Sardans et al., 2013 Acta Oecologica; Rivas-Ubach et al., 2012 PNAS) being these changes more intense even than changes in foliar N and P concentrations (Sardans et al., 2012 PEES). Even so, the study of foliar K concentrations in the ecological stoichiometry context in

both seasonal and under drought conditions is still scarce and warrants more attention (Sardans et al., 2012 Functional Ecology).

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The relatiohships between water status in plants and herbivore activity have been already reviewed showing the importance of soil water status in tree resistance against herbivore attack (Rouault et al., 2006 Annals of Forest Science). Also, some studies observed that herbivores enhanced their activity in response to the increase in concentration of soluble nitrogen in foliage (Larsson et al., 1989 and 1993; Rouault et al., 2006 Annals of forest Science). Others observed that the nutritional foliar quality is affected by drought and indirectly it stimulates the insect foliar feeding (White et al., 1984 Oecologia; Rouault et al., 2006 Annals of forest Science). The great variation in C:N:P biomass stroichiometries in plants, both in time and space may be thus a significant factor, among others, of host selection for herbivores trying to choose foliage with high nutritional contents (Gusewell and Koerselman, 2002; Oleksyn et al., 2002; Lindroth et al., 2002; Raubenheimer and Simpson 2003 The Journal of Experimental Botany; Sardans et al., 2012 Biogeochemistry). Additionaly, it has seen that folivory rates are more directly related to the proportion of foliar structural compounds than foliar N concentrations, usually avoiding high proportions of these compounds (Choong et al., 1992; Williams et al., 1998). Even so, these plant-herbivore relationships remain unclear and need further study (Raubenheimer and Simpson 2003 The Journal of Experimental Botany).

Most elements such as C, N and P do not actuate as themselves but mainly as molecular compounds (Peñuelas and Sardans 2009 Nature) such as lignine and cellulose in lignified structures or other different defensive compounds against herbivore attack (Bennett & Wallsgrove 1994 New Phytologist; Kelser and Baldwin 2001 Science). Furthermore, the plant stoichiometric changes may thus influence the coevolution of

insects with the defensive chemical compounds of plants (Raubenheimer and Simpson 2003 The Journal of Experimental Botany) but the relationship between overall plant stoichimoetry and metabolism with herbivore attack is still unclear and warrants study.

The effects of drought on foliar stoichiometry and metabolism have been reported in several studies (Sardans et al., 2011 Chemoecology, Sardans et al., 2012 PEES, Rivas-Ubach et al., 2012 PNAS) where others have reported the plant molecular responses to herbivores (Ali and Agrawal 2012, references there in). Plants are capable to respond to herbivory producing chemical defenses such as alkaloids, terpenes and phenolics (Bennett & Wallsgrove 1994 New Phytologist; Kelser and Baldwin 2001 Science) or even changing the concentrations of compounds from the primary metabolism as a result of tissue predation or infection (Widarto et al., 2006, Lafa & Fungate 2011, Ehness et al., 1997, Sardans et al., 2013 Plant Biology). Generally, the study of plant responses to herbivore predation has been focused to the identification of single compounds of families of metabolites (Sardans et al., 2013 Plant Biology) but the use of the new emerging metabolomic techniques to apply in the field of ecology and plant physiology (ecometabolomics) aim to study the entire metabolomes of organisms, the total set of metabolites present in an organism in a specific moment (Fiehn 2002) and their changes with abiotic and biotic environmental changes (Peñuelas and Sardans 2009 Chemical Ecology; Sardans et al., 2011 Chemoecology; Sardans and Peñuelas 2012 Plant Physiology; Peñuelas et al., 2013 Global Change Biology; Rivas-Ubach et al., 2013 MEE). Some recent plant ecometabolomic studies have allowed showing which metabolites change their concentrations in a plants throughout year Mediterranean seasons (Rivas-Ubach et al., 2012 PNAS), in plants submitted to a biotic stress such as herbivore predation (Leiss et al., 2009; Jansen et al., 2009) or a non-biotic stress such as drought (Charlton et al., 2008 Metabolomics; Lugan et al., 2009 Plant

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Cell Environment; Rivas-Ubach et al., 2012 PNAS) from the total set of plant metabolism, even though the number of studies is still scarce to understand the general trends of the shit in metabolism of plants in front of stresses. The link between the shifts of foliar stoichiometries and the shifts of foliar metabolome troughout year seasons and under field drought experiments has been recently demonstrated (Rivas-Ubach et al., 2012 PNAS), so that nutritional quality of the food for herbivores may shift too.

The folivory rates can thus be influenced directly by food-quality and indirectly by plant water status, we hypothesize that shifts on both foliar elemental stoichiometry and metabolomics of plants produced by drought have an effect on the herbivore activity. The shifts of foliar composition induced by drought could thus induce a long term cascade effect producing trophic web changes due the shifts in the food composition for herbivores.

We sampled once per season leaves of *Quercus ilex* trees from a mature forest in Catalonia (North-East Iberian Peninsula) exposed to field conditions of moderate experimental drought as those projected for the immediate decades. All the samples were used for metabolomic and elemental analyses. Also, the folivory rates were calculated for each sampled individual. The present study tries to understand together the stoichiometric and metabolomic responses of *Q. ilex*, the most dominant tree forming the Mediterranean basin forests, across the marked year seasons and under drought experimental conditions and the further relationships with herbivore attack trying thus to comprehend at the same time how the elemental and metabolic concentrations of plants may affect the food selection by herbivores. We used *Q. ilex* forest to test our hypothesis that drought conditions, produced by drought experimental plots, may shift the folivory activity due the shift on the foliar metabolism. It could thus indicate a hint of a near future change in trophic webs of Mediterranean forests.

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Material & Methods

Study site

This study was carried out in a natural *Quercus ilex* forest in the Prades mountains in southern Catalonia (41˚13' N, 0˚55'E). All sampled plots were in south-southeast face and with a 25% of slope at 930m altitude above sea level. Climate is mesic-Mediterranenan with a marked summer drought for 3 months. Vegetation consists of a forest dominated by *Quercus ilex* (20.8 m^2ha^{-1} of trunk basal area at 50 cm), followed by *Phillyrea latyfolia* (7.7 m^2ha^{-1} of trunk basal area at 50 cm) and *Arbutus unedo*. There are also several species adapted to drought conditions such as *Juniperus oxycedruys*, *Erica arborea* and *Cistus albidus*, and sporadic individuals of deciduous species such as *Acer monspesulanum* and *Sorbus torminalis*. Environmental conditions such as soil moisture, air humidity, air and soil temperatures and precipitation were monitored each half-hour in all plots (see Ogaya & Peñuelas 2007 for details).

Experimental design

Four plots in mature *Q.ilex* forest (15 x 10 m.) were established in March 1999 with a 15m of distance between them (Ogaya et al. 2003). Two plots received drought treatment and the other two were left as control plots. Drought treatments were assigned randomly to different plots and not contigously. Drought treatment consists to cover approximately a 30% of soil surface with a 14m long and 1 m wide PVC strips placed from top-edge to bottom-edge at 0.5-0.8 m above the soil excluding a representing rainfall fraction and a 0.8-1 m deep ditch was dug alone the entire top edge of the

150 treatment plots to intercept runoff water. All intercepted water by the strips was
151 channeled to the bottom edge of drought plots. Drought treatment results on average
152 reduction of 18% in the relative soil moisture (Barbeta et al., 2013 GCB).
153
154 Sampling of leaves.
155 Five individuals of *Q. ilex* were randomly selected from each plot as study cases (n = 5
156 x 4 = 20). Leaves were sampled once for each year season; February in winter, May in
157 spring, August in summer and November in autumn. A little branch exposed to sun was
158 removed of each tree with a pole and a fraction of the youngest leaves was frozen into
159 liquid nitrogen for the stoichiometric and metabolomic analyses. The rest of the leaves
160 were kept in bags at 6-8 ˚C in order to determine water content and to take pictures for
161 the analyses of the herbivore consumption rates.
162 Herbivore consumption rate calculation.
163 Five-ten randomly selectedamong the youngest leaves of each tree were placed on a flat
164 white surface and were photographed with a Nikon D80 and Nikkor AF-S 18-135/3.5-
165 5.6 G DX objective in order to calculate the percentage of folivory. The predated area of
166 leaves was calculated with Adobe Photoshop CS2 (Adobe Systems Incorporated, San
167 Jose, California, USA). The assigned consumed area value to each individual tree was
168 the mean of its 15 leaves analyzed. After that, the predation values for each tree were
169 standardized by the total foliar biomass of *Q. ilex* of its plot. All values were thereafter
170 transformed for normality (arcsin(square root(percentage))).
171
172 Foliar processing for elemental and metabolomic analyses.
173
174 The processing of the leaves is explained in detail in Rivas-Ubach et al., 2013 MEE.
175 Briefly, frozen leaves in liquid nitrogen were lyophilized, kept into plastic cans and

maintained at -20˚C. Samples were ground with a ball mill at 1600 rpm for 6 min. (Mikrodismembrator-U, B. Braun Biotech International), obtaining a fine sample powder that was therafter kept at -80˚C until metabolite extract preparation.

Elemental analysis

For the C and N analyses; for each sample, 1.4 mg of powder were weighted and introduced into a tin microcapsule. C and N concentrations were determined by elemental analysis using combustion coupled to gas chromatogrtaphy with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).

The macroelements (P, K) determination was performed using acid digestion into microwave with high pressure and temperature control. A widely used acid digestion method was performed (Sardans et al. 2010); 250 mg of leaf powder were weighted into a Teflon tubes and 5mL of Nitric acid and 2mL of H_2O_2 were added. A *MARSXpress* microwave (CEM, Mattheus, USA) was used to perform the acid digestions (Chemical analyses details are explained in Supporting information). All the digested concentrations were added into 50 ml flasks and dissolved with Milli-Q water until 50mL. After digestions, the P and K concentrations were determined by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (The Perkin-Elmer Corporation, Norwalk, USA).

Metabolite extraction for NMR analyses.

The extraction of foliar metabolites for the NMR analyses is described in detail in Rivas-Ubach et al., 2013 MEE. First, two centrifuge tube sets of 50 mL were labeled, set A was used for metabolite extraction and set B was used for lyophilization. Additionally, a set of crystals jars was also labeled to keep the organic fraction. After that, 200 mg of powdered leaf material of each sample was introduced into the

corresponding tube of set A. Six mL of water–methanol (1:1) and 6 mL of chloroform were added to each tube. Samples were vortexed for 15 s and then sonicated for 2 min at room temperature. All tubes were centrifuged at 1 100 × g for 15 min. Four mL of each fraction (aqueous and organic) were collected independently; aqueous fractions were collected into the centrifuge tubes set B and organic fractions were collected into crystal jars. This procedure was repeated twice to perform two extractions on the same sample. Aqueous samples, previously redissolved in water (<15% methanol), were lyophilized. Once lyophilized, 4 mL of water were added to each tube, vortexed and centrifuged at 23, 000 x g for 3 minutes. Samples were frozen at -80ºC and lyophilized again. Organic fractions were placed in a round-bottomed evaporation flask and dried in a rotary vacuum evaporator. Finally, 1mL of KH_2PO_4-NaOD–buffered D_2O (pH 6.0) was added to the dried aqueous fractions, and 1 mL of chloroform-D was added to the dried organic fractions. All concentrations were resuspended with micropipette and transferred into 2 mL centrifuge tubes and centrifuged at 23, 000 x *g* for 3 minutes. The supernatants were transferred into NMR sample tubes.

Metabolite extraction for LC-MS.

The metabolite extraction was done by following t'Kind et al., 2008 Jounal of Chromatography B with little modifications. Two sets of Eppendorf tubes were labeled (set A and set B). The set A was to perform the extractions and the set B to keep the extracts.

One hundred mg of sample powder of each sample was weighted into the corresponding Eppendorf tube of set A. One mL of MeOH/H_2O (80:20) was added to each tube. Tubes were vortexed for 15 minutes and 5 minutes sonicated at room temperature. After that, tubes were centrifuged at 23, 000 x *g* for 5 minutes. 0.6 mL of

supernatant was collected for each tube and introduced into the corresponding Eppendorf set B. This procedure was repeated to perform two extractions of the same initial sample. Once the two extractions were performed, the set B of tubes were centrifuged at 23, 000 x *g* rpm for 5 minutes. The supernatants were collected by crystal syringes, filtered with microfilters and introduced into a labeled set of HPLC vials. The vials were kept frozen at -80ºC until LC-MS analysis.

LC-MS analyses.

LC-MS chromatograms were obtained using a Dionex Ultimate 3000 HPLC system (ThermoFisher Scientific, USA/Dionex RSLC, Dionex, USA) coupled to a LTQ Orbitrap XL high resolution mass spectrometer (ThermoFisher Scientific, USA) equipped with a HESI II (Heated electrospray ionization) source. Chromatographic method was performed on a Hypersil gold column (150 × 2.1 mm, 3µ particle size; Thermo Scientific). Column temperature was set up at 30ºC. Acetonitrile (A) and water (0.1% acetic acid) (B) were used as the mobile phase. Both mobile phases (A) and (B) were filtrated and degassed for 10 min in an ultrasonic bath prior to use. With 0.3mL flow rate, the gradient elution started at 10% A (90%) in 5 min stable, next 20min a gradient to 10% B (90% A) started, next 5 minutes were to recover the initial proportions gradually (10% A; 90% B) and 5 additional minutes were to wash and stabilize the column to inject next sample. 5µl was the injection volume of samples. The heated electrospray ionization (HESI) was used for MS detection. All samples were injected twice, once with ESI operating in negative ionization mode (-H) and once in positive ionization mode (+H). The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) in full scan mode with a 50-1000 m/z mass

range and high mass resolution (60 000). The resolution and sensitivity of the Orbitrap were controlled by injection of mixed standard after analyzing of each 10 samples and resolution was also checked by the help of lock masses (phthalates). Blanks were also analyzed during sequence.

NMR analyses.

^{1}H NMR, 2D 1H–1H-correlated spectroscopy (COSY), heteronuclear multiple bonds coherence (HMBC), heteronuclear single quantum coherence (HSQC) experiments were recorded at 298.0 Kon a Bruker AVANCE 600 spectrometer equipped with an automatic sample changer and a multinuclear triple resonance TBI probe (BrukerBiospin, Rheinstetten, Germany) at a field strength of 14.1 T (600.13 MHz ^{1}H frequency). Following the introduction to the probe, samples were allowed to equilibrate (2 min) prior to the shimming process to ensure good magnetic field homogeneity. All liquid sample handling, automation and acquisition were controlled using TopSpin 3.1 software (BrukerBiospin, Rheinstetten, Germany).

For the water/methanol extract samples, one-dimensional (1D) ^{1}H NMR spectra were acquired with suppression of the residual water resonance. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation (55 dB) at the H_2O frequency during the recycle delay (2s). Each ^{1}H NMR spectra consisted of 128 scans requiring 8 min acquisition time with the following parameters: 0.29 Hz/point, pulse width (PW) = 90◦ (12.7 _s), and relaxation delay (RD) = 2.0 s. FIDs were Fourier transformed with LB = 0.2 Hz. For the chloroform extract samples, standard pulse-acquisition 1D ^{1}H-NMR spectra were acquired. The acquisition parameters were also set at 32 k data points, 16 ppm of spectral width and 128 transients, resulting in a total acquisition time of ~8 min per sample. The COSY spectra

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were acquired with 2.0 s relaxation delay, 7211 Hz spectra width in both dimensions. The window function for COSY spectra was sine-bell (SSB = 0). The HMBC spectra were obtained with 1 s relaxation delay, using 5411 Hz spectral width in F2 and 33,202 Hz in F1. For HSQC spectra, the 1 s relaxation delay was used, 9615 Hz spectral width in F2 and 15,091 Hz in F1. All 2D spectra were calibrated at 0.00 ppm to TSP and were acquired using standard Bruker pulse sequences and routine conditions (Kim *et al*. 2010; Rivas-Ubach *et al*. 2012).

NMR metabolite elucidation.

The NMR spectrometer described for the fingerprinting was used for the acquisition of the 2D NMR experiments on random selected samples of the water-methanol and of the chloroform extracts. The probe temperature was set to 298.0 K and the software utilized to acquire and process the experiments was also TopSpin3.1 (BrukerBiospin). For the water–methanol extract samples, 2D experiments were carried out with standard presaturation of the residual water peak during the relaxation delay. Spectra were referenced to TSP (^{1}H and ^{13}C at δ 0.00 ppm) in the case of polar samples and to the residual $CHCl_3$ (δ_H 7.260 ppm and δ_C 77.00 ppm for ^{1}H and ^{13}C, respectively) in the case of nonpolar samples.

The representative both polar and non-polar 1H NMR spectra of *Q. ilex* is shown in Figure S1and S2. The 1D 1H selective total correlation spectroscopy (TOCSY) experiments, as well as 2D experiments, such as, 1H-1H TOCSY, 1H-1H–correlated spectroscopy 1H-13C heteronuclear single-quantum correlation, and 1H-13C heteronuclear multiple-bond correlation, allowed the identification of the metabolites. All elucidated metabolites were further confirmed by reported literature data (Fan & Lane 2008; Fan 1996; Walker *et al*. 1982; Breitmaier*et al*. 1979; Iles *et al*. 1985;

Bolinger*et al.* 1984; Brown 1989; Corse &Lundin 1970; Ulrich *et al.* 2007; Sacchi*et al.* 1997; Jie&Lam 1995; Llusia *et al.* 2008; Gunstone*et al.* 1995; The AOCS Lipid Library 2012). Table S1 and S2 summarize the chemical shifts and coupling constants of all the identified metabolites present in *Q. ilex*, including amino/organic acids, carbohydrates, phenolic compounds and non-polar metabolites.

LC-MS chromatograms processing

The raw data files from the Orbitrap were processed by MzMine 2.10 (Pluskal et al., 2010 BMC Bioinformatics). Chromatograms were base line corrected, deconvoluted, aligned and filtered before exporting the numerical database to CSV. (see Table S3 for details). Metabolite assignation was done by the exact mass and retention time from measurement of standards in MS and MS^n modes of Orbitrap (see Table S4 for details). The different assigned variables corresponding to the same molecular compounds were added up.

NMR bucketing.

The processing of ^{1}H-NMR spectra is detailed in Rivas-Ubach et al., 2013MEE. Briefly, before the extraction of ^{1}H NMR numerical databases, all spectra were phased, base line corrected and referenced to the internal standard (TSP for polar and TMS for nonpolar samples) resonance at δ 0.00 ppm with TOPSPIN 3.1. A variable-size bucketing was thus applied to all ^{1}H-NMR spectra with AMIX software (Bruker Biospin, Rheinstetten, Germany), scaling the buckets relative to the internal standard (TMS or TSP). The output was a data set containing the integral values for each ^{1}H-NMR spectral peak accounted for in the described pattern. The buckets corresponding to the same molecular compound were added up.

329

330 Statistical analyses

331 To test the differences between seasons and climatic treatments in foliar elemental
332 sotichiometry and metabolome, the *Q.ilex* foliar LC-MS and NMR metabolomic
333 fingerprints were subjected to PERMANOVA analysis (Anderson et al., 2008) using the
334 Euclidean distance, with season (spring, summer, autumn and winter) and climatic
335 treatment (control and drought) as fixed factors and folivory as a covariable. The
336 number of permutations was set up at 999. The PERMANOVA analysis was conducted
337 with PERMANOVA+ for PRIMER v.6 software (Anderson et al., 2008).

338 Additionaly, to understand how the foliar stoichiometry and metabome of *Q.ilex*
339 shift among the studied factors (seasons, climatic treatment), the foliar stoichiometric
340 and metabolomic fingerprint were also subjected to principal component analysis
341 (PCA). The seasonal PCA included the fingerprints of all seasons together (Figure.1).
342 Spring and summer seasons were additionally submitted to another PCA analysis
343 separately (Figure.2) since these are the seasons presenting higher foliar herbivore
344 activity in the Mediterranean basin. The PCA analyses were performed by the "*pca*"
345 function of "*mixOmics*" package of R (R Development Core Team 2008). Scores
346 coordinates of variables were subjected to One-way ANOVAs to find statistical
347 differences among groups (See Supporting Information of Rivas-Ubach et al., 213).
348 Kolmogorov-Smirnov test was performed to each variable to test variable normality, all
349 assigned and elucidated metabolites presented normal distribution, and any not
350 identified metabolomic that did not present normality was removed from the dataset.

351 An additional PCA conducted with summer data and excluding the herbivore
352 consumption rates were also performed (Figure S3). The PC1 scores of this PCA
353 including the whole elemental, stoichiometric and metabolomic variation of summer

cases were plotted versus the herbivore consumption rates (Figure.4). GLM analysis were used to explain the folivory as a function of the climatic treatment and the metabolome variation (PC1 scores); folivory (continuous) was set up as dependent variable while climatic treatment (categorical) and PC1 scores of the PCA (continuous) were set up as independent variables. Statistica v8.0 (Statsoft) was used to perform one-way ANOVAs, post hoc test of scores coordinates of the PCAs and general lineal model (GLM) analysis.

Results

Elemental, stoichiometric and metabolism shifts across year seasons and drought experiment.

PERMANOVA analysis performed with all elemental, stochiometric and metabolomic data (assigned and not assigned metabolites) showed that the different year seasons, drought treatments and the folivory rates presented different foliar chemistry and metabolism (Folivory: Pseudo-F = 2.4832, $p<0.001$; Season: Pseudo-F = 2.4749, $p<0.001$ and Treatment: Pseudo-F = 3.1031, $p<0.001$).

The seasonal PCA analysis analyzed with all elemental, stocichiometric and metabolomic data plotted by PC1 and PC2 (Figure.1) showed more than 50% of variance among the fourth first PC axis (PC1= 15.2%, PC2= 14.7%, PC3= 14.1% and PC4= 12.7%). Different seasons were markedly explained by PC1 ($p<0.001$), PC3 ($p<0.05$) and PC4 ($p<0.001$). Droughted trees differed from control tress in PC1 ($p<0.001$), PC2 ($p<0.05$) and PC4 ($p<0.001$). Post-hoc analysis of the score coordinates of the different cases showed that summer leaves stoichiometries and metabolomes differed significantly from the other seasons in the PC1 axis.

The C, N, P, K foliar concentrations and their respective ratios (C/N, C/P, C/K, N/K and K/P) except N/P changed with the seasons (Figure.1). The highest K/P and K/N concentration ratios were found in summer, coinciding with the highest K foliar concentrations. N and P foliar concentrations were highest in autumn, presenting thus the lowest C:N and C:P ratios (See Table S5 and Sardans et al., 2013 Acta Oecologica for more details).Mainly all assigned and elucidated metabolites in leaves of *Q. ilex* shifted across different seasons (Table S5).. The main changes were between summer and spring, two critical seasons in the Mediterranean basin. Generally, summer leaves presented higher concentrations of polyphenolics and sucrose whereas spring leaves presented higher concentrations of amino acids, some related compounds of amino acid and sugar metabolism (RCAAS) and some sugars such as pentoses and disaccharides, products directly related to growth. The effects of experimental drought were also detected at seasonal PCA plot (Figure.1; Table S6). In the cases plot of the PCA analysis, droughted trees tended to be distributed to the same direction than the summer ones; summer is the driest season in Mediterranean climates.

Total concentrations of amino acids, sugars and polyphenolics.

The assigned and elucidated variables of the different metabolite families (amino acids, sugars and phenolics) were added up. Factorial ANOVAs showed that in spring and in winter trees presented higher concentrations of foliar amino acids (Figure.3a). Drought treatment affected trees in all seasons increasing the concentrations of total sugars, even though there were not significant differences in summer (Figure.3b). Winter showed higher concentrations of sugars than the rest of the seasons due the increases in droughted trees (Figure.3b). Summer trees showed higher concentrations of total

phenolics and drought treatments increased the phenolic concentrations significantly in summer and winter trees (Figure.3c).

Folivory and drought.

The PCAs conducted to investigate the relationships of drought on folivory in spring and summer cases showed also separation between control and droughted trees in the multidimensional space (Figure 2). In the case of spring, the first four PCs explained the 51% of the total variability. PC1 and PC2 explained the difference between control and droughted trees with a 18.5% and 16.6% respectively of the total variance (Figure.2a), one-way ANOVAs on the PC score coordinates showed values of $p<0.05$ in both axis (Figure.2b). PC3 (16%) and PC4 (14.7%) did not explain variation between control and droughted trees ($p>0.05$). In the PCA plotted with summer cases, the four first PCs explained more than 66% of the total variability (Figure.2c) but only PC1 (18.7%) explained significant difference between control and droughted trees ($p<0.001$) (Figure.2d).

The whole GLM conducted with summer data and with folivory as dependent variable and climatic treatment and PC1 scores of the PCA as independent variables showed statistical significance ($p < 0.001$; $R^2=0.60$) with not significant *PC1 scores* ($p=0.53$) and significant *treatment* ($p<0.05$) effects. It implies that the existence of a significant relationship between folivory and the PC1 scores coordinates of the PCA is mainly due the fixed effect of drought.

Discussion

Seasonality and drought

As expected, the foliar metabolomes of *Q. ilex* showed differences across the year seasons (PERMANOVA p<0.001; Figure. 1). PC1 of the seasonal PCA could detect significant stoichiometric and metabolomic shifts between summer and the rest of the seasons although the differences between seasons were not as marked as found in past studies with the Mediterranean shrub *Erica multiflora* (Rivas-Ubach et al., 2012 PNAs). Trees in spring season, the Mediterranean growing season, showed high concentrations of amino acids, some RCAAS and some sugars such as pentoses and disaccharides in accordance with past studies (Rivas-Ubach et al., 2012 PNAs), even though the total concentration of amino acids did not differ from winter (Figure.3a), the coldest season of the Mediterranean basin, and foliar N:P ratios did not show the lowest values as expected in the frame of the growth rate hypothesis (Rivas Ubach et al., 2012 PNAs) which proposes that organisms with high rates of growth need high contest of P (low N:P ratios) to meet the demand for protein sythesis (Elser et al., 2003; Zhang and Han 2010). Even so, our results are in accordance with other studies in plants. The high foliar concentrations of amino acids in winter trees could be explained by the accumulation under cold-stress. Some amino acids such as proline and glycine are able to buffer the NADP+/NADPH ratio in plants (Xu et al., 2013 Plant Biology (15:2, 292-303)) consequently to the low photosynthetic activity in winter and provide reducing agents to support generation of ATP in mitochondria, consequently helping growth recovery (Hare & Cress 1997; Xu et al., 2013 Plant Biology (15:2, 292-303)). On the other hand, Matzek and Vitousek 2009 did not find significant relationships between N:P with increasing growth in 14 different species of pines, and concluded that terrestrial plants need to invest in other fundamental functions besides growth such as storage and defense which often require high investments in N and P.

452 The concentrations of the different detected sugars were distributed differently
453 among seasons (Figure.1) but winter droughted plants showed the highest concentration
454 of total concentrations of sugars (Figure.3b) that may be also related to the sugar
455 synthesis in response to cold-stress, the allocation from other organs (wood,
456 lignotubers) to leaves or the further preparation for the growth season (Xu et al., 2013
457 Plant Biology (15:2, 292-303); Grimaud et al., 2013 Journal of Proteomics). *Q. ilex* is an
458 evergreen tree of slow growth; they present large lignotubers, swollen woody structures
459 at the stem base (James 1984) that are capable to store essential nutrients and
460 metabolites such as carbohydrates to ensure rapid regrowth after severe stresses
461 (Canadell and Zedler 1995). Our stoichimetric and metabolomic results suggest that
462 terrestrial trees, with big woody structures such as trunks and/or lignotubers, are able to
463 store large amounts of different nutrients allowing to resist severe environmental
464 disturbances by supplying essential resources to photosynthetic organs (Canadell and
465 Zedler 1995, Galiano et al., 2012 Tree physiology) and as a consequence it seems to be
466 able buffering the metabolomic changes on foliar ontogeny throughout the different
467 marked seasons.

Codi de camp canviat

468 The largest stoichiometric shift between seasons seemed to be related to drought
469 stress (Figure.1). Potassium concentrations were higher in summer trees, the driest
470 season, presenting thus higher foliar K:P and lower N:K and C:K concentration ratios
471 (Sardans et al., 2013 Acta Oecologica) thus coinciding with other studies in
472 Mediterranean seasons (Sardans et al., 2011 Forest Ecology and Management; Sardans
473 et al., 2012 Functional Ecology). K has demonstrated to be involved in the plant–water
474 relationship (Babita et al., 2010) through plant osmotic control (Babita et al., 2010; Laus
475 et al., 2011) and improving the foliar stomata function (Khosravifar et al., 2008). These
476 results demonstrated the importance of the role of K in ecological stoichiometric studies

in terrestrial plants (Sardans & Peñuelas 2007 Functional Ecology; Sardans et al., 2012 Functional Ecology; Rivas-Ubach et al., 2012 PNAs; Sardans et al., 2013 Acta Oecologica). Moreover, these shifts in K concentrations in summer leaves were accompanied by higher concentrations of sucrose that could also actuate as osmolyte, together with K, avoiding water losses through osmotic control (Ingram and Bartels 1996 annual review of plant physiology and plant molecular biology).

Interestingly enough, PC1 and PC2 of the seasonal PCA also showed differences between the induced experimental droughts with respect to control trees (Figure.1; PERMANOVA $p<0.001$). Droughted trees tended to follow the same direction than summer trees on the layout plotted by PC1 vs. PC2 in accordance with the past studies (Rivas-Ubach et al., 2012 PNAs) showing thus a certain foliar elemental-metabolomic response to drought independently of plant ontogeny. Summer and droughted trees of all seasons tended to present higher concentrations of flavonoids (Figure.3c), the largest group of naturally occurring polyphenols (Strack and Wray 1994 The Flavonoids: Advances in Research Since 1986 (llibre)), but the flavonoid composition differed between summer season and drought treatment. Summer trees presented higher concentrations of quinic acid, catechin, luteolin among other polyphenolics while droughted trees had higher concentrations of catechin, quercitol, homoorientin and quercitin (Figure.1; Table S6). These differences should be related to the different effect of drought treatment at the different seasons (Figure.3c). A great number of biological activities have been described for flavonoids, especially their role as antioxidants (Bruda and Oleszek 2001 J Agric Food Chem; Kwang-Geun 2003 J Agric Food Chem). The antioxidant activity results mainly as they actuate as electron donors (RiceEvans et al., 1997) and the role altering the kinetics of peroxidation (Arora et al., 2000). Quercitol has been related to osmotic stress avoidance under drought stress in *Quercus*

species (Passarinho et al., 2006; Spiess et al., 2012). Our study did not show significant differences ($p = 0.12$) of choline in droughted trees but it showed a tendency of increasing concentrations in stressed plants. Choline is also involved in osmotic protection (McNeil et al., 2001 PNAs). As expected in a water-limited Mediterranean ecosystem, the oxidative stress on plants tends to increase under drought conditions (Prince et al., 1989 Free Rad Res Commun; Dat et al., 2000 Cell Mol Lif Science; Peñuelas et al., 2004 New Phytologist; Munné-Bosch and Peñuelas 2004 Plant Science) and seems that it will have a significant impact on Mediterranean plant metabolomes since drought periods will become more frequent and intense in the following decades as predicted by climate change projections (IPCC 2007).

Furthermore, droughted trees presented also higher concentrations of total sugars concentrations (Figure. 3c), supporting the idea of sugars acting as osmolytes to prevent water losses (Ingram and Bartels 1996 annual review of plant physiology and plant molecular biology). Control trees in summer did not show significant lower foliar concentrations of sugars with respect to the droughted ones (Figure.3b), even though droughted trees showed a tendency to present higher concentrations. Summer is a water limited season in the Mediterranean basin, and the small shift of sugar foliar concentrations between control and droughted shifts could be explained mainly by the natural drought-stress of this season by increasing the foliar sugar concentrations of control trees.

Folivory

In the Mediterranean basin, spring and early summer are the main year periods presenting the highest herbivore activity by insects (Powell and Logan 2005; Bonal et al., 2010 Ecological Entomology) and leaves of trees present the accumulated folivory

of the year in the middle of summer. Interestingly, the herbivore activity was higher in droughted trees in both spring and summer seasons (Figure.2 and 4). GLM analyses of summer season showed that the relationship between folivory and PC1 scores coordinates of the additional PCA (foliar stoichiometric and metabolomic shifts between control and droughted trees) (Figure S3) is mainly due the fixed effect of drought since there is not any relationship inside groups (control, drought) ($p>0.05$) (Figure.4). Our results suggest thus that the stoichiometric and metabolomic shifts are mainly caused by the experimental drought and it produced later an indirect effect increasing the folivory rates. Our stoichiometric results did not show significant relationships between foliar N concentrations and folivory rates as reported in other studies (Larsson et al., 1989 and 1993; Rouault et al., 2006 Annals of forest Science) showing that folivory is not only related to foliar N concentrations(Choong et al., 1992; Williams et al., 1998). As discussed above, droughted trees tended to present significant higher concentrations of sugars and polyphenols (flavonoids) than controls (Figure.3). It has been demonstrated that flavonoids such as quercitin act as phagoestimulants for insect herbivores (Diaz Napal et al., 2010 Journal of Chemical Ecology; Kosonen et al., 2012 Journal of Chemical Ecology).The higher concentrations of sugars and some flavonoids found in foliar droughted *Q. ilex* trees seem to increase the herbivore activity (Figure.4), even though the number of studies is still scarce, our results also showed a higher folivory activity in droughted trees supporting the idea of high palatability of some flavonoids not directly related to folivory defense but as antioxidant function (Diaz Napal et al., 2010 Journal of Chemical Ecology; Kosonen et al., 2012 Journal of Chemical Ecology). Also, droughted leaves presented higher concentrations of sugars (Figure.4), contributing thus with higher concentrations of rapid energy for herbivores. Even so, not all the assigned polyphenols are only related to antioxidant protection in

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plants. Several studies discussed the effect of polyphenolics as a plant chemical defense against herbivore predation (Berg 2002 Oecologia; Lokvam and Kursar 2005 Journal of Chemical Ecology; Kosonen et al., 2012 Journal of Chemical Ecology; Rani and Pratyusha 2013 Jornal of Asia-Pacific Entomology). Kosonen et al., 2012 discussed that the effects of some polyphenolics could be toxic for specialist herbivores but increases the palatability of plants for generalist herbivores and suggests that climate change is able to decrease the herbivore damage caused by specialists and increases that caused by generalists. Adding to it, studies suggest the use of phenolic acids as pest management (Rani and Pratyusha 2013). Moreover, the effects of polyphenols in plants have been shown also in mammals, p.eg, Berg 2003 showed that elevated concentrations of catechin had negative effects on the consumption rates of plants by collared lemmings. In our study, mainly all the assigned polyphenols has been associated to drought-stress since mostly assigned flavonoids presented antioxidant functions but droughted trees also presented higher significant foliar concentration of catechin (Figure.2a,c) supporting thus the idea of also plant-induced defenses which are not only produced by drought but also by higher herbivore attack rates.

It has been thus demonstrated that seasonal drought affected plant stoichiomtrey and metabolism, and the indirect effect of experimental drought in folivory activity by also shifting the metabolism (Figure.4). The further predicted severe and more repeated droughts in the Mediterranean basin (IPCC 2007) may have thus an indirect impact on trophic webs by shifting the consumption rates of herbivores due the foliar stoichiometric and metabolomic shifts (Figure.4) conducing to more intensive and extensive pest outbreaks and possible changes in the food distribution between specialists and generalists herbivores (Kosonen et al., 2012)

Conclusions.

· Foliar N and P did not show significant shifts between year seasons; K did, and thus showed its importance in naturally summer drought-stress. The insignificant variation of N and P in *Q. ilex* may be explained by the buffering effect of lignotubers.

· Drought stress produced increases the concentrations of sugars and polyphenolic compounds with antioxidant function in leaves of *Q. ilex*. Our results suggest that these shifts turn stimulating the herbivore foliar consumption making thus that plants respond also to herbivore attack by increasing polyphenolic compounds with defensive functions.

· Coupling stoichiometric with metabolomic techniques have been proved to be useful to show the molecular responses of plants to stresses such as drought and even more to understand which mechanisms and functions underlay the plant responses and finally allow to interpret further implications throughout trophic webs.

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Figures

Figure.1 PCA conducted with the elemental, stoichiometric and metabolomic variables in *Q. ilex* leaves using PC1 versus PC2. (A). Panel of stoichiometric and metabolomic variables. C/N/P/K ratios and Folivory are shown in red. Colors indicate different metabolomic families: blue, sugars; green, amino acids; orange, RCAAS; cyan, nucleotides; violet, phenolics; lightorange, non-polar metabolites; darkblue, NMR overlapped signals; brown, terpenes. A number has been assigned to each metabolite forming part of the NMR overlapped signals: Sucrose (Suc; 1), α-Glucose (αG; 2), β-Glucose (βG; 3), Deoxy-Hexose (Deoxy-Hex), Hexose (Hex), Pentose (Pent), Disacharide (Disach), Aspartic acid (Asp.ac), Lactic acid (Lac.ac; 11), Succinic acid (Succ.ac), Citric acid (Cit.ac; 4), Piruvate (Pir), Malic acid (Mal.ac), Gallic acid (Gall.ac), Alanine (Ala; 5), Isoleucine (Ile; 6), Threonine (Thr), Valine (Val; 7), Leucine (Leu), Phenilalanine (Phen), Proline (Pro), Arginine (Arg), Tryptophan (Trp), Tyrosine (Tyr), Quercitol (Q.OH; 8), Quinic acid (Q.ac; 9), Choline (Chol; 10), Catechin (Cate), Epicatechin (Epica), Epicallocatechin (Epiga), Homoorientin (Hom), Quercitin (Quer), Rhamnetin (Rham), Kampferol (Kamp), Luteolin (Lut), Chlorogenic acid (Chlo.ac), N-acetyl group (12), Polyphenol (Poly; 13), Phenolic group (Phe.gr), Pyridoxine (Pyri), Caryophyllene (Caryo), α-Humulene (αHum), Fatty acids (FA), Unsaturated fatty acids (UFA), Polyunsaturated fatty acids (PUFA), Diacylglicerids (DGA), Triacylgricerid 1 (TGA1), Triacylglicerid 2 (TGA2) Aldehid group (Ald), Acetyl group (Acetyl), Linoleyl Fatty acid (Linoleyl FA), Polyphenol derived 1 (P.1), O1 – O10: NMR overlapped signals: O1, 5+10+2+1; O2, 10+2+1; O3, 10+3+1; O4, 4+13; O5, 6+7; O6, 11+Unknown; O7, 11+12; O8, 8+9; O9, 8+9+2+3+1; and O10, 8+1, not assigned metabolites are represented by small grey points. (B) Panel of samples categorized by season and drought treatment. Seasons are indicated by different colors (green, spring; red, summer; yellow, autumn; and blue, winter). Climatic treatment is indicated by geometric figures: circles, controls; triangles, drought. The different black geometric figures indicate the mean of PC1 vs. PC2 scores for treatments (control trees, circle and droughted trees, cross). Arrows outside plots indicate the mean PC for each season (color arrows) or treatment (black arrows). The statistically significant differences between seasons were detected by Bonferroni post hoc tests and are indicated by letters ($p < 0.05$).

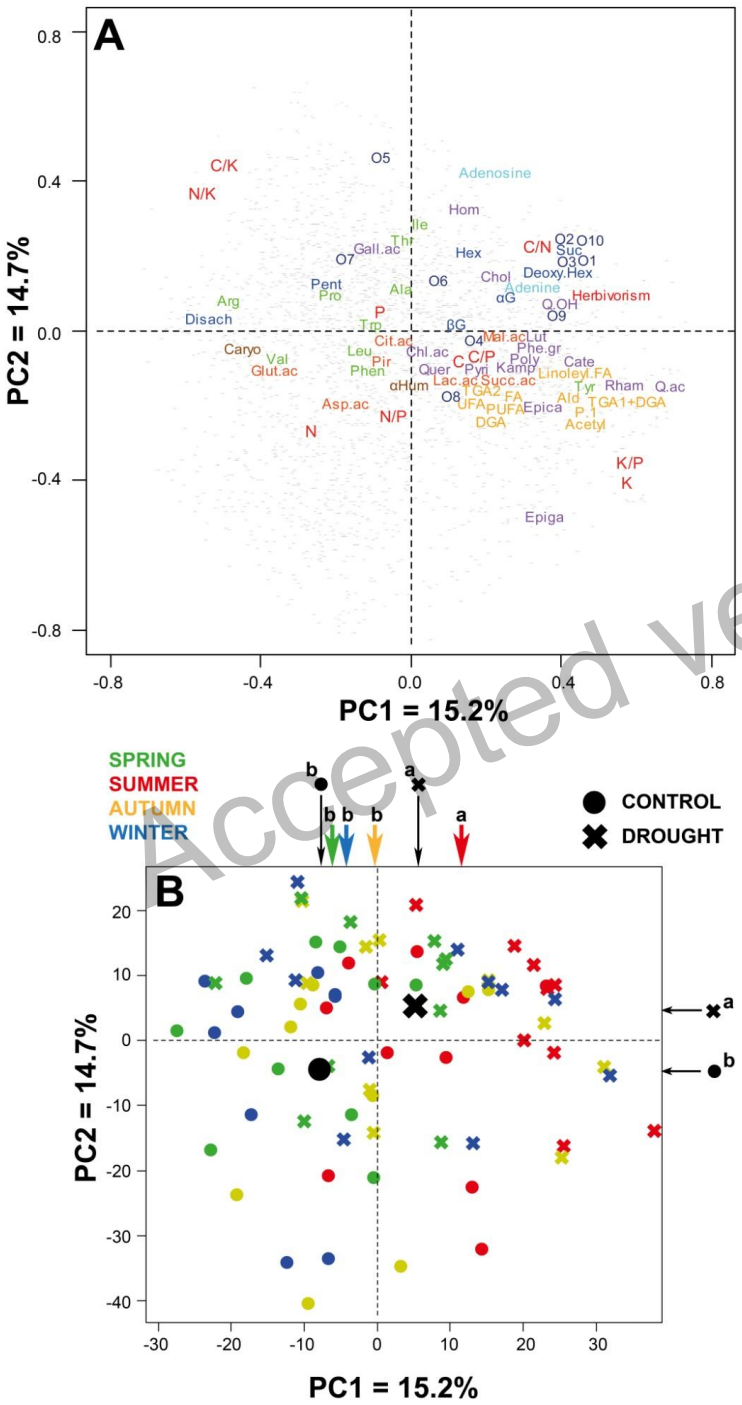
Figure.2 Plots of the PCAs conducted with the metabolomic and stoichometric variables of spring and summer seasons, (A) variables plot of spring season, (B) cases plot of spring season, (C) variables plot of summer season and (D) cases plot of summer season. C/N/P/K ratios and herbivorism are shown in red. Colors indicate different metabolomic families: blue, sugars; green, amino acids; orange, RCAAS; cyan, nucleotides; violet, phenolics; lightorange, non-polar metabolites; darkblue, NMR overlapped signals; brown, terpenes; not assigned metabolites are represented by small grey points. Variable labels are described in Figure.1. Control trees are indicated by blue color and circles, and droughted trees are indicated by orange color and crosses. Arrows outside plots indicate the mean PC for each treatment. The statistically significant differences between seasons were detected by Bonferroni post hoc tests and are indicated by letters ($p < 0.05$).

Figure.3 Histograms of the total foliar concentration of amino acids (A), sugars (B) and polyphenolics (C). Empty columns represent control plots and black columns represent

droughted plots. The statistically significant differences between seasons and treatments were detected by Bonferroni post hoc tests ($p < 0.05$). Seasonal statistical differences are indicated by different letters and climatic treatment statistical differences are indicated by asterisks.

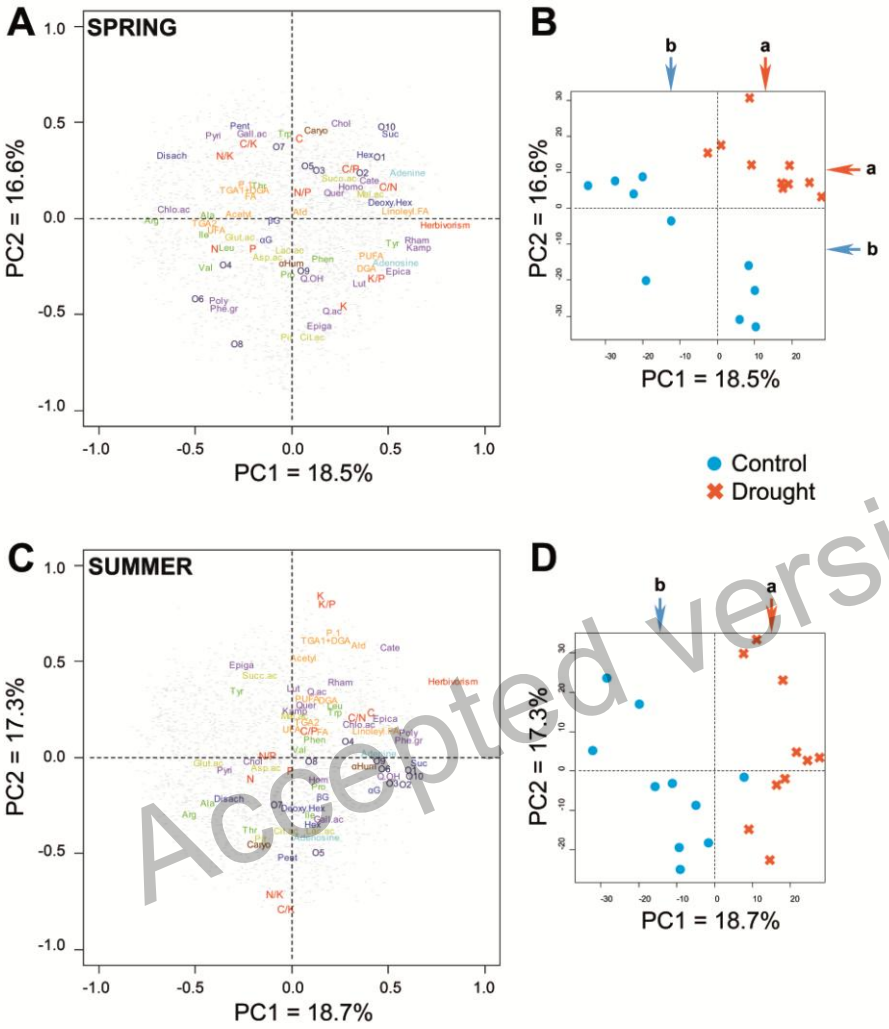
Fiure.4 PC1 scores of a PCA analysis excluding the folivory rates conducted with summer metabolomic and stoichiometric data (Fig. S1) versus the foliar consumption rates. Grey circles represent control trees and grey crosses represent droughted trees. Black circle represents the mean of control trees ±SE and black cross represents the mean of droughted trees ±SE.

776 Fig.1 (COLOR FIGURE)
777

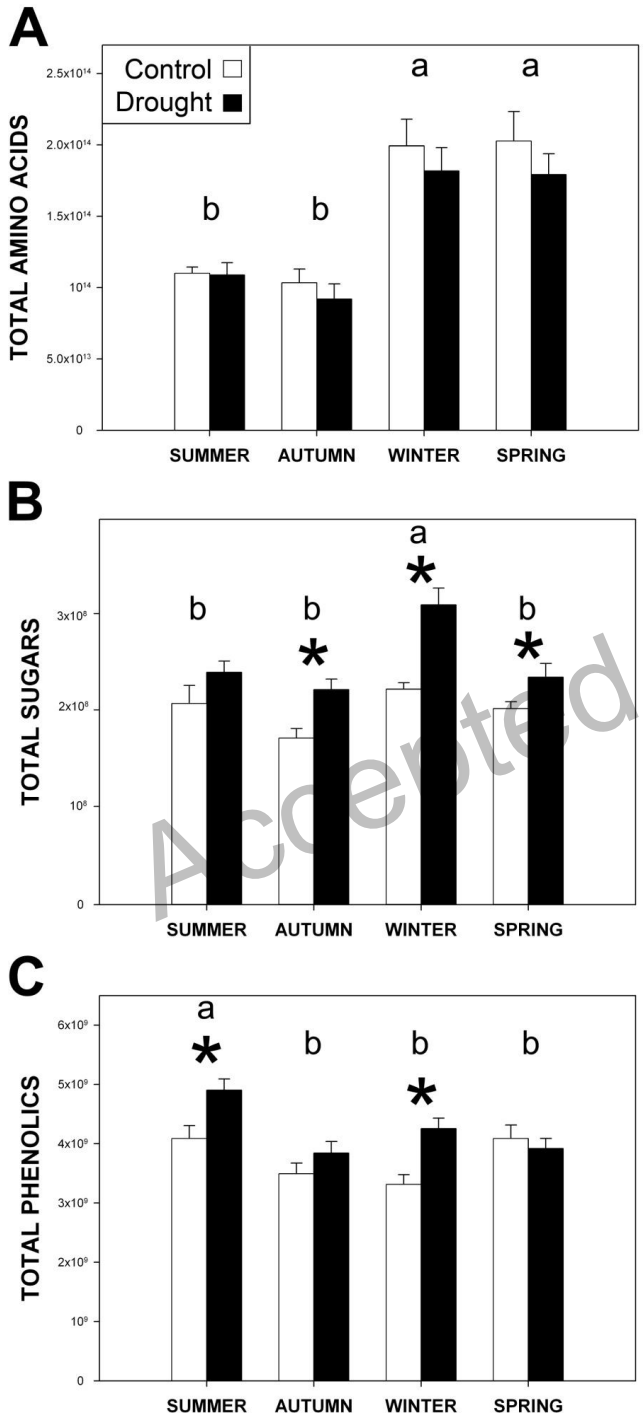


778

Fig.2 (COLOR FIGURE)

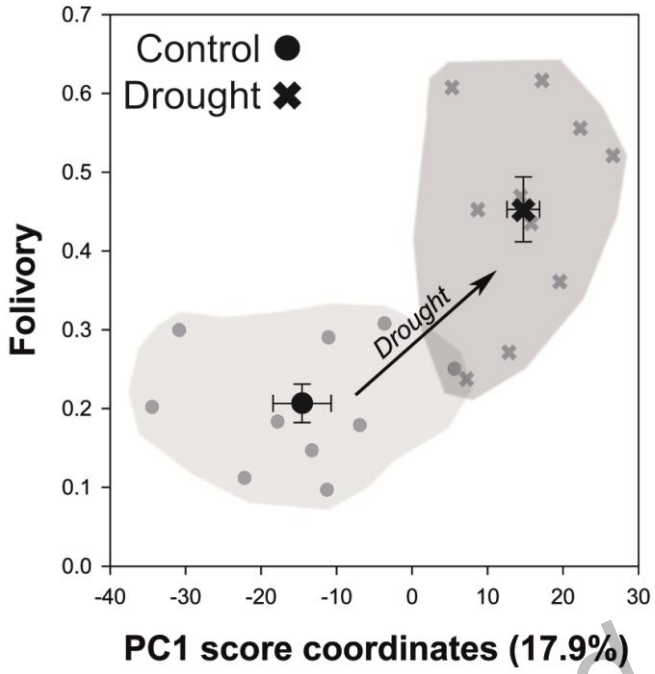


794 Fig.3 (BLACK & WHITE FIGURE)
795



796

Fig.4 (BLACK & WHITE FIGURE)



SUPPORTING INFORMATION

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Chemical analyses

C and N analyses: For each sample, 1.4 g of powder were weighted and introduced into 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 analyses: The P and K analyses determination were performed using acid digestion into microwave with high pressure and temperature control. Briefly, 250 mg of leaf powder were weighted into a Teflon tube and 5mL of Nitric acid and 2mL of H_2O_2 were added (7). A **MARSXpress** microwave (CEM, Mattheus, USA) was used to perform the acid digestions; temperature was increased until 130˚ with a 10min ramp, samples were maintained at this temperature for 5min, then a 10min ramp increases temperature to 200˚C and samples were maintained during 20min. To finish digestions temperature was increased to 220˚C with a 5min ramp and maintained for 20min more.

All the digested contents were put into 50 mL flasks and dissolved with Milli-Q water until 50mL. After digestions, the P and K were determined by *Optima 2300RL* ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (The Perkin-Elmer Corporation, Norwalk, USA).

NMR Elucidation.

The polar and nonpolar metabolic profiles of *Quercus ilex* leaf are shown in Figure S1a and S1B respectively and in Tables S1 and S2 of the supporting information. The detailed ^{1}H NMR metabolic profile of the polar extract is shown in Figure S2. The signals in the region between 3.2 and 5.9 ppm correspond mainly to sugars. Among them, α-glucose and β-glucose, with anomeric proton resonances at 5.29 ppm (d, J = 3.8 Hz) and at 4.70 ppm (d, J = 8.2 Hz) respectively, were identified. The disaccharide sucrose was also observed, with its characteristic anomeric proton doublet of the glucose unit at 5.45 ppm (d, J = 3.8 Hz). The singlet at 3.19 ppm which correlates to multiplets at 3.99 and 3.51 ppm via COSY (bonded respectively to carbon atoms at 53.8, 69.6 and 58.0 ppm correlates via HSQC) corresponds to choline, which is an important osmolyte. In the aliphatic region between 2.2 and 1.7 ppm, the secondary metabolites quinic acid and quercitol were identified, showing very intense signals. Among amino acids, asparagine was identified, by the doublet corresponding to its Hα carboxylic at 4.06 ppm (bonded to a carbon atom with 51.0 ppm chemical shift) and which correlates via COSY two doublet of doublets signals, corresponding to the protons of the methylene group at 2.93 and 2.99 ppm (bonded to a carbon atom at 34.5 ppm correlates via HSQC). Also glutamate, valine, alanine, isoleucine, and leucine were identified. Some organic acids such as formic acid (sharp singlet at 8.50 ppm), malic acid, citric acid, acetic acid and lactic acid were identified as well. The triplet at 1.33 ppm (t, J = 7.17 Hz), which correlates via COSY to a quartet at 4.36 ppm (t, J = 7.17 Hz), typically corresponds to N-acetyl group. Finally, signals at the 6.5 - 7.4 ppm region, area mainly of aromatic compounds, were also observed. All the identifications were based on the ^{1}H and ^{13}C NMR complete or partial assignment of the molecules based on 1D and 2D NMR experiments and on the comparison with reported data. For the complete description see Table S1 of the Supporting Information.

Figure S1B shows the ^{1}H NMR metabolic profile of the nonpolar extracts. Saturated and unsaturated fatty acid chains were identified, which come from free fatty acids, fatty alcohols, diacylglycerols and triacylglycerols. Also, *p*-coumaric acid derivatives were observed in the aromatic region. The complete description for nonpolar metabolites is shown in Table S2 of the Supporting Information.

902 **Table S1.** NMR assignments of the major metabolites in samples of H_2O/MeOH (1:1)
903 extracts. Samples dissolved in D_2O (pH 6.0) and ^1H and ^{13}C NMR chemical shifts
904 referenced to TSP.

Metabolite				NMR chemical shift (d)			Assignment
Id.	Name	Atom id.		^1H		^{13}C	[a, b, c]
		spect.	molec.	(ppm)	m,[d] *J* (Hz)[e]	(ppm)	
1	a-glucose[1, 2, 3]	1.1	1	5.292	d; 3,81	92.62	a [COSY, TOCSY, HSQC, HMBC], b, c
		1.2	2	-	-	-	
		1.3	3	3.68	m*	73.04	
		1.4	4	3.445	m*	70.45	
		1.5	5	3.879	m*	72.78	
		1.6	6	3.945	m*	61.2	
2	b-glucose[1, 2, 3]	2.1	1	4.699	d; 8,16	96.35	a [COSY, TOCSY, HSQC, HMBC], b, c
		2.2	2	3.294	dd; 9,3; 8,3	73.91	
		2.3	3	3.591	m*	77.04	
		2.4	4	*	*	*	
		2.5	5	3.529	m*	76.23	
		2.6	6	3.806	m*	61.5	
3	sucrose[1, 2, 4]	3.1	1	3.728	s	61.6	a [COSY, TOCSY, HSQC, HMBC], b, c
		3.2	2	*	*	*	
		3.3	3	4.256	d; 8,82	76.8	
		3.4	4	4.084	t; 8,69	74.42	
		3.5	5	3.941	m	81.76	
		3.6	6	3.865	d, 3,4	62.9	
		3.1'	1'	5.45	d; 3,78	92.66	
		3.2'	2'	3.613	m	71.48	
		3.3'	3'	3.816	t; 9,5	72.82	
		3.4'	4'	3.514	t; 9,4	72.78	
		3.5'	5'	3.881	m	69.68	
		3.6'	6'	3.858	d; 3,4	60.54	
4	alanine[1, 2, 4-6]	4.1	1	-	-	177.97	a [COSY, HSQC, HMBC], b, c
		4.2	2	3.842	*	52.51	
		4.3	3	1.525	d; 7,3	16.54	
5	isoleucine[1, 2, 4, 5]	5.1	1	-	-	*/**	a [COSY, HSQC], b, c
		5.2	2	3.61	d*/**	*/**	
		5.3	3	1.998	m*/**	*/**	
		5.4	4	1.252	m	23.85	
		5.5	5	0.9348	t; 7,3	**	
		5.6	6	1.019	d*/**	*/**	
6	threonine[1, 2, 4, 7]	6.1	1	-	-		a [COSY, HSQC], b, c
		6.2	2	3.58	*	*/**	
		6.3	3	4.25	m	66.32	
		6.4	4	1.3201	d; 6,40	20.61	
7	citrate[1, 2, 5]	7.1	1	-	*	*	a [COSY, HSQC], b, c
		7.2	2a	2.58	d*	45.6	

		7.3	2b		2.551	d*	45.6		
		7.4	3		-		*		
8	N-acetyl group	8.1	1		-	*	*		a [COSY]
		8.2	2		4.378	q; 7,12	*		
		8.3	3		1.352	t; 7,12	*		
9	quinic acid[10, 11]	9.1	1		-	-	*		a [COSY, TOCSY, HSQC, HMBC], b, c
		9.2	2		-	-	77.49		
		9.3a	3a		1.996	dt; **	37.3		
		9.3b	3b		2.073	dd; **	37.3		
		9.4	4		4.182	dt; **	70.4		
		9.5	5		3.584	dd; **	63.9		
		9.6	6		4.042	m	63.1		
		9.7a	7a		1.898	dd; **	41.0		
		9.7b	7b		2.096	ddd; ***	41.0		
10	choline[1, 14]	10.1	1		3.947	m	65		a [COSY, HSQC, HMBC], b, c
		10.2	2		3.514	m	64.7		
		10.3	3		3.214	s	65.6		
11	lactate[1, 2, 5]	11.1	1		-	*	*		a [COSY], b, c
		11.2	2		4.145	q*	69.1		
		11.3	3		1.341	d*	20.6		
12	Quercitol[17]	12.1	1		3.774		68.75		a [COSY, TOCSY, HSQC, HMBC], b, c
		12.2	2		3.572	t;9,5	74.33		
		12.3	3		3.736		70.79		
		12.4	4		3.956		72.15		
		12.5	5		4.039		68.34		
		12.6a	6a		2.004	dt;13,9;3,36	33.2		
		12.6b	6b		1.819	td;13,9;3,36	33.2		
13	Lysine[1,2,3,19-21]	13.1	-		-	-	*		a [COSY, TOCSY, HSQC, HMBC], b, c
		13.2			*	*	*		
		13.3a			1.869	m	32.15		
		13.3b			*	*	32.15		
		13.4a			*	*	*		
		13.4b			*	*	*		
		13.5a			1.747	m	29.11		
		13.5b			*	*	29.11		
		13.6			3.0423	t;7,5	41.78		
14	Dimethylamine (DMA)[1,2]	14.1	1		2.7353	s	35.97		a [HSQC], b,c
		14.2	2		2.7353	s	35.97		
15	valine[1,2]	15.1	1		-	-	*		a [COSY], b, c
		15.2	2		3.61	d*	*		
		15.3	3		2.282	m	33.37		
		15.4a	4a		1.006	d*	17.62		
		15.4b	4b		1.054	d*	*		
16	acetic acid[18]	16.1	1		1.9539	s	20.26		a,b,c
17	ethanol[19-21]	17.1	1		1.1957	t;7,0	19.40		a [COSY]
		17.2	2		3.6689	q;*	59.90		

	5'-AMP		H8		8.6038	s			
	methanol	15.1	1		3.3693	s	51.5		
	5'-ADP		H8		8.5527	s			
	NAD		A8/A2		8.472	s			
	formate[1, 2, 8, 16]	24.1	1		8.487	s	*		a,b,c

* Overlapped signal.
** Not enough signal.
[a] From 1D ^{1}H NMR chemical shift and 2D ^{1}H-^{1}H and ^{1}H-^{13}C NMR couplings.
[b] Comparison with ^{1}H and ^{13}C NMR data from references indicated.
[c] Comparison with ^{1}H and ^{13}C NMR data from the *Biological Magnetic Resonance Data Bank.* (http://www.bmrb.wisc.edu/).[17]
[d] Multiplicity: singlet (s), doublet (d), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), ddd (doublet of doublet of doublets), m (multiplet).
[e] 1H-1H coupling constant.

Table S2. NMR assignments of the major metabolites in samples of chloroform extracts. Samples dissolved in $CDCl_3$ and ^1H and ^{13}C NMR chemical shifts referenced to the residual solvent peak.

NMR Peak/Spectral Region					**Assignment**[18-24]
d ^{1}H (ppm)	**m, *J*** (Hz)	**d ^{13}C** (ppm)		**id.** spect.	**metabolite (atom id.)/group**
0,864-0,899	t	14.11		A	-CH3 ; FA[a] chains
0,934-0,963	t	14.38		B	-CH3 ; Linoleyl FA chains
1,235-1,272	br	29,05-29,90		C	-CH2 ; FA chains
1.371	t; 7,2	13.84			CH3-CH2-OR; acetyl group
1.569	m	33.52			HO-CH2-CH2- ; FAl
1,582-1,638	m	24.98		D	-CO-CH2-CH2- ; FA chains
2,018-2,099	m	26.95		E	=CH-CH2- ; UFA[b] chains
2,242-2,365	t	34.28		F	-CO-CH2- ; FA chains
2,330-2,364	t	33.92		G	-CO-CH2- ; FFA[f]
2.76	*	25.6			=CH-CH2-CH=; linoeyl and linolenyl chain
2,784-2,826	m	25.67		H	=CH-CH2-CH= ; PUFA[c] chains
3.630	t	63.12			HO-CH2- ; FAl
4.371	q; 7,2	61.65			CH3-CH2-OR; acetyl group
3.746	dd	68.38		I	-CH2OH; 1,2-DGA[d]
3.889	dd	68.38		I	-CH2OH; 1,2-DGA
4.153	dd	62.07		I	-CH2O-; TGA-1[e]
4.210	dd	62.7		I	-CH2O-; 1,2-DGA
4.290	dd	62.07		I	-CH2O-; TGA-1
4.389	dd	62.7		I	-CH2O-; 1,2-DGA
4.493	dd	61.58		I	-CH2O-; TGA-2[f]
5.114	*	72.08		J	-CHO-; TGA-2
5.264	*	68.85		K	-CHO-; TGA-1
5,294-5,417	m	127,70-132,00		L	-CH= ; UFA chains
5.310	*	70.18		M	-CHO-; 1,2-DAG
6.293	d; 16,0	116.28		N	PCAD-2[l](2)
6.833	d; 8,5	115.8		N	PCAD-2 (6/8)
7.431	d; 8,5	129.91		N	PCAD-2 (5/9)
7.598	d; 16,0	143.85		N	PCAD-2 (3)
-		166.86			PCAD-2 (1)
-		127.05			PCAD-2 (4)
-		157.4			PCAD-2 (7)

a.Unknown 1; b.FA: Fatty Acid; c.Unknown 2; d.FAl: Fatty Alcohol; e.UFA: Unsaturated Fatty Acid; f.FFA: Free Fatty Acid; g.PUFA:Polyunsaturated Fatty Acid; h.1,2-DGA: 1,2-Diacylglycerol; i.TGA-1: Triacylglycerol 1; j.TGA-2: Triacylglycerol 2; k.PCAD-1: *p*-Coumaric Acid Derivative 1; l.PCAD-2: *p*-Coumaric Acid Derivative 2.
* Overlapped signal.

Table S3. LC-MS Chromatogram Processing. Chromatograms obtained by LC-MS Orbitrap were processed by MzMine 2.0 (Pluskal et al., 2010). The following table resumes the different process and parameters applied to foliar *Q. ilex* chromatograms.

		(+H) Chromatograms		(-H) Chromatograms
1	**BaseLine correction**			
	Chromatogram Type	TIC		TIC
	MS Level	1		1
	Smoothing	10E7		10E7
	Asymetry	0.001		0.001
2	**Mass detection** (Exact Mass)			
	Noise Level	5×10^5		4×10^5
3	**Chromatogram builder**			
	Min time span	0.05		0.05
	Min 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
	Min 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
	Ions Excluded from Database	< 75 83.05 102.05 114.09 227.17 607.29 Between 0.0 and 1 minute Between 28.5 and 30 minutes		<85 119.035 223.082 391.196 159.25 186.186 Between 0.0 and 1,1 minute Between 27.0 and 30 minutes

Table S4. Metabolite assignation by LC-MS. The assignation of metabolites was done by standards. The following table resume the retention time (RT) and mass (m/z) of the assigned metabolites in both ionization modes positive and negative.

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	Deoxy-Hexose	1.42	163.0618
Negative	Disacharide	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	Kampferol	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	Piruvate	1.65	87.0089
Negative	Quercitin	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	a-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	Phenilalanine	1.91	166.086
Positive	Proline	1.49	116.07
Positive	Pyridoxine	1.38	170.0812
Positive	Quercitin	13.72	303.0498
Positive	Rhamnetin	15.95	317.0653
Positive	Tryptofan	2.49	205.097
Positive	Tyrosine	1.54 - 1.77	182.081
Positive	Valine	1.53	118.086

Table S5. One-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Q.ilex* leaves for seasons (Spring, Autumn, Winter, Spring). For the RMN variables, marked by asterisks (*), integral mean value (mM respect initial TSP or TMS concentration (0.01%). For the LC-MS variables, marked by crosses (†), integral mean value of deconvoluted total intensities.

	SEASON									
Variable	SUMMER		AUTUMN		WINTER		SPRING		F	p_value
C/N (mg/gr)	42.29829	a	39.16987	b	42.18622	a	43.55859	a	3.58477	0.017607
N/P (mg/gr)	13.62614		12.90552		13.58659		12.65678		1.11242	0.349497
C/P (mg/gr)	571.9214	a	502.6113	b	570.9746	a	550.5556	ab	2.84708	0.043165
C/K (mg/gr)	77.1155	b	84.7965	b	116.2141	a	109.6029	a	9.68552	0.000018
K/P (mg/gr)	7.869561	a	6.490965	b	5.374841	b	5.393000	b	6.07256	0.000932
N/K (mg/gr)	1.856670	d	2.202410	cd	2.785448	ab	2.533329	bc	6.69686	0.000458
N (mg/gr)	11.88250	b	12.97053	a	11.96500	b	11.59750	b	4.61285	0.005130
P (mg/gr)	0.885445	b	1.033817	a	0.892095	b	0.930850	b	4.13922	0.009028
K (mg/gr)	6.862471	a	6.437835	a	4.656478	b	4.922463	b	8.64768	0.000053
C (mg/gr)	496.7660	b	501.5189	a	501.2745	a	501.2185	a	2.54490	0.062412
aG *	6.017010		5.627649		5.823781		6.496155		1.04830	0.376300
BG *	4.150334	b	4.488954	ab	4.179343	b	5.279338	a	3.53452	0.018712
Suc *	8.975502	a	5.342097	b	7.530463	a	4.695855	b	10.77758	0.000006
Deoxy-Hex †	11312267	a	10177827	ab	9503434	bc	8283399	c	5.24351	0.002438
Hex †	83095558	b	67722637	b	122136094	a	79844162	b	8.52596	0.000061
Pent †	6887232	b	7031891	b	8693196	a	6937933	b	3.27374	0.025675
Disach †	14132326	c	22054521	b	26564959	a	30164246	a	26.29805	0.000000
Ile *	0.800511	ab	0.606389	b	0.931038	a	0.727391	b	3.71506	0.015041
Thr *	0.437575	ab	0.325627	b	0.441336	a	0.376471	ab	4.00130	0.010654
Ala †	3567865	a	2011520	d	2927839	ab	2775083	b	6.76460	0.000424
Val †	7.486852E+13	b	7.411317E+13	b	1.522798E+14	a	1.600834E+14	a	23.08100	0.000000
Leu †	10421698	b	8477753	b	23590504	a	11044350	b	36.63706	0.000000
Phen †	9931861	b	16308864	b	30909308	a	26103989	a	16.71035	0.000000
Pro †	3865724	c	5529905	c	10163191	b	14756314	a	13.30101	0.000000
Arg †	1761996		2530282		3002304		2594525		1.27475	0.289214
Trp †	7733855	b	1136389	b	94926108	a	11048345	b	63.73468	0.000000
Tyr †	2071404	a	1457088	b	2042432	a	628666	c	12.04537	0.000002
Adenine †	5498695	b	4228099	b	9963002	a	4429600	b	3.56145	0.018111
Adenosine †	18420795	a	10891533	b	10847325	b	11418633	b	2.98152	0.036640
Choline †	1.540490	a	1.043819	b	1.295577	ab	1.471539	ab	1.64612	0.185925
Quercitol *	27.25285		24.22621		24.74710		25.69583		0.78888	0.503864
Polyphenol *	15.39138	a	11.97778	b	15.74066	a	14.14173	ab	2.95328	0.037923
Phenolyc group *	21.28824	a	16.30085	b	22.17597	a	19.24353	ab	3.08414	0.032335
Quinic acid †	1.531992E+09	a	1.290526E+09	b	1.113824E+09	c	1.116493E+09	c	10.83631	0.000005
Catechin †	1.953379E+09	a	1.541045E+09	b	1.696778E+09	b	1.957675E+09	a	5.56307	0.001679
Chlorogenic acid †	86723.69		82417.51		93842.30		88329.01		0.05330	0.983657
Epicatechin †	3887947		2982179		2703103		2438935		1.03349	0.382744
Epigallocatechin †	42592593		47130408		36217531		35222684		1.53078	0.213484
Gallic acid †	3790056		3212265		3511846		3789569		0.81244	0.490959
Homoorientin †	63611951		55919822		55675633		57063388		0.82253	0.485512
Kampferol †	25974094		22547824		22040560		26668875		1.05755	0.372323
Luteolin †	140290.3	a	47045.9	b	73569.5	b	112639.2	a	4.36493	0.006892
Pyridoxine †	697662.3	a	172048.3	b	343072.7	b	206236.2	b	5.93158	0.001096
Quercitin †	8599422		8508000		9478483		8926141		0.30910	0.818735
Rhamnetin †	5939953		4893556		5395996		4495051		0.56062	0.642668
Asp.ac †	5210008	c	7241072	b	7503356	b	9116571	a	12.35343	0.000001
Lac.ac †	6369585		6621801		6110383		6424977		0.45129	0.717134
Succ.ac †	16570697	a	12678641	b	15682285	a	17165800	a	3.85842	0.012653
Cit.ac †	158063505	b	223779176	a	135408263	b	147593431	b	14.86171	0.000000
Glut.ac †	30798422	b	41275713	ab	33262197	b	50578093	a	5.15844	0.002694
Pir †	10597297	b	14067267	a	11211737	b	9607654	b	6.99700	0.000326
Mal.ac †	288704758	ab	266570535	b	228916618	c	301694902	a	7.49909	0.000186

a-Humulene	1506341	b	2605868	b	9315889	a	2894857	b	5.20789	0.002542
Caryophyllene	31458.1	b	262900.0	ab	385043.0	a	304277.0	a	2.96437	0.037414
Overlapping1 *	43.43188	a	31.36382	b	38.04842	a	30.10467	b	5.55420	0.001683
Overlapping2 *	11.12391		9.68886		10.23706		9.25318		1.47215	0.228837
Overlapping3 *	12.12945	a	9.24635	d	11.22037	abc	10.07431	cd	3.73908	0.014556
Overlapping4 *	3.051565	a	2.270426	c	3.490667	b	2.933038	a	7.78369	0.000134
Overlapping5 *	0.432328	ab	0.314493	b	0.485281	a	0.424704	ab	2.35644	0.078432
Overlapping6 *	2.263595	ab	1.949481	b	2.497122	a	2.061469	ab	2.08579	0.109099
Overlapping7 *	0.406019	b	0.400394	b	0.555244	a	0.502439	ab	3.21761	0.027410
Overlapping8 *	15.11812		13.89496		13.14657		15.23863		1.00354	0.395996
Overlapping9 *	19.20380		16.80289		17.58093		18.05957		0.95513	0.418413
Overlapping10 *	12.41667	a	8.81738	bc	11.00373	abc	8.87950	bc	5.00243	0.003215
UFA *	22.74433	b	27.29784	a	17.95084	c	23.60898	b	6.57121	0.000522
TGA2 *	5.447353	bc	6.747529	a	4.312369	c	5.568492	b	5.83152	0.001219
TGA1+DGA *	2.546622	a	1.237474	b	1.187863	b	1.532120	b	13.31292	0.000000
PUFA *	8.631549	a	9.566383	a	5.288433	b	9.476447	a	18.50884	0.000000
P *	3.755380	a	2.835052	b	1.839125	c	2.868086	b	8.76008	0.000046
Linoleyl FA *	7.683955	a	7.735614	a	5.941504	b	7.695507	a	4.10045	0.009415
FA *	116.2851	ab	129.7729	a	106.1379	b	107.6017	b	3.21462	0.027510
DGA *	13.18280	a	14.18169	a	8.40722	b	14.29089	a	15.86459	0.000000
Ald *	0.432162	a	0.399634	a	0.268933	b	0.318003	b	6.75223	0.000425
Acetyl *	6.316552	a	4.948125	b	4.383811	b	5.384305	ab	4.51887	0.005705
Herbivorism	0.329726	a	0.265019	a	0.368654	a	0.284742	a	1.89654	0.137305

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Table S6. One-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Q.ilex* leaves for treatments (Control, Droguht) with data from all seasons. For the RMN variables, marked by asterisks (*), integral mean value (mM respect initial TSP or TMS concentration (0.01%). For the LC-MS variables, marked by crosses (†), integral mean value of deconvoluted total intensities.

	(ALL YEAR) TREATMENT					
	CONTROL		DROUGHT		F	p
C/N	40.84257	b	42.85607	a	4.04553	0.047788
N/P	13.48905		12.89829		1.62914	0.205659
C/P	548.2077		551.0343		0.02034	0.886955
C/K	98.65126		95.48032		0.20098	0.655192
K/P	6.124151		6.438726		0.35378	0.553726
N/K	2.429144		2.261255		0.94057	0.335168
N	12.32400		11.85590		2.58445	0.112011
P	0.927450		0.941341		0.15366	0.696148
K	5.549705		5.875869		0.58808	0.445507
C	498.1220	b	502.2862	a	8.66700	0.004282
aG	5.437045	b	6.568781	a	10.93840	0.001433
BG	4.295323		4.760449		2.53508	0.115439
Suc	4.840646	b	8.510523	a	40.47144	0.000000
Deoxy-Hex	9279617	b	10363487	a	3.35992	0.070667
Hex	77861033	b	99328335	a	5.82383	0.018188
Pent	7345000		7440337		0.03515	0.851776
Disach	23872520		22599121		0.44361	0.507375
Ile	0.699775	b	0.838697	a	3.67636	0.058898
Thr	0.408277		0.383679		0.72229	0.398030
Ala	3017774		2639069		1.99605	0.161739
Val	1.229186E+14		1.086165E+14		1.14476	0.287990
Leu	12616352		14296263		0.91341	0.342202
Phen	18206920	b	23602431	a	3.46258	0.066590
Pro	8482520		8755692		0.02748	0.868762
Arg	3120388	a	1806060	b	8.83947	0.003934
Trp	23320247		34947376		1.28109	0.261209
Tyr	1329974	b	1777840	a	3.79370	0.055091
Adenine	3237191	b	8940312	a	17.73076	0.000068
Adenosine	13303293		12526731		0.12016	0.729804
Choline	1.209234		1.477316		2.43858	0.122484
Quercitol	22.91172	b	28.14730	a	14.51730	0.000278
Polyphenol	13.49634	b	15.21025	a	2.91242	0.091930
Phenolyc group	18.03568	b	21.60112	a	5.76095	0.018802
Quinic acid	1.237052E+09		1.289336E+09		0.54184	0.463907
Catechin	1.640474E+09	b	1.944040E+09	a	12.05120	0.000852
Chlorogenic acid	88017.60		87772.54		0.00015	0.990310
Epicatechin	2765944		3246753		0.58999	0.444773
Epigallocatechin	43916566	a	36396699	b	2.79446	0.098650
Gallic acid	3728851		3428421		0.98913	0.323070
Homoorientin	53809793	b	62489855	a	4.62151	0.034713
Kampferol	23043107		25650127		1.29770	0.258166
Luteolin	90516.89		97517.36		0.11088	0.740054
Pyridoxine	447097.6	a	264729.2	b	2.93551	0.090675
Quercitin	7963006	b	9825966	a	6.09596	0.015765
Rhamnetin	4830741		5547896		0.73926	0.392566
Asp.ac	7682559		6842992		2.36981	0.127801
Lac.ac	6184678		6577590		1.63022	0.205510
Succ.ac	14838437		16300829		1.93747	0.167951
Cit.ac	175769858	a	154931129	b	2.83074	0.096527

Glut.ac	41941804		35880529		2.05088	0.156165
Pir	11980111		10677113		2.74878	0.101398
Mal.ac	267766088		275398005		0.33713	0.563188
a-Humulene	3037257		5188794		1.65242	0.202483
Caryophyllene	321757.2	a	167701.9	b	2.87427	0.094046
Overlapping1	27.50030	b	43.97410	a	57.15315	0.000000
Overlapping2	8.06413	b	12.08737	a	65.29019	0.000000
Overlapping3	8.94536	b	12.38988	a	36.19355	0.000000
Overlapping4	2.811879		3.060969		1.51780	0.221654
Overlapping5	0.374319	b	0.454084	a	2.83613	0.096164
Overlapping6	2.071991		2.313843		2.04606	0.156595
Overlapping7	0.460949		0.471099		0.05271	0.819017
Overlapping8	15.03701		13.66212		1.89619	0.172445
Overlapping9	16.00664	b	19.81695	a	16.43988	0.000118
Overlapping10	7.74314	b	12.81551	a	66.89548	0.000000
UFA	23.52921		22.27179		0.57681	0.449854
TGA2	5.721362		5.316509		0.81310	0.369983
TGA1+DGA	1.601675		1.650365		0.05315	0.818272
PUFA	8.245113		8.236293		0.00021	0.988454
P	2.757615		2.891207		0.19493	0.660067
Linoleyl FA	7.024638		7.503652		1.08310	0.301221
FA	115.5635		114.3353		0.03754	0.846876
DGA	12.46900		12.56230		0.01125	0.915819
Ald	0.336018		0.373348		1.39237	0.241591
Acetyl	5.241531		5.274866		0.00657	0.935597
Herbivorism	0.239194	b	0.384876	a	23.07889	0.000007

Table S7. One-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Q.ilex* leaves for treatments (Control, Droguht) for spring season. For the RMN variables, marked by asterisks (*), integral mean value (mM respect initial TSP or TMS concentration (0.01%). For the LC-MS variables, marked by crosses (†), integral mean value of deconvoluted total intensities.

	(SPRING) TREATMENT					
	CONTROL		DROUGHT		F	p
C/N	42.13999		44.97719		2.88940	0.106379
N/P	12.51845		12.79511		0.12874	0.723914
C/P	524.2908		576.8204		2.08225	0.166197
C/K	106.8274		112.3785		0.15283	0.700433
K/P	5.310746		5.475253		0.05085	0.824125
N/K	2.555357		2.511301		0.01736	0.896624
N	11.97600		11.21900		2.69176	0.118226
P	0.961484		0.900215		0.89784	0.355911
K	5.061831		4.783095		0.20510	0.656050
C	499.6720		502.7650		1.12580	0.302704
aG	6.663098		6.329213		0.28998	0.596831
BG	5.244602		5.314074		0.01207	0.913728
Suc	4.204184	b	5.187526	a	4.55348	0.046864
Deoxy-Hex	7904360		8662437		0.57054	0.459815
Hex	65242324	b	94446000	a	3.81013	0.066679
Pent	6536739		7339127		1.02193	0.325459
Disach	31365468		28963024		0.58868	0.452878
Ile	0.778965		0.675817		1.10134	0.307858
Thr	0.383486		0.369456		0.11490	0.738561
Ala	3080988		2469177		1.74753	0.202742
Val	1.701922E+14		1.499746E+14		0.67852	0.420878
Leu	11827592		10261108		1.13914	0.299941
Phen	24766576		27441401		0.24506	0.626562
Pro	17157745		12354883		1.15379	0.296946
Arg	3105428		2083622		1.32647	0.264502
Trp	8570908		13525781		1.52197	0.233188
Tyr	523316.7		734016.0		1.77800	0.199026
Adenine	2350238	b	6508963	a	6.36123	0.021303
Adenosine	12515700		10321567		0.27156	0.608637
Choline	1.030264	b	1.912814	a	5.05701	0.037287
Quercitol	27.24363		24.14804		2.07703	0.166702
Polyphenol	17.10965	a	11.17381	b	16.25200	0.000783
Phenolyc group	23.47285	a	15.01421	b	19.26894	0.000353
Quinic acid	1.224914E+09	a	1.008072E+09	b	4.64068	0.045019
Catechin	1.820540E+09		2.094809E+09		1.79155	0.197399
Chlorogenic acid	121371.1	b	55286.9	a	5.67969	0.028387
Epicatechin	2443714		2434155		0.00014	0.990802
Epigallocatechin	39292016		31153353		1.42901	0.247438
Gallic acid	3975557		3603580		0.43490	0.517944
Homoorientin	53188447		60938330		0.82319	0.376236
Kampferol	24826282		28511468		0.60366	0.447276
Luteolin	135093.3		90185.1		0.78247	0.388052
Pyridoxine	203383.2		209089.2		0.00725	0.933090
Quercitin	7591675		10260606		2.85583	0.108288
Rhamnetin	4580448		4409654		0.02095	0.886514
Asp.ac	10336980	a	7896162	b	4.87660	0.040434
Lac.ac	6650317		6199636		0.49327	0.491455
Succ.ac	15443406		18888193		2.20263	0.155076
Cit.ac	164267024		130919838		2.97362	0.101762

Glut.ac	53688341		47467846		0.67814	0.421006
Pir	10730868		8484440		1.60898	0.220795
Mal.ac	294997176		308392627		0.77799	0.389382
a-Humulene	3430045		2359669		0.64010	0.434108
Caryophyllene	222886.7		385667.2		0.95832	0.340584
Overlapping1	27.88787	b	32.32146	a	3.09087	0.095724
Overlapping2	8.745718		9.760637		2.17383	0.157652
Overlapping3	9.72601		10.42262		0.56357	0.462529
Overlapping4	3.390062	a	2.476015	b	5.72069	0.027892
Overlapping5	0.391762		0.457645		0.93632	0.346048
Overlapping6	2.629504	a	1.493434	b	13.83636	0.001568
Overlapping7	0.451908		0.552970		0.88695	0.358773
Overlapping8	19.63276	a	10.84450	b	33.38865	0.000018
Overlapping9	19.25143		16.86770		2.15477	0.159385
Overlapping10	7.60305	b	10.15596	a	8.37474	0.009672
UFA	24.29475		22.92321		0.60755	0.445839
TGA2	5.768940		5.368044		0.56792	0.460830
TGA1+DGA	1.606155		1.458085		0.71968	0.407388
PUFA	9.361127		9.591766		0.17339	0.682036
P	2.949732		2.786440		0.25368	0.620603
Linoleyl FA	7.394525		7.996490		1.32084	0.265484
FA	107.4735		107.7300		0.00156	0.968890
DGA	14.14589		14.43590		0.13253	0.720067
Ald	0.325804		0.310202		0.16006	0.693811
Acetyl	5.571168		5.197442		1.14426	0.298889
Herbivorism	0.231760		0.337723		2.83447	0.109524

Table S8. One-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Q.ilex* leaves for treatments (Control, Droguht) for summer season. For the RMN variables, marked by asterisks (*), integral mean value (mM respect initial TSP or TMS concentration (0.01%). For the LC-MS variables, marked by crosses (†), integral mean value of deconvoluted total intensities.

	(SUMMER) TREATMENT					
	CONTROL		DROUGHT		F	p
C/N	41.66038		42.93619		0.34966	0.561660
N/P	13.77995		13.47232		0.09652	0.759615
C/P	569.3676		574.4752		0.01958	0.890258
C/K	81.59398		72.63693		1.07595	0.313341
K/P	7.235808		8.503313		1.55022	0.229069
N/K	1.969276		1.744063		0.86426	0.364851
N	11.97600		11.78900		0.09529	0.761102
P	0.880722		0.890169		0.02510	0.875882
K	6.317420		7.407521		1.84736	0.190877
C	494.0180	b	499.5140	a	3.65572	0.071929
aG	5.233182	b	6.800838	a	4.70655	0.043682
BG	4.007798		4.292870		0.33796	0.568219
Suc	6.52583	b	11.42518	a	21.92706	0.000185
Deoxy-Hex	11665376		10959158		0.42219	0.524053
Hex	84067267		82123850		0.01415	0.906632
Pent	7127894		6646569		0.32928	0.573187
Disach	15342556		12922096		2.77051	0.113326
Ile	0.744576		0.856447		0.37981	0.545423
Thr	0.500795		0.374355		2.99581	0.100586
Ala	4158994	a	2976737	b	5.07479	0.036992
Val	7.523658E+13		7.450046E+13		0.00411	0.949575
Leu	8918857		11924538		1.70889	0.207583
Phen	8742734		11120989		0.71659	0.408379
Pro	3580016		4151432		0.53599	0.473527
Arg	2477675	a	1046317	b	7.68947	0.012541
Trp	5981268		9486441		1.55074	0.228994
Tyr	2093373		2049436		0.00983	0.922129
Adenine	2826604	a	8170786	b	4.40172	0.050284
Adenosine	21049369		15792222		1.09683	0.308822
Choline	1.764591		1.316388		1.54677	0.229567
Quercitol	23.21481	b	31.29090	a	6.48586	0.020237
Polyphenol	12.98213	b	17.80063	a	6.58725	0.019415
Phenolyc group	17.21579	b	25.36068	a	10.23488	0.004971
Quinic acid	1.484741E+09		1.579244E+09		0.52287	0.478912
Catechin	1.734808E+09	b	2.171950E+09	a	7.92429	0.011462
Chlorogenic acid	43031.3	b	130416.1	a	3.01184	0.099746
Epicatechin	2170140	b	5605754	a	3.28101	0.086807
Epigallocatechin	47459630		37725555		1.47933	0.239589
Gallic acid	3909556		3670557		0.11656	0.736744
Homoorientin	61644091		65579812		0.28566	0.599558
Kampferol	25383765		26564422		0.06162	0.806765
Luteolin	136963.8		143616.8		0.01792	0.894986
Pyridoxine	1005105	a	390220	b	3.44019	0.080084
Quercitin	7823142		9375702		0.77178	0.391244
Rhamnetin	5209374		6670531		0.47712	0.498542
Asp.ac	5597393		4822622		1.13350	0.301104
Lac.ac	6191870		6547300		0.27299	0.607707
Succ.ac	17165656		15975739		0.42050	0.524873
Cit.ac	157678450		158448559		0.00167	0.967844

Glut.ac	37458234		24138609		2.05407	0.168942
Pir	10913495		10281099		0.16723	0.687411
Mal.ac	277676155		299733361		1.19513	0.288713
a-Humulene	1184114		1828568		0.53673	0.473225
Caryophyllene	48176.16		14740.12		1.92285	0.182478
Overlapping1	33.00544	b	53.85831	a	17.70423	0.000529
Overlapping2	8.73067	b	13.51716	a	13.77425	0.001598
Overlapping3	10.08155	b	14.17735	a	9.24243	0.007042
Overlapping4	2.816203		3.286928		1.31184	0.267062
Overlapping5	0.430013		0.434643		0.00171	0.967429
Overlapping6	1.862727	b	2.664463	a	5.94843	0.025315
Overlapping7	0.438479		0.373560		0.59953	0.448808
Overlapping8	14.62969		15.60654		0.22186	0.643287
Overlapping9	16.48671	b	21.92088	a	6.17741	0.022993
Overlapping10	9.64605	b	15.18729	a	15.26398	0.001033
UFA	23.61925		21.86941		0.45029	0.510719
TGA2	5.571362		5.323343		0.12293	0.729944
TGA1+DGA	2.507704		2.585541		0.01841	0.893590
PUFA	8.687640		8.575459		0.01487	0.904284
P	3.484175		4.026586		0.55731	0.464985
Linoleyl FA	7.363597		8.004314		0.68387	0.419085
FA	114.9628		117.6074		0.03839	0.846865
DGA	12.99469		13.37092		0.08809	0.770019
Ald	0.371887	b	0.492436	a	3.42695	0.080620
Acetyl	6.428194		6.204910		0.04476	0.834815
Herbivorism	0.206635	b	0.452817	a	26.35006	0.000070

Figure S1. Examples of a 1D ^{1}H NMR spectra of both H_2O/MeOH (1:1) and chloroform extracts of *Quercus ilex* leaves. Assignments of the main peaks are indicated. Samples dissolved in D_2O (pH 6.0) and referenced to TSP and samples dissolved in $CDCl_3$ and referenced to the residual solvent peak ($CHCl_3$). Spectrum acquired at 600 MHz magnetic field and at 298.0 K.

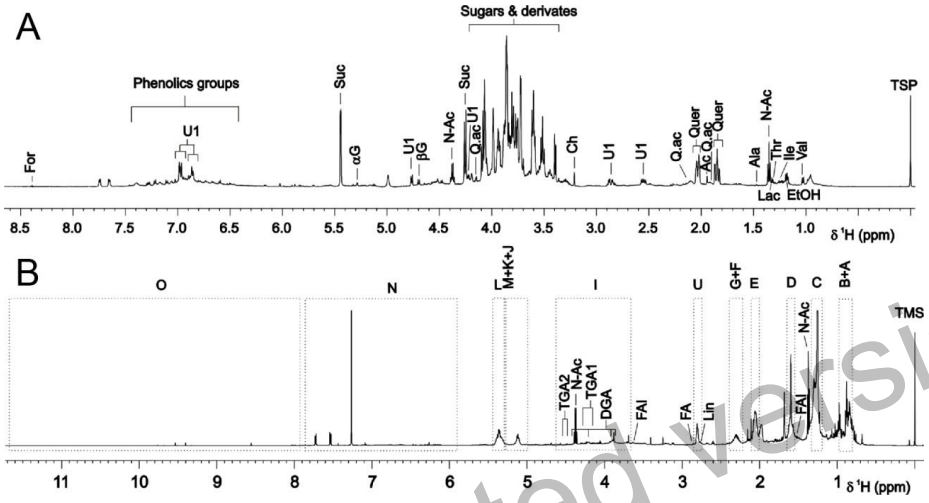


Figure S2. Detailed example of a 1D ^{1}H NMR spectrum of the H_2O/MeOH (1:1) extract of *Quercus ilex* leaves with the assignment of the main peaks. Sample dissolved in D_2O (pH 6.0) and referenced to TSP. Spectrum acquired at 600 MHz magnetic field and at 298.0 K.

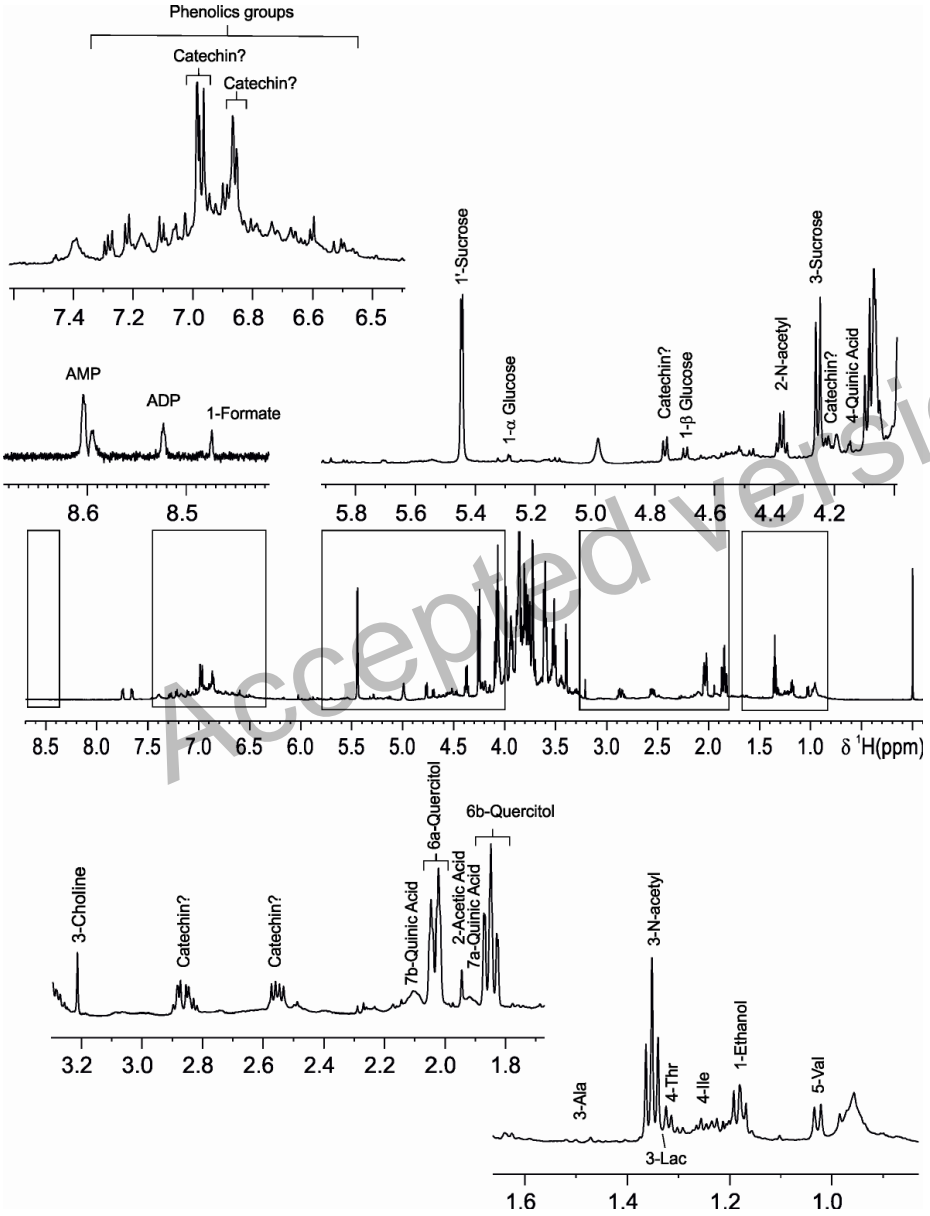
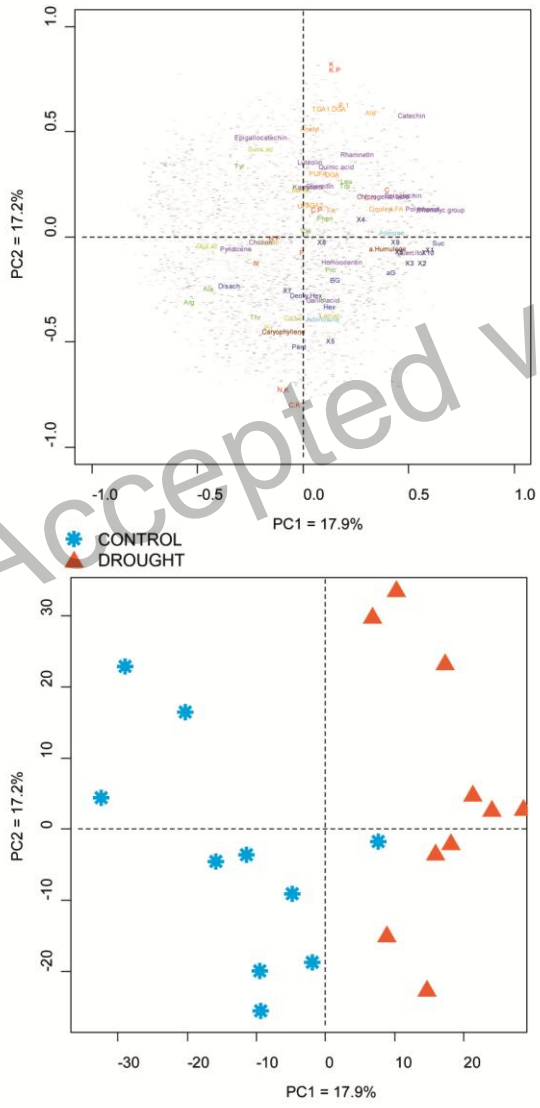


Figure S3. Plots of the PCAs conducted with the metabolomic and stoichometric variables of all seasons excluding folivory as variable. Colors indicate different metabolomic families: blue, sugars; green, amino acids; orange, RCAAS; cyan, nucleotides; violet, phenolics; lightorange, non-polar metabolites; darkblue, NMR overlapped signals; brown, terpenes; not assigned metabolites are represented by small grey points. Variable labels are described in Figure.1 of the main text. Control trees are indicated by blue color and asterisks, and droughted trees are indicated by orange color and triangles.



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