Hormone and carbohydrate regulation of defense secondary metabolites in a Mediterranean forest during drought

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Tables and figures in main body: seven figures, all should be published in colour

Supplemental file: four figures and four tables

Declarations of interest: none

Abstract

Plants have evolved complex physiological mechanisms to regulate production of defense secondary metabolites (SMs) to cope with environmental stress such as drought. Yet, these mechanisms remain under-studied in mature trees, limiting our ability to understand effects of climate change on tree productivity and survival. We investigated defense SMs and their relations to growth, nonstructural carbohydrates and phytohormones across seasons, in a Mediterranean forest dominated by two tree species with different resistance to drought. Our results showed seasonality of leaf SMs, with a strong accumulation of SMs during cold and dry conditions in winter. The drought-resistant species *Phillyrea latifolia* had high levels of SMs in both leaves (ca. 10-20%) and stem phloem (4-6%), but experimental drought decreased SMs and soluble sugars in stem phloem in spring and summer. By contrast, the drought-susceptible species *Quercus ilex* had relatively low and constant levels of SMs, irrespective of drought. We further showed that *P. latifolia* leaves had generally higher levels of jasmonic acid and lower salicylic acid than *Q. ilex* leaves, potentially leading to differences in SMs and growth between the two co-occucrring species. Our study suggests that species- and organ-specific dynamics of defense SMs are driven by phytohormones and carbohydrates in mature trees, and that *P. latifolia* may become less resistant to abiotic and biotic stress due to reduced phloem defense SMs and carbohydrates in a drying climate.

Keywords: carbon allocation; drought; growth-defense trade-offs; jasmonic acid; nonstructural carbohydrates; phytohormones; salicylic acid; secondary metabolites; tree mortality and community shift

Introduction

Climate change is expected to increase the frequency and intensity of drought, heat waves, and fires that are likely to threaten forest ecosystem services, including disruption of biogeochemical cycles and loss of biodiversity (Anderegg et al., 2020; McDowell et al., 2020). Of particular concern are drought-induced tree mortality and associated decline in forest productivity, particularly in dry regions of the US (Trugman et al., 2020), Asia (Liu et al., 2013), and Europe (Peñuelas et al., 2018; Senf et al., 2020). Drought may reduce the ability of trees to defend against abiotic and biotic stresses (McDowell et al., 2011). In this context, considerable research has focused on drought impacts on carbohydrate metabolism and in particular nonstructural carbohydrates (NSCs) (Hesse et al., 2021; Jin et al., 2018; Pivovaroff et al., 2021; Rowland et al., 2015; Schönbeck et al., 2018; Zhang et al., 2020). In contrast, very few field studies have investigated impacts of drought on production of secondary metabolites (SMs; Trowbridge et al., 2021), which are a crucial component of plant antioxidant (Agati et al., 2020) and antiherbivore defense (Huang et al., 2020a).

Production of defense SMs under drought is thought to be regulated by carbohydrate availability. Under moderate drought conditions, carbon supply to trees exceeds sink demand, because drought leads to more rapid and sharper decreases in growth than in leaf photosynthesis (sink limitation; Muller et al., 2011; Palacio et al., 2014). This sink limitation may lead to an increase in carbohydrate availability for production of SMs. However, as drought progresses, photosynthesis continues to decrease and cannot meet carbohydrate demands for maintenance metabolism, leading to source limitation for production of defense SMs (Herms and Mattson, 1992; McDowell et al., 2011). This may be particularly important in the Mediterranean where stomata frequently close to reduce transpiration but also limit photosynthetic activity. The extent to which drought affects NSCs and their utilization for defense SMs may vary depending on the season and organ types. Drought-induced sink limitation to growth and increases in NSCs and SMs may occur mainly in the growing season. However, severe drought in summer may lead to source limitation and high carbohydrate demands for maintenance (e.g., osmoregulation; Blumstein et al., 2023; Hajek et al., 2022), thereby reducing the availability of carbohydrates for production of SMs. Drought impacts on NSCs and SMs are further complicated by organ-specific responses to drought. Drought may reduce leaf export and phloem transport of

carbohydrates (Hesse et al., 2018; Joseph et al., 2020; Ruehr et al., 2009), leading to lower carbohydrate availability for production of SMs in stem phloem and roots than in leaves (Mundim and Pringle, 2018).

In addition to carbohydrates, phytohormones have been identified as the central regulators of growth and defense, as well as their trade-offs (Huot et al., 2014; Panda et al., 2021; Züst and Agrawal, 2017). Modulation of phytohormones has also been associated with growth and defense regulation under drought stress, such as accumulation of abscisic acid (ABA) and jasmonic acid (JA) as a signaling mechanism to close stomata, inhibit growth, and induce antioxidant and antiherbivore defense (De Ollas and Dodd, 2016; Nguyen et al., 2016; Riemann et al., 2015). Furthremore, the antagonistic interactions between JA and salicylic acid (SA) are known to mediate plant defense (Pieterse et al., 2012; Thaler et al., 2012). However, studies on phytohormonal regulation of tree defense are mostly based on short-lived model species, such as *Arabidopsis* and crop plants under short-term stress. There is a substantial knowledge gap for long-lived organisms, including trees, that must acclimate and adapt to periodic and episodic environmental stress conditions.

To begin filling this gap, we conducted a quantitative analysis of diverse polyphenolics (flavonoids and secoiridoids), NSCs (soluble sugars and starch), and phytohormones (ABA, JA and SA) in leaves and stem phloem of two co-occurring tree species (*Quercus ilex* and *Phillyrea latifolia*) in a rainfall exclusion experiment. Polyphenolics have been shown to be prominent antioxidants that defend against UV, heat, fire, and drought stresses in Mediterranean tree species (Laoué et al., 2022; Santacruz-García et al., 2021), including *Q. ilex* (Tienda-Parrilla et al., 2022) and *P. latifolia* (Gori et al., 2021; Gori et al., 2019) studied here. It should be noted that *P. latifolia* can synthesize and store SMs not only in parenchyma and epidermal cells but also in glandular trichomes, and that accumulation of SMs in glandular trichomes can enhance resistance of *P. latifolia* to high solar radiation (Tattini et al., 2000; Tattini et al., 2005). Polyphenolics were used as an indicator of defense SMs because our previous work showed that both *Q. ilex* and *P. latifolia* do not store terpenes (Llusia and Penuelas, 1998). The two tree species, *Q. ilex* and *P. latifolia*, are the dominant tree species in this region and known to differ in their resistance and resilience to drought. Both species experienced crown defoliation, growth reduction and/or stem mortality following extreme natural drought (Barbeta et al., 2015) and

during experimental sustained drought (Barbeta et al., 2013; Liu et al., 2015; Ogaya et al., 2020), but drought impacts tend to be more pronounced in *Q. ilex* than in *P. latifolia*. Thus, *Q. ilex* appears to be more vulnerable to climate stress than *P. latifolia*.

The objective of this study is to investigate how drought affects defense SMs and their relationships to carbohydrates and phytohormones in co-cocurring mature trees. Specifically, we addressed the following hypotheses: 1) experimental drought causes an increase in SMs and NSCs in the leaves, especially in the growing season (i.e. spring); 2) experimental drought decreases levels of SMs and NSCs in the stem phloem, especially in the driest season (i.e. summer). We further hypothesized that 3) phytohormones are potentially associated with SMs and their responses to experimental drought.

Materials and methods

Study site and experimental design

The experiment was conducted in a holm oak (*Q. ilex*) forest, located in the Prades Mountains in southern Catalonia, Spain (41°21'N, 1°2'E) at an elevation of 930 m. The region is characterized by a typical Mediterranean climate, with the majority of vegetation growth occurring during the wet spring and autumn months. Across the 20-year experiment, mean annual air temperature was 12.4°C and mean annual precipitation was 610 mm. The tree community of the forest is dominated by *Q. ilex*, *P. latifolia* and *Arbutus unedo*, with understory species, such as *Erica arborea* and *Juniperus oxycedrus*. The structure consists of 6–8 m tall, dense multi-stemmed stools, as a result of coppicing activity prior to 1950. Soils comprise Dystric Cambisol over Paleozoic schist, ranging in depth from 35 to 100 cm. Eight 15 × 10 m plots (four plots received ambient rainfall and four received reduced rainfall) were established in March 1999; PVC strips were placed 0.5–0.8 m above the soil surface, to reduce rainfall by *c*. 30%. Rainfall was reduced by ca. 30%, within anticipated precipitation reductions of ~20% to ~40% in Mediterranean regions under the RCP8.5 warming scenario (Brogli et al., 2019; Tuel and Eltahir, 2020). Upslope runoff was intercepted by *c.* 0.8 m deep trenches along the upper edges of the plots. In each plot, soil moisture was measured continuously at four randomly selected positions (at soil depth of 25 cm) using automatic sensors connected to a data logger (ECH2O, METER, Pullman, WA, USA). Overall, the rainfall reduction treatment resulted in c. 15% lower soil moisture

throughout the experimental period (Bogdziewicz et al., 2020). Leaf water potential was measured at around midday in each of the seasons on twigs of two trees per species in each plot, using a Scholander pressure chamber (Fig. S1; PMS Instrument Co., Corvallis, OR, USA). We identified and labeled all living stems with a diameter larger than 2 cm at breast height (c. 530 and 880 stems for *Q. ilex* and *P. latifolia*, respectively), and measured changes in stem diameter every winter using a metric tape, as described in Liu et al. (2015) and Ogaya et al. (2020).

Sampling and processing

For each treatment, plant samples were collected from four plots. In each plot, we selected four trees for each of the two dominant tree species (*Q. ilex* and *P. latifolia*), and collected current-year leaf from the upper canopy and stem phloem at around noon on 30-31 May 2018 (spring), 30-31 August 2019 (summer), 12-13 November 2019 (autumn), and 21-22 February 2019 (winter), respectively. In total, this yielded 512 samples (2 treatments × 4 plots × 2 species × 4 trees × 2 organ types × 4 seasons). Samples were flash-frozen in liquid N_2, transported on dry ice to the Max Planck Institute for Biogeochemistry (Jena, Germany), and stored at -80 °C prior to processing. Samples were freeze-dried and weighed for measurement of biomass. Leaf samples of *P. latifolia* from each plot were pooled to make a composite sample due to low leaf biomass. We define stem phloem as the inner bark separated from the thin dead bark and xylem. Samples were ground to fine powder using a ball mill (Retsch MM400, Haan, Germany) and stored at -20 °C prior to extraction and analysis of soluble sugars, starch, secondary metabolites, and phytohormones.

Soluble sugars and starch

Soluble sugars and starch were extracted following Landhäusser et al. (2018). Approximately 10 mg of freeze-dried fine powdered samples were extracted using 85% ethanol, and then vortexed and incubated at 90 °C for 10 min. After centrifugation at 13000 *g* for 1 min, the supernatant was collected, diluted and stored at -20 °C prior to analysis. Glucose, sucrose, and fructose of the supernatant were analyzed using an High Performance Liquid Chromatography coupled to a Pulsed Amperometric Detection (HPLC-PAD). Concentrations of glucose, sucrose, and fructose were quantified based on linear calibration curves from their respective authentic standards. Soluble sugars were calculated as their sum. For starch analysis, the remaining pellets of the

sugar extraction were oven-dried at 40 °C, digested with α-amylase (Sigma-Aldrich), vortexed, and incubated at 85 °C for 30 min. After centrifugation at 13000 *g* for 1 min, an aliquot of the supernatants was transferred to a new vial and digested using amyloglucosidase (Sigma-Aldrich) at 55 °C for 30 min. The supernatant aliquot was then washed with chloroform to remove interfering enzymes, diluted, and analyzed again using HPLC-PAD to determine concentrations of hydrolyzed glucose. Starch content was calculated as glucose equivalents, by multiplying by a factor of 0.9 (Landhäusser et al., 2018). Oak standards were measured as a quality control; measurement error for individual soluble sugars and starch was < 0.6 mg/g.

Polyphenolics

We extracted polyphenolics as described by Huang et al. (2017). Freeze-dried fine powdered samples (*c.* 10 mg and 30 mg for leaf and stem phloem, respectively) were extracted using methanol, bead-beaten for 40 s at 6.0 ms, vortexed, and centrifuged at 11 000 *g* for 10 min. The supernatant was collected and the remaining pellet was re-extracted using the same procedure (methanol) to improve extraction efficiency. The supernatants were combined and then stored at -20°C prior to analysis.

Interspecific comparisons of SMs require accurate identification and quantification of metabolites in different tree species and organ types (Tables S1, S2). To do so, we identified and quantified the major defense compounds in leaves and stem phloem of the two tree species using a combination of HPLC-UV-ESI-Ion-Trap-MS and UPLC-Q-TOF-MS. An aliquot of methanol extracts (40 µl) was injected into HPLC-UV-ESI-MS (Esquire 6000, Bruker Daltronics, Bremen, Germany). Compounds were separated using a Nucleodur Sphinx RP column (250 × 4.6 mm, 5 µm particle size; Macherey Nagel, Dueren, Germany), with mobile phases of 0.2% (v/v) formic acid (A) and acetonitrile (B), with A–B gradient elution profiles at a flow rate of 1 ml/min over *c.* 25 min. The elution profile was: 0 to 5min, 0% B; 5 to 20 min, 0% to 45% B; 20 to 20.1 min, 45% to 100% B; 20.1 to 23 min, 100% B; 23 to 28 min, 0% B. Major phenolic compounds were selected for subsequent identification and quantification using UV wavelengths at 280 and 330 nm. ESI-MS was performed in either positive or negative mode at 60 V skimmer voltage, 4200 V capillary voltage, 35 psi nebulizer pressure, 11 l/min drying gas at 330 °C, and -121V capillary exit potential. Compounds were identified by comparing the fragmentation patterns with previous studies (Guimarães et al., 2013; Quirantes-Piné et al., 2013; Song et al., 2021) and/or

five commercial standards (catechin, quercetin 3-glucoside, oleuropein, procyanidin B1, verbascoside; Tables S1, S2).

Compounds that displayed a large UV peak, but could not be clearly identified using ESI-Ion-Trap-MS were further investigated using UPLC-Q-TOF-MS. An aliquot of methanol extracts (2 µl) was injected into UPLC-Q-TOF-MS, and compounds were separated using a Dionex Ultimate 3000 RS Pump system (Thermo Fisher Scientific) equipped with a Zorbax Eclipse XDB-C18 column (100x2.1 mm,1.8mm; Agilent Technologies). The mobile phases consisted of 0.1% (v/v) formic acid in water (A) and acetonitrile (B), with a flow rate of 0.3 mL/min. The column temperature was maintained at 25°C. The elution profile was: 0 to 0.5min, 5% B; 0.5 to 11 min, 5% to 60% B; 11.1 to 12 min, 100% B; 12.1 to 15 min, 5%B. The LC system was coupled to a timsTOF mass spectrometer (Bruker Daltonics) equipped with a turbospray ion source (capillary voltage, 3,500 V). Nitrogen was used as drying gas (8 L/min, 230°C) and nebulizer gas (2.8 bar). Analysis was carried out in negative ionization mode, scanning a mass range from m/z 50 to 1,500. Sodium formate adducts were used as internal calibrators. The derived molecular formula was used to identify SMs, based on previous studies included in the SciFinder database. The major SMs that may induce trade-offs with growth were quantified based on linear calibration curves from seven commercial standards, including catechin, coumaric acid, apigenin 7-O-glucoside, quercetin 3-glucoside, oleuropein, procyanidin B1 and verbascoside (Tables S1, S2).

Phytohormones

Phytohormones were extracted, identified, and quantified as described by Huang et al. (2017), with slight modifications. The extraction procedures used for the phenolic compounds were also used for phytohormones, except that freeze-dried ground powder (*c.* 20 mg and 30 mg for leaf and stem phloem, respectively) was extracted using methanol containing 40 ng/ml of stable isotope-labeled internal standards, including D_5-indole-3-acetic-acid (D_5-IAA, Olchemin, Olomouc, Czech Republic), D_6-abscisic acid (D_6-ABA, Toronto Research Chemicals, Toronto, CA), D_6-jasmonic acid (D_6-JA, HPC Standards GmbH, Cunnersdorf, Germany), and D_4-salicylic acid (D_4-SA, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The remaining pellet was re-extracted using methanol without internal phytohormone standards, and the supernatants were combined and then stored at -20°C prior to analysis.

We identified and quantified phytohormones using a UPLC coupled to a triple quadrupole MS (QTRAP 6500^{+}, Sciex, Foster City, CA, USA), where phytohormones were separated using a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies), at a flow rate of 1.1 ml/min, with mobile phases of 0.05% (v/v) formic acid (A) and acetonitrile (B) comprising linear gradient elution profiles of 0 to 0.5 min, 10% B; 0.5 to 4.0 min, 10–90% B; 4.0 to 4.02 min, 90–100% B; 4.02 to 4.50 min, 100% B, 4.50 to 4.51 min, 100–10% B; and, 4.51 to 7.00 min, 10% B. ABA, JA, and SA were ionized in negative mode; IAA was ionized in positive mode in separate chromatographic analysis, with collision energy set at 19V and declustering potential at 31V. The parent ion → product ion (m/z) of each hormone was: 263.0 → 153.2 for ABA; 269.0 → 159.2 for D_6-ABA; 209.1 → 59.0 for JA; 215.0 → 59.0 and 214.0 → 59.0 for D_6-JA; 136.9 → 93.0 for SA; 140.9 → 97.0 for D_4-SA; 176 → 130 for IAA; and, 181 → 135, 181 → 134, and 181 → 133 for D_5-IAA. Concentrations of ABA, JA, SA, and IAA were calculated as the ratio of the peak area of the analyte to that of the corresponding stable isotope-labeled internal standard multiplied by the concentration of the standard. Linearity was confirmed from analysis of serial dilutions of internal standards. IAA concentrations are below the limits of detection in both tree species and thus not reported here. Data were processed using Analyst 3.0.3 software (Sciex).

Data analysis

Prior to statistical analyses, leaf biomass, stem diameter and metabolite concentrations of the four individual trees were averaged for each plot. Leaf mass and stem diameter were used as a proxy of leaf and stem size, respectively. We calculated changes in leaf biomass over a 30-day period in spring as an indicator of springtime leaf growth, and the 2018-2019 annual increment in stem diameter as an indicator of stem growth. Data normality and variance equality were checked using Shapiro–Wilk and Levene's tests, respectively. Differences in metabolites (SMs, soluble sugars, starch, NSCs, ABA, JA and SA) among species, treatment and seasons were tested by three-way ANOVA (Table S3, S4). Drought effects on leaf and stem size/growth, and metabolites were tested using Student's t-test. Data were log-transformed to meet normality, homoscedasticity and/or sphericity when needed.

To determine the relationships of SMs to soluble sugars, starch, and different phytohormones, we used Pearson's correlation and calculated coefficients for each species in each season. We pooled

253 treatment data (control + drought plots) for each species and preformed principal component
254 analysis (PCA) to summarize and visualize relationships among biomass/stem diameter and all
255 metabolic data across species and treatments. All data were standardized prior to PCA. All
256 statistical analysis was conducted in R (version 3.6.3, R Development Core Team, 2020).

257 **Results**

258 *Environmental conditions*

259 Our field site in Catalonia is characterized by strong seasonality of solar radiation, air
260 temperature and precipitation (Fig. 1). Daily solar radiation and mean air temperature had similar
261 seasonal fluctuations: they increased from early spring (March) to mid summer (July) and then
262 declined until winter (Fig. 1a, b). Precipitation also showed great seasonal variation (Fig. 1c),
263 with 90 % of precipitation (c. 890 mm) in spring (March to May) and autumn (September to
264 November), and almost no precipitation in summer (June to August) and winter (December to
265 February).

266 *Leaf biomass and stem diameter*

267 *P. latifolia* had significantly lower leaf biomass and springtime leaf growth than *Q. ilex*,
268 regardless of drought treatment (Fig. 2a, b). *P. latifolia* also had smaller stem diameter sizes than
269 *Q. ilex*, while the two tree species showed similar annual stem diameter growth (Fig. 2c, d). For
270 both species, experimental drought had no significant effects on leaf biomass and stem diameter.

271 *Seasonal dynamics of secondary metabolites in leaves and stem phloem of both species*

272 We identified and quantified a diverse array of SMs in leaves and stem phloem of both species
273 (Tables S1, S2). The major SMs in *P. latifolia* leaves were hydroxycoumarin glucoside, flavones
274 (e.g., apigenin and luteolin derivatives), and in particular secoiridoids (e.g., oleuropein). The
275 stem phloem of *P. latifolia* contained secoiridoids (e.g., oleoside dimethyl ester) and
276 phenylpropanoids (e.g., verbascoside and osmanthuside J). The major SMs in *Q. ilex* leaves were
277 flavan-3-ols (e.g., catechin and procyanidins) and flavonols (e.g., quercetin and kaempferol
278 derivatives); *Q. ilex* stem phloem contained flavan-3-ols (e.g., catechin and procyanidin) and
279 flavonols (e.g., taxifolin-hexoside).

We found much larger differences in concentrations of leaf and phloem SMs between species than within species (Fig. 3; Table S3, S4). Concentrations of leaf and phloem SMs were significantly higher in *P. latifolia* (up to 18% and 5%, respectively) than in *Q. ilex* (7% and 0.6%, respectively) (Fig. 3a). In both species, concentrations of leaf SMs varied with seasons, and increased from autumn to winter (Fig. 3a). Experimental drought had no significant effects on concentrations of total SMs in leaves (Fig. 3a; Table S3), although there was a modest decrease in specific groups of SMs, like flavan-3-ols in *Q. ilex* (Fig. S2) and flavones in *P. latifolia* (Fig. S3). In stem phloem, concentrations of SMs remained relatively constant throughout the year (Fig. 3b). Experimental drought significantly decreased concentrations of SMs (secoiridoids; Fig. S3) in spring and summer for *P. latifolia* ($P<0.05$), but not for *Q. ilex* (Fig. 3b).

Seasonal dynamics of soluble sugars and starch in leaves and stem phloem of both species

The two co-occurring tree species showed significant species-specific seasonal varations in concentrations of soluble sugars and starch (Fig. 4a, d; Tables S3, S4). In spring, leaves and stem phloem had significantly higher concentrations of soluble sugars in *P. latifolia* than in *Q. ilex* ($P<0.05$; Fig. 4a, d). For *P. latifolia*, soluble sugars strongly declined from spring to summer in leaves, and from autumn to winter in both leaves and stem phloem. By contrast, for *Q. ilex* soluble sugars remained relatively constant in leaves and strongly increased from spring to summer in the stem phloem (Fig. 4a, d). As a result, the interspecific differences in soluble sugars were diminished in summer and autumn, and were reversed in winter (Fig. 4a, d). More specifically, *P. latifolia* leaves had generally higher concentrations of glucose and lower concentrations of sucrose compared to *Q. ilex* leaves. In the stem phloem, however, *P. latifolia* had lower concentrations of glucose and sucrose than *Q. ilex* across seasons (Fig. S4).

Concentrations of leaf starch were generally higher in *P. latifolia* than in *Q. ilex*, with the latter having extreme low concentrations in summer (< 5 mg/g; Fig. 4b). In contrast to leaves, stem phloem had lower concentrations of starch in *P. latifolia* than in *Q. ilex* (Fig. 4e). In both species, concentrations of phloem starch were lowest in summer and autumn, and strongly increased from autumn to winter (Fig. 4b, e). Concentrations of leaf NSCs were significantly higher in *P. latifolia* than in *Q. ilex* during the growing season (spring and early summer), but the differences diminished in autumn and winter. By contrast, concentrations of phloem NSCs were similar

between the two species during the growing season, but were significantly lower in *P. latifolia* than in *Q. ilex* in winter.

Experimental drought had no significant effects on concentrations of soluble sugars in leaves of both species (Fig. 4a). In the stem phloem, however, concentrations of soluble sugars were significantly reduced in spring and summer for *P. latifolia*, but not for *Q. ilex* (Fig. 4d). There were no significant effects of drought on the concentrations of starch in leaves and stem phloem of both species across seasons (Fig. 4b, e).

Seasonal dynamics of phytohormones in leaves and stem phloem of both species

Concentrations of phytohormones showed seasonal variations and species- and tissue-specific differences (Fig. 5; Tables S3, S4). In spring, leaf ABA levels were higher in *P. latifolia* than in *Q. ilex*, whereas the opposite was found for phloem ABA levels (Fig. 5a, d). Leaves of *P. latifolia* had generally higher concentrations of JA and lower concentrations of SA compared to leaves of *Q. ilex* across seasons (Fig. 5b, c; Table S3). Leaf hormone levels showed strong seasonal varations for both species, and were highest in spring and lowest in autumn or winter (Fig. 5a-c). Under experimental drought, *Q. ilex* showed a slight but nonsignificant increase in leaf SA and ABA as well as in phloem ABA, whereas *P. latifolia* showed no changes in leaf phytohormones (Fig. 5a-c). Note that both JA and SA were present at very low concentrations in stem phloem (Fig. 5e, f) and did not differ between the two species (Table S4).

Correlations of SMs to soluble sugars, starch, and phytohormones within and between species

SMs and soluble sugars were not correlated in leaves of both species (Fig. 6a, b), but positively correlated in stem phloem (Fig. 6c, d), especially for *P.latifolia*. Relationships between SMs and phytohormones differed between species. In *Q. ilex*, SMs were generally negatively correlated with phytohormones in leaves (Fig. 6a), particularly with ABA and SA in spring. In *P. latifolia*, however, there were no consistent relationships between SMs and phytohormones in leaves (Fig. 6b) and stem phloem (Fig. 6d).

Principal component analysis showed that the two tree species were separated on the first axis (PC1) reflecting trade-offs between SMs and leaf biomass/stem diameter across seasons (Fig. 7). Across species and treatments, there were positive relationships between SMs and soluble sugars

in spring (Fig. 7a, e); however, these relationships were absent or even reversed in the other seasons. The relationships between SMs and starch were relatively consistent but differed between organs, being generally positive in leaves (Fig. 7; upper panels) and negative in stem phloem (Fig. 7; lower panels). Strikingly, leaf SMs were found to be consistently and positively correlated with leaf JA and negatively correlated with leaf SA in all seasons (Fig. 7; upper panels). In stem phloem, however, SMs were negatively correlated with ABA, but not with JA and SA (Fig. 7; lower panels).

Discussion

Species-specific SMs and their seasonal dynamics

Understanding the role of SMs in tree responses to environmental changes requires accurate identification of SMs. By using UPLC-Q-TOF-MS, we found that the two tree species have different types of SMs: *P. latifolia* has flavones (e.g. luteolin-hexoside) and secoiridoids (e.g. oleuropein and its precursor oleoside), whereas *Q. ilex* has flavan-3-ols (e.g. catechin), quercetin and kaempferol deriviatives. These compounds are known to have multiple functions, such as anti-herbivore defense by decreasing the suitability of tissues for insects (Hammerbacher et al., 2020; Konno *et al.*, 1999), antioxidant defense by scavenging reactive oxygen species (ROS) and photoprotection by screening UV-B radiation (Agati *et al*., 2020; Agati and Tattini, 2010; Brunetti *et al*., 2015).

Quantification of SMs based on multiple standards showed that concentations of SMs (flavones and secoiridoids) in the drought-resistant species *P. latifolia* (up to 20% of biomass) were much higher than those of SMs (flavan-3-ols and flavonols) in the drought-susceptible species *Q. ilex*. High levels of SMs may be attributed to the presence of glandular trichomes for *P. latifolia*, but this is not the case for *Q. ilex*. Our result may help explain why *P. latifolia* experienced less defoliation and mortality following extreme nature drought (Barbeta *et al.*, 2015) and herbivory and diseases than *Q. ilex* (Paine and Lieutier, 2016). However, high production of SMs may be associated with the low growth capacity of *P. latifolia*, leading to a trade-off between resistance and growth. This may reflect an evolutionary adaptation of the drought-resistant *P. latifolia* to harsh environmental conditions and high herbivory/oxidative stress that prevail in Mediterranean semi-arid climates (Di Ferdinando *et al.*, 2014).

The seasonal dynamics of SMs may in part be driven by their demands for screening UV-B radiation and alleviating oxidative damage caused by environmental stress like excess light, high or low temperatures, and drought (Fig. 1; Agati et al., 2020; Agati and Tattini, 2010; Brunetti et al., 2015). In *P. latifolia*, concentrations of leaf SMs increased during hot and dry summer (Fig. 3), consistent with previous studies on *P. latifolia* (Gori et al., 2019) where phenylpropanoids accumulated in response to high temperatures. However, this is not case for *Q. ilex* in summer, where production of SMs could potentially be limited by carbohydrate supply (Huang et al., 2020b), as indicated by a severe depletion of leaf starch (Fig. 4). In both species, accumulation of SMs was observed in leaves during dry and cold winter (Fig. 3). We propose that accumulation of SMs may be an important physiological mechanism to enhance leaf tolerance to photoinhibition in winter (Chang *et al*., 2021), as reflected by relatively low photochemical efficiency (Ogaya and Peñuelas, 2003; Ogaya *et al.*, 2011).

Homeostatic levels of leaf defense SMs and NSCs under experimental drought

We hypothesized that experimental drought would lead to an increase in leaf SMs, especially during the growing season. However, contrary to our hypothesis, concentrations of total leaf SMs showed a slight and nonsignificant decrease in *Q. ilex* and remained relatively constant in *P. latifolia* (Fig. 3), even when leaf water potential showed a great decline in summer (Fig. S1). These results differ from a few glasshouse studies on tree seedlings, in which drought led to significant changes in leaf content of phenolics (Aranda et al., 2018; Mundim and Pringle, 2018). In our study, these mature trees have acclimated to the long-term drought treatment and that droughted trees may have reduced canopy area (Barbeta et al., 2013; Liu et al., 2015) and adjusted root distribution (Barbeta et al., 2015), thereby leading to no significant changes in leaf-level chemical characteristics. This is also supported by non-significant effects of experimental drought on leaf growth (Fig. 2) and NSCs (Fig. 4), as has been observed in other tree species after long-term acclimation to rainfall manipulations (Pivovaroff et al., 2021; Rowland et al., 2015; Schönbeck et al., 2018).

Within-species variations in SMs were not associated with those in soluble sugars in leaves, most likely due to the multifunctional nature of soluble sugars being not only substrates but also osmolytes (Blumstein et al., 2023; Hajek et al., 2022) and reserves for survival (Herrera-Ramírez et al., 2021; Huang et al., 2021). Another explanation is that turnover of SMs and soluble sugars

may have very different temporal dynamics, and that SMs had more constant seasonal dynamics than soluble sugars. Substantial amounts of SMs like flavan-3-ols in *Q. ilex* and secoiridoids in *P. latifolia*, were produced in spring to protect developing and vulnerable organs (*sensu* optimal defense theory, Barton et al., 2019; McKey, 1974). These compounds may be stored for a long time until being mobilized or metabolized during stress (Panda et al., 2021), whereas soluble sugar levels are known to be determined by the source-sink balance that can vary from hours and days to seasons (Furze et al., 2018; Martínez-Vilalta et al., 2016; Tixier et al., 2018). Thus, our results highlight that the temporal dynamics of SMs and NSCs should be accounted for when interpreting the physiological linkages between SMs and NSCs, especially for trees operating at seasonal and annual timescales.

Species-specific reduction in phloem defense SMs and soluble sugars under experimental drought

Consistent with our hypothesis, experimental drought reduced levels of total SMs and NSCs in the stem phloem of *P. latifolia* during the growing season (spring and early summer; Fig. 3). Given that we measured concentrations of SMs and NSCs including those that are being transported in the sieve tubes and also those stored in parenchyma cells, we propose two possible mechanisms: 1) reduced soluble sugars may indicate locally reduced availability of substrates for production of SMs in the stem phloem (Erbilgin et al., 2021; Huang et al., 2020b; Wiley et al., 2016). This is supported by significant relationships between SMs and soluble sugars in *P. latifolia* stem phloem from spring to autumn; 2) reduced levels of phloem SMs and soluble sugars in *P. latifolia* may indicate reduced leaf export and phloem transport of soluble compounds (e.g., oleoside dimethyl ester; Table S2) under experimental drought (Savage et al., 2016). This explanation is consistent with results of isotopic labeling studies showing that drought can limit transport of recent photosynthates belowground (Dannoura et al., 2018; Hagedorn et al., 2016; Hesse et al., 2018; Joseph et al., 2020; Ruehr et al., 2009). Our study thus provides experimental evidence that drought may reduce defense and carbohydrate metabolisms in the stem phloem of mature *P. latifolia* trees, implying that with more frequent and severe future spring/summer drought this tree species may be less resistant to oxidative stress (Tattini et al., 2015) and biotic attacks by phloem-feeding insects (Huang et al., 2020a).

In contrast to *P. latifolia*, *Q. ilex* did not reduce levels of phoem SMs and NSCs under experimental drought. The exact mechanisms leading to the differential effects of drought on phloem SMs in *P. latifolia* and *Q. ilex* remain uncertain, but are probably related to the concentration of solutes in the stem phloem. Compared to *P. latifolia*, *Q. ilex* trees transport and/or store significantly smaller amounts of SMs in stem phloem. The relatively high concentrations of phloem SMs (*c.* 5 % on a dry weight basis) in *P. latifolia* trees may theoretically lead to high phloem sap viscosity, making phloem transport of soluble SMs and carbohydrates susceptible to increased drought stress. This may explain why experimental drought decreased levels of phloem SMs in *P. latifolia* in spring and summer, but not in winter when phloem soluble sugars decreased to very low levels (*c.* 0.05 %). These results demonstrate that drought impacts on phloem defense and carbohydrate metabolism are seasonal and species-specific, and suggest phloem transport as an important mechanism that needs to be examined by isotopic labeling and tracing (Huang et al., 2019).

The potential role of phytohormones in regulating growth and production of SMs within and between species

While studies have investigated the hormonal regulation of defense SMs in crop and model plant species (Huot et al., 2014; Panda et al., 2021; Züst and Agrawal, 2017), little information is available for mature trees. We found that stem phloem had extremely low levels of JA and SA across seasons, while leaf hormone levels were highest in spring and declined over time (Fig. 5). Similarly, a recent study found in Scots pine and Norway spruce that leaf hormones declined over time during summer drought (Pashkovskiy et al., 2022). These results suggest that phytohormones may play an important role in regulating leaf metabolism during the early stage of development. Interestingly, experimental drought induced a slight but nonsignificant increase in ABA and SA in *Q. ilex* leaves (Fig. 5), which was significantly correlated with SMs, especially in spring (Fig. 6). Future studies should investigate whether increased ABA in *Q. ilex* leaves may induce stomatal closure and reduce photosynthesis (De Ollas and Dodd, 2016; Nguyen et al., 2016), and whether SA-induced abiotic stress responses (i.e. production of antioxidant enzymes; Khan et al., 2015) may induced trade-offs with production of defense SMs (Thaler et al., 2012).

The divergence in leaf growth and defense SMs between the co-occurring species appears to be associated with phytohormones (Fig. 7). Compared to *Q. ilex* leaves, *P. latifolia* leaves had significantly higher levels of ABA in spring, potentially leading to lower growth capacity (Fig. 2; Nguyen et al., 2016). It should be noted that the two species have similar timing of leaf flushing (late April) and at the time that we collected leaf samples (mid-May), flower senescence occurred in *P. latifolia* but not in *Q. ilex*. Thus, the high levels of leaf ABA may be associated with flower senescence in *P. latifolia* (Arrom and Munné-Bosch, 2012; Tripathi and Tuteja, 2007). Interestingly, *P. latifolia* leaves also had significantly higher levels of JA than *Q. ilex* leaves, which may potentially induce greater production of SMs (Wasternack and Strnad, 2019). In addition, JA appears to antagonistically interact with SA between species (Pieterse et al., 2012; Thaler et al., 2012). Thus, we propose that differentiation in phytohormone production is a potential mechanism driving the divergence in leaf growth, phenology and defense SMs in this forest community. If this holds true for other tree species, investigations of phytohormones would have strong implications for assessing and predicting species-specific defense capacity.

Conclusion and outlook

Our field drought experiment combined with quantitative metabolomics provide evidence about how drought affects the seasonal dynamics of SMs and their potential interactions with carbohydrates and phytohormones in co-occurring mature trees. We show that the two dominant tree species have different types of SMs, and that the drought-resistant species *P. latifolia* contained greater levels of SMs compared to the drought-susceptible species *Q. ilex*. The observed seasonality of SMs may be explained by the seasonal variations in climate. Experimental drought had no significant effects on levels of SMs, NSCs and phytohormones in the leaves of both tree species, even when leaf water potential was strongly reduced in dry summer. The homeostatic leaf-level chemical characteristics corroborate the acclimation of mature trees to long-term drought. By contrast, experimental drought reduced levels of SMs and soluble sugars in the stem phloem of *P. latifolia* but not of *Q. ilex*, especially in spring and summer. This suggests that increasing drought stress may reduce the resistance of *P. latifolia* trees to abiotic and biotic stresses. Our results provide mechanistic insights into organ- and species-specific dynamics of SMs, which may represent an important adaptation component in

this forest community and may further shape its structure and function under ongoing climate change and biotic attacks (Peñuelas et al., 2018).

We acknowledge that our drought treatment (i.e. 30% rainfall exclusion) was relatively moderate, and suggest that future studies should impose more severe drought treatments to examine the relationships of SMs to NSCs and phytohormones. Future studies should investigate the linkage of production of SMs to carbon economy, hydraulic strategies (Limousin et al., 2022) and phenology across tree species under experimental drought. Phytohormoal regulation of defense SMs should be examined for a greater range of tree species and organ types, to unravel the key regulatory mechanisms that determine the defense capacity of different tree species under climate change.

Supplementary Information

Figure S1 Leaf water potential in *Quercus ilex* and *Phillyrea latifolia*.

Figure S2 Concentrations of secondary metabolites in leaves and stem phloem of *Quercus ilex.*

Figure S3 Concentrations of secondary metabolites in leaves and stem phloem of *Phillyrea latifolia*.

Figure S4 Concentrations of glucose, sucrose, and fructose in leaves and stem phloem of *Quercus ilex* and *Phillyrea latifolia*.

Table S1 Method of identification and quantification of the major secondary metabolites in leaves of *Quercus ilex* and *Phillyrea latifolia*.

Table S2 Method of identification and quantification of the major secondary metabolites in stem phloem of *Quercus ilex* and *Phillyrea latifolia*.

Table S3 Summary of the three-way ANOVA testing effects of species, treatment, seasons, and their interactions on different metabolic pools in leaves.

Table S4 Summary of the three-way ANOVA testing effects of species, treatment, seasons, and their interactions on different metabolic pools in the stem phloem.

Acknowledgements

We thank Dr. Henriette Uthe for her assistance with analysis of the phytohormones, and Savoyane Lambert, Anett Enke and Iris Kuhlmann for their help with sample processing and analysis of NSCs. J.H. acknowledge support from the Max Planck Society, and Deutsche Forschungsgemeinschaft (437772218).

Author contributions

J.H., H.H., and J.P. contributed to the plan and design of the work. R.O. collected samples in the field. J.H., M.R., H.H., and J.G. proformed compound analysis. J.H. and I.S analysed the data. J.H. wrote the manuscript and all authors contributed to revisions.

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Figure legends

Figure 1 Daily solar radiation (a), mean air temperature (b) and precipitation (c) during the study period (2018–2019). Grey lines indicate the sample dates. Thick lines are smoothing splines with a span value of 0.7.

Figure 2 Leaf mass (a) and springtime leaf mass increment (b), stem diameter (c) and annual stem diameter increment (d) in *Quercus ilex* (green) and *Phillyrea latifolia* (blue) under control (filled circles and solid lines) and drought (open circles and dashed lines) treatment. Shaded areas represent 95% confidence intervals for each species. Each point represents the mean of four plots, and error bars are SE.

Figure 3 Concentrations of total secondary metabolites (SMs; the sum of individual SMs) in leaves (a) and stem phloem (b) in *Quercus ilex* (green) and *Phillyrea latifolia* (blue) under control (filled circles and solid lines) and drought (open circles and dashed lines) treatment. Shaded areas represent 95% confidence intervals for each species. Each point represents the

768 mean of four plots, and error bars are SE. Significant differences between control and drought
769 plots were indicated by asterisks (Student's test, $P<0.05$). Levels of statistical significance (P-
770 values) of species, treatment, seasons, and their interactions on SMs are in shown Table S3 and
771 S4.

772 Figure 4 Concentrations of soluble sugars, starch, and nonstructural carbohydrates (NSCs) in
773 leaves (upper panels) and stem phloem (lower panels) in *Quercus ilex* (green) and *Phillyrea*
774 *latifolia* (blue) under control (filled circles and solid lines) and drought (open circles and dashed
775 lines) treatment. Shaded areas represent 95% confidence intervals for each species. Each point
776 represents the mean of four plots, and error bars are SE. Levels of statistical significance (P-
777 values) of species, treatment, seasons, and their interactions on sugars, starch and NSCs are
778 shown in Table S3 and S4.

779 Figure 5 Concentrations of abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) in
780 leaves (upper panels) and stem phloem (lower panels) in *Quercus ilex* (green) and *Phillyrea*
781 *latifolia* (blue) under control (filled circles and solid lines) and drought (open circles and dashed
782 lines) treatment. Shaded areas represent 95% confidence intervals for each species. Each circle
783 represents the mean of four plots, and error bars are SE. Significant differences between control
784 and drought plots were indicated by asterisks (Student's test, $P<0.05$). Levels of statistical
785 significance (P-values) of species, treatment, seasons, and their interactions on ABA, JA and SA
786 are shown in Table S3 and S4.

787 Figure 6 Coefficients for relationships of secondary metabolites (SMs) to soluble sugars, starch,
788 abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) in leaves (upper panels) and
789 stem phloem (lower panels) for *Quercus ilex* (left panels) and *Phillyrea latifolia* (right panels).
790 Data are not shown for SA in leaves of *Phillyrea latifolia* and JA in the stem phloem of both
791 species due to very low concentrations. Significant relationships were indicated by asterisks
792 ($P<0.05$).

793 Figure 7 Principal component analysis showing the distribution of the variation among secondary
794 metabolites (SMs; blue arrows), leaf biomass or stem diameter (green arrows), soluble sugars
795 and starch (brown arrows), phytohormones (red arrows; abscisic acid, ABA; jasmonic acid, JA;
796 salicylic acid, SA) in leaves (upper panels) and stem phloem (lower panels). Each circle

represents the mean of four individual trees of each species (*Quercus ilex*, green; *Phillyrea latifolia*, blue) for each plot (control, filled circles; drought, open circles). Ellipses represent the 95% confidence interval of the bi-variate distribution.