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A decade of rain exclusion in a Mediterranean forest reveals trade-offs of leaf chemical defenses and drought legacy effects

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Increasing aridity in the Mediterranean region will result in longer and recurrent drought. These changes could strongly modify plant defenses, endangering tree survival. We investigate the response of chemical defenses from central and specialized metabolism in *Quercus pubescens* Willd. to future Mediterranean drought using a long-term drought experiment *in natura* where trees have been submitted to amplified drought (~ −30% annual precipitation) since April 2012. We focused on leaf metabolites including chlorophylls and carotenoids (central metabolism) and flavonols (specialized metabolism). Measurements were performed in summer from 2016 to 2022. Amplified drought led to higher concentrations of total photosynthetic pigments over the 2016–2022 period. However, it also led to lower AZ/VAZ and flavonol concentrations. Additionally, chemical defenses of *Q. pubescens* responded to previous precipitation where low precipitation 1 year and/or 2 years preceding sampling was associated to low concentrations of VAZ, flavonol and high neoxanthin concentrations. Our study indicates that the decline of flavonol concentration under long-term drought is counterbalanced by a higher production of several central metabolites. Such results are potentially due to an adjustment in tree metabolism, highlighting the importance of performing long-term experimental studies *in natura* for assessing drought legacy effects and thus forest adaptation to climate change.

Keywords Adaptation, Central and specialized metabolites, Climate change, Drought, Flavonols, Long-term stress, Pigments, Rainfall exclusion, Trade-offs

The Mediterranean region is a critical climate-change hotspot due to the expected rapid increase of temperatures and drought episodes[1]. In this region, climatic models forecast a decrease in annual precipitation of about 30%, longer summer periods and general warming between 1 and 5 °C by the end of the century, especially during the warm season where temperature increase could reach up to 7 °C[2]. Such increases in drought episodes will lead to tree mortality thereby threatening forest ecosystems[3,4]. Although Mediterranean forests have demonstrated great resistance and resilience to water stress, the expected rapid climate change could dramatically modify tree growth, survival and species distribution[5].

One of the most important consequences of drought stress is a reduction in net photosynthesis through stomatal closure resulting in an excess of energy and a high accumulation of reactive oxygen species (ROS) eventually causing DNA damage and cell death[6,7]. To limit photosynthetic damage and oxidative stress associated with water limitation and other abiotic constraints, plants have evolved diverse mechanisms including the production of a vast diversity of chemical defenses which are classified as central and specialized metabolites.

Plant central metabolites—also known as primary or universal metabolites—are essential for growth and development and for universal metabolic processes such as photosynthesis whereas plant specialized metabolites (formerly named "secondary metabolites") are specifically involved in plant environmental interactions, stress responses and survival and account for supplementary plant defenses[8,9]. Among central metabolites, chlorophylls are crucial pigments involved in photosynthesis which can be strongly affected by water deficit[10,11]. In addition, carotenoid pigments such as β-carotene, lutein or those from the xanthophyll cycle (violaxanthin, zeaxanthin,

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antheraxanthin; hereafter referred to as VAZ) act as ROS scavengers[12,13]. Among carotenoids, the de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin (denoted by increasing the ratio AZ/VAZ) is a major reaction indicator of the dissipation of excess energy in the antennae of the photosynthetic apparatus which plays a crucial role to cope with photo-oxidation in water-stressed photosynthetic tissues[14]. The proportion between leaf carotenoids and chlorophylls, expressed as the ratio car/chl $a+b$, allows to provide some valuable information on the plant physiological status[15].

In addition to central antioxidant metabolites, specialized metabolites are widely spread in the plant kingdom where they play essential roles in plant defense. The major classes of plant specialized metabolites are terpenoids, phenolic compounds and alkaloids. Among phenolic compounds, flavonoids account for the most widespread group and include flavonols, well known for their role during drought stress, particularly as antioxidants and UV-screening, thus conferring protection against oxidative stress and photoprotection[16,17].

Foliage defenses deployed against short and mild-term exposure to drought are well documented in various plant species both in natural and controlled conditions[18–21] while long-term exposure, especially *in natura*, remains poorly explored[22,23]. Studies on *Quercus pubescens* Willd.—known for its resistance to relatively long drought periods[20,24,25] and highly spread in the northern Mediterranean basin (over 307,000 hectares in Southern France; IGN 2023)[26]—reveal that amplified drought during the 1st[25], 3rd, 4th[20,27] and 10th year[23,28] in the field clearly halts tree functioning through limited CO_2 fixation. This leads to moderate modifications of leaf metabolome with only a clear drop in storage of some antioxidant metabolites (related to central and specialized metabolism). The common approach of all these studies is the comparison of tree response under natural and restricted precipitation during the current year, but neglects whether this response is influenced by precipitation from previous years[29] or by precipitation during the growing season, which strongly influences plant growth[30,31]. Indeed, past drought events can induce lagged effects in subsequent seasons and years. Such legacy effects have been widely studied on tree growth[32–36] and more recently on tree metabolism[37,38]. The recent study of Eisenring et al.[37] highlighted that extreme drought events can alter the phytochemical profiles of beech leaves for at least 2 years post-drought. Moreover, such drought legacy effects on leaf chemical are likely to affect tree-herbivores interactions and thus forest health. Likewise, while monitoring of tree growth over the years is a common approach to study tree growth pattern over climatic variability[39–41], monitoring of central and specialized metabolism over several years needs to be more deeply explored, especially in forest ecosystems. Focusing on plant metabolism and the potential impact of recurrent stress conditions might be very helpful in understanding plant response to predicted climate scenarios.

The present study aims to assess the response of central and specialized metabolites of *Q. pubescens* to long-term amplified drought expected in the Mediterranean area. For this purpose, we (i) compared *Q. pubescens* response growing under natural drought (ND) and amplified drought (AD) from the 5th to the 11th year of rain restriction, (ii) analyzed the impact of previous precipitation on leaf metabolic concentrations, and (iii) correlated central and specialized metabolites where a negative correlation would be indicator of a putative trade-off between central and specialized leaf defenses. Based on several studies on the impact of drought across several years on tree growth and metabolism of coniferous and deciduous species[18,34,42], we hypothesized that long-term recurrent drought would lead to metabolic adjustments which in turn could be beneficial for tree resistance to future increasing drought episodes.

Materials and methods

Study site, sampling design and campaigns

Q. pubescens was studied at the O_3HP (the Oak Observatory at the Observatoire de Haute-Provence), an *in natura* experimental site located in the French Mediterranean area (5°42′44″E, 43°55′54″N) which is part of the European research infrastructures AnaEE-France (Analysis and Experimentation on Ecosystems) and AnaEE-ERIC (European Research Infrastructure Consortium). The study site is located in a forest dominated by *Q. pubescens* and is characterized by a supra-Mediterranean sub-humid bioclimate with an average annual precipitation of 784 mm and annual mean temperature of 13.1 °C for the period 1993–2022. The O_3HP has been equipped since April 2012 with a dynamic rainfall exclusion system consisting of a roof placed at 6 m above the canopy (Fig. 1a,b) which is electronically deployed in order to exclude ~ 30% of annual precipitation on a forest plot of 300 m^2 referred to as "amplified drought" plot (AD) according to future climate forecasts for the Mediterranean region (Giorgi and Lionello[43], i.e. − 30 ± 10%). For this purpose, rain is excluded during some rain events between April and October (approximatively 20 days) with a total rain exclusion from the beginning of July to the end of September thus simulating a drier and longer summer season[44]. Thus, while mean annual rainfall is 784 mm, rainfall in the AD plot ranges between 500–550 mm and excluded water is drained off-site via gutters. Soil water content was lower in the amplified drought plot as shown in previous studies conducted on the same experimental site in 2012, 2019 and 2020[25,44]. A footbridge provides access to the canopy (Fig. 1c). Trees from the AD plot are compared to trees from the adjacent plot (300 m^2) which receives natural rainfall and is referred to as the "natural drought" (ND) plot.

Metabolite and physiological traits were studied for the summer period within each drought condition (AD and ND). Central and specialized metabolites were characterized for seven years (2016–2022), 2016 and 2022 corresponding to tree response after 5 to 11 years of rain exclusion, respectively. The exact sampling dates were the following: July 26th 2016, August 2nd 2017, July 18th 2018, July 17th 2019, July 8th 2020, July 15th 2021 and July 21st 2022. Ombrothermic diagrams highlight drought intensity every year, monthly cumulative precipitation, and mean temperatures (Fig. S1). A detailed description of climatic conditions over 2016–2022 is given in the results section. Physiological traits were studied during a more restricted period, from 2020–2022. The exact dates of physiological trait measurements were July 8th–9th 2020, July 12th–13th 2021 and July 18th–21st 2022.

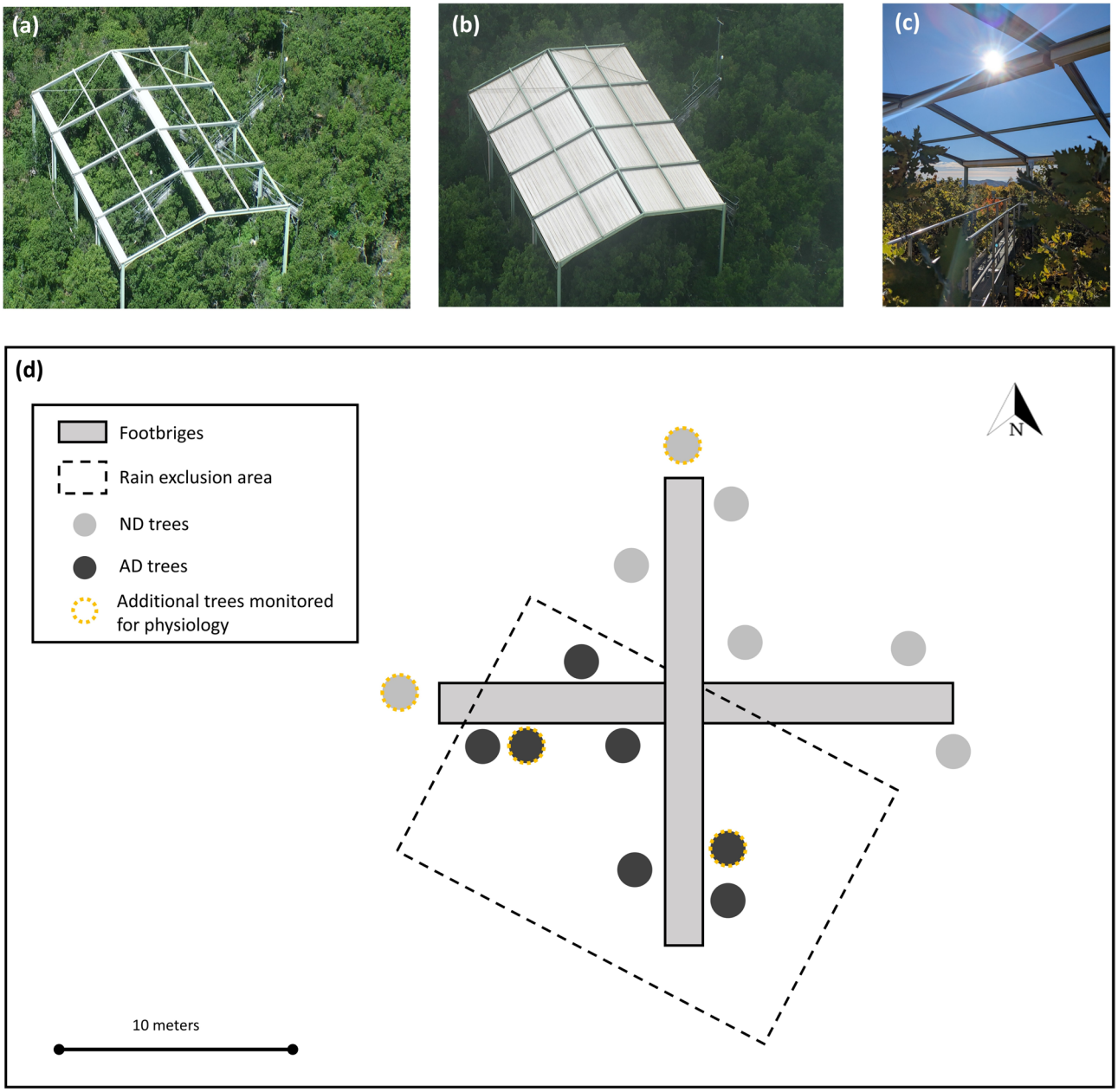

Fig. 1. Experimental site at the Oak Observatory at the Observatoire de Haute Provence (O_3HP). Photo of the experimental site at the O_3HP with the exclusion device (**a**) opened and (**b**) closed. (**c**) Photo on the footbridge. (**d**) Scheme of the experimental site showing the distribution of the seven sampled trees. Trees under natural drought (ND) are shown in light grey and trees under amplified drought (AD) in dark grey. Trees circled in yellow are additional trees monitored for physiological measurements (seven trees by drought condition) and those not circled were used for metabolite analysis (five trees by drought condition).

Physiological traits

Water and CO_2 gas exchanges were measured on 7 trees per condition (on 3 leaves per tree) between 10 a.m. and 1 p.m. (Fig. 1d) using sun-exposed mature leaves from the top canopy. Measurements were performed using an open-system gas analyzer (CIRAS-3, PP Systems, Amesbury, MA, USA) providing net CO_2 assimilation (A_n, µmol CO_2 m^{-2} s^{-1}) and water stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}). The reference CO_2 concentration was maintained at 400 µmol mol^{-1}, the leaf chamber temperature was set to 27 °C, and the photosynthetically active radiation (PAR) was set to 1000 µmol m^{-2} s^{-1}.

Stem water potential was determined using stems containing 3 to 5 mature leaves on 4–7 trees per drought condition. Predawn water potential (Ψ_{pd}) was measured using a Scholander pressure chamber (PMS Instrument Co., USA; range 0–7 MPa) in the morning hours before sunrise.

Analysis of central (photosynthetic pigments) and specialized metabolites (flavonols)

Central and specialized metabolites were measured on 5 trees per condition. For each tree, 5 to 10 sun-exposed mature leaves (other than those used for physiological measurements) were harvested from the top of the canopy during each sampling campaign around midday. Leaves were immediately frozen in liquid nitrogen and stored at − 80 °C until analysis. Permission for leaf sampling was given by the O_3HP scientific director who is co-author of this paper and who belongs to IMBE who manages the site on behalf of CNRS. Sampling methods were carried out in accordance with relevant guidelines for collecting this oak species which does not require permissions or licenses.

Regarding central metabolites, photosynthetic pigments of each tree were extracted using 20–50 mg of leaf powder (from 5 to 10 leaves previously ground in liquid nitrogen) and 2 mL of cooled methanol. The fresh mass of the sample used for pigment extraction was determined by weighing the tube + methanol before and after adding the leaf powder. Another aliquot of leaf powder was also weighed and immediately dried in an oven at 80 °C to determine the ratio fresh mass/dry mass for each sample in order to express the final results on a dry mass basis. Among photosynthetic pigments, we separated and quantified chlorophylls (chlorophyll *a* and *b*), carotenoids from the xanthophyll cycle (VAZ: violaxanthin + antheraxanthin + zeaxanthin) neoxanthin, lutein, and β-carotene by high-performance liquid chromatography (HPLC) as described in Havaux et al.[45]. In brief, the methanolic extract was centrifuged for 15 min at 4 °C. Then, 100 µL were injected in the HPLC system which consisted in a Waters 600E system controller, a Waters 717plus autosampler and a Waters 2998 photodiode array detector. Pigment separation was done with a Waters Nova Pak column 3.9 × 300 mm, 4 µm, 60 Å. The flow rate was 1.5 mL. Elution starts in solvant A (acetonitrile/water/triethylamine (900/100/1, v/v/v) for 1 min. Then, a linear gradient was imposed to reach 100% solvant B (ethylacetate) after 15 min. At time 16 min, solvent B was changed for solvent A. The total run time was 22 min. Pigments were detected at 450 nm and were quantified with standards (chlorophylls, lutein, zeaxanthin, β-carotene) obtained from Sigma-Aldrich or Extrasynthese. The calibration curve of lutein was used for the quantification of neoxanthin, antheraxanthin and violaxanthin.

For specialized metabolites, flavonols were extracted for each tree using 10 mg dry matter (DM) (from 5 to 10 leaves previously ground using freeze-drying) and 1 mL of methanol containing 1% formic acid. The extract was processed using an Acquity UPLC-DAD-ESI-TQD system (Waters, USA). A C18 BEH column was used for separation, with an elution rate of 0.4 mL min^{-1} at a constant temperature of 30 °C. Injection volume was set to 2 µL. Chromatographic solvents are composed of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The chromatographic gradient was 3% of B for 3 min and then 17 min linear gradient until 90% B, followed by column cleaning at 90% B for 3 min and then 6 min equilibration at 3% B giving a 25 min total runtime. Flavonols were detected at 350 nm and verification of their identity or structure was accomplished using the triple quadrupole mass detector in negative ionization mode. The identity of flavonols was confirmed using external standards of monoglycosylated flavonols (quercetin and myricitrin) by triple quadrupole mass detector in negative ionization mode. Parameters of the electrospray source were as follows: capillary voltage 2.9 kV, cone voltage 35 V, cone temperature was maintained at 150 °C, and desolvation temperature at 400 °C. External quantification with mono glycosylated flavonols (quercetin and myricitrin) was applied. To finish, flavonols and photosynthetic pigments were quantified in µg g_{DM}^{-1}.

Statistical analyses

To characterize metabolite and precipitation variability from 2016 to 2022 and between natural and amplified drought conditions (ND and AD) a principal component analysis (PCA) was carried out using FactoMineR and factoextra packages[46,47] (R software v.4.0.3; R Core Team, 2020[48]) followed by a two-way PERMANOVA (with 9999 permutations) using vegan package[49]. In order to assess the relationship between metabolite concentrations and precipitation, Pearson's correlations ("cor.test" function in R) were performed with drought conditions pooled together using the following precipitation data: (i) total annual precipitation 1-year before sampling (from 1st January to 31st December), (ii) total annual precipitation 2-years before sampling (from 1st January to 31st December), (iii) precipitation during the growing season of the sampling year (from March 15th to leaf harvest date) and (iv) precipitation from 1st January to 15th March of the sampling year.

To highlight differences in photosynthetic pigments, flavonols and physiological traits (A_n, g_s, Ψ_{pd}) over years and drought conditions we performed a two-way ANOVA followed by post-hoc Tukey tests. Data were previously checked for normality and homoscedasticity and data were log-transformed if necessary. Since the interaction between 'years' and 'drought' was not significant, differences between years were evaluated with drought conditions pooled and the drought effects were shown with all years pooled.

Results

Climatic conditions across years and drought conditions

The quantification of metabolites was performed for the summer period of seven years (from 2016 to 2022), that is from the 5th to the 11th year of rain restriction since precipitation restriction started in April 2012. Climatic data during 2014 and 2015 were considered in this study in order to determine whether precipitation 1 year and 2 years preceding the measurements could impact metabolite production. During the entire period of 9 years (2014–2022), ombrothermic diagrams revealed (i) five dry years (2015, 2017, 2020, 2021 and 2022) including the 2 driest years (2017 and 2020) with 3–5 dry months (Fig. 2a, Fig. S1) and (ii) four wet years (2014, 2016, 2018 and 2019) where total annual precipitation was well above 784 mm (Fig. 2a). Between 2014 and 2022, from 12 to 47% of annual precipitation was excluded every year (Fig. 2a; Table 1).

Annual mean temperatures were quite similar between years except 2022 where it was 14.7 °C, that is, 1.6 °C above the annual mean temperature recorded in this site (i.e. 13.1 °C, over the 1993–2022 period) (Fig. 2b). The monthly distribution of rainfall displayed different patterns according to the year (Fig. S1). Both 2017 and

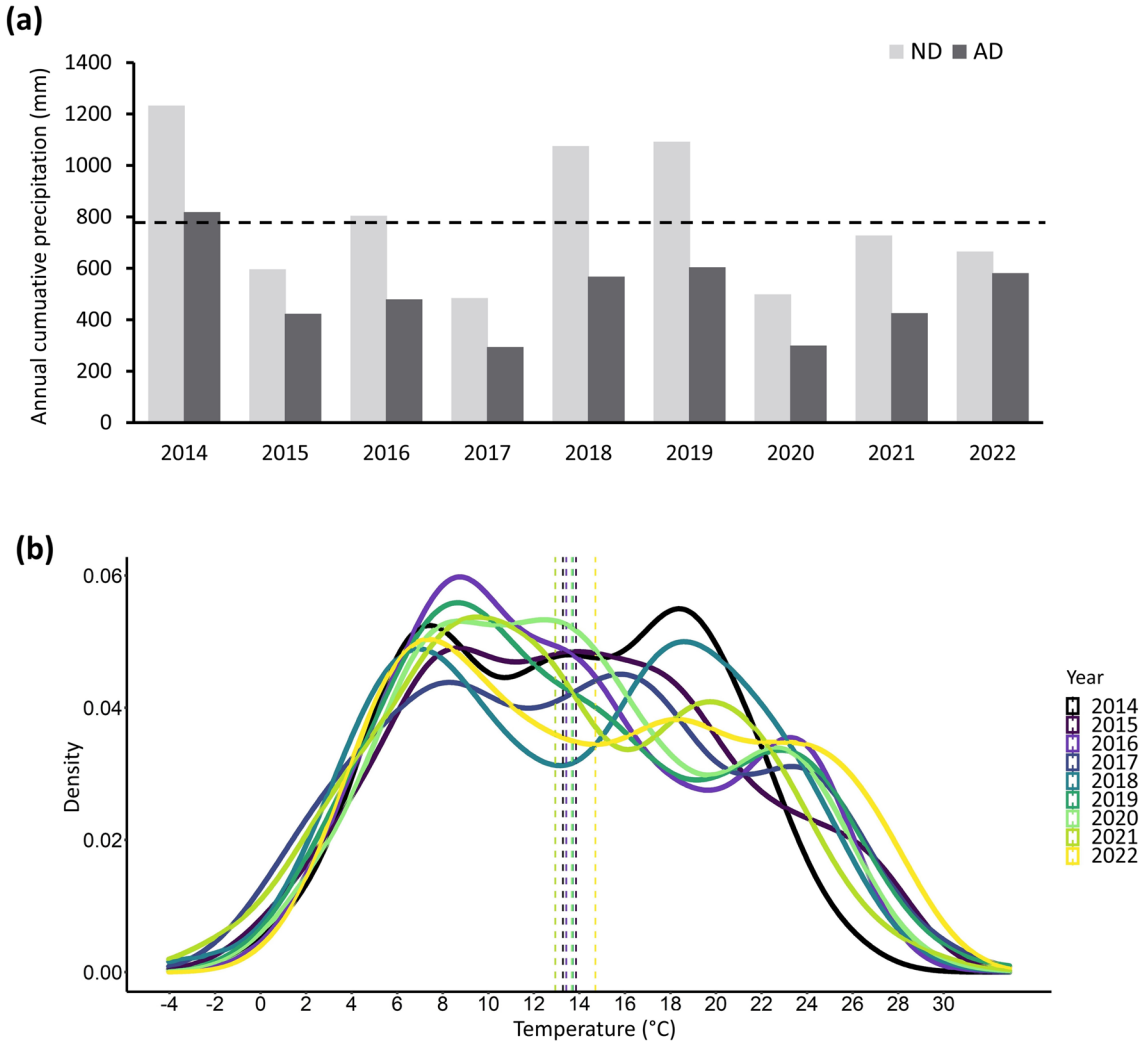

Fig. 2. Climatic conditions on the experimental site from 2014 to 2022. (**a**) Annual cumulative precipitation for natural (ND, light gray) and amplified (AD, dark gray) drought plots for each year from 2014 to 2022. The dotted black line represents the annual mean precipitation calculated for the period 1993–2022 (784 mm). Under ND, when precipitation bars are below the 784 mm annual mean, the year is considered as "dry year" while when it is above the annual mean the year is considered as "wet year". (**b**) Density of temperature values for each year. The mean temperature for each year is represented by the dotted line and highlighted in the corresponding colour.

Year	Precipitation from 1st January to 15th March (mm)		Growing season cumulative precipitation (mm)		Growing season mean temperature (°C)	Percentage of precipitation excluded (%)
	ND	AD	ND	AD		
2014	326	326	334	215	15.4	34
2015	144	144	160	92	16.8	29
2016	123	123	146	41	15.9	40
2017	82	43	218	84	17.7	39
2018	181	154	342	174	16.1	47
2019	62	44	213	144	15.7	45
2020	63	62	254	120	15.3	40
2021	115	107	243	158	14.4	41
2022	36	36	174	173	16.9	12

Table 1. Precipitation during both the period from 1st January to 15th March and during the growing season (i.e. from 15th March to harvest date), mean temperature during the growing season for 2014–2022 period and percentage of precipitation excluded between natural and amplified drought plots over a year.

2022 were naturally very dry displaying the highest number of dry months (up to 5 dry months under ND) and accordingly, trees under AD were also exposed to a high number of dry months (5–7) while in 2018 trees were exposed to only two months of natural drought (Fig. S1).

Regarding climatic conditions during the growing season (from March 15th to harvest date), precipitation was on average of 232 mm for the period 2014 to 2022. The wettest growing season occurred in 2018 with 342 mm of precipitation while the driest growing season occurred in 2016 with only 146 mm (Table 1). Under AD, 43% of precipitation was excluded during the growing season on average, while up to 72% precipitation was excluded in 2016 and no rain was excluded in 2022 since recorded temperatures were particularly high and precipitation was very low in spring 2022 (Table 1).

Physiological traits across years and drought conditions

Regarding physiological traits, only net CO_2 assimilation (A_n) tended to decrease by 50% under AD ($0.05 < P < 0.1$; Fig. 3a) which represents a remarkable decline considering that A_n values under ND were rather low ranging from 3 to 11 µmol CO_2 m^{-2} s^{-1} in 2020 to 2021. Tree physiology was especially slowed-down in 2022 since net CO_2 assimilation was negative (Fig. 3a) and stomatal conductance (g_s) (Fig. 3b) and predawn water potential (Ψ_{pd}) reached the lowest values (Fig. 3c), reflecting the extreme dry and warm conditions this year (Table 1).

Variation in photosynthetic pigments and flavonols across years and drought conditions

The PCA analysis followed by two-way PERMANOVA revealed two main principal components (PC) explaining 33.5% (PC1) and 14.4% (PC2) of the variance in metabolite concentrations and cumulated precipitations (Fig. 4). This analysis highlighted a net effect of both drought conditions and year ($P < 0.001$; Fig. 4a). According to these axes, significant differences in metabolite concentrations were pointed out between trees growing under ND and AD where trees under AD featured a higher concentration of photosynthetic pigments (lutein, neoxanthin and β-carotene and the total chlorophyll) which explained the positive part of PC1 and low concentrations of flavonols (quercetin derivatives) and AZ/VAZ represented in the negative part of PC1. Differences between drought conditions were also visible, to a lesser extent, across PC2, where trees under AD seemed to feature lower concentrations of kaempferol and myricetin derivatives.

The total annual precipitation 2 years preceding sampling was the most contributing variable to PC2 followed by the total annual precipitation 1-year preceding sampling (Fig. 4b). Precipitation from January to March as well as during the growing season (from 15th March to harvest date) poorly contributed to the variation in the dataset (Fig. 4b). The PCA also revealed a particular discrepancy between tree metabolite concentrations and precipitation in 2022, and the other years, where 2022 was a particularly dry and warm year and trees featured the lowest concentrations of both central and specialized metabolites.

Two-way ANOVA (Figs. 5, 6) also revealed significant differences in metabolite concentrations between drought conditions and across years and confirmed most differences described above. Trees under AD exhibited significantly higher concentrations in total photosynthetic pigments (Fig. 5a)—including a 15% rise of total chlorophyll concentrations own to the increase in both chlorophyll *a* and *b* (Fig. S2) although the ratio Chl *a*/*b* did not change (Fig. 5b,c). In addition, the concentration of three carotenoids, namely neoxanthin, β-carotene and lutein, significantly raised under AD (up to 16% for neoxanthin) (Fig. 5d–f). By contrast, the ratio AZ/VAZ significantly dropped by 23% under AD which was due to a clear drop of zeaxanthin concentration and also to a significant increase in violaxanthin under AD (Fig. 5h; Fig. S3). The pool of metabolites from the xanthophyll cycle (VAZ) was not impacted by AD (Fig. 5g). The ratio of carotenoid to total chlorophyll concentrations (Car/Chl) marginally ($P < 0.1$) decreased under AD (Fig. 5i). It should be noted that the total chlorophyll concentration was particularly high in 2018 due to very high chlorophyll *b* concentration (Fig. S2).

Regarding specialized metabolites, total flavonol concentrations significantly dropped under AD independently of the year (Fig. S4), especially quercetin derivatives (quercetin galloyl glucose, quercetin-3-*O*-glucose and quercetin pentose; Fig. S5) but also isorhamnetin-3-glucuronique (Fig. S5) and myricetin derivatives (myricitrin; Fig. S5) (Fig. 6).

While total flavonol concentrations remained quite stable over the years (Fig. S4), the total photosynthetic pigment concentrations significantly dropped in 2022 due to the decrease in the total chlorophyll, lutein and VAZ concentrations (Fig. 5b,f,g). Interestingly, the total photosynthetic pigments and flavonols were significantly and negatively correlated ($r = -0.41$, $P < 0.001$) likely indicating a trade-off between central and specialized metabolism (Fig. 7).

Correlations between metabolite concentration and precipitation

The PCA revealed that both total photosynthetic pigments and flavonol concentrations were especially associated with the total annual precipitation 2-years before sampling and 1-year before sampling, rather than the precipitation during the growing season (Fig. 4). Low values of annual precipitation during the 2 years before sampling correlated to low concentrations of VAZ ($r = 0.34$, $P < 0.01$; Fig. 8b), AZ/VAZ ($r = 0.27$, $P < 0.05$; Fig. 8c), and total flavonols ($r = 0.41$, $P < 0.001$; Fig. 8f). Furthermore, low annual precipitation 1-year before sampling was associated to high concentration in neoxanthin ($r = -0.24$, $P < 0.05$; Fig. 8g) and low concentrations in VAZ ($r = 0.23$, $P < 0.05$; Fig. 8h), AZ/VAZ ($r = 0.34$, $P < 0.01$; Fig. 8i) and total flavonol ($r = 0.31$, $P < 0.01$; Fig. 8l). The total carotenoid and chlorophyll concentrations did not correlate with either precipitation 1-year or precipitation 2-year before sampling. Precipitation during the growing season did not correlate with either total photosynthetic pigment or flavonol concentrations (Fig. S6a,b). By contrast, high precipitation from 1st January to 15th March was associated to high total photosynthetic pigment concentrations (Fig. S6e), especially to high chlorophyll *b* concentrations (Fig. S6h) but did not correlate with flavonol concentrations (Fig. S6f).

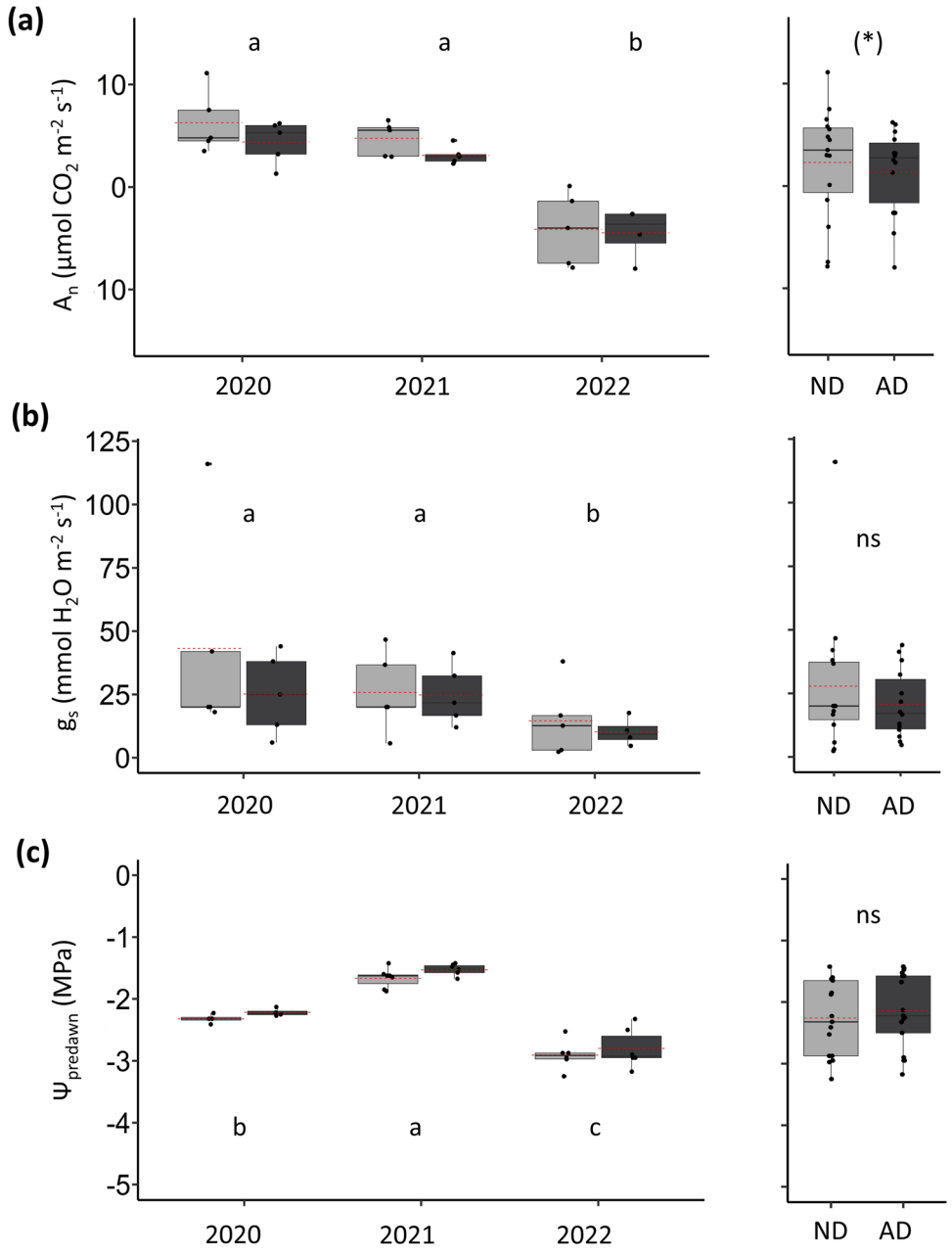

Fig. 3. Physiological leaf traits. (**a**) Net CO_2 assimilation (A_n), (**b**) stomatal conductance to water (g_s) and predawn water potential ($\Psi_{predawn}$) through years (2020, 2021 and 2022) and drought conditions (natural drought (ND) in light gray and amplified drought (AD) in dark gray). Differences are tested with a two-way ANOVA. Since interaction between year and drought was not significant, significant differences across years and drought conditions are noted on separate graphs. Significant differences are denoted using letters (a > b > c) for the years and asterisks for the drought condition: (*): $0.05 < P < 0.1$. Ns is for not significant results. The horizontal black lines inside the boxes are the medians and the horizontal red dashed lines are the means (n = 4–7 trees).

Discussion

In the present study, we investigated the ability of *Q. pubescens*—a deciduous and largely distributed Mediterranean species characterized by its drought resistance—to cope with long-term precipitation restriction simulating future aridity conditions in the Mediterranean region. The original approach consisted of analyzing how both the central and specialized defenses of this species were modulated *in natura* through long-term amplified drought (AD) and the effects of cumulated precipitation across several years.

The rainfall exclusion in our field site (12–47% annual precipitation reduction since April 2012) implied a decline of net photosynthesis under AD from 2020 to 2022 which had already been shown during our previous

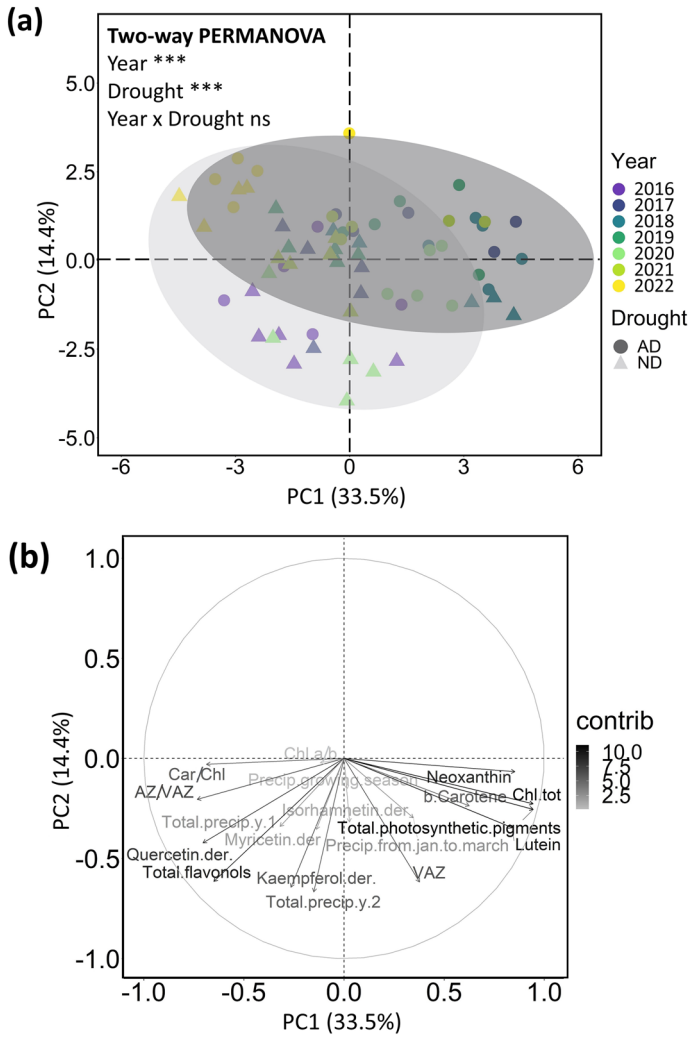

Fig. 4. Principal component analysis (PCA) for the first two PCA axes of (**a**) individuals and (**b**) variables (metabolites and precipitation patterns), observed under natural drought (ND) and amplified drought (AD) from 2016 to 2022, with n = 5 for each condition. AZ/VAZ, de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin; Car/Chl, ratio of total carotenoid to total chlorophyll concentrations; Chl. tot, total chlorophyll (chl. $a+b$); der., derivatives; Precip. growing season, precipitation during the growing season (from 15th March to harvest date); Precip jan. to march, precipitation from 1st January to 15th March of leaf harvest year; Total precip. y-1, Total annual precipitation 1-year before sampling; Total precip. y-2, Total annual precipitation 2-years before sampling.

studies on *Q. pubescens* in the same experimental site in 2014 and 2015[20]. Note that net photosynthesis was below zero in 2022 probably due to the very high temperatures recorded in July associated with water scarcity, pointing out the most severe climate stress on trees recorded so far for this experimental site[20,23,25]. However, no difference occurred between both drought conditions regarding either trait, g_s and Ψ_{pd} (measured in July 2020, 2021 and 2022). Two strategies are argued to explain these results. First, *Q. pubescens* is described as an anisohydric species which only partially closes stomata during drought stress[50], thus allowing to maintain some transpiration and photosynthesis rates even at very low leaf water potentials[24,51] such as those recorded in this study (up to − 3.25 MPa). Second, *Q. pubescens* also possess a highly efficient hydraulic system as previously suggested by Nardini and Pitt[24] allowing to maintain water content in leaves and impeding water potential drop. These two capabilities, namely partial stomatal closure and avoidance of the drop in water potential during drought stress, could explain why stomatal conductance and predawn water potential values remained similar between both drought conditions. It is worth noting that the low rates of net photosynthesis, while stomatal conductance remained stable under amplified drought, could indicate that photosynthesis is constrained by non-stomatal limitations (e.g. metabolic impairment)[52]. Such a result is supported by our previous study where a high internal CO_2 concentration (C_i) was measured in summer 2021 under amplified drought thus reflecting damage to the photosynthetic apparatus[23,53].

Regarding leaf metabolite concentrations, both 'drought conditions' and 'years' explained most of the variance (Fig. 4). Importantly, among central metabolites, photosynthetic pigments were highly accumulated under AD. Some carotenoids, namely neoxanthin, β-carotene and lutein were triggered under AD during the 2016–2022

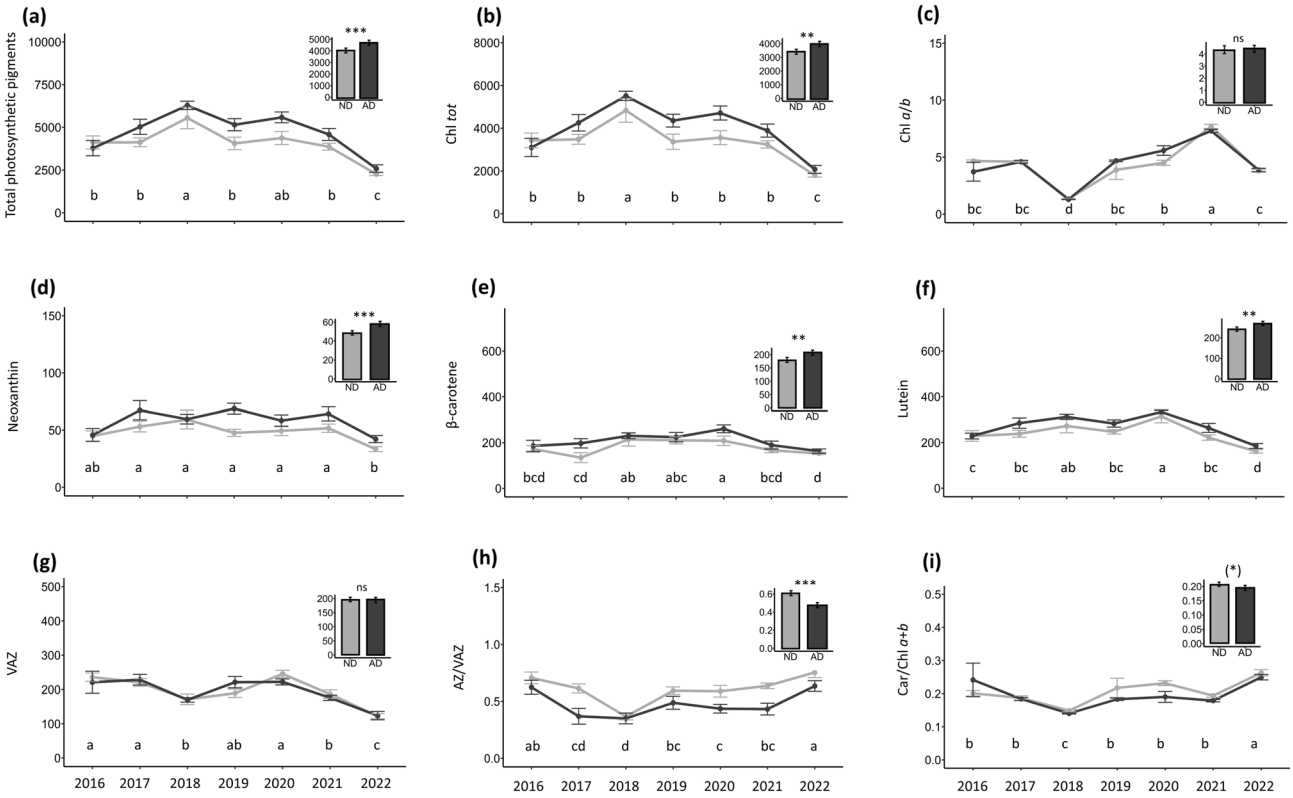

Fig. 5. Leaf concentrations of photosynthetic pigments (μg g_{DM}^{-1}) according to years (from 2016 to 2022) and drought conditions. (**a**) Total photosynthetic pigments (carotenoids and chlorophyll). (**b**) Total chlorophyll (Chlorophyll *a* and *b*). (**c**) Ratio of chlorophyll *a* to *b* (Chl *a*/*b*). (**d**) Neoxanthin (carotenoid). (**e**) β-carotene (carotenoid). (**f**) Lutein (carotenoid). (**g**) Xanthophyll cycle (including three carotenoids: violaxanthin, antheraxanthin and zeaxanthin; VAZ). (**h**) De-epoxidation of violaxanthin to antheraxanthin and zeaxanthin (AZ/VAZ). (**i**) Ratio of carotenoid to total chlorophyll concentrations (Car/Chl *a* + *b*). Differences between years were tested with post hoc Tukey tests and are indicated with different letters (a > b > c > d). Differences between drought conditions were indicated by asterisks with *0.05 < P < 0.1, **0.001 < P < 0.01 and ***P < 0.001. Ns is for not significant results. Values are mean ± se (n = 5 trees).

period, contrasting with Saunier et al.[20] who observed a drop in neoxanthin and lutein concentrations during the 4th year of AD (in 2015). These metabolites play a crucial role against drought-related oxidative stress by acting as ROS scavengers[54–56]. Additionally, the total chlorophyll concentration also increased under AD through the 2016–2022 period. More specifically, the ratio Car/Chl tended to decrease under AD indicating a reduction in the concentration of carotenoids compared to chlorophylls. Such result could indicate a potential loss in photoprotection since carotenoids dissipate excess light energy but also a loss in protection of the photosynthetic apparatus from oxidative damage[57]. Although the ratio of Chl *a*/*b* was similar between both drought conditions—suggesting that the efficiency of photosynthetic process was not totally altered by AD—*Q. pubescens* trees seem to prioritize the maintenance of chlorophyll concentration to avoid a drastic drop (more than 50%) of net photosynthesis under amplified drought conditions, despite potentially increased photodamage. It is worth noting that the accumulation of some carotenoids coupled with the increase in total chlorophyll concentration did not impede the photosynthesis decrease (− 50%) under AD over the 2020–2022 period. Also, the drop of net CO_2 assimilation in summer was even stronger (− 60%) during the 4th year of rain restriction[20] when such metabolic adjustments (accumulation of some carotenoids and chlorophylls) had not yet been observed, which could indicate that in our study the upregulation of the synthesis of these central metabolites could help to cope with long-term water deficit maintaining photosynthesis. These observations are in line with Gallé et al.[58] who highlighted that *Q. pubescens* is able to preserve its photosynthetic apparatus during extreme drought through maintaining the concentration of the photosynthetic pigments (Chl *a* + *b* and associated carotenoids, e.g. β-carotene, lutein and neoxanthin). Although these results give evidence of *Q. pubescens* acclimatation to harsher climatic conditions, it is worth noting that the ratio AZ/VAZ, which refers to the de-epoxidation state of the xanthophyll cycle, strongly dropped under AD in our study. Such a decrease denotes a loss in dissipating excess energy required for protecting the photosynthetic apparatus[59]. In addition, zeaxanthin concentrations also significantly dropped under AD. This carotenoid plays an important role as an antioxidant[60,61] and its decrease could indicate the presence of oxidative stress under AD as suggested in Laoué et al.[23] and endanger *Q. pubescens* functioning under very-long term precipitation decline.

Regarding specialized metabolites, the total flavonol concentration strongly dropped under AD over the 2016–2022 period according to results from previous years (2014 and 2015; Saunier et al.[27]). Myricitrin

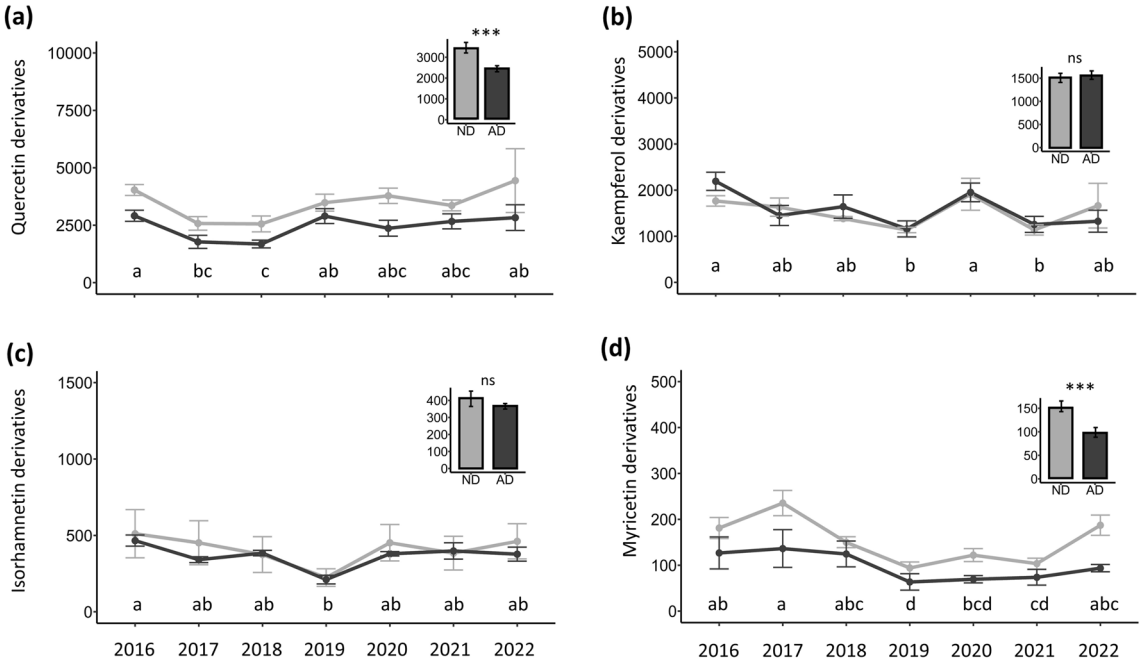

Fig. 6. Leaf concentrations of flavonols (μg g_{DM}^{-1}) according to years (from 2016 to 2022) and drought conditions. (**a**) Quercetin derivatives. (**b**) Kaempferol derivatives. (**c**) Isorhamnetin derivatives. (**d**) Myricetin derivatives. Differences between years were tested with post hoc Tukey tests and are indicated with different letters (a > b > c > d). Differences between drought conditions were indicated by asterisks with ***$P < 0.001$. Ns is for not significant results. Values are mean ± se (n = 5 trees).

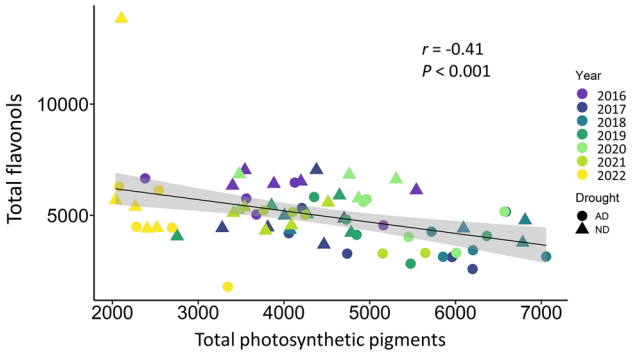

Fig. 7. Linear Pearson correlation between total photosynthetic pigment and total flavonol concentrations (μg g_{DM}^{-1}). Solid line represents the regression line, both drought conditions pooled together with the shaded areas around the lines indicating the 95% of confidence interval. Pearson correlation coefficient (*r*) and *P*-values are indicated (n = 5 trees).

derivatives displayed the highest decrease (− 33%), followed by quercetin derivatives (− 28%). Due to their chemical structure, quercetin and its derivatives possess the highest antioxidant activity compared to other flavonols[16]. Although the glycosylated forms of these compounds possess a poorer antioxidant activity[62,63], the drop of some flavonol derivatives under AD could reflect their antioxidant activity leading to their oxidation and thus in their consumption[64,65].

Our results indicate that *Q. pubescens* prioritizes the synthesis of central metabolites essential for photosynthesis (i.e. higher chlorophyll and carotenoid concentrations) rather than specialized defenses. This trade-off between specialized and central metabolites is also supported by the negative correlation between total flavonols and total photosynthetic pigments. Contrasting to our study, long-term amplified drought (more than 10 years) in a semi-arid Mediterranean site triggered foliar concentrations of flavonoids in the evergreen Mediterranean species (*Quercus ilex* L.)[22]. *Q. ilex* was however localized in a semi-arid Mediterranean site (average precipitation 610 mm) and submitted to a more drastic rain exclusion (compared to our study) leading to tree mortality after only 5 years of amplified drought. The effective role of flavonoids as antioxidants is currently intensively discussed and they have recently been described to act as a supplementary source of antioxidants when central antioxidants are unable to effectively neutralize ROS, especially in plants suffering from severe photooxidative

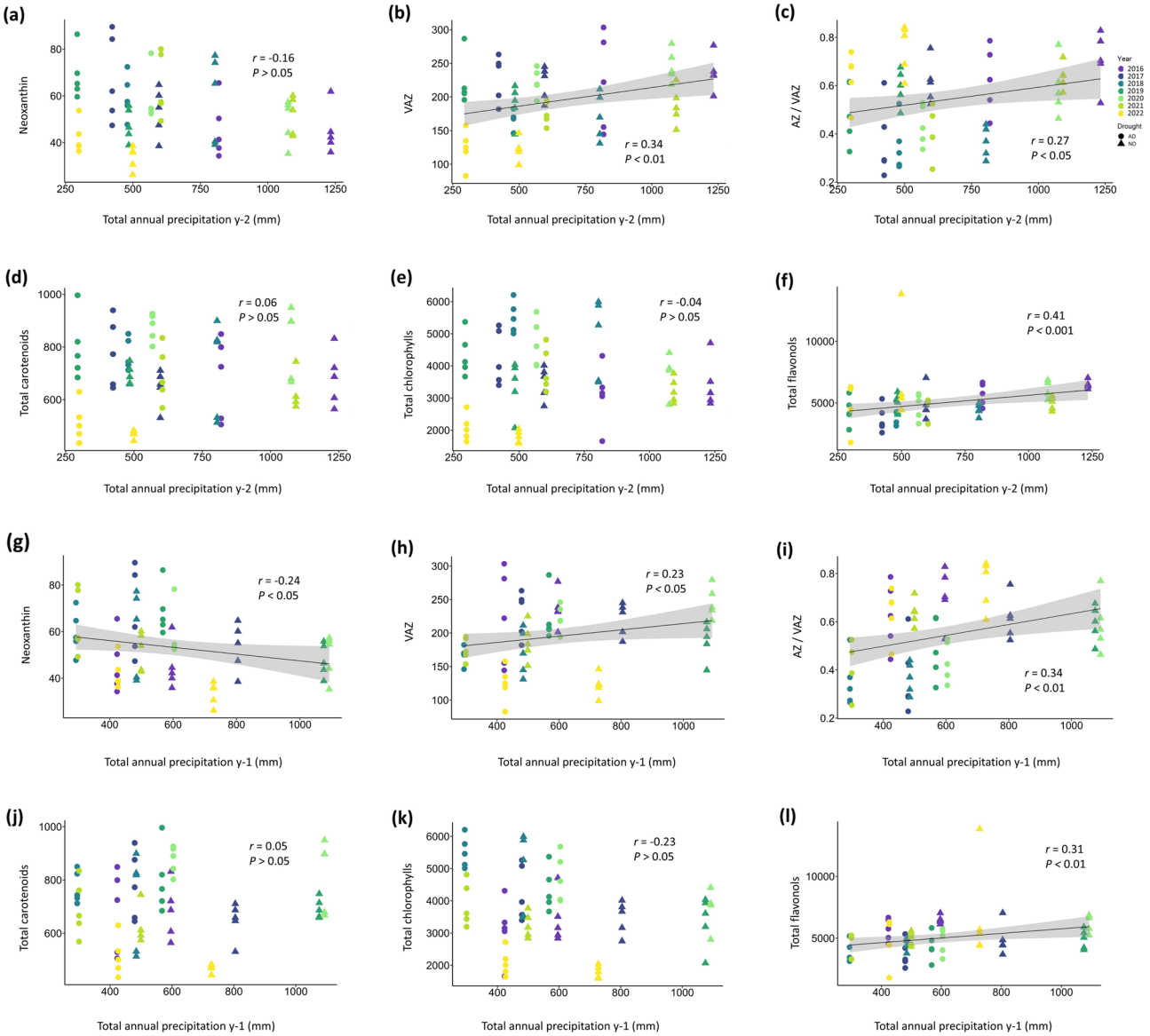

Fig. 8. Linear Pearson correlations between the total annual precipitation 1 and 2-years before sampling and metabolite concentrations (μg g_{DM}^{-1}). Relationship between neoxanthin, VAZ, AZ/VAZ, total carotenoid, total chlorophyll and flavonol concentrations and the total annual precipitation 2-years before sampling (**a**–**f**) as well as between the total annual precipitation 1-year before sampling and (**g**–**l**). Solid line represents the regression line for both drought conditions pooled together with the shaded areas around the lines indicating the 95% of confidence intervals. Pearson correlation coefficient (*r*) and *P*-values are indicated.

stress[66]. This suggests that central antioxidant defenses of *Q. pubescens* may help trees to withstand long-term (10 years) amplified drought conditions present in our supra-Mediterranean study, although the drop in some central antioxidant compounds (i.e. zeaxanthin) and in AZ/VAZ coupled with lower carbon assimilation are still indicative of a moderate stress level. It also suggests that harsher aridity conditions like the one present in the semi-arid Mediterranean site are required to upregulate specialized defenses, as reported in *Q. ilex*[22].

Precipitation during the growing season had no effect on either the concentration of central or specialized metabolites. However, it is worth noting that precipitation months prior to the growing season (i.e. from 1st January to 15th March) strongly influenced the concentration of chlorophyll *b*. Since chlorophyll synthesis is intimately linked to water availability[67], such relationship could explain the very high concentration of Chl *b* in 2018 where precipitation during this period was particularly high (i.e. 181 mm) compared to the other years. The total annual precipitation 2-years before sampling contributed the most to metabolite concentration profile of the sampling year, followed by the total annual precipitation 1-year before sampling (Fig. 4). Thus, poor precipitation cumulation was associated to poor concentrations of flavonols, VAZ and AZ/VAZ (Fig. 8). This result demonstrates that previous precipitation significantly drives the metabolic response of trees the current years and highlights a potential legacy effect in response to previous drought stress. Drought legacy effects refer to the lagged effects on plant physiology and more generally it includes alteration of tree growth by inducing

for example crown dieback or damage to water transport system[68,69]. In forest ecosystems, these drought legacy effects can negatively impact forest tree functioning for years[36]. However, there are little information regarding tree metabolite adjustments induced by recurrent drought over a long-term period (i.e. several years). Recently, Eisenring et al.[37] showed that extreme drought events past years (up to two years) can alter phytochemical profiles of beech leaves (especially specialized metabolites) which in turn affect tree- herbivore interaction potentially endangering forest health. Regarding deciduous *Quercus* species, drought stress over two years triggers adaptation processes of *Quercus robur* L. mainly by increasing protective osmolyte concentration such as quercitol and mannitol[18]. In our study, low precipitation levels during both 1- and 2-years before sampling were strongly linked to low concentrations of VAZ and flavonols, in line with their drop when trees grow under amplified drought conditions. This result reinforces the idea that consecutive and long-term drought episodes strongly modulate plant chemical defenses[70] and can potentially impact tree growth as observed in Mediterranean holm oak forest[4,71,72]. However, it should be pointed out that severe drought conditions during the previous year also had a positive impact on some antioxidant defenses as shown by neoxanthin accumulation at low precipitation level suggesting that neoxanthin possess a remarkable antioxidant activity[73,74].

In conclusion, our study provides valuable insights into how *Q. pubescens* will respond to future limited rain by analyzing the modulations in central (photosynthetic pigments) and specialized (flavonols) metabolites under intermediate to long-term scales (from the 5th to the 11th year of rain restriction). Despite a potential loss in photoprotection and antioxidant defenses due to the respective decrease in AZ/VAZ and flavonol concentrations under amplified drought, the accumulation of other carotenoids (namely neoxanthin, β-carotene and lutein) and of chlorophylls, demonstrates a remarkable ability of *Q. pubescens* to adapt and maintain its photosynthetic efficiency and antioxidant defenses, potentially allowing the species to withstand long-term amplified drought. Nevertheless, further investigations should address whether this shift in defense metabolism (upregulation of some central metabolites with a detrimental effect on flavonol concentration) could be dramatic for the species by favoring biotic stresses since these metabolites prevent invasion through plant signaling and act as a direct toxic substances against insects or pathogens[75]. Another remarkable result of our study is that rainfall from the previous and two previous years were important drivers of metabolite concentrations, where previous dry years led to a decrease in VAZ and flavonol concentrations whereas precipitation of the previous year favored neoxanthin concentrations. As a whole, long-term (> 10 years) and very long-term (> 20 years) experiments in the field allow us to assess drought legacy effects on Mediterranean forest defenses.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions

EO and CF conceived the study with input from JL and MH. JL performed fieldwork and collected the data with the help of EO and JPO. MH and BK did the pigment quantification. IR monitored climatic data since the implementation of the experimental site and compiled these data with the help of JL and JPO. JL and EO analyzed the data and wrote the manuscript with input and advice from MH, BK, JPO, IR and CF.

Competing interests

The authors declare no competing interests.

Additional information

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