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Long-term rain exclusion in a Mediterranean forest: response of physiological and physico-chemical traits of *Quercus pubescens* across seasons

Justine Laoué[1,*], **Michel Havaux**[2], **Brigitte Ksas**[2], **Béatrice Tuccio**[3], **Caroline Lecareux**[1], **Catherine Fernandez**[1] and **Elena Ormeño**[1,*]
[1]*Aix Marseille Univ., Univ Avignon, CNRS, IRD, IMBE, Marseille, France,*
[2]*Aix Marseille Univ., CEA, CNRS UMR 7265 BIAM, CEA/Cadarache, Saint-Paul-lès-Durance, France, and*
[3]*Aix Marseille Univ., CNRS, ICR, UMR 7273, Marseille, France*

Received 19 May 2023; revised 21 July 2023; accepted 4 August 2023.
*For correspondence (e-mail justine.laoue@gmail.com; elena.ormeno-lafuente@imbe.fr)

SUMMARY

With climate change, an aggravation in summer drought is expected in the Mediterranean region. To assess the impact of such a future scenario, we compared the response of *Quercus pubescens*, a drought-resistant deciduous oak species, to long-term amplified drought (AD) (partial rain exclusion *in natura* for 10 years) and natural drought (ND). We studied leaf physiological and physico-chemical trait responses to ND and AD over the seasonal cycle, with a focus on chemical traits including major groups of central (photosynthetic pigments and plastoquinones) and specialized (tocochromanols, phenolic compounds, and cuticular waxes) metabolites. Seasonality was the main driver of all leaf traits, including cuticular triterpenoids, which were highly concentrated in summer, suggesting their importance to cope with drought and thermal stress periods. Under AD, trees not only reduced CO_2 assimilation (−42%) in summer and leaf concentrations of some phenolic compounds and photosynthetic pigments (carotenoids from the xanthophyll cycle) but also enhanced the levels of other photosynthetic pigments (chlorophylls, lutein, and neoxanthin) and plastochromanol-8, an antioxidant located in chloroplasts. Overall, the metabolomic adjustments across seasons and drought conditions reinforce the idea that *Q. pubescens* is highly resistant to drought although significant losses of antioxidant defenses and photoprotection were identified under AD.

Keywords: climate change, cuticular waxes, drought, ecophysiology, *Quercus pubescens*, rainfall exclusion, seasonality, targeted metabolomics.

INTRODUCTION

The Mediterranean region is an important climate change ‘hot spot’ combining dry and hot summer periods which are expected to accentuate in the future (Lionello & Scarascia, 2018). Climate models forecast a significant reduction in annual precipitation reaching −30% or even exceeding −40% in some parts of the southern Mediterranean with the highest water scarcity during summer months (Zittis et al., 2019). These changes occur rapidly and will increase in intensity, frequency, and severity, prolonging the summer drought, thereby endangering the growth and survival of Mediterranean forest ecosystems (Peñuelas et al., 2018). One of the most important challenges in ecology is thus to anticipate the plant responses to a drier climate.

Plant’s physico-chemical leaf traits are strongly influenced by seasonal variations in temperature, light, humidity, precipitation, and phenology (Liebelt et al., 2019; Saunier et al., 2018, 2022). Adjustments in these traits remain crucial to cope with summer drought stress (Brunetti et al., 2019). Plants exhibit a range of physical traits, which allow adaptation to dry conditions, mainly by reducing water loss (De Micco & Aronne, 2012). The most commonly regarded physical trait for plant performance under drought is the specific leaf area (SLA) (Wellstein et al., 2017). Several studies have reported that leaves with low SLA imply high thickness as a mean to cope with dry conditions (Ramírez-Valiente et al., 2017, 2020), while higher SLA (and thus thinner leaves) coupled with larger leaves can reflect an acquisition resource-use strategy where light capture, photosynthesis, and resource assimilation are maximized (Pérez-Ramos et al., 2013; Reich, 2014).

Among chemical traits, plants synthesize and store a large variety of carbon-based metabolites classified as central (or universal) metabolites, which play crucial roles for basic plant functioning, growth, and survival and as specialized metabolites which mediate plant interaction with the environment and represent supplementary defenses against biotic and abiotic stresses (Fang et al., 2019). One of their major roles is to get rid of reactive oxygen species (ROS) overproduced upon excess of UV radiation, temperature, and water deficit (Havaux & Kloppstech, 2001; Hernández et al., 2012; Ormeño, Viros, et al., 2020; Reddy et al., 2004; Shepherd & Wynne Griffiths, 2006). Such metabolites include carotenoids, tocochromanols, plastoquinones, and phenolic compounds well known to act as ROS scavengers (Das & Roychoudhury, 2014; Esteban et al., 2015; Havaux, 2020; Ksas et al., 2015; Laoué et al., 2022; Muñoz & Munné-Bosch, 2019). In addition to their role as direct ROS scavengers, phenolic compounds also contribute to UV protection and can act as signaling molecules (Laoué et al., 2022; Peer & Murphy, 2006; Santos-Sánchez et al., 2019). Leaf pigments (e.g., chlorophylls and carotenoids) are essential for photosynthesis and photoprotection by harvesting and transferring light energy (Maoka, 2020; Moreno et al., 2021). In particular, carotenoids from the xanthophyll cycle (violaxanthin, antheraxanthin, and zeaxanthin) contribute to dissipate the excitation energy within the antenna of photosystem II, which accumulates in excess when stomatal closure limits photosynthesis (Demmig-Adams, 1990). Plastoquinones (PQ) are classified in this study as central metabolites since they play an essential role in photosynthesis, and plants deficient in PQ are not viable (Havaux, 2020). Moreover, PQ have also an antioxidant function (Hundal et al., 1995). In addition, cuticular waxes are located in the outer leaf layer and play a major role in evapotranspiration limitation through their hydrophobic properties (Holloway, 1994; Schuster et al., 2016). They are thus key plant defenses against drought (Shepherd & Wynne Griffiths, 2006). In an evolutionary context, plants have developed water-resistant cuticles to colonize lands (Edwards, 1996). Cuticular waxes are predominantly formed by long-chain aliphatic waxes including fatty acids and their derivatives (alkanes, alkanols, and aldehydes) as well as triterpenoids, highly concentrated in some species (Bueno et al., 2020; Shepherd & Wynne Griffiths, 2006; Simões et al., 2020). Triterpenoids confer mechanical stability to aliphatic waxes, especially at elevated temperature, and thereby reduce thermal stress (Schuster et al., 2016). Regarding the *Quercus* genera, the study of cuticular waxes has been documented in sclerophyllous species (Bueno et al., 2020; Martins et al., 1999; Ormeño, Ruffault, et al., 2020; Simões et al., 2022), while in deciduous species, only *Quercus robur* L. cuticular waxes have been characterized so far (Gülz & Müller, 1992).

Although changes in leaf metabolome can denote the capacity of plants for adaptation to climate change (Sardans et al., 2011), their modulation due to chronic and long-term drought (over years) remains poorly documented in natural conditions. Rivas-Ubach et al. (2014) reported a clear overaccumulation of leaf phenolic compounds in *Quercus ilex* L. after 10 years of partial rain exclusion, while Saunier et al. (2022) showed a drop of few phenolic compounds after 3–4 years of partial rain exclusion in *Quercus pubescens* Wild. Responses of cuticular waxes to long-term drought in the field have only been investigated in a site dominated by the sclerophyllous species *Quercus coccifera* L. (Ormeño, Ruffault, et al., 2020; Ormeño, Viros, et al., 2020), while this trait remains unexplored in deciduous *Quercus* species. Nevertheless, several studies under controlled conditions reported that drought enhances the accumulation of cuticular waxes in the model plant *Arabidopsis* and various crop species (Kosma et al., 2009; Xue et al., 2017).

In the present work, we studied the response of *Q. pubescens* to long-term amplified drought (AD) (i.e., after 10 years) over seasons in a Mediterranean forest with a focus on chemical, physical, and physiological traits. We targeted numerous specialized metabolites, with a focus on cuticular waxes (never documented so far for *Q. pubescens*) and phenolic compounds, but also tocochromanols. Among tocochromanols, hydroxy-plastochromanol (PC-OH) was used as a proxy of the leaf oxidative stress since it is specifically formed upon the action of the singlet oxygen ($^1O_2$) (Szymańska et al., 2014). We also targeted a few metabolites from the central or universal metabolism (chlorophylls, carotenoids, and plastoquinones). Physical leaf traits encompassed leaf size and SLA. Physiological traits were assessed through the measure of net photosynthesis, stomatal conductance, internal CO_2 concentration, water-use efficiency (WUE) as well as midday and predawn water potential, which are associated with plant response to water stress (Damesin & Rambal, 1995; Gallé et al., 2007).

Previous studies on the same experimental site showed a strong impact of middle-term (3–4 years) AD on physiological functioning of *Q. pubescens* with a slight decrease in the production of some carotenoids and phenolic compounds (Saunier et al., 2018; Saunier et al., 2022). Accordingly, we hypothesized that long-term (10 years) AD could compromise physiological functioning and production of plant central and specialized metabolites, especially during the driest season. In line with Gallé et al. (2007), which highlighted that *Q pubescens* withstands extreme drought by preserving its photosynthetic apparatus through maintenance of photosynthetic pigments, we hypothesized that these central metabolites could at least be maintained or even be triggered during long-term summer drought, allowing recovery of photosynthetic activity in autumn.

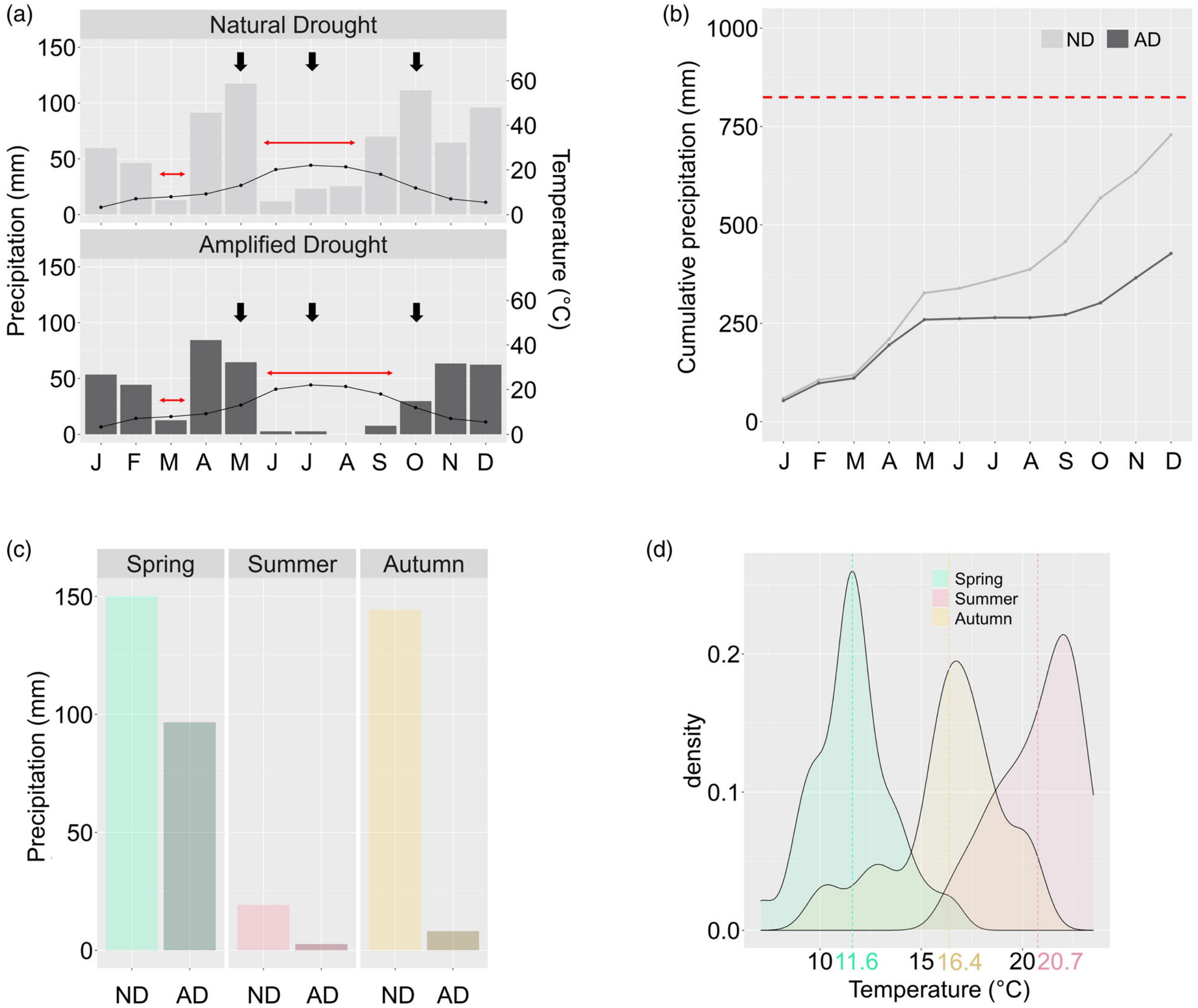

Figure 1. Climatic conditions on the experimental site in 2021. (a) Ombrothermic diagram for natural (light gray) and amplified (dark gray) drought plots where bars represent monthly cumulative precipitation (P, mm) and curves represent mean monthly temperature (T, °C) with P = 2 T. When temperature curves are above precipitation bars, it corresponds to dry periods indicated by red horizontal arrows. Vertical black arrows indicate field campaigns in May (spring), July (summer), and October (autumn). (b) Cumulative precipitation for natural (light gray) and amplified (dark gray) drought plots. The dotted red line represents the annual mean precipitation calculated for the period 1967–2000 (830 mm). (c) Sum of precipitation during the 30 days preceding each sampling campaign in spring (green), summer (pink), and autumn (yellow) under amplified drought (AD) and natural drought (ND). (d) Density of temperature values for each season where the mean temperature during the 30 days preceding each sampling campaign is highlighted in the corresponding color.

RESULTS

Physiological and physical traits: Seasonality and drought responses

Net CO_2 assimilation changed according to seasonality and drought (Figure 2A). The highest A_n was reached in spring and autumn (10.29 ± 0.68 and 9.58 ± 1.06 μmol CO_2 m^{-2} sec^{-1}, respectively), while in summer, it decreased by 61% (4.06 ± 0.49 μmol CO_2 m^{-2} sec^{-1}) associated with the decrease in precipitation (Figure 1A). Amplified drought significantly impacted the A_n leading to an overall decrease of 16% compared with ND (with a decrease up to 42% in summer), which was associated with a slight decline of g_s ($R^2 = 0.39$; $P < 0.001$; Figure S1; Figure 2A,B). The internal CO_2 concentration (C_i) increased over the seasons and was marginally higher ($P < 0.1$) in leaves under AD, especially in summer with an increase up to 41% (Figure 2C). Regarding the WUE, a decrease is observed in autumn only, independently of the drought conditions (Figure 2D). Both Ψ_{md} and Ψ_{pd} exhibited the highest values in spring (-1.39 ± 0.11 and -0.53 ± 0.03 MPa, respectively) (Figure 2E,F). The lowest Ψ_{md} was reached in summer (-3.09 ± 0.15 MPa) with no difference between conditions since precipitation was very low in both plots

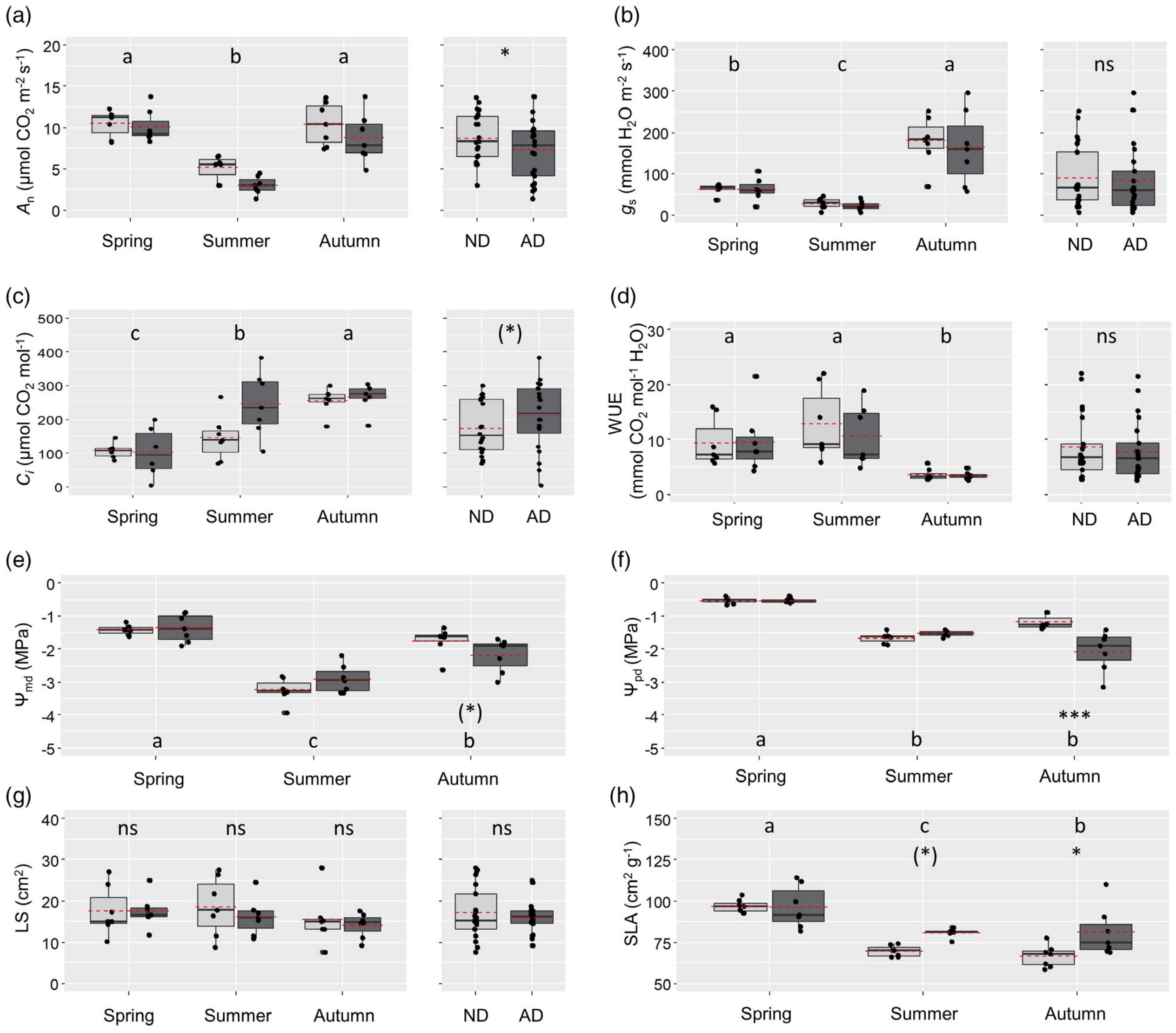

Figure 2. Physiological (a–f) and physical (g, h) leaf traits through seasons (spring, summer, and autumn) and drought conditions (natural drought (ND) in light gray and amplified drought (AD) in dark gray). When interaction between season and drought was not significant (a–d, g), significant differences across seasons and drought conditions are noted on separate graphs. When significant interactions occurred (e, f, and h), differences are highlighted in a single graph. Significant differences are denoted using letters (a > b > c) for the seasons and asterisks for drought condition: (*): $0.05 < P < 0.1$, *: $0.01 < P < 0\ 0.05$, **: $0.001 < P < 0.01$, and ***$P < 0.001$. Ns is for non-significant results. The horizontal black lines inside the boxes are the medians and the horizontal red dashed lines are the means ($n = 7$). A_n, assimilation; C_i, internal CO$_2$ concentration; g_s, stomatal conductance, WUE, water-use efficiency; Ψ_{md}, midday water potential; Ψ_{pd}, predawn water potential; LS, leaf surface; and SLA, specific leaf area.

(19 mm in ND and 3 mm in AD; Figure 1C). Water potentials were lowered under AD in autumn only: Ψ_{md} tended to drop from −1.75 to −2.18 MPa and Ψ_{pd} significantly decreased from −1.19 to −2.06 MPa (Figure 2E,F). Rain precipitation was indeed almost completely excluded (−94%) in the AD plot the month before autumn sampling (Figure 1C).

Regarding physical traits, leaf size remained stable across seasons and drought conditions although we noticed a non-significant larger leaf size under ND (18.58 cm^2) compared with AD (16.16 cm^2) in summer. Leaf size was the same in spring (17.45 cm^2 under ND and 17.56 cm^2 under AD) (Figure 2G). SLA significantly decreased through seasons with the lowest values in autumn (66.60 ± 2.56 cm^2 g^{-1} in ND plot; Figure 2H), and such decrease was less marked under AD in both summer ($P < 0.1$) and autumn ($P < 0.05$).

Chemical traits: Seasonality and drought responses

We quantified 57 metabolites classified within five major groups including photosynthetic pigments (chlorophylls and carotenoids), plastoquinone, tocochromanols (plastochromanol and α-tocopherol), phenolic compounds (flavonols, flavanols, and phenolic acids), and cuticular waxes (alkanes, alkanols, aldehyde, fatty acids, tocopherols, phytosterol, and triterpenoids) (Table 1) belonging to different biosynthetic pathways (Figure S2). Independently of the season and the drought condition, cuticular waxes were dominated by triterpenoids (37%) followed by fatty acids (28%) (Table 2).

The metabolic profiles across seasons and drought conditions were analyzed on 1) the five major metabolic groups, 2) subgroups within the five major metabolite groups, and 3) single metabolites. Principal component analysis (PCA) analysis revealed that major metabolic groups and subgroups (Figure 3A), but also single metabolites (Figure 3B), were only modulated by seasonality (PCA, PERMANOVA, $P < 0.001$). The PCA revealed two main principal components (PCs), which explained 61.3% of the metabolite concentration variations (Figure 3A). Carotenoids, plastoquinones, tocochromanols, fatty acids, and tocopherols (from cuticular waxes) were the most contributing variables to the first axis while aldehyde (only one was detected) and triterpenoids explained the variability across the second axis. At the single compound level, PCA revealed that spring leaves were characterized by high concentrations of the photosynthetic pigment carotenoids (lutein, β-carotene, and VAZ) and chlorophylls, as well as high storage of phenolic acids (feruloyl quinic acid), alkanols, and to a less extent flavonols (Figure 3). Contrastingly, summer leaves featured high concentrations of cuticular waxes, mainly triterpenoids and aldehyde (octacosanal) (Figure 3). Autumn leaves were also rich in cuticular waxes, mainly tocopherols (α-tocopherol), long-chain fatty acids (pentacosanoic, hexacosanoic, and octacosanoic acids), and the major triterpenoid (lupeol) (Figure 3B). Autumn leaves also featured high concentrations of tocochromanols (PC-8, PC-OH, and α-tocopherol), plastoquinones, and flavanols (catechin) (Figure 3).

Parametric analysis (two-way ANOVA; Table 2; Tables S1 and S2) revealed that storage of metabolite groups, subgroups, and single metabolites was driven by seasonality, but some differences between drought conditions could also be highlighted. All metabolite groups showed seasonal differences with the highest concentrations of photosynthetic pigments and phenolic compounds in spring, while plastoquinones and tocochromanols were most concentrated in autumn (they also accumulated in summer but to a lesser extent compared with autumn) and cuticular waxes in both summer and autumn (Table S1). Among photosynthetic pigments, chlorophyll and carotenoid concentrations decreased over the seasonal cycle with the highest concentrations in spring (4263 ± 205 and 915 ± 29 $\mu g\ g^{-1}{}_{DM}$, respectively) (Table 2). By contrast, plastoquinones and tocochromanols were approximately 10-fold higher in autumn compared with spring. Among phenolic compounds, only flavanols increased in summer mainly due to the increase in catechin (Table S2), while phenolic acids exhibited the highest storage in both spring (1976 ± 161 $\mu g\ g^{-1}{}_{DM}$) and summer (1800 ± 114 $\mu g\ g^{-1}{}_{DM}$) and then dropped in autumn (1027 ± 47 $\mu g\ g^{-1}{}_{DM}$). Flavonols—the main phenolic compound subgroup (Table 2)—were mainly concentrated in spring (7358 ± 490 $\mu g\ g^{-1}{}_{DM}$), especially quercetin and kaempferol derivatives compared with summer and autumn where they showed similar concentrations (summer: 5650 ± 500 $\mu g\ g^{-1}{}_{DM}$; autumn: 5716 ± 400 $\mu g\ g^{-1}{}_{DM}$) (Table 2; Table S2). Total cuticular waxes reached its highest concentration in summer (4725 $\mu g\ g^{-1}{}_{DM}$) and autumn (4351 $\mu g\ g^{-1}{}_{DM}$) (Figure S3). Triterpenoids were the most concentrated waxes with an average of 1607 $\mu g\ g^{-1}{}_{DM}$ all seasons pooled (Figure S3; Table 2) and were the only subgroup—with aldehyde—to reach the highest concentrations in summer (2182 ± 287 $\mu g\ g^{-1}{}_{DM}$), mainly attributed to the significant rise in β-amyrin and lupeol (Table S2). Otherwise, alkanes, fatty acids, and cuticular wax tocopherols displayed the lowest concentrations in spring and were similarly concentrated in summer and autumn (Table 2). β-sitosterol did not show any change across seasons (Table S2).

Regarding metabolic changes according to drought conditions, only photosynthetic pigments tend to increase under AD (Table S1). Among these photosynthetic pigments, two metabolite subgroups, namely chlorophylls (chlorophyll *a* and *b*) and plastochromanols, significantly increased by 12% and 8%, respectively, under AD (Table 2; Table S2). The increase in plastochromanol concentrations was due to the increase in both PC-8 and PC-OH (Table S2). The increase in PC-8 concentrations occurred only in summer (Table S2). Also, AD led to a rise in two single carotenoids, namely neoxanthin (+16%) and lutein (+11%), and in cuticular waxes (docosanol, docosanoic acid, and δ-tocopherol) (Table S2). Contrastingly, three phenolic compound concentrations (myricitrin, quercetin galloyl glucose, and *p*-coumaric acid glycosylated derivative) decreased under AD from −14% to −32% as well as one cuticular wax (octacosanal; −23%) and pigments from the xanthophyll cycle (VAZ; −10%) (Table S2).

DISCUSSION

In the present study, the physiological, physical, and chemical trait adjustments related to both seasonality and long-term rain exclusion were evaluated in *Q. pubescens*, a drought-resistant species (Damesin & Rambal, 1995; Saunier et al., 2018; Struve et al., 2009). Seasonality appeared as the main driver of all traits, but AD was also associated with some significant changes, allowing to anticipate the strategies used by this species to cope with future drier climate (Figure 4).

Physiological traits

There was a strong effect of seasonality on physiological traits in 2021 with main stress occurring during summer as

Table 1 Metabolite groups and subgroups targeted in this study and belonging to central and specialized metabolism in the whole leaf and leaf surface (cuticular waxes), biosynthetic pathways, main functions, and references.

Leaf part	Metabolism	Metabolite group	Metabolite subgroup	Biosynthetic pathways	Main functions	References
Whole leaf	Central	Photosynthetic pigments	Carotenoid	MEP	Light harvesting for photosynthesis Photoprotection related to xanthophyll cycle Antioxidant Signaling molecule	Esteban et al. (2015) and Moreno et al. (2021)
			Chlorophyll	MEP	Light-absorbing for photosynthesis Electron transporters in the photosynthesis	Kim et al. (2013)
	Central	Plastoquinones	Plastoquinones	MEP and shikimate	Electron transporters in the photosynthesis Antioxidant	Havaux (2020) and Hundal et al. (1995)
	Specialized	Tocochromanols	Hydroxy-plastochromanol	MEP and shikimate	Oxidation marker	Szymańska et al. (2014)
			Plastochromanol	MEP and shikimate	Antioxidant	Havaux (2020)
			Tocopherols	MEP and shikimate	Antioxidant	Muñoz and Munné-Bosch (2019)
	Specialized	Phenolic compounds	Flavonols	Shikimate	Antioxidant UV-B protection Signaling molecule	Laoué et al. (2022) and Santos-Sánchez et al. (2019)
			Flavanols	Shikimate	Antioxidant UV-B protection Signaling molecule	Laoué et al. (2022) and Santos-Sánchez et al. (2019)
			Phenolic acids	Shikimate	Antioxidant Signaling molecules	Santos-Sánchez et al. (2019)
Cuticular waxes leaf surface	Specialized	Cuticular waxes	Alkanes	Fatty acids derivatives	Cuticular transpiration barrier	Batsale et al. (2021) and Jetter and Riederer (2016)
			Alkanols	Fatty acids derivatives	Cuticular transpiration barrier	Batsale et al. (2021) and Jetter and Riederer (2016)
			Aldehyde	Fatty acids derivatives	Cuticular transpiration barrier	Batsale et al. (2021) and Jetter and Riederer (2016)
			Fatty acids	Fatty acids derivatives	Cuticular transpiration barrier	Batsale et al. (2021) and Jetter and Riederer (2016)
			Phytosterol	MVA	Maintenance of membrane homeostasis	Rogowska and Szakiel (2020)
			Triterpenoids	MVA	Cuticle stabilization during thermal stress Toxicity for herbivores	Schuster et al. (2016) and Tsubaki et al. (2013)
			Tocopherols	MEP and shikimate	Antioxidant Maintenance of the integrity of long-chain fatty acids	Muñoz and Munné-Bosch (2019)

MEP, methyl-D-erythritol phosphate (or non-mevalonate) pathway; MVA, mevalonate pathway.

Table 2 Results of two-way ANOVA performed on metabolite subgroup concentrations (μg $g^{-1}{}_{DM}$) according to season and drought conditions ($n = 7$).

		Spring (Sp)		Summer (Su)		Autumn (Au)		Two-way ANOVA		
Metabolite group	Metabolite subgroup	ND	AD	ND	AD	ND	AD	Season (Sp, Su, Au)	Drought (ND, AD)	S × D
Photosynthetic pigments	Carotenoids	893.38 ± 30.04	936.64 ± 27.33	645.28 ± 25.86	700.19 ± 34.07	525.77 ± 13.42	497.47 ± 50.75	*** (a, b, c)	ns	ns
	Chlorophylls	4032.43 ± 221.68	4493.39 ± 188.53	3366.46 ± 192.10	4034.64 ± 233.46	2693.77 ± 120.95	2732.86 ± 361.65	*** (a, b, c)	* (B, A)	ns
Plastoquinones	Plastoquinones	192.58 ± 26.80	195.17 ± 11.22	713.50 ± 45.29	727.99 ± 35.37	1819.21 ± 170.61	1909.93 ± 213.64	*** (c, b, a)	ns	ns
Tocochromanols	Plastochromanols	3.22 ± 0.37	4.01 ± 0.41	9.74 ± 0.68	13.76 ± 0.38	46.76 ± 2.63	46.54 ± 5.19	*** (c,b,a)	* (B, A)	ns
	Tocopherols	75.50 ± 5.92	82.16 ± 5.48	308.64 ± 54.88	237.51 ± 13.08	580.64 ± 20.57	624.90 ± 73.31	*** (c, b, a)	ns	ns
Phenolic compounds	Flavonols	7635.31 ± 309.91	7080.10 ± 669.80	5387.32 ± 408.91	5913.13 ± 591.99	5489.95 ± 379.80	5942.50 ± 418.39	** (a, b, b)	ns	ns
	Flavanols	795.99 ± 110.27	764.18 ± 56.35	1358.87 ± 229.86	1372.73 ± 158.75	1946.73 ± 404.05	1564.39 ± 231.49	*** (b, a, a)	ns	ns
	Phenolic acids	1866.00 ± 158.39	2086.64 ± 163.67	1834.10 ± 109.22	1765.27 ± 118.00	1084.70 ± 59.95	969.62 ± 34.13	*** (a, a, b)	ns	ns
Cuticular waxes	Alkanes	109.11 ± 10.52	116.71 ± 14.15	138.73 ± 11.33	137.87 ± 16.09	159.02 ± 16.90	166.14 ± 20.82	* (b,ab, a)	ns	ns
	Alkanols	1632.35 ± 220.10	1679.84 ± 190.28	724.84 ± 54.88	783.77 ± 64.99	845.76 ± 84.49	920.40 ± 38.88	*** (a, b, b)	ns	ns
	Aldehyde	56.35 ± 9.08	44.23 ± 8.70	104.12 ± 9.84	71.58 ± 8.39	57.92 ± 5.91	53.43 ± 7.02	*** (b, a, b)	** (A, B)	ns
	Fatty acids	610.82 ± 33.82	844.52 ± 154.58	1493.33 ± 163.01	1222.80 ± 144.29	1458.30 ± 99.26	1576.48 ± 148.87	*** (b, a, a)	ns	ns
	Tocopherols	22.52 ± 4.19	41.21 ± 7.83	78.61 ± 7.03	91.66 ± 12.29	98.03 ± 8.56	87.94 ± 7.48	*** (b, a, a)	ns	ns
	Phytosterol	124.31 ± 12.07	157.04 ± 26.99	131.30 ± 8.04	106.71 ± 5.48	111.93 ± 7.49	111.13 ± 7.20	ns	ns	ns
	Triterpenoids	986.04 ± 55.41	1237.42 ± 158.69	2151.83 ± 392.04	2212.61 ± 182.77	1604.06 ± 206.56	1451.93 ± 156.50	*** (c, a, b)	ns	ns

P-values are indicated by *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, ***$P < 0.001$. Lowercase letters denote differences between seasons ordered as follows in the parenthesis: (spring, summer, and autumn), with a > b > c. Uppercase letters denote significant differences between drought conditions ordered as follows in the parenthesis: (ND, AD), with A > B.

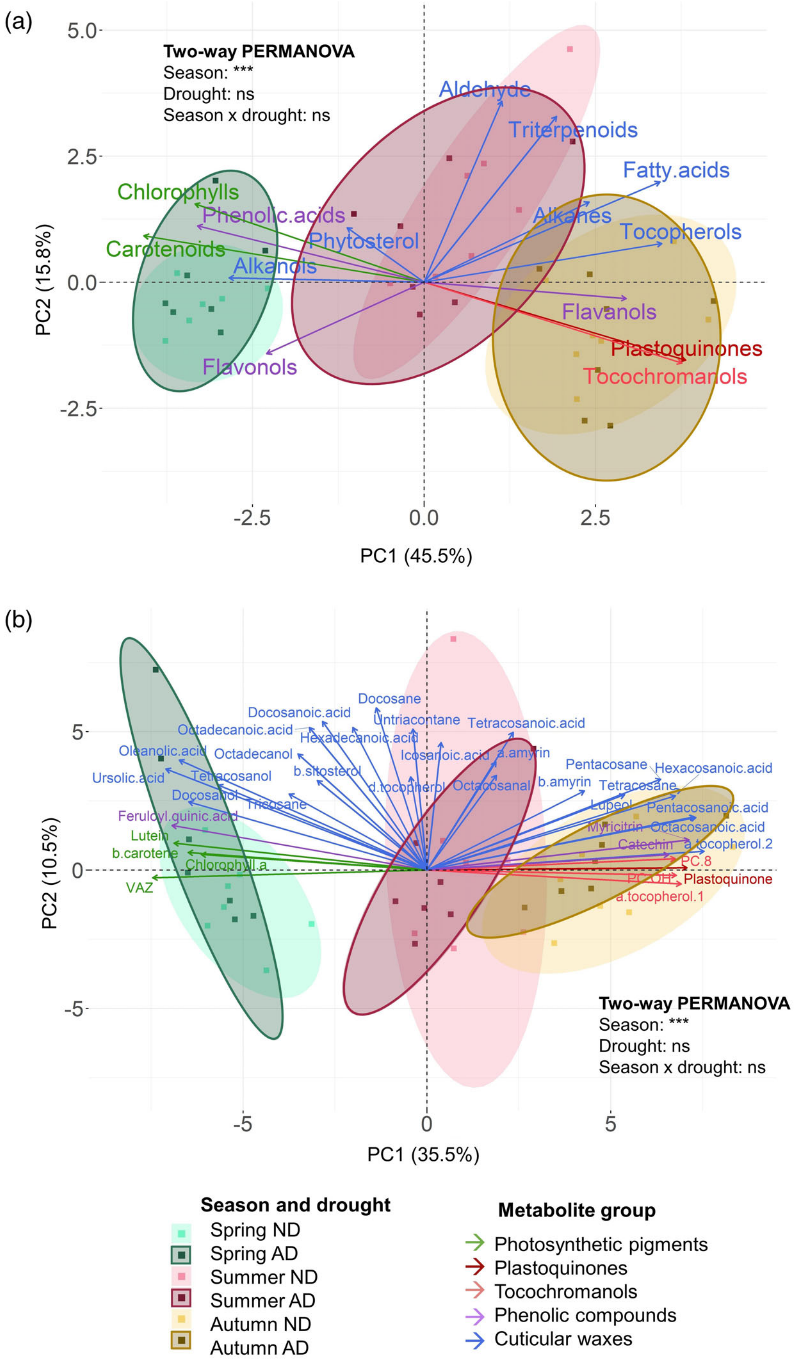

Figure 3. Principal component analysis (PCA) for the first two PCA axes of (a) the metabolite groups and subgroups, and (b) the 30 most contributive single metabolites (among the 57 single metabolites analyzed). Metabolite groups (photosynthetic pigments, plastoquinones, tocochromanols, phenolic compounds, and cuticular waxes) are indicated with arrows in different colors. Seasons are represented in green (spring), pink (summer), and yellow (autumn). Drought conditions are represented in light (ND) and dark circled colors (AD), with $n = 7$ for each condition.

denoted by the lowest values of A_n (4 μmol CO_2 m^{-2} sec^{-1}), g_s (25 mmol H_2O m^{-2} sec^{-1}), and Ψ_{md} (−3 MPa). These results were also reported by Saunier et al. (2018) in 2015 in the same field site, that is, after 4 years of rain exclusion. However, 2015 was a drier year (598 mm annual precipitation) compared with 2021 (729 mm), and accordingly, the values of all these physiological traits were slightly lower in 2015, especially in summer (A_n: 1.3 μmol CO_2 m^{-2} sec^{-1}, g_s: 3.8 mmol H_2O m^{-2} sec^{-1} and Ψ_{md}: −3.6 MPa; Saunier et al., 2018). The C_i

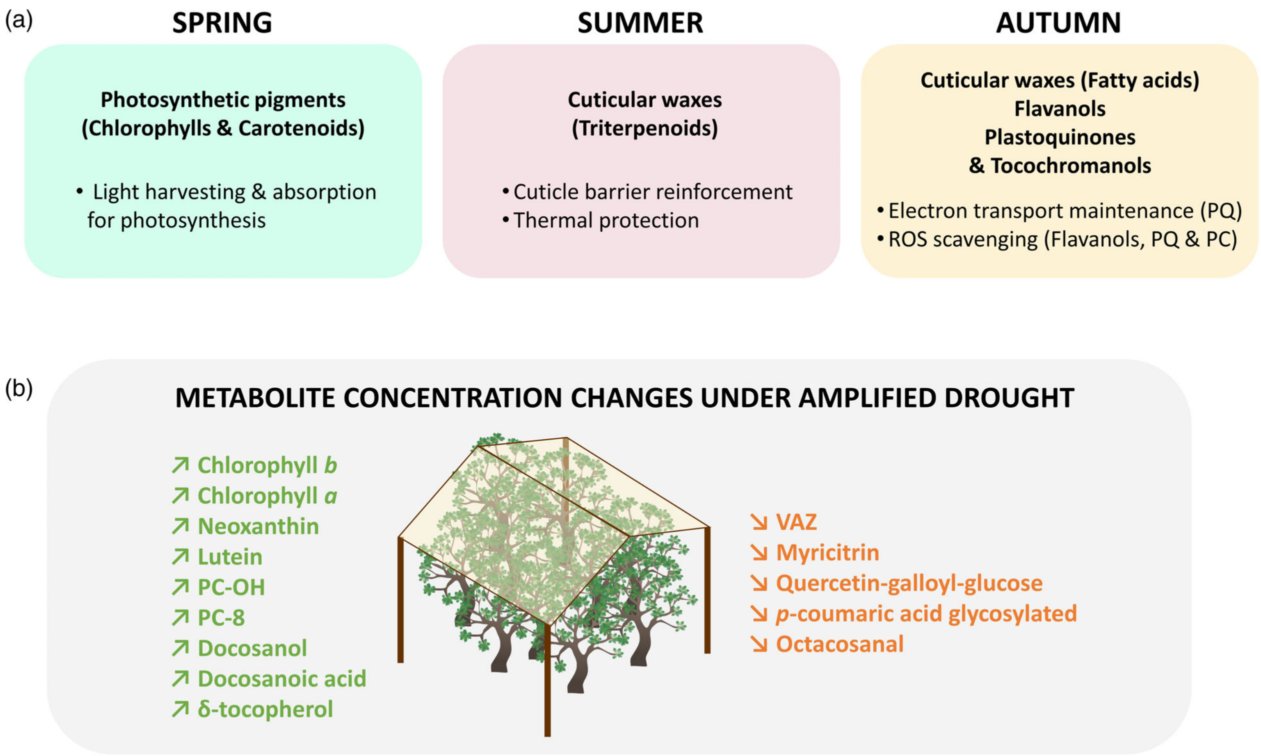

Figure 4. Overall conclusion of the main chemical trait responses of *Q. pubescens* leaves through (a) seasonality where the most concentrated groups (or subgroups) within each season are represented with their main function and (b) amplified drought (AD) where green arrows indicate a significant increase and orange arrows a significant decrease in single metabolites in comparison with natural drought (ND). PQ, Plastoquinones; PC-OH: Hydroxy-plastochromanol; PC-8, Plastochromanol-8. See Table 1 for bibliographic references regarding metabolite functions.

was lowest in spring, intermediate in summer, and highest in autumn, indicating a higher CO_2 assimilation in spring through photosynthesis (denotated by higher A_n in spring) (Flexas et al., 2001). During autumn, even if A_n was similar to spring, C_i was highest due to stomatal aperture (i.e., higher g_s). *Q. pubescens* is known to possess a highly efficient hydraulic system allowing to both maintain a high relative leaf water content and display relatively high Ψ_{md} (−3 MPa) and Ψ_{pd} (−1.6 MPa) in summer compared with sclerophyllous oak Mediterranean species (e.g., *Q. ilex* with $-4 > \Psi_{pd} > -6$ MPa; Limousin et al., 2022; Tognetti et al., 1998). These physiological traits indicate that *Q. pubescens* adapts to water scarcity through a drought-avoidance strategy (Nardini & Pitt, 1999).

Amplified drought led to lower water potentials but only in autumn, while Saunier et al. (2018) reported a decrease in summer as well. Rain exclusion was indeed weak in summer 2021 since precipitation was naturally scarce, thus limiting differences between the AD and ND plots. By contrast, precipitation was particularly important in autumn 2021 where 94% of precipitation was excluded in the AD plot the month before our sampling campaign, exacerbating the differences between the two plots. Stomatal conductance was not significantly limited by AD, although values tended to be lower under rain restriction (−23% in summer). Contrastingly, g_s strongly dropped in summer during the first and fourth year of rain exclusion in the same field site (Genard-Zielinski et al., 2018; Saunier et al., 2018). In the cited studies, June precipitation was higher than in 2021, which implies that the total rain exclusion in the AD plot allowed to exacerbate differences between the two plots. Amplified drought impacted A_n at any season with the main decrease occurring in summer (−42%) according to our hypothesis although such decrease was not associated with significant stomatal closure. Saunier et al. (2018) reported a higher drop in A_n during autumn (−85%) compared with summer (−60%) 2015, due to very low g_s. Along with the decline in A_n in summer 2021, the C_i was higher under AD. The change in C_i with drought is used as an indicator of stomatal or non-stomatal limitations to photosynthesis (Flexas, 2002). With increasing stress, C_i initially declines and later undergoes an increase as drought severity intensifies (Lawlor, 1995). In our study, when trees were exposed to higher stress level in summer ($\Psi_{md} < -3$ MPa), the low values of g_s (< 22 mmol H_2O m^{-2} sec^{-1} under AD) coupled with higher C_i indicate that non-stomatal limitations to photosynthesis were predominant at this level of stress as reported in several studies (Flexas, 2002; Marino et al., 2018; Medrano, 2002).

Physical traits

SLA was the highest in spring and lowest in summer, indicating a higher leaf density in summer and probably higher leaf thickness too as a mean to limit water loss during the driest season (Galmés et al., 2013; Wright et al., 2004). High SLA in spring is often observed since leaves are actively growing, possess a poor leaf density, and thus display high photosynthetic rates (Shipley et al., 2005; Szőllősi et al., 2010). Regarding the response of trees to AD, such seasonal decreases in SLA were of a lower magnitude during both, summer, and autumn, suggesting that the growth process is halted by harsher long-term drought conditions. Contrasting with our study, a previous study on *Q. pubescens* in the same field site did not report high SLA under AD after 4 years of rain exclusion (Saunier et al., 2018) although the authors only measured SLA in autumn. In agreement with our study, high SLA was reported under moderate (Ψ_{md} − 2.9 MPa) and severe (Ψ_{md} − 4.8 MPa) drought stress in a greenhouse study (4-year-old potted plants) (Genard-Zielinski et al., 2014). While low SLA under drought stress is regarded as a drought-adaptation strategy (Poorter et al., 2009), such response was not observed in *Q. pubescens* in this long-term rain exclusion experiment. Moreover, contrasting with our result, increases in both SLA and leaf surfaces have been associated with a resource-acquisition strategy where light capture and photosynthesis are maximized (Blanco-Sánchez et al., 2022; Griffin-Nolan et al., 2019; Pérez-Ramos et al., 2013). *Q. pubescens* did not show this pattern since leaves did not possess higher leaf surface under AD thus limiting A_n maximization.

Chemical traits

Seasonality was the main driver of leaf metabolome as previously shown for *Q. pubescens* (Saunier et al., 2018) and other Mediterranean species (Gori et al., 2019, 2020; Rivas-Ubach et al., 2014; Tixier et al., 2020). In our study, the metabolic profile differed among seasons with a strong investment in (i) photosynthetic pigments in spring, (ii) cuticular waxes in both summer and autumn, and (iii) tocochromanols, plastoquinones, and flavanols in autumn (Figure 4A).

The highest leaf photosynthetic pigment concentrations (chlorophylls and carotenoids) in spring are in agreement with the highest A_n during favorable climatic conditions with high soil water availability (Ψ_{pd} values −0.5 MPa; Epron & Dreyer, 1993) and average temperatures of 12°C (Epron & Dreyer, 1993; Gratani et al., 2000). Indeed, during spring, the main growing season, plants strongly invest in photosynthetic processes to maximize growth (Rivas-Ubach et al., 2012, 2014). In addition to photosynthetic pigments, a few specialized metabolites including the antioxidants phenolic acids and flavonols also accumulated in spring, as shown by Saunier et al. (2018) after 3 and 4 years of rain exclusion. This observation supports the protective role of flavonols and phenolic acids to cope with strong light irradiation at the beginning of the vegetative cycle and the associated oxidative stress (Ferreyra et al., 2021; Laoué et al., 2022; Roeber et al., 2021).

Despite the very marked effect of seasonality on leaf metabolome, some metabolite subgroups and single metabolites were sensitive to AD (Figure 4B). The high accumulation of PC-OH under AD indicates that trees subjected to rain exclusion displayed higher oxidative stress since this compound is specifically formed upon the action of the singlet oxygen ($^1O_2$), the main ROS in plants (Szymańska et al., 2014). Such accumulation was not reported after 4 years of AD (Figure S4). Carotenoids (neoxanthin and lutein) and PC-8 were also more accumulated under AD. These metabolites confer protection against drought-related oxidative stress since they act as ROS scavengers (Cazzaniga et al., 2016; Rastogi et al., 2014). Saunier et al. (2018) reported opposite results with a decrease in neoxanthin and lutein in 2015, after 4 years of AD. Also, carotenoid accumulation coupled with the increase in chlorophyll *b* and chlorophyll *a* under AD could allow to maintain efficient light harvesting and thus prevent a drastic decline in photosynthesis (Esteban et al., 2015; Kim et al., 2013) as the one observed in Saunier et al. (2018) (i.e., 60% in summer and 85% in autumn). According to our hypothesis, the higher accumulation of major photosynthetic pigments and chloroplastic antioxidants confirms that *Q. pubescens* is able to resist to long-term drought by preserving its photosynthetic apparatus as suggested by Gallé et al. (2007) for extreme drought in the same species. Contrasting with these carotenoids (neoxanthin and lutein), VAZ decreased under AD. Since the xanthophyl cycle is involved in dissipating excess of energy thus limiting ROS generation (García-Plazaola et al., 2008; Xu et al., 2020), a decrease in their concentration under AD indicates a loss of photoprotection which was already observed after 4 years of AD (Saunier et al., 2018). Zeaxanthin also functions as an antioxidant supplementing the action of tocopherol (Havaux et al., 2005; Havaux & García-Plazaola, 2014). The consumption of zeaxanthin and the decrease in VAZ could therefore indicate significant oxidative stress conditions in the oak leaves under AD.

Regarding phenolic compounds, a major group of plant-specialized metabolites, two flavonoids (myricitrin and quercetin galloyl glucose) and one phenolic acid (*p*-coumaric acid glycosylated derivative) were less stored under AD compared with ND. Such decrease could be a consequence of their antioxidant action under AD resulting in their oxidation and thus in their consumption

(Hernandez et al., 2006; Takahama, 2004). These metabolites possess the highest antioxidant potential followed by carotenoids and tocopherols (Catoni et al., 2008), indicating that trees under AD are likely to be more prone to oxidant pressure. These results contrast with the evolutionary concept proposed by Close and McArthur (2002) where the abundance of phenolic compounds is expected to increase as a mean to reduce the oxidative pressure through ROS quenching. Literature shows contrasting sensitivities of phenolic compound concentrations to water scarcity. Short-term water stress (over a few weeks), often tested under controlled conditions, causes either a significant increase (Fini et al., 2012; Nakabayashi et al., 2014; Nichols et al., 2015) or decrease in leaf phenolic concentrations (Król et al., 2014; Rahimi et al., 2018). Under long-term AD (over years) in the same field site, Saunier et al. (2022) also reported decreases in some phenolic compound concentrations after 3 and 4 years of rain exclusion. Contrastingly, Rivas-Ubach et al. (2014) mentioned that several phenolic compounds increased after 10 years of drought stress in *Q. ilex,* thus reinforcing its antioxidant system. Notwithstanding, Rivas-Ubach et al. (2014) also noted tree mortality after only 5 years of AD, while we did not observe mortality after 10 years since water stress is less pronounced in our experimental site. These contrasting results can be explained by the different climatic conditions between the two experimental sites. Our study was conducted in a humid/subhumid vegetation type (receiving annual mean precipitation of 830 mm), while the study on *Q. ilex* was conducted in a semi-arid/subhumid forest corresponding to a drier Mediterranean climate with an annual mean precipitation of 610 mm (Peñuelas et al., 2018) where trees are exposed to higher drought-related oxidative stress. Stimulation (in *Q. ilex*) or partial loss (in *Q. pubescens*) of the phenolic storage under water scarcity could also reflect a higher ability of *Q. ilex* to resist to future drought pressure in the Mediterranean basin as pointed out by Vuillemin (1982).

Regarding cuticular waxes, triterpenoids (the major cuticular waxes) exhibited the highest concentration in summer, but other subgroups (fatty acids, tocopherols, and alkanes) showed the highest concentrations during both summer and autumn. According to the model of Riederer and Schneider (1990) and Riederer and Schreiber (1995), cuticular waxes consist of ordered crystalline wax domains (long-chain aliphatics) and amorphous domains (more polar and cyclic waxes) in between. Recent advances indicate that cuticular triterpenoids do not directly act as a barrier for cuticular transpiration (Jetter & Riederer, 2016; Schuster et al., 2016), but rather prevent thermal damage by stabilizing the cuticle structure (Schuster et al., 2016; Tsubaki et al., 2013). They can contribute up to 60% of the total cuticular wax concentrations in some *Quercus* species (Bueno et al., 2020) and represent 37% of the cuticular fraction of *Q. pubescens* leaves. Triterpenoids could thereby confer protection against thermal stress during hot summer, thus contributing to leaf protection during concurrent periods of drought and thermal stress in *Q. pubescens*. Seasonal variation of leaf waxes has been described in other deciduous trees including *Fagus sylvatica* L. (Prasad & Giilz, 1990) and *Quercus robur* (Gülz & Müller, 1992), the latter displaying also higher cuticular wax accumulation in summer and autumn. From an ecological perspective, it is worth noting that adult individuals of *Q. robur* and sclerophyllous *Quercus* species (*Q. suber* and *Q. coccifera* L.) display leaf cuticular wax concentrations in the same range (35–200 μg cm^{-2}; Bueno et al., 2020; Gülz & Müller, 1992; Simões et al., 2022) as *Q. pubescens* (90 μg cm^{-2}), indicating that cuticular cover is not higher in *Quercus* sclerophyllous species, which possess thicker and harder leaves compared with broad-leaf deciduous *Quercus* species.

There was no clear impact of long-term drought on cuticular waxes since trees growing under AD exhibited higher concentrations of only three minor cuticular waxes (docosanol, docosanoic acid, and δ-tocopherol) compared with ND while octacosanal storage decreased. Unlike natural species, the effect of drought on cuticular wax concentrations has been widely studied on the model plant *Arabidopsis thaliana* (Aharoni et al., 2004; Kosma et al., 2009; Yang et al., 2011) and on crop species for which an increase in wax content is associated with water shortage and enhancement of drought tolerance (for a review see Xue et al., 2017). For natural species, the study of leaf cuticular wax concentrations under different drought conditions has only been documented for *Pinus pinaster* under greenhouse conditions (Le Provost et al., 2013) and in leaf litter of *Q. coccifera* in the field (Ormeño, Ruffault, et al., 2020; Ormeño, Viros, et al., 2020). Unlike our study, these two latter studies revealed an increase in cuticular wax concentrations with rising water deficit. Despite *Q. pubescens* did not exhibit overaccumulation of cuticular wax concentrations under rain exclusion in our study, the fact that the major cuticular waxes (triterpenoids) exhibited the highest concentrations in summer still supports the importance of triterpenoids to stabilize the cuticle during thermal stress in summer (Jetter & Riederer, 2016; Schuster et al., 2016; Shepherd & Wynne Griffiths, 2006). Besides cuticle-based strategies, plants possess many other adaptations to limit water loss (Jenks et al., 2007). For example, *Q. pubescens* features a deep root system, which allows to maintain hydraulic conductivity, leaf water content, relatively high assimilation rates, and stomatal conductance (Damesin & Rambal, 1995; Genard-Zielinski et al., 2014; Nardini & Pitt, 1999; Struve et al., 2009).

Autumn leaves exhibited higher concentrations of several antioxidants such as plastoquinones, tocochromanols, and flavanols. In addition to their role as photosynthetic electron carriers, plastoquinones also play important functions in various processes, such as light acclimation, and act as potent antioxidants by scavenging ROS (Havaux, 2020; Ksas et al., 2018). Plastoquinones, which were strongly accumulated in autumn, can scavenge superoxide and hydrogen peroxide (Khorobrykh & Tyystjärvi, 2018). The accumulation of plastoquinones and three other antioxidants (PC-8, α-tocopherol, and the flavanol catechin) suggests a strong antioxidant activity to both limit oxidative stress still present in autumn and maintain protection of the photosynthetic apparatus in spite of the decline in photosynthetic pigment concentrations (Bernatoniene & Kopustinskiene, 2018; Kumar et al., 2020; Muñoz & Munné-Bosch, 2019).

To conclude, our study provides information about the response of physiological and physico-chemical traits of *Q. pubescens* across seasons and under a long-term rain exclusion simulating future drought in the Mediterranean region. *Q. pubescens* leaf chemical traits, especially tackled in this work, were mainly driven by the seasonality with the highest investment in i) photosynthetic pigments in spring, ii) cuticular wax compounds in both summer and autumn, and iii) tocochromanols, plastoquinones, and flavanols in autumn. *Q. pubescens* also displayed some metabolomic adjustments in response to AD consisting of higher concentrations of pigments and chloroplastic antioxidants (PC-8), which could allow to preserve the photosynthetic apparatus from oxidative stress during drought stress. However, AD also led to lower concentrations of antioxidant defenses (i.e., flavonoids and phenolic acids) as well as a loss of photoprotection own to the decrease in xanthophyll cycle-related pigments. Although cuticular wax concentrations did not clearly change over long-term rain exclusion, the high concentration of triterpenoids in summer under both ND and AD confirms that these metabolites confer thermal protection of the leaf cuticle. Further research should be conducted to evaluate whether leaf physical traits, such as stomatal and trichome density, and anatomical features, could contribute to drought resistance in *Q. pubescens*.

EXPERIMENTAL PROCEDURES

Experimental site and species

This study was conducted at O_3HP (Oak Observatory at the 'Observatoire de Haute Provence'), a long-term experimental *in natura* forest site belonging to both AnaEE-France (Analysis and Experimentation on Ecosystems) and AnaEE-ERIC (European Research Infrastructure Consortium). The site is located at Saint-Michel-l-'Observatoire at 60 km north of Marseille in southern France (5°42′44″ E, 43°55′54″ N) at an elevation of 650 m above sea level. The study site is characterized by a supra-Mediterranean humid bioclimate with an average annual precipitation of 830 mm and annual mean temperature of 11.9°C for the period 1967–2000. The forest is mainly composed of *Q. pubescens,* which represents approximately 90% of the biomass, followed by approximately 10% of *Acer monspessulanum* L. trees.

The implementation of a rainfall exclusion system at O_3HP since April 2012 allows to simulate future climate predictions in the Mediterranean region by reducing precipitation and increasing the length of the dry season in an AD plot of 300 m^2, which is compared with an adjacent plot (300 m^2) where trees grow under natural drought (ND) (Figure S5). The exclusion system runs from April–May to September–October following a rainfall prediction curve for 2100 with most exclusion between the beginning of July and the end of September. Three measurement campaigns were carried out in 2021: spring (May 17–19), summer (July 12–14), and autumn (October 11–13). During the year 2021, annual mean temperature was 12.3°C; Figure 1D) and it was a naturally dry year with 4 dry months (see Ombrothermic diagram Figure 1A) and an annual precipitation of 729 mm in the ND (subhumid bioclimate; Thompson, 2020). As a whole, a total of 41% of precipitation was excluded in the AD plot (thus reaching 427 mm) simulating a semi-arid bioclimate and extending the dry period from 4 to 5 months in the AD plot (Figure 1A). When focusing on the previous 30 days preceding each campaign (following the approach of Sytiuk et al., 2023), it can be checked that the major rain exclusion occurred in autumn (with 94% of precipitation excluded), followed by spring (−86%) and summer (−36%) since summer 2021 was naturally very dry (19 mm), and so rain exclusion could not be further amplified (Figure 1C). By contrast, spring and autumn were relatively wet with a mean precipitation of 150 and 144 mm, respectively (Figure 1C).

Chemical, physical, and physiological traits were assessed on 14 Downy oak trees (seven under each drought condition) over the seasonal cycle (Figure S5). Leaves samples came from the top of the canopy. For all chemical analyses (see below), leaf samples were collected around midday and immediately frozen in liquid nitrogen then stored at −80°C.

Physiological and physical traits

Gas exchanges were measured on three leaves from a sun-exposed branch at the top of the canopy of each tree using an open-system gas analyzer for high-precision detection of CO_2 and H_2O gasses (CIRAS-3, PP Systems, Amesbury, MA, USA). The reference CO_2 concentration was set to 400 μmol mol^{-1}, temperature of the leaf chamber was set to 27°C, and photosynthetically active radiation (PAR) was set to 1000 μmol m^{-2} sec^{-1}. Traits measured were the net CO_2 assimilation (A_n, μmol CO_2 m^{-2} sec^{-1}), stomatal conductance (g_s, mmol H_2O m^{-2} sec^{-1}), internal CO_2 concentration (C_i, μmol mol^{-1}), and WUE (mmol CO_2 mol^{-1} H_2O). Stem water potential was measured using stems containing three to five leaves. Both water potential at midday (Ψ_{md}) and predawn (Ψ_{pd}) were measured with a Scholander pressure chamber (PMS Instrument Co. USA; range 0–7 MPa). Regarding morphological traits, we focused on the SLA (cm^2 g^{-1}) calculated as leaf size (or leaf area) per unit dry mass, and leaf size which was calculated by scanning 10–15 leaves by tree and using ImageJ software to calculate the area in cm^{-2}.

Chemical traits

Leaf powder was obtained by grinding 5–10 leaves in liquid nitrogen. Photosynthetic pigments were extracted from leaf powder (20 to 50 mg) using 2 ml of methanol and were quantified by high-performance liquid chromatography (HPLC). Chlorophylls and several carotenoids were analyzed, including those from the xanthophyll cycle (violaxanthin + antheraxanthin + zeaxanthin, hereafter, also referred as VAZ), neoxanthin, lutein, and β-carotene. Their concentrations (μg $g_{DM}{}^{-1}$) were calculated from calibration curves performed using carotenoid pigment standards (Extrasynthèse, France) and chlorophyll *a* and *b* standards (-Sigma-Aldrich, USA). More details of the method are given in Havaux et al. (2007).

Plastoquinones (PQ) and tocochromanols, including α-tocopherol, plastochromanol-8 (PC-8), and hydroxy-plastochromanol (PC-OH), were extracted from leaf powder (20 to 50 mg) mixed in 2 mL of ethyl acetate. After centrifugation, the supernatant was filtered and evaporated on ice under a stream of N_2. The residue was recovered in methanol/hexane (17:1 vol) and analyzed by HPLC (see Ksas et al., 2015, 2018) with UV absorbance and fluorescence detection. The column was a Macherey-Nagel Nucleosil 100–5 C18. PC-8 was detected by fluorescence at 330 nm with an excitation at 290 nm. Plastoquinones consisted of the pool of reduced and oxidized forms of Plastoquinone-9 and were referred to as plastoquinones (PQ) in the present study. Quantification was achieved using PQ and PC-8 standards, which were a kind gift from Dr. J. Kruk (Krakow, Poland) and α-tocopherol standard was purchased from Sigma.

Phenolic compounds were extracted using 10 mg (DM) (from 5–10 leaves, previously lyophilized and frozen-ground) and 1 mL of methanol containing 1% formic acid. The extract was homogenized for 5 min in an ultrasonic bath and centrifuged at 12000 tr min-1 for 5 min. Analyses were performed with an Acquity UPLC-DAD-ESI-TQD system (Waters, USA). UPLC separation was carried out using a C18 BEH column (2.1 mm × 150 mm, 1.7 μm, Waters, USA). Elution rate was set to 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. Photodiode array was set from 190 to 600 nm, and phenolic compounds' wavelength detection was chosen depending on compound characteristic spectra (350 nm, 320 nm, or 280 nm). Their identity or structure was confirmed with the 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 desolvatation temperature at 400°C. External quantification with mono glycosylated flavonols (quercetin and myricitrin) and hydroxycinnamic acid standards (caffeoyl acid, chlorogenic acid, and coumaric acid) was applied.

Cuticular waxes were extracted by immersing precisely defined leaf circles (9.54 cm^2) obtained from three to five leaves into 3 ml of cyclohexane and chloroform (70:30 vol) under constant shaking for 2 min using a vortex. Cuticular wax removal was checked through scanning electron microscopy (Figure S6). Then, 2.5 ml of the extract was recovered. Tetracosane D50 (C24; 98%; Sigma-Aldrich) was added as an internal standard, and the total solvent was evaporated. Those cuticular wax extractions were then resuspended into 250 μl of acetonitrile and derivatized with 20 μl of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich) and 20 μl of pyridine (Sigma-Aldrich) to improve the separation of metabolites. The extract was filtered with a PTFE syringe filter (0.2 μm, 30 mm diameter). To allow derivatization, the mixture was heated for 30 min at 70°C. Each derivatized sample was analyzed by injecting 1 μl in an Agilent 7890 gas-chromatography/mass-spectrometry (GC–MS) equipped with an ALS7693 automatic injector, a MSD5977A Network mass detector (70 eV), and an HP5-MS apolar column (30 m × 0.25 mm × 0.25 μm; J&W Agilent Technologies). Cuticular waxes were identified through the Agilent ChemStation software by comparing their mass spectra, retention times, and Kovats retention index with libraries (NIST 2020 and ADAMS 2008), published data, and experimental data for some available standards (alkanes C8-40, lupeol, α-amyrin, β-amyrin, α-tocopherol, and δ-tocopherol). Identified compounds were quantified against the internal standard (tetracosane D50). Results were expressed by dry matter (μg $g_{DM}{}^{-1}$) by oven-drying leaf circles (used for extractions) at 65°C for 48 h.

Statistical analysis

For analysis of chemical traits, we first performed a PCA followed by a two-way PERMANOVA to assess whether chemical traits were modulated by seasonality and drought. Then, we used a two-way ANOVA analysis followed by post hoc tests to highlight whether leaf metabolites responded differentially according to seasons and drought conditions. All analyses were performed using groups and subgroups although detailed results on single metabolites are shown in Supporting Information.

For assessing adjustments of morphological and physiological traits across seasons and drought conditions, we performed two-way ANOVAs followed by post hoc Tukey tests. When significant interactions occurred between seasonality and drought, we performed a one-way ANOVA followed by Tukey tests to evaluate the effect of seasons on ND and AD separately and Student tests to evaluate differences between drought conditions (ND and AD) within each season. When interaction between seasonality and drought was not significant, differences between seasons were evaluated with drought conditions pooled and the drought effects were shown separately with all seasons pooled.

Before analyses, normality and homoscedasticity were checked and data were log-transformed if necessary. All data analyses were performed using R v.4.0.3 (R Core Team, 2020) using car, agricolae, and multcomp packages for two-way ANOVA analysis, Tukey, and Student tests. The PCA was performed using FactoMineR and factoextra packages (Husson et al., 2010; Kassambara, 2020) and the two-way PERMANOVA using vegan (Oksanen et al., 2013) with 9999 permutations.

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AUTHOR CONTRIBUTIONS

EO and CF conceived the study with input from JL. JL performed fieldwork and collected the data with the help of EO. JL did the cuticular wax quantification with the help of CL. MH and BK did the PQ, PC, and pigment quantification. JL and EO analyzed the data and wrote the manuscript with input and advice from BT, CF, CL, MH, and BK.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Correlation between stomatal conductance (g_s) and net CO_2 assimilation (A_n) with data from all seasons and drought conditions.

Figure S2. Biosynthetic pathways of all metabolites targeted in the study.

Figure S3. Seasonal variation of cuticular waxes (divided into subgroups) of *Q. pubescens* leaves with drought conditions pooled.

Figure S4. Concentration of plastochromanol-OH (PC-OH) in summer 2015.

Figure S5 Experimental site and sampling plan.

Figure S6. Scanning electron microscopy (SEM) images of the leaf abaxial surface of *Q. pubescens*.

Table S1. Results of two-way ANOVA performed on metabolite group concentrations ($\mu g\ g^{-1}{}_{DM}$) according to season and drought conditions (n = 7).

Table S2. Results of two-way ANOVA performed on single metabolite concentrations ($\mu g\ g^{-1}{}_{DM}$) according to season and drought conditions (n = 7).

Captions.

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