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RESEARCH ARTICLE

The interplay between drought and phosphorus scarcity shapes resilience to stress in *Quercus* spp. by modulating metabolomic profiles

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Abstract

Background and aims Plant resilience to drought conditions is modulated by physiological and metabolic responses, including the accumulation of antioxidant metabolites. These mechanisms can be in influenced by nutrient availability, especially phosphorus (P). In European mixed forests, native species with high resource demands often compete with more efficient alien species, whose advantages under abiotic stress can enhance their invasiveness. This study investigates how drought and P availability affect the resilience of two *Quercus* species differing in stress tolerance and resource use efficiency.

Methods We conducted a mesocosm experiment in which the native *Q. robur* (English oak, EO) and alien *Q. rubra* (Red oak, RO) were grown under well-watered or drought conditions, with or without P supplementation. Leaf gas exchange and stem water potential were measured, leaves and soil were chemically characterized, and metabolite biosynthesis was investigated using an omics approach.

Results During drought, the metabolism of P-supplied EO shifted toward the accumulation of phenylpropanoids, flavonoids, saikosaponins, mannitol, and long-chain fatty acids, which are compounds known for their antioxidant, osmoprotectant, and membrane-stabilizing functions. Conversely, RO plants displayed a more conservative metabolic profile, with limited changes in response to P supply. Under P deficiency, RO accumulated secondary metabolites such as phenylpropanoids and alkaloids, highlighting its ability to withstand combined nutrient and water stress.

Conclusions Our results reveal contrasting adaptive strategies: EO is more susceptible to drought stress, but its resilience can be modulated by P availability, whereas RO maintains more stable physiological and metabolic functions, consistent with higher nutrient-use efficiency.

Keywords Climate change · Water stress · Nutrient deficiency · Oak · Metabolism · Resilience

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Introduction

Ecosystems increasingly face more frequent and prolonged extreme conditions due to climate change

(IPCC 2023). Since drought is one of the leading environmental stress factors, understanding how plants respond to water deficit conditions is crucial to predict future patterns of plant redistribution (Dyderski et al. 2018). Under water deficit conditions, plants reduce stomatal conductance (gs) and leaf transpiration (E) to limit the negative effects of soil dryness (Whitehead and Beadle 2004). Osmotic adjustment is one of the plant strategies that help sustain gas exchanges and maintain stomata open as the soil becomes drier (Dichio et al. 2003). The modulation of leaf gas exchange under dry soil conditions reduces net photosynthesis (An), leading to excessive production of reactive oxygen species (ROS), which cause oxidative damage to proteins, DNA and lipids (Cruz De Carvalho 2008; Tariq et al. 2017).

The shift in metabolomic processes regulates the plant response to water deficit, including leaf gas exchange and cell turgor, which are mainly affected by primary metabolites, as well as secondary metabolites accumulation. During drought, primary metabolites such as sugars (e.g., sucrose, glucose, fructose, raffinose, and mannitol) can osmoregulate cells and contribute to synthesizing antioxidants, such as ascorbic acid (Rodríguez-Sánchez et al. 2010; Raza et al. 2025). When the risk of photodamage and the impairment of leaf photochemistry arises, increased levels of amino acids (e.g., proline or glutamine) can stabilize the cellular membranes and mitigate the negative effects of ROS accumulation (Ain-Lhout et al. 2001; Hu et al. 2015). Increasing metabolites with antioxidant and ROS-scavenging activities is a common plant strategy to limit oxidative damage and improve cell membrane stability (Sobrino-Plata et al. 2024). Many secondary metabolites act as osmoprotectants, reducing water loss and protecting the photosynthetic apparatus from damage (Escandón et al. 2021). To preserve green leaf tissues, plants accumulate phenolic compounds (e.g., flavonoids) and terpenoids, which act as antioxidants and ROS scavengers. Plants can also improve their tolerance to drought by increasing tannin levels as soil water content declines, especially under less favorable conditions (Top et al. 2017; Arab et al. 2020).

Osmotic adjustment, secondary metabolite accumulation and antioxidant mechanisms in plants can be influenced by nutrient availability (Yang et al. 2018). According to Kleiner et al. (1992), fertilized chestnut oaks exhibit greater osmotic adjustment than those growing in nutrient-poor soils, while nitrogen (N) and phosphorus (P) deficiencies promote the accumulation of flavonoids (Stewart et al. 2001). Phosphorus, a key component of molecules such as nucleic acids, phospholipids and ATP, is a macronutrient in plant metabolism (Amtmann and Blatt 2009). Phosphorus deficiency inhibits photosynthesis, respiration, and the uptake and assimilation of nitrate in plants (Pilbeam et al. 1993). According to the literature, P stress promotes the accumulation of organic acids and phenolics (e.g., flavonoids), but it can reduce the levels of most sugars (Hermans et al. 2006; Liu et al. 2016; Zhang et al. 2021). Conversely, Tariq et al. (2018) reported that *Alnus cremastogyne* Burkill seedlings exposed to high P availability under drought conditions showed improved photosynthetic rates and enhanced antioxidant defense, with reduced lipid peroxidation. The activity of antioxidant enzymes such as superoxide dismutase, catalase and peroxidase, along with the accumulation of osmolytes and soluble proteins, can be improved by P input. However, drought events may cause nutrient deficiencies even in plants with high soil nutrient availability, as nutrient uptake declines with decreasing soil moisture. In the soil matrix, the phosphate ion primarily moves by diffusion. Thus, dry conditions limit its movement toward the root surface (da Silva et al. 2011).

The availability of nutrients and water regulates plant growth, species distribution, and competition within ecosystems, ultimately influencing forest composition. Introducing alien species can further affect native biodiversity and biogeochemical processes (Angeloni et al. 2006). In Europe, the Northern American species *Quercus rubra* L. (Red Oak, RO) was introduced by humans at the end of the seventeenth century as an ornamental and valuable timber tree (Nicolescu et al. 2020). Due to its remarkable capacity for natural regeneration, RO is considered potentially invasive, or even fully invasive, in some European countries. In northern Italy, this alien species has colonized much of the native vegetation in the Po River plain (*Querco-carpinetum* cenosis), a forest predominantly composed by *Quercus robur* L. (English Oak, EO) and *Carpinus betulus* L. (Terzuolo et al. 2006). The introduction of RO in the mixed broadleaved forests of Northwestern Italy can change the soil characteristics, affecting the bio-cycling of nutrients, especially the

availability of P. In well-developed soils characterized by low P availability, introducing RO could further slowdown P turnover, potentially driving the ecosystem towards a no-return threshold for restoring the original forest composition (Bonifacio et al. 2015). Since RO is more effective in dominating nutrient-poor soils and dry conditions (Major et al. 2013; Lavnyy and Savchyn 2016) than EO, drought periods and nutrient deficiency may exacerbate competitive interactions between the two species.

Rolando et al. (2025) observed that not only EO was less tolerant to drought, but it also exhibited a slower recovery from repeated severe drought events compared to RO, with its vulnerability being particularly pronounced in nutrient-limited environments. Their findings further indicate that P supplementation mitigates the adverse effects of drought on EO, improving plant recovery.

In this context, the aim of this study is to investigate the metabolomic responses of *Quercus robur* (English Oak, EO) and *Quercus rubra* (Red Oak, RO) to the combined drought and P scarcity stress factors. Specifically, we hypothesize that i) the P supply enhances the gas exchange rates of EO, with limited effect on RO, due to interspecific differences in resource use efficiency, therefore ii) EO will exhibit better drought tolerance when soil P availability is increased, as higher soil P levels may enhance photosynthetic rates and antioxidant defense mechanisms, which help mitigate oxidative damage; iii) in contrast, the RO response to water scarcity will be not significantly influenced by nutrient availability, since the alien species has inherently low water and nutrient demands and is able to adopt adaptive strategies to cope with low soil fertility and water availability, iv) consequently, increasing P availability may improve the competitive strength of the native EO in relation to RO. To test these hypotheses, we conducted a mesocosm experiment in which seedlings of both EO and RO were transplanted into nutrient-poor soil collected from an area predominantly colonized by RO. To simulate different environmental conditions, we manipulated soil fertility by adding P and applied two distinct water management regimes to assess the effects of varying water availability and nutrient input on plant growth and metabolism.

Materials and methods

Plant and soil material

The experiment was conducted in a greenhouse at the Department of Agricultural, Forest and Food Science (DISAFA) of the University of Turin, Italy. In December 2021, two-year-old *Quercus rubra* L. (RO) and *Quercus robur* L. (EO) seedlings provided by a nursery were transplanted into 3.4 L cylindrical pots (28 cm height $\times$12.5 cm diameter). A total of 40 plants (20 RO and 20 EO) were kept under partially controlled conditions until the beginning of the experiment.

The soil used in the experiment (0–20 cm layer) was collected from La Mandria Natural Park in Northwestern Italy (N 45.153213, E 7.581204), an area currently dominated by RO but formerly covered by EO and *Carpinus betulus* L.. The soil was classified as Oxyaquic Fragiudalfs (USDA, Soil Taxonomy) with the following initial characteristics: pH 4.4, organic carbon 29 g kg^{-1}, total nitrogen (N) 2.2 g kg^{-1}, available P 5.2 mg kg^{-1} (P_{av}), 4.2 mg $N\text{-}NO_3^-$ kg^{-1}, and 36 mg $N\text{-}NH_4^+$ kg^{-1}. During the experiment, plants were maintained in a glass greenhouse under partially controlled climatic conditions, with an average air temperature of 25.4 $\pm$4.8 °C and relative humidity of 67.7 $\pm$14.6%.

Experimental design and treatments

Nutrient inputs consisted of two conditions: one representing the original nutrient-poor soil (-P), and another where plants received 39.3 mg P kg^{-1} provided as KH_2PO_4 (+ P), on May 18 th 2023 (Day of year—DOY- 138). Twenty-five days after nutrient inputs, 10 EO and 10 RO plants were regularly well-watered (denoted as WW), while the remaining 20 plants were subjected to water deficit conditions (denoted as D), followed by rewatering (RW). The experimental design followed a split-plot layout with five replications (R). The main plots consisted of the two plant species (SP, EO and RO), the subplots included two water management regimes (WM), and the sub-subplots involved two nutrient input levels (NI). The WW condition was maintained through daily irrigation, ensuring the field capacity. At the same time, 70% of the water lost from the previous day was restored in D plants until DOY 178 (15

days of drought). The D plants were then rewatered to reach and keep the field capacity of approximately 80% until DOY 193 (15 days of rewatering). All pots were weighed daily to adjust the WM accordingly.

Soil analysis

Soil samples were collected on DOYs 163 and 178 to assess variations in total carbon (TC), total nitrogen (TN), and available P (P_{av}) following the drought period. Only two soil samplings were carried out during the plant growth to minimize disturbance of the rhizosphere during the water stress phase. Samples were dried and sieved at 2.0 mm. A portion was ground to 0.5 mm for TC and TN analysis using high-temperature combustion in an elemental analyzer (Vario Isotope Select; Elementar Analysensysteme GmbH, Hanau, Germany). Available P was quantified according to Olsen et al. (1954). The extracted P was then measured colorimetrically using the malachite green method (Ohno and Zibilske 1991).

Measurement of leaf gas exchange and stem water potential

Leaf gas exchanges (gs, An and E) were measured on fully expanded leaves exposed to direct sunlight using a portable infrared gas analyzer (ADC-LCPro + system, The Analytical Development Company Ltd, Hoddesdon, UK). CO_2 levels were maintained at greenhouse conditions (400–450 ppm). Leaf gas exchange was monitored every two days in the morning (between 10:00 am and 12:30 pm) on three to five plants in each treatment (one leaf per plant) for the whole duration of the experimental trial. The Ψ_{stem} was measured on leaves collected before imposing water stress conditions (DOY 163), after 4 (DOY 167) and 11 (DOY 174) days of drought, at the end of the water scarcity phase (DOY 178), and after 15 days of rewatering (DOY 193).

Leaves were placed in humidified, aluminum foil-wrapped plastic bags for about 15 min prior to excision. After excision, the leaves were allowed to equilibrate for an additional 10 min before the water potential was measured using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). On DOY 178, the relative chlorophyll content (SPAD values) was measured with a SPAD meter (SPAD 502 Plus Chlorophyll Meter, Spectrum, Plainfield, IL, USA) to assess chlorophyll levels after drought.

The Performance Index (PI) parameter was measured in each plant using a continuous excitation Pocket PEA fluorometer equipped with black leaf clips (Hansatech Instruments Ltd, King's Lynn, UK). Leaves were dark-adapted for at least 30 min before measurement.

Chemical characterization of leaves

Leaves collected on DOYs 163 (watering initial phase), 167, 174, 178 (drought) and 193 (rewatering) were dried and grounded to 0.5 mm. Carbon (C) and Nitrogen (N) contents were determined by high-temperature combustion using an elemental analyzer (Vario Isotope Select; Elementar Analysensysteme GmbH, Hanau, Germany). For P analysis, samples were digested with 12.5 mL of H_2SO_4 and 2.5 mL of $HClO_4$, and quantified colorimetrically using the malachite green method (Ohno and Zibilske 1991).

Metabolomics analysis of leaves

The metabolomics analysis was conducted only on DOY 178 plants, at which time leaves were collected, frozen in liquid nitrogen and stored at −20ºC. Lyophilized plant tissue (100 mg) was dissolved in 1 mL of 80% methanol (v/v) acidified with 0.1% formic acid (v/v) and mechanically extracted using a Polytron PT 1200E homogenizer for 3 min at maximum power. The extracts were centrifuged at 5000 g for 15 min and filtered through a 0.22 µm cellulose membrane filter into vials for liquid chromatographic analysis. Untargeted profiling of samples was performed using the 6560-drift tube-ion mobility-quadrupole-time of flight-high resolution mass spectrometer (DTIM-UHPLC-QTOF-HRMS; Agilent Technologies, Santa Clara, CA, USA). The chromatographic separation was achieved under a water-acetonitrile (both LC–MS grade, from Sigma-Aldrich, Milan, Italy) gradient elution (6–94% acetonitrile in 32 min), flow rate of 0.2 mL/min and injection volume of 6 µL, using 0.1% (v/v) formic acid as phase modifier on an -Agilent Poroshell 120 PFP column (100 mm ×2.1 i.d., 1.9 µm particle size) -Agilent Zorbax Eclipse plus C18 analytical column (50 ×2.1 mm, 1.8 µm). The QTOF mass analyzer operated in positive mode (ESI +) for both MS and MS/MS acquisition with nitrogen

as both sheath gas (12 L/min and 315 ◦C) and drying gas (14 L/min and 250 ◦C). The nebulizer pressure was 45 psi, nozzle voltage was 350 V, and the capillary voltage was 4.0 kV. For MS acquisition, the full scan mode was performed within range of the m/z 100–1200 (1 spectra/s), mass resolution of 30,000 full width at half maximum (FWHM), m/z = 200. The data-dependent mode was performed for precursor fragmentation (10, 20, and 40 eV) and acquisition of MS/MS data from QC samples, with a mass resolution of 30,000 (FWHM), selecting 8 precursors per cycle (1 Hz, m/z 80–1200, positive polarity, and active exclusion after 2 spectra). The Processing of the chromatograms was carried out using the MassHunter Qualitative Analysis software (version B.06.00, Agilent Technologies). The collected data (.d files) were processed using MS-DIAL software (version 4.70) for automatic peak finding, LOWESS normalization, and annotation via spectral matching, exploiting the comprehensive BMDMS-NP, Fiehn/Vaniya natural product library, and GNPS databases. The mass range 100–1200 m/z was searched for features with a minimum peak height of 10,000 cps, using an accurate mass tolerance for peak centroiding of 0.05 and 0.1 Da, for MS and MS/MS, respectively. Retention time information was excluded from the calculation of the total score. The identification step was based on mass accuracy, isotopic pattern, and spectral matching. These criteria were used to calculate a total identification score, using a minimum cut-off value of 70%, considering the most common HESI + adducts.

The data annotation and MS/MS structural confirmations were conducted using MS-DIAL software—version 4.90—(Tsugawa et al. 2015) for automated peak finding (against pooled QC) and putative annotation via spectral matching using the publicly available databases (e.g., BMDMS-NP, Fiehn/Vaniya natural product library, and GNPS). Compound annotation was carried out considering mass accuracy, isotopic profiles, and MS/MS spectral matching (where available), using a total identification score with a minimum cut-off of 70% to reach level 2 of confidence in identification (Blaženović et al. 2019). The mass range of 100–1200 m/z with a minimum peak height of 10,000 cps was considered, using accurate mass tolerances of 0.05 Da for MS and 0.1 Da for MS/MS.

Data collection and statistical analysis

Plant physiological data were recorded every two days from June 11 (163 DOY) to July 11 (DOY 193), 2023, to evaluate the status of WW, D and RW plants. The watering conditions before imposing drought (DOY 163), two representative time points during water stress (DOYs 167 and 174), the end of drought (DOY 178), and rewatering (DOY 193) were chosen to determine leaf gas exchanges, Ψ_{stem} and chemical characterization of leaves (C, N, P).

All the metabolomics (DOY 178) and soil (DOYs 163 and 178) data were statistically analyzed. For variables measured at multiple time points, the time (T) factor was incorporated into the analysis.

All the statistical analyses were conducted using R 4.3.1 (R Code Team, 2022).

The statistical analysis was organized into three distinct phases: baseline measurements (DOY 163), drought conditions (DOYs 167, 174, 178), and rewatering (DOY 193). First, a two–way variance (ANOVA) analysis with a linear model was performed for each physiological parameter and leaf chemical element recorded at DOY163, using the *stats* package (R Core Team 2023). The same statistical approach was applied to soil results, separating DOY 163 and DOY 178. During the drought phase, a four-way ANOVA with a mixed effect model was performed on each physiological parameter and leaf chemical element measured using the *nlme* package (Pinheiro et al. 2007). The fixed factors in this model were SP, WM, NI and T, while replicates (R) – represented by 3 out of 5 replicates – were treated as a random factor.

For the rewatering phase, a three-way ANOVA with a linear model was conducted on each parameter recorded on DOY 193 and for SPAD and performance index measurements recorded on DOY 178, again employing the *stats* package (R Core Team 2023).

The distribution of measured variables was checked for normality and homogeneity of variance using the Shapiro–Wilk and Levene's tests, respectively. When ANOVA assumptions were violated, data were log-transformed, and the tests were repeated on the transformed data. The post–hoc test performed after each ANOVA was the Tukey's honestly significant difference (HSD) at a significance

level of *p-value* < 0.05, with the *multcomp* packages (Hothorn et al. 2008).

Afterwards, a multivariate analysis of variance (MANOVA) was conducted to assess the influence of SP, WM and NI on leaf gas exchange parameters and Ψ_{stem} measured at the end of water stress (DOY 178) and rewatering (DOY 193). The same analysis was conducted on chemical data from leaves sampled on DOYs 178 and 193.

Finally, the apparent P recovery (APR) of EO and RO leaves was calculated as follows:

$$APR\% = \frac{\left(P\ pool_{+P\ DOY_x}\right) - \left(\overline{P\ pool}_{-P\ DOY_x}\right)}{Input\ P} * 100$$

where $P\ pool_{-N+P\ DOY_x}$ was the content of P in the total leaf biomass of +P plants (mg plant^{-1}), $\overline{P\ pool}_{-N-P\ DOY_x}$ was the average P content in the total leaf biomass of -P plants (mg plant^{-1}), and Input P was the amount of inorganic P supplied to each plant (120 mg P plant^{-1}).

The metabolomics data were analyzed using the software Mass Profiler Professional 12.6 (Agilent Technologies). The raw data were log2 transformed, 75 th percentile normalized, and baselined against the median of each compound. Sample patterns were investigated using unsupervised hierarchical cluster analysis (HCA) with Euclidean distance and Ward's linkage rule. Furthermore, a supervised ANOVA multi-block orthogonal partial least squares (AMOPLS) was carried out using the package rAMOPLS on R (v 4.2.1.) to select Variable Importance in Projection (VIP^2) markers associated with different treatments. The AMOPLS statistical significance was set at $\alpha = 0.01$ and validated with 100 permutation processes. The results were expressed as the Relative Sum of Squares (RSS), representing the percentage of variability attributed to each factor; RSS p-value, statistical significance; and block contribution, in percentage, associated with each effect. Afterwards, the VIP markers associated with different factors were considered to build the pathways analysis, comparing [WW +P] *vs.* [WW -P], [D -P] *vs.* [WW -P], and [D +P] *vs.* [WW -P], separating for EO and RO species. The metabolite changes were defined using log Fold Change analysis comparing all the treatments with control well-watered and under phosphorus deficiency.

Results

Soil

On DOY 163 (watering phase), the soil total C (TC) under EO -P (22.71 ±2.71 g kg^{-1}) was similar to that of RO -P soil (23.39 ±2.95 g kg^{-1}) (Fig. 1a). However, with P input, TC differed between EO (25.03 ±4.07 g kg^{-1}) and RO (22.74 ±2.57 g kg^{-1}) with a remarkable SP*NI interaction effect on DOY 163. Conversely, drought nullified TC differences between EO +P and RO +P, though notable differences were still observed between soils under different species and low nutrient availability. Even in the absence of statistical significance, a decreasing trend in soil TC under WW -P appeared to emerge for RO, decreasing from DOY 163 (23.39 ±2.95 g kg^{-1}) to DOY 178 (19.28 ±1.96 g kg^{-1}). Conversely, under -P conditions, the drought event led to an increase in soil TC (24.68 ±3.08 g kg^{-1}) compared to WW at DOY 178.

The SP and NI did not impact the soil TN during the watering phase, but nutrient content in the soil was affected by the interaction of SP*WM*NI during drought. The main difference was observed between EO D -P (1.72 ±0.18 g kg^{-1}) and RO D -P (2.00 ±0.19 g kg^{-1}), while P addition suppressed the differences observed between SP in D plants (Fig. 1b). On DOY 178, as reported for soil TC, the TN content in RO D -P was higher than in RO WW -P soil (1.64 ±0.11 g kg^{-1}).

The available P pool was below 5 mg kg^{-1} in -P soils, independently of WM and T. Nutrient availability increased with the addition of P, ranging between 28.58 ±13.44 mg kg^{-1} in RO WW to 43.10 ±10.96 mg kg^{-1} in EO D. In fact, ANOVA highlighted a significant effect of NI at both DOY 163 and 178 (Fig. 1c).

Physiological parameters

During the initial watering phase, the Ψ_{stem} values of EO -P and EO +P were −0.40± 0.07 MPa and −0.38± 0.10 MPa, respectively (Fig. 2a). Similar values were measured for RO -P and RO +P without statistical differences on DOY 163. Drought reduced the Ψ_{stem} in both species, up to −2.5 MPa. After 15 days of rewatering, Ψ_{stem} of D plants was comparable to that of WW. However, ANOVA analysis highlighted a remarkable effect of SP and NI, revealing that the Ψ

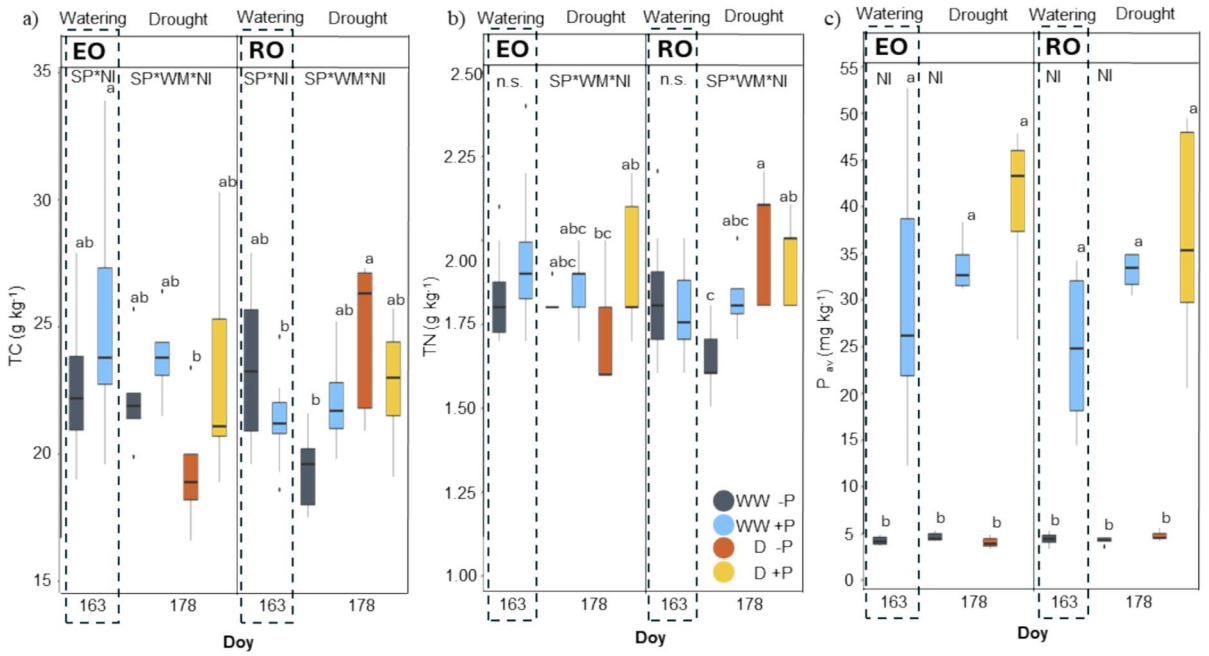


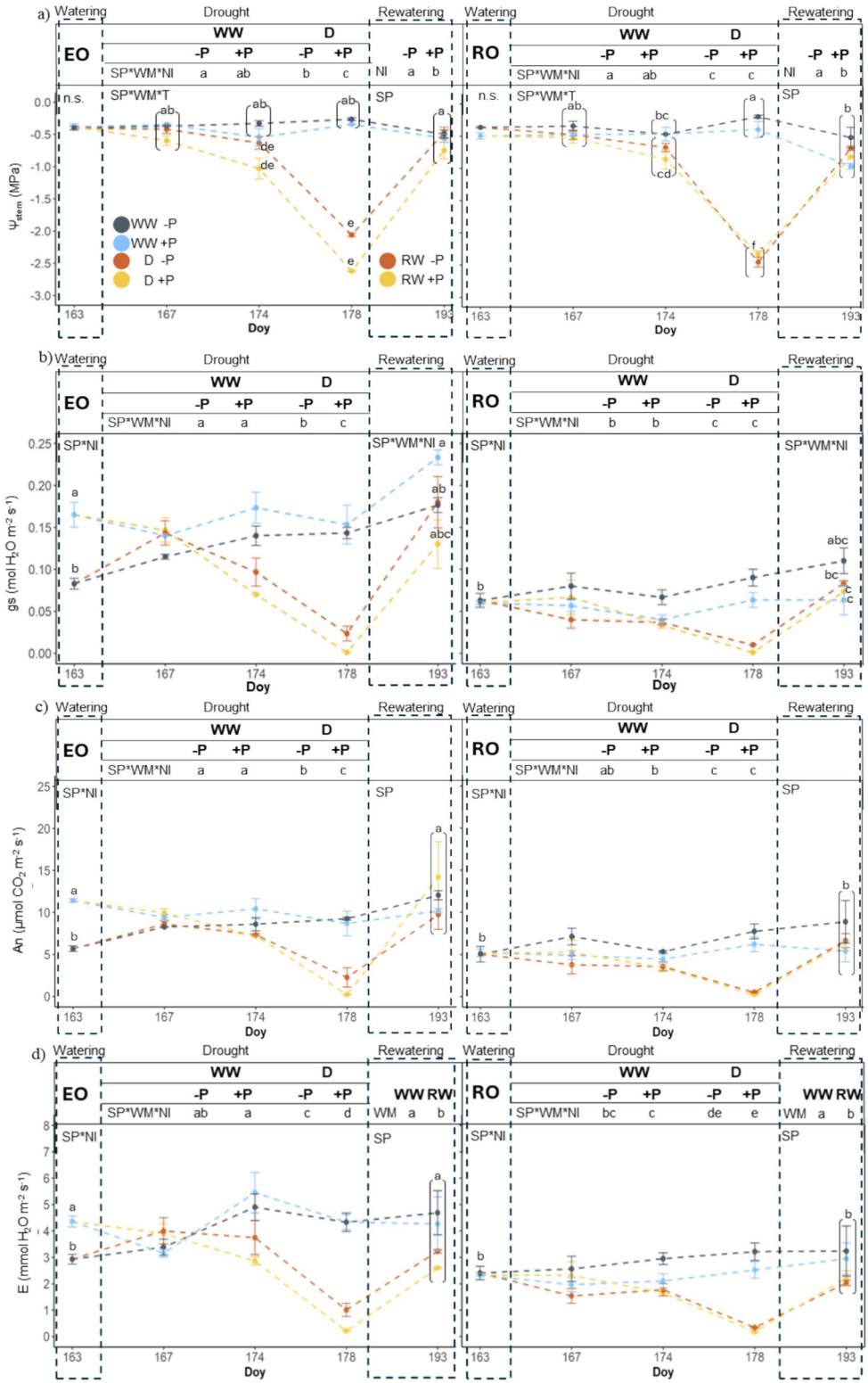
Fig. 1 **a** total carbon (TC), **b** total nitrogen (TN) and **c** available phosphorous (P_{av}) in soils affected by two different species (SP; English Oak, EO and Red Oak, RO) under different water management (WM; well-watered, WW and drought conditions, D) with different nutrient input (NI), at the initial watering phase (DOY 163) and the last day of drought imposition (DOY 178). According with ANOVA analysis and Tukey's post - hoc, the different letters show significant among factor (p<0.05)

$_{stem}$ of EO was higher than that of RO. Regardless of species, RW + P plants had lower Ψ_{stem} than RW -P.

After 25 days from nutrient input (DOY 178), and prior to the imposition of drought conditions, adding P increased the gs by 112.5% compared to EO -P (Fig. 2b). Conversely, gs in RO was not affected by NI. The gs in water-stressed EO and RO + P was 0.00 ± 0.00 mol H_2O m^{-2} s^{-1}, while EO D -P and RO D -P exhibited minimal values of 0.02 ± 0.02 mol H_2O m^{-2} s^{-1} and 0.01 ± 0.00 mol H_2O m^{-2} s^{-1}, respectively. ANOVA analysis showed a significant effect of SP*WM*NI during D conditions, highlighting the role of P input in reducing gs in drought-stressed EO. Moreover, the SP*WM*NI interaction revealed interspecific differences between EO WW and RO WW from DOY 167 to 178. After 15 days of rewatering, both EO and RO fully recovered their gs (DOY 193).

Similarly, at DOY 163, P input did not affect the An of RO, but it increased the An of the native species by 101.1% compared to EO -P (Fig. 2c). The minimum An in both SP was measured on DOY 178. During water stress conditions, ANOVA denoted a significant effect of SP*WM*NI interaction, indicating interspecific differences in WW + P plants. Regardless of the time, P addition decreased An in EO, while no effect was detected on RO. On DOY 178, the An of EO D+ P plants (0.21 ± 0.07 µmol CO_2 m^{-2} s^{-1}) was lower than that of EO D -P (2.25 ± 2.00 µmol CO_2 m^{-2} s^{-1}). The rewatering restored An in D plants to levels comparable to WW plants in both species. ANOVA showed that SP was the only factor affecting An on DOY 193, with EO exhibiting higher values than RO.

On DOY 163, P input affected all gas exchanges, including E (Fig. 2d). Results showed that P addition increased E in EO but did not affect RO. Drought conditions reduced E to 1.01 ± 0.44 mmol H_2O m^{-2} s^{-1} in EO -P, and 0.33 ± 0.04 mmol H_2O m^{-2} s^{-1} in RO -P. The lowest values were observed on DOY 178 in + P plants, with EO D showing 0.20 ± 0.02 mmol H_2O m^{-2} s^{-1} and RO D at 0.17 ± 0.06 mmol H_2O m^{-2} s^{-1}. After 15 days of rewatering, E in RW plants remained lower than in WW, regardless of SP and NI. ANOVA identified remarkable differences between SP, showing that E in EO was higher than in RO, consistent with the other gas exchange parameters.


a)
b)
c)
d)
Watering
Drought
Rewatering
WW
D
EO
RO
-P
+P
SP*WM*NI
SP*WM*T
SP*NI
SP
n.s.
WW -P
WW +P
D -P
D +P
RW -P
RW +P
Doy
Ψstem (MPa)
gs (mol H2O m-2 s-1)
An (µmol CO2 m-2 s-1)
E (mmol H2O m-2 s-1)

◂**Fig. 2** **a** Stem water potential (Ψ_{stem}), **b** stomatal conductance (gs), **c** net photosynthesis (An) and **d** leaf transpiration (E) of two different species (SP; English Oak, EO and Red Oak, RO) under different water management (WM; well-watered, WW and drought conditions, D; followed by rewatering, RW) with different nutrient input (NI) during time (T). According with ANOVA analysis and Tukey's post – hoc the different letters show significance between treatments (p=0.05). The statistical analysis was performed separating the watering (DOY 163), drought (DOY 167, 174, 178) and rewatering phase (DOY 193)

On DOY 178, ANOVA on SPAD and PI revealed significant effects of SP and WM (Figure S1a and b). Both parameters were higher in EO (39.1 ±3.4 SPAD; 4.5 ±1.9 PI) compared to RO (36.1 ±3.0 SPAD; 3.6 ±1.7 PI). Regardless of SP, D plants exhibited reduced SPAD (36.2 ±3.8) and PI (2.7 ±1.1) compared WW plants (SPAD: 38.9 ±2.5; PI: 5.5 ±1.3).

MANOVA on DOY 178 data showed that SP (*p-value* = 4.54e-04), WM (*p-value* = 3.76e-12), NI (*p-value* = 8.84e-08) and all their interactions significantly affected leaf gas exchanges and Ψ_{stem} (Table S1). Interspecific differences were observed between well-watered EO and RO, irrespective of NI. Profile plots separated EO WW -P and EO WW +P from RO WW -P and RO WW +P (Figure S2a). In contrast, the physiological status of EO D+ P was similar to RO D+ P, while EO and RO under combined water and nutrient stress were classified separately. On DOY 193, results displayed a remarkable effect of SP (*p-value* = 7.95e-05), WM (*p-value* = 2.80e-03) and NI (*p-value* = 3.22e-03), as well as WM*NI (*p-value* = 3.21e-02) and SP*WM*NI (*p-value* = 1.82e-03; Table S1). In the profile plot, EO RW +P grouped with RO RW -P and RO RW +P, while during rewatering, RO WW +P also joined this group. EO RW -P exhibited a physiological status similar to EO WW, independent of NI (Figure S2b).

Leaves chemical characterization

At DOY 163 (watering initial phase), the C content in -P plants (EO -P: 451.1 ±5.4 g kg^{-1}; RO -P: 456.1 ±5.1 g kg^{-1}) was higher than in +P plants (EO: 447.8 ±8.1 g kg^{-1}; RO: 451.0 ±6.4 g kg^{-1}). Notably, NI was the only factor with a remarkable effect on C content at DOY 163 (Fig. 3a).

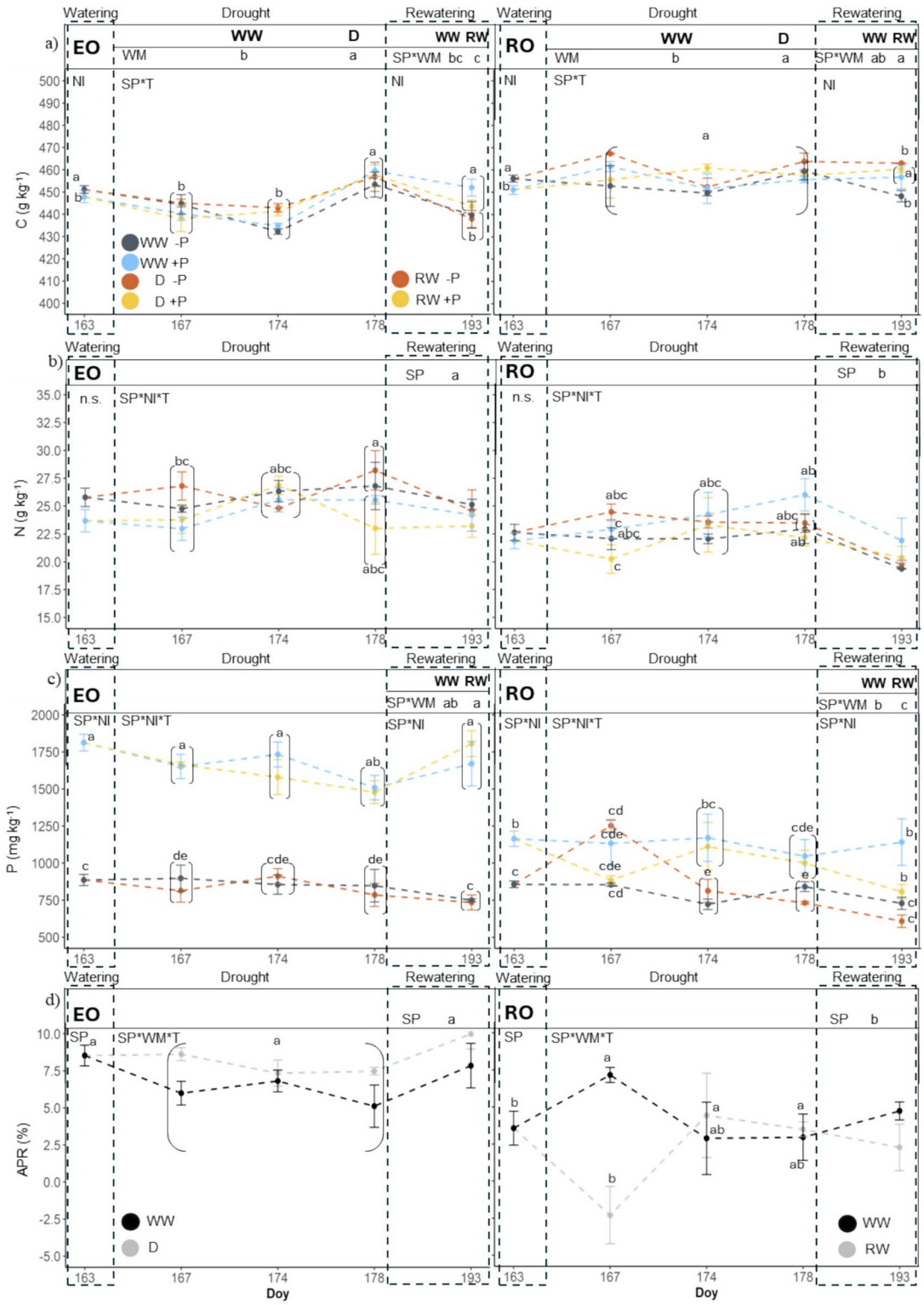
From DOY 167 to 178, EO increased its leaf C content regardless of WM and NI, reaching the highest value in WW +P (459.6 ±4.7 g kg^{-1}) on DOY 178. No differences in C content were observed in RO, during drought. Although the SP*WM interaction did not significantly affect C content during drought, ANOVA indicated that WM alone had a significant effect, with D leaves exhibiting higher C content than WW plants. As reported for DOY 163, +P plants had higher C content than -P plants after rewatering.

At DOY 163, the N content in EO -P leaves (25.8 ±2.6 g kg^{-1}) was similar to that in RO -P (22.6 ±2.3 g kg^{-1}) (Fig. 3b). P addition did not affect N content in both SP. However, from DOY 163 to 178, ANOVA displayed a remarkable effect of SP*NI*T. In EO, the main differences were observed between 167 and 178, where -P plants showed an increase in N content from 24.8 ±0.6 g kg^{-1} to 26.8 ±3.7 g kg^{-1} in WW plants and from 26.8 ±2.2 g kg^{-1} to 28.2 ±3.1 g kg^{-1} in D plants. During the same period, RO +P increased N content to 26.0 ±2.5 g kg^{-1}in WW plants and 22.2 ±1.3 g kg^{-1}in D plants. After rewatering, the N content differed between species, with EO leaves having higher nutrient levels than RO.

At DOY 163, the P content in the leaves of -P plants was 885.8 ±99.5 mg kg^{-1} for EO and 856.8 ±62.8 mg kg^{-1} for RO (Fig. 3c). NI significantly affected the nutrient content in the plants, resulting in a higher P level in EO (1812.4 ±169.2 mg kg^{-1}) compared to RO (1163.9 ±154.9 mg kg^{-1}). ANOVA highlighted statistical differences between EO WW +P and RO WW -P.

During drought conditions, the P content of EO +P remained higher than that of RO +P, regardless of WM. However, after rewatering, RO RW showed a lower P content than RO WW. At the same time, EO displayed no significant differences between RW and WW, maintaining higher values than RO regardless of NI.

After P addition, EO absorbed more P than RO. Specifically, at DOY 163, the APR for EO was 8.5 ±1.8%, while the apparent P recovery for RO was 3.6 ±3.2% (Fig. 3d). Drought did not affect the APR of EO, but it reduced the RO APR to −2.3± 3.4% on DOY 167. Consequently, from DOY 174 to 178, the RO APR values returned to the levels observed at DOY 163. Following rewatering, ANOVA revealed a


Watering
Drought
Rewatering
a)
EO
RO
WW
D
WW RW
WM b
a
SP*WM bc c
SP*WM ab a
NI
SP*T
C (g kg-1)
WW -P
WW +P
D -P
D +P
RW -P
RW +P
b)
n.s.
SP*NI*T
SP a
SP b
N (g kg-1)
c)
SP*NI
P (mg kg-1)
d)
SP a
SP*WM*T
SP
APR (%)
WW
D
RW
163
167
174
178
193
Doy

◂**Fig. 3** **a** Carbon (C), **b** nitrogen (N) and **c** phosphorous (P) leaves content, **d** apparent P recovery (APR) of two different species (SP; English Oak, EO and Red Oak, RO) under different water management (WM; well-watered, WW and drought conditions, D; followed by rewatering, RW) with different nutrient input (NI) during time (T). According with ANOVA analysis and Tukey's post – hoc the different letters show significance between treatments (p=0.05). The statistical analysis was performed separating the watering (DOY 163), drought (DOY 167, 174, 178) and rewatering phase (DOY 193)

significant effect of plant species on APR, consistent with the observations at DOY 163.

At the beginning of the experiment, the C/N ratio of EO leaves (17.5 ±1.8 in -P) was lower than that of RO (20.4 ±2.2 in -P). ANOVA did not reveal a significant effect of NI on the C/N ratio at the initial watering phase. The interspecific difference persisted during drought and after rewatering (Fig. 4a).

The C/P ratio of EO -P leaves (516.8 ±53.4) was comparable to that of RO -P leaves (536.1 ±40.8) at DOY 163 (Fig. 4b). However, P addition resulted in differences between species, with a decline of the ratio of EO + P down to 248.7.1 ±26.5, while RO + P measured 390.1 ±58.3. ANOVA displayed a significant effect of the interaction SP*NI at DOY 163, during the drought phase, and after 15 days of rewatering. From DOY 167 to 178, the C/N ratio increased regardless of SP, WM and NI.

At DOY 163, the nutrient-stressed native and alien species exhibited similar N/P ratios. The P input decreased the N/P ratio to 13.1 ±2.2 in EO and 18.9 ±2.9 in RO (Fig. 4c). ANOVA indicated a significant effect of SP*NI during drought and after rewatering, confirming the earlier observations based on DOY 163 results. Moreover, on DOY 193, the significant effect of SP*WM revealed that RO RW had a higher N/P ratio than both EO RW and WW plants.

MANOVA on DOY 178 data showed that SP (p-*value* = 1.61e-03), NI (p-*value* = 1.64e-04), and their interaction (p-*value* = 2.23e-03) significantly affected the leaves chemical characterization (Table S2). Interspecific differences were observed between well-watered EO and RO, irrespective of NI.

Phosphorus input induced differences in EO, regardless of water management; however, this trend was not observed in RO, as RO -P and RO + P clustered together independently of water availability (Figure S3a). On DOY 193, results displayed a remarkable effect of SP (p-value = 9.00e-05), NI (p-value = 2.18e-06) and their interaction (p-value = 1.63e-03), as well as SP*WM (p-value = 2.88e-02; Table S2). Consistent with the observations from leaves collected on DOY 178, the MANOVA conducted on DOY 193 indicated that phosphorus affected the chemical composition of EO leaves, whereas a minor effect was observed in RO leaves (Figure S3b).

Metabolic response of EO and RO under nutrient and drought stress

The metabolomics analysis was conducted on the DOY 178 plant stage using an untargeted approach, allowing us to annotate 1744 metabolites putatively. The list of identified features, including pathway classification, specific metabolite ontology, their abundances, and MS1 isotopic and MS/MS spectra (where they are confirmed), is listed in the supplementary Table S3. Analyzing features obtained from the profiling of EO and RO leaves under nutrient and drought stress, the approach perfectly identified secondary metabolites as the most abundant class, followed by primary metabolites (e.g., fatty acids and lipids, carbohydrates, amino acids, and nucleotides, Table S3).

To assess the overall similarities/dissimilarities among samples influenced by different factors (i.e., water management, nutrient supplementation, and species), a hierarchical clustering analysis (HCA) was performed. As reported in Figure S4, the species factor resulted in the most differences, splitting into two branches. Concerning EO species, the HCA analysis clusters samples based on both nutrient supplementation and water management, suggesting specific metabolite profiles and abundances along treatments. Conversely, RO species reported less linearity between metabolic response and treatments, confirming our more resilient species hypothesis. Based on the unsupervised results, highlighting the species factor as the primary contributor to the overall variance, a supervised ANOVA multi-blocking orthogonal projection to latent structures discriminant analysis (AMOPLS-DA) was performed to focus on the detail about the nutrient and water management factors on the data variance, for both EO and RO species separately (Table 1).

Our results reported that the WM factor exerted the greatest influence on EO and RO species, showing

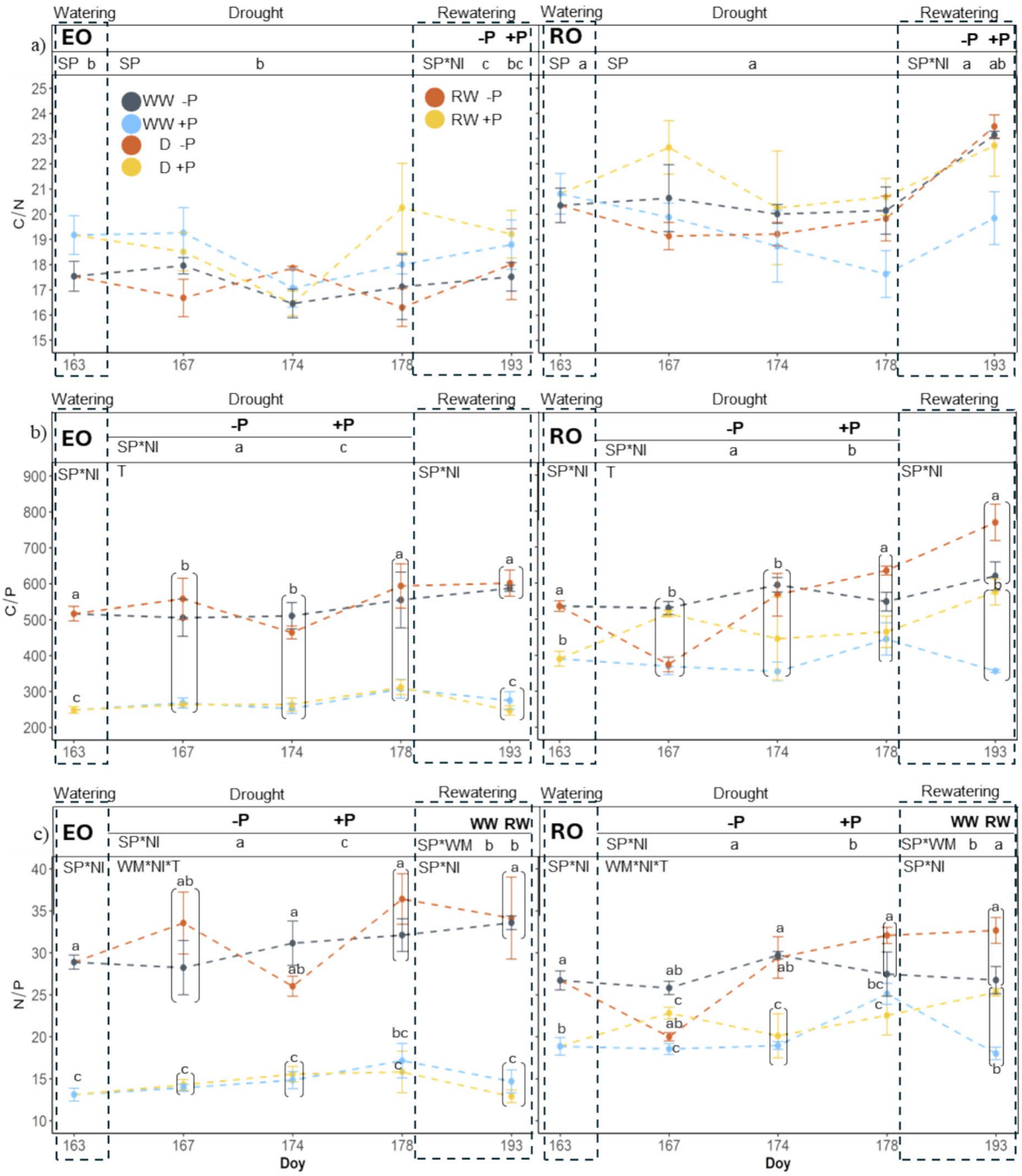


Fig. 4 **a** C/N,**b** C/P, **c** N/P ratio leaves of two different species (SP; English Oak, EO and Red Oak, RO) under different water management (WM; well-watered, WW and drought conditions, D; followed by rewatering, RW) with different nutrient input (NI), during time (T). According with ANOVA analysis and Tukey's post – hoc the different letters show significance between treatments (p=0.05). The statistical analysis was performed separating the watering (DOY 163), drought (DOY 167, 174, 178) and rewatering phase (DOY 193)

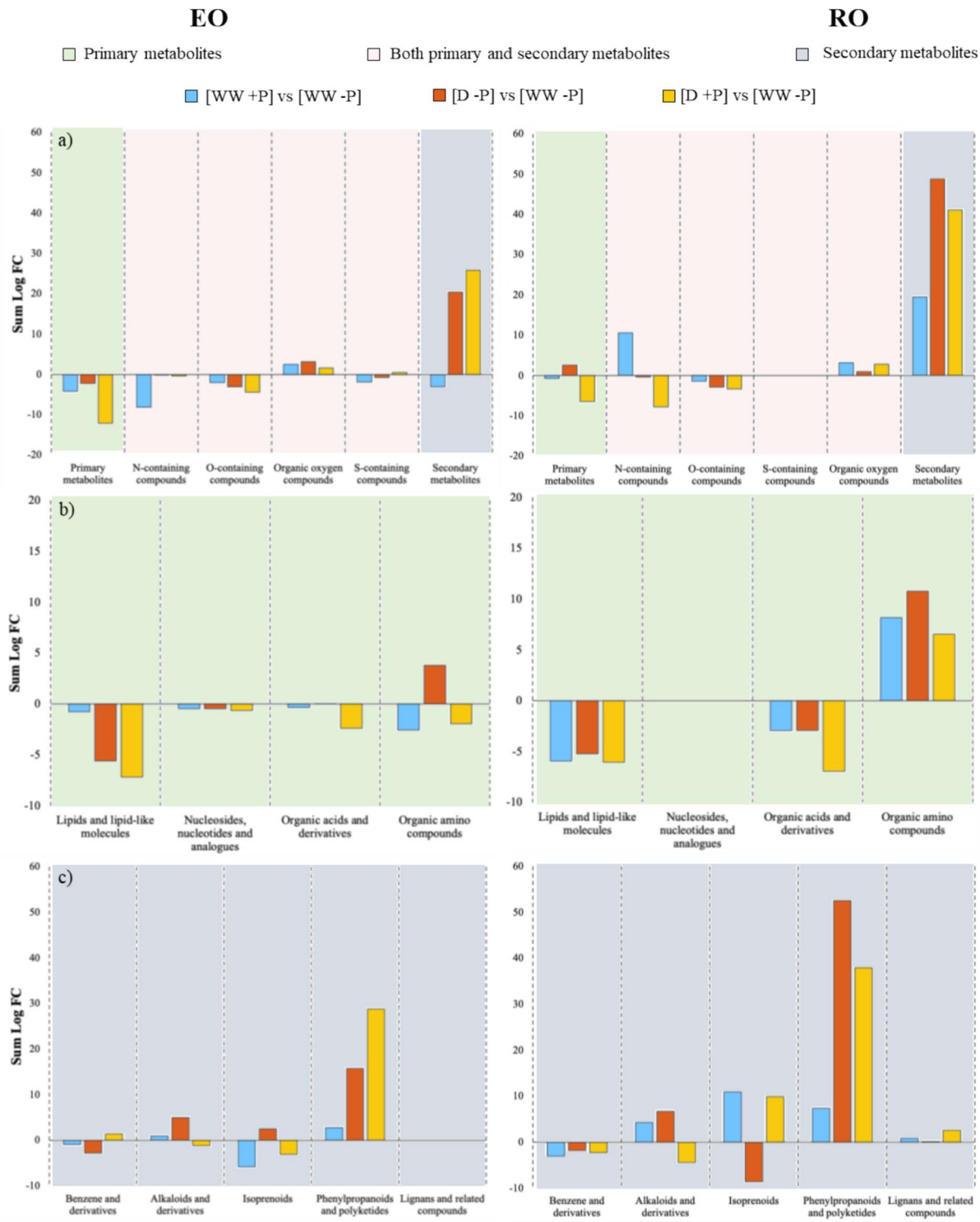
Table 1 Relative variability and block contributions of the ANOVA multi-blocking orthogonal projection to latent structures discriminant analysis (AMOPLS) of two Quercus spp metabolomic data. species (English Oak, EO; Red Oak, RO) affected by different water management (WM), phosphorus nutrient input (NI), and interaction between WM x NI. RSS: Relative sum of squares, Tp1–3: predictive components, To: orthogonal component

EO AMOPLS model							
Effect Name	RSS	RSS p-value	R2Y p-value	Tp1	Tp2	Tp3	To1
WM	20.9%	0.01	0.01	97.2%	0.2%	1.4%	18.4%
NI	14.1%	1.00	0.01	0.8%	99.1%	1.7%	22.7%
WM x NI	7.6%	1.00	0.01	1.0%	0.3%	94.5%	27.8%
Residuals	57.5%	-	-	1.1%	0.4%	2.4%	31.0%
RO AMOPLS model							
WM	12.8%	0.01	0.01	95.6%	1.7%	4.4%	23.1%
NI	8.2%	1.00	0.01	1.4%	94.6%	4.7%	24.7%
WM x NI	6.0%	1.00	0.01	1.4%	1.8%	85.8%	25.5%
Residuals	73.0%	-	-	1.5%	1.9%	5.1%	26.7%

20.9% and 12.8% relative sum of squares (RSS) values, respectively. The discrimination between well-watered and drought-stress plants was perfectly made by component 1 (tp1). Nutrient supplementation and its interaction with WM were the secondary factors that influenced metabolic response in both species and discriminated using components 2 and 3 (tp2 and tp3), respectively. Interestingly, the supervised AMOPLS model highlighted a higher percentage of the observed metabolomic differences that could not be explained by our factors in RO than EO species, supporting the hypothesis of better correlation between treatments and metabolic response in EO as firstly suggested by HCA. Afterwards, the variable importance in the projection (VIP) analysis was carried out to identify biomarkers associated explicitly with differentiating samples based on factors. The 50 most discriminant metabolites per factor were selected and reported in the supplementary table, together with log fold change values for both EO (Table S4) and RO (Table S5). The specific metabolic pathways were then built using these VIP markers, considering biosynthetic pathways (Fig. 5a) and further going into detail about primary (Fig. 5b) and secondary metabolites biosynthesis (Fig. 5c).

Regarding the modulation of EO biosynthesis pathways, phosphorus supplementation under WW conditions down-modulated primary and secondary metabolite synthesis and nitrogen-, oxygen- and sulfur-containing metabolites (Fig. 5a). The most affected metabolite classes, belonging to primary metabolites, were organic amino compounds, including gamma-amino acids, valine, and methionine, as well as their derivatives, and lipids and derivatives, such as medium-chain fatty acids, steroidal saponins, and cucurbitacin glycosides (Fig. 5b; Table S4). Secondary metabolites were isoprenoids and benzene derivatives (Fig. 5c), highly represented by terpene glycosides and pyrroloindoles belonging to the two metabolic classes. In contrast, the drought stress up-modulated the biosynthesis of secondary metabolites, where the accumulation of phenylpropanoids, alkaloids, and isoprenoids was mainly represented (Fig. 5c). Specifically, coumarins, flavonoid-*O*-glycosides, flavones, and their derivatives were mostly up-accumulated for the class of phenylpropanoids. Additionally, lipid and fatty acid synthesis were also affected, reporting a reduction in steroids and short- and medium-chain fatty acids, alongside an increase in long-chain fatty acid synthesis, like 2-decyl-3-hydroxypentanedioic acid (Fig. 5b). Interestingly, phosphorus supplementation under drought-stress conditions led to an increased abundance of specialized metabolites in plant tissues (Fig. 5a), fully addressing the synthesis of phenylpropanoids, mostly represented by coumarin compounds. This evidence suggests a synergistic effect of phosphorus input in enhancing endogenous bioactive compounds to cope with abiotic stress.

Concerning the modulation of RO biosynthesis pathways, nutrient and water management factors produced similar modulation patterns, differing



mainly in the abundance of specific classes of metabolites. Indeed, the most relevant metabolite classes were secondary metabolites, followed by nitrogen-containing compounds and primary metabolites (Fig. 5a and b). Regarding secondary metabolites biosynthesis, drought stress selectively

◂**Fig. 5** Pathway analysis of two *Quercus* spp. species (English Oak, EO; Red Oak, RO) affected by different water management – i.e., well-watered (WW) and drought (D) and phosphorus nutrient input (P), in comparison to the initial experimental conditions (WW –P). **a** Biosynthesis of primary and secondary metabolites, as well as compounds potentially associated with both metabolic pathways, in EO and RO. **b** Superclass of primary metabolites and (**c**) superclass of secondary metabolites primarily affected in EO and RO under different water and nutrient availability conditions

increased the accumulation of phenylpropanoids (neoflavans, hydrolysable tannins, and angular pyranocoumarins) and alkaloids (tropane and matrine alkaloids) while reducing isoprenoids as a response strategy, mostly triterpenoids. At the same time, phosphorus input under the same condition maintained a high level of phenylpropanoids and was accompanied by the up-modulation of isoprenoids synthesis (Fig. 5c).

Profiling of phenylpropanoids composition of EO and RO under nutrient and drought stress

Since the primary metabolic response of EO and RO under nutrient and water management stress factors was secondary metabolite accumulation, a deep clarification of specific subclasses of phenylpropanoid compounds was plotted based on log fold change values derived from the pairwise comparison between treatments and control (Fig. 6). Regarding the EO species (Fig. 6a), drought conditions, and at a higher extent combined with the phosphorus supplementation, increased flavonoid *O*-glycosides (luteolin 7-glucuronide and scutellarin are the most abundant), coumarins and derivatives (phebalosin), flavones, and hydrolyzable tannins. Specifically, the enhanced production of coumarins under drought + phosphorus was also confirmed by a clear down-accumulation of coumaric acid, their synthetic precursors. Focusing on RO species (Fig. 6b), drought stress induced the accumulation of flavonoid *O*-glycosides (myricetin 3,7,3',4'-tetramethyl ether and icariin), neoflavans, isoflavones, and high hydrolyzable tannins (schizantherin E and schisandrin C). As previously observed, the RO species resulted in less sensitivity to the phosphorus input under both well-watered and drought conditions.

Discussion

Interspecific plant differences due to nutrient status under watering conditions

To understand the responses of EO and RO to water stress under varying levels of P availability, it is essential to assess the nutritional and physiological status of well-watered species under -P and + P soil. Twenty – five days after the P supply, before imposing a mild and long drought event, the stem water potential (Ψ_{stem}) and gas exchange value of EO and RO were consistent with the previous findings of Gori et al. (2023), on well-watered *Q. ilex* and *Quercus* spp., respectively.

Our results showed that Ψ_{stem} was unaffected by P input, while increased gas exchange levels were observed for EO + P, in line with the findings from Rolando et al. (2025). The relationship between net photosynthesis and P has been studied in plants: P can modulate the assimilation of N for the synthesis of ribulose-1,5-bisphosphate carboxylase, the primary enzyme that catalyzes the first step of carbon dioxide fixation in photosynthesis (Spreitzer and Salvucci 2002; Warren et al. 2005). Moreover, P is a component of the adenosine triphosphate synthesis (ATP) and nicotinamide adenine dinucleotide (NADPH), which are, respectively, the primary sources of energy and reducing agents needed to drive the biochemical transformations of carbon dioxide into organic compounds in the Calvin cycle (Farooq et al. 2012). As a matter of fact, Kayoumu et al. (2023) observed that P shortage reduces the photosynthetic capacity and leaf transpiration. Therefore, increasing EO gas exchanges following P input could suggest that this soil element is the limiting factor for EO growth. Conversely, gas exchanges of RO were not modulated by soil P addition, probably because the alien species did not experience a P deficit, even in the depleted soil. This may be attributed to RO having a higher P use efficiency than EO, as previously observed (Rolando et al. 2025). This interspecific difference in resource utilization capacity could also be extended to photosynthesis. In fact, under adequate soil water content and regardless of P availability, the gas exchange rates of RO are lower than those of EO, suggesting a reduced CO_2 assimilation of RO.

The native species absorbed and allocated more P in leaves than the alien species; thus, the apparent P

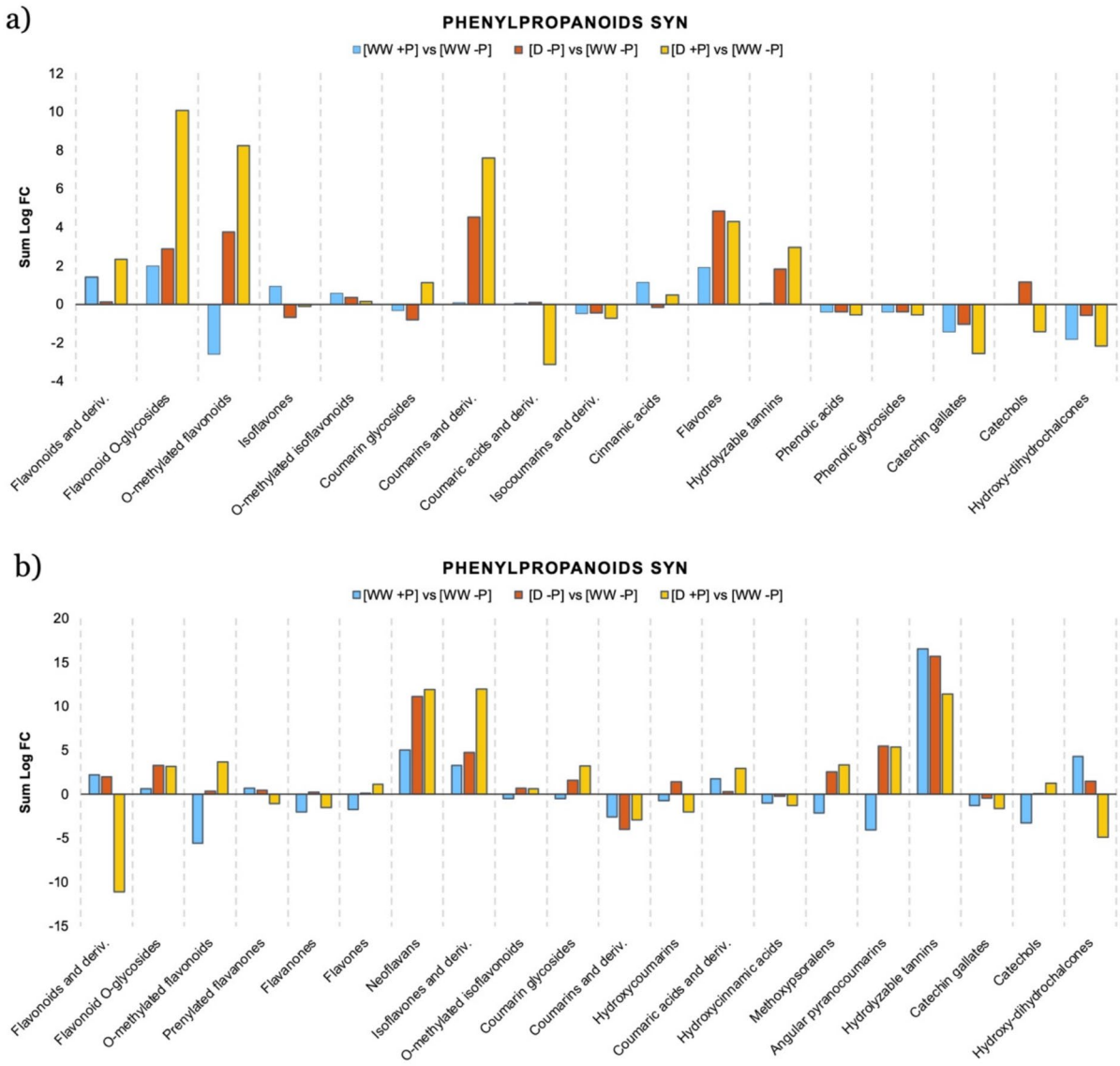


Fig. 6 Phenylpropanoids profiling analysis of two *Quercus* spp. species (English Oak, EO; Red Oak, RO) affected by different water management – i.e., well-watered (WW) and drought (D) and phosphorus nutrient input (P) for (**a**) EO and (**b**) RO species. syn = synthesis, deriv. = derivatives

recovery confirmed that EO's P demand was higher than RO's. Nevertheless, the soil P_{av} pool did not show significant differences between +P soils under different species. This may be related to the dramatic soil low P levels: although a large amount of P (110 kg/ha) was added to mitigate nutrient deficiency in plants, a large part of the added P may have been retained by the mineral phase and released when needed, leading to equalize the P_{av} pool in soil, independently of P species (Rolando et al. 2025). Evidence of this process is provided by the increase in total organic soil C, which suggested root exudation under our experimental conditions. Root exudates (e.g., organic acids, enzymes, phenolic compounds) can enhance P availability in soils, mobilizing P from stable pools (DeLuca et al. 2015; Menezes-Blackburn et al. 2016; Rafi et al. 2019). Preece et al. (2021) observed the same trend in +P soil under *Q. Ilex*.

According to our first hypothesis, EO exhibited increased gas exchange following P input, likely due to its lower resource use efficiency than RO. This confirms that P is a limiting factor for EO growth and its competitive ability against RO in highly weathered soils.

Effects of drought on plants as a function of soil P addition

The combined application of physiological measurements and untargeted metabolomics shed light on plant response to drought and nutrient stresses, indicating that both EO and RO triggered contrasting effects at both physiological and metabolic levels, primarily depending on water availability.

Water deficits disrupt crucial processes in plants, such as photosynthesis, transpiration, and nutrient uptake, with the effects varying depending on the severity of stress, plant genotype, and growth stage (Kirkham 2005). Under drought conditions, plants often produce ROS, leading to oxidative damage, carbohydrate and protein synthesis disruption, and lipid peroxidation, which can ultimately cause cell death (Feng et al. 1994; Blum 2005). In response to these stresses, plants have evolved various adaptive mechanisms to mitigate damage, such as limiting transpiration through stomatal closure, accumulating osmoprotective solutes in the cytoplasm, and activating pathways that enhance water uptake, improving stomatal conductance, and increasing water-use efficiency (Mitchell et al. 2008; Peñuelas and Staudt 2010).

Concerning EO, drought significantly reduced the stem water potential, but the decrease was less pronounced under P-limited conditions. This finding is consistent with previous studies, such as those by Burman et al. (2009), which highlighted that P fertilization could help mitigate the negative effects of water stress by promoting plant growth and leaf area expansion, increasing water demand and accelerating soil water consumption. However, the increased water consumption in P-fertilized plants could lead to a more significant reduction in water status during drought stress. Despite this, plants with higher P availability tend to show better recovery after rehydration, improved photosynthetic efficiency, and higher enzymatic activity, which is supported by our findings of improved gas exchange in EO with P supplementation during the initial watering phase (Burman et al. 2009).

As drought progressed, EO plants showed a clear reduction in net photosynthesis, mainly when P was present, coinciding with stomata's closure, limiting CO_2 uptake. In contrast, EO -P plants maintained higher gas exchange rates during drought, indicating a potential trade-off between maintaining higher photosynthesis and tolerating stress. Although during watering conditions, P supply promoted photosynthesis by enhancing the biosynthesis of the primary source of energy (ATP), under drought, plants closed stomata, limiting the absorption of CO_2 from the atmosphere. This could be due to an imbalance between ATP and CO_2 concentrations, affecting the photosynthetic process.

These physiological changes in EO were mirrored in the metabolomic profile, where we observed a shift towards synthesizing primary and secondary metabolites that could help mitigate oxidative damage and osmotic stress under the reduced water availability conditions (Street et al. 2006; Peñuelas and Staudt 2010).

In this species, drought-induced stress led to an increase in the biosynthesis of phenylpropanoids, particularly hydroxylated phenylpropanoids (e.g., [6-[3,4-dihydroxy-2,5-bis(hydroxymethyl)oxolan-2-yl]oxy-3,4,5-trihydroxyoxan-2-yl]methyl and E)−3-(4-hydroxy-3-methoxyphenyl) prop-2-enoate and dihydroxy B-ring-substituted flavonoids (e.g., luteolin 7-glucuronide and scutellarin). These metabolites are well-known for their antioxidant properties, as they scavenge ROS and help reduce oxidative damage (Gould et al. 2002; Hernandez et al. 2006; Agati et al. 2007; Hernández et al. 2009; Agati and Tattini 2010; Ferreres et al. 2011; Pollastri and Tattini 2011).

Interestingly, adding P to drought-stressed EO led to the upregulation of primary and secondary metabolites, which act as antioxidants and osmoprotectants (Dos Santos et al. 2022; Ahmad et al. 2023). In particular, P addition under drought conditions overstimulated the production of phenolic compounds, including flavonoid O-glycosides, O-methylated flavonoids and coumarins. These compounds likely contribute to maintaining turgor pressure by decreasing osmotic potential in vacuoles, which helps mitigate the effects of water stress (Almeida et al. 2020). In addition to phenylpropanoids, an accumulation of saikosaponins, a class

of triterpene saponins, was detected. Saikosaponins are known for their ability to scavenge ROS and protect cellular membranes from peroxidation, which is especially important under drought stress (Liu et al. 2005; Zhu et al. 2009).

This finding supports the hypothesis that EO, when supplied with P, activates metabolic pathways that enhance its ability to cope with drought, possibly by increasing the production of stress-related metabolites.

Among primary metabolites, increased levels of mannitol were found in the metabolic profile of EO under water stress and P supplementation. Mannitol helps stabilize proteins and cellular structures by interacting with the hydration shell around them and scavenging ROS produced in higher quantities under drought stress (Patel and Williamson 2016). Similarly, the metabolomic data also revealed an accumulation of 1,3,5-tricaffeoylquinic acid, a bioactive compound involved in antioxidant defense. Previous studies have shown that caffeic acids and their derivatives, such as 1,3,5-tricaffeoylquinic acid, are crucial in protecting plants from oxidative stress (Harrison et al. 2008; Kim et al. 2012). This metabolite may help protect EO from ROS damage, complementing the physiological data that suggest that EO's photosynthetic system is less impaired under drought when P is available.

Moreover, the input of P reprogrammed lipid and fatty acid metabolism in drought-stressed EO. The observed increase in long-chain fatty acids in EO +P under drought conditions has been previously interpreted as a plant strategy to maintain membrane integrity, potentially limiting cellular turgor loss (Ullah et al. 2022). Fatty acids are a crucial component of cellular membranes (Ruiz-Lopez et al. 2015), and their accumulation contributes to membrane rigidification (López-Pérez et al. 2009).

In addition, EO's metabolomic profile revealed increased levels of gibberellic acid (GA). This plant hormone is known to be involved in response to various abiotic stresses, including water scarcity. GA helps regulate membrane permeability, osmolyte accumulation, and antioxidant systems, all enhancing stress tolerance (Shah et al. 2023). Our findings of increased GA accumulation under drought with P supplementation suggest that P might promote EO's ability to manage stress through the modulation of plant growth and metabolism, which is reflected in the enhanced photosynthetic activity observed during the initial watering phase.

On the other hand, RO data displayed a different response from EO for both physiological and metabolomic analyses under drought conditions. Regardless of P availability, water stress negatively impacted stem water potential and gas exchange values. Similarly, P supply did not lead to a higher production of defense secondary metabolites compared to P-limited conditions. In the absence of P, the metabolomic analysis displayed a remarkable increase in phenylpropanoids and alkaloids, suggesting that RO has a metabolic system capable of handling both water and nutrient limitations, possibly by activating specific stress-related pathways even under low P conditions.

However, even without drought imposition, high levels of hydrolyzable tannins were detected in RO +P leaves. This points out the plant's intrinsic capability to upregulate the synthesis of hydrolyzable tannins under adequate soil nutrient availability (Salminen et al. 2004). Hydrolyzable tannins play a key role in plant defense by deterring herbivory and inhibiting the growth of pathogens, which may confer a competitive advantage in nutrient-rich environments where biotic interactions are intensified (Al-Khayri et al. 2023). Additionally, their antioxidant properties can contribute to cellular protection, enhancing overall stress resilience.

The lack of a strong effect of P on RO nutrient uptake during drought, particularly in terms of P accumulation, reinforces the idea that RO is less dependent on P compared to EO. This contrasts with the more pronounced effects of P on EO physiological and metabolic responses. In EO, P input appeared to support a more regulated drought response, contributing to the stabilization of key physiological functions and the activation of protective metabolic pathways.. Conversely, metabolic changes in RO were less influenced by P supplementation, suggesting that RO may operate at a baseline metabolic level that allows it to thrive in a wider range of environmental conditions.

The metabolic reprogramming of both species under water-limited conditions was consistent with soil–plant nutrient bio-cycling. As previously shown by metabolomic data, EO was more responsive than RO to P addition, reflecting a greater P leaf accumulation. On the other hand, P content in RO -P leaves was not linked to P input. Nevertheless, these results were observed regardless of water availability.

In contrast to previous studies, the unaltered P concentration of RO leaves under water and nutrient-limited conditions and the increased content of soil total organic carbon suggested a higher root exudation responsible for P mobilization from the stable pool (DeLuca et al. 2015; Menezes-Blackburn et al. 2016; Rafi et al. 2019). This could be one of the competitive advantages of RO in dry and low fertility soils, as a strategy to gain an advantage over the native vegetation. Root exudates can help mobilize nutrients in the rhizosphere, offering a competitive advantage in nutrient-poor environments (Gargallo-Garriga et al. 2015). Moreover, the fact that RO leaves did not show a significant increase in P accumulation under drought conditions supports the idea that RO may have evolved to be less reliant on P input compared to EO, possibly due to its ability to regulate nutrient uptake and allocation more efficiently. This behavior aligns with the metabolic shifts observed in RO, where the accumulation of secondary metabolites likely supports its stress tolerance mechanisms under drought and nutrient limitation.

Along with the increase of soil total C, the same trend was observed for total N in RO soil under both water and nutrient-limited conditions. However, these values were higher than those observed for EO under the same conditions. This could be due to EO's greater N uptake than RO during drought. In fact, in the absence of both water and P supply, N content in the leaves of RO tended to be lower than that of EO.

Effect of rewatering on plants subjected to drought as a function of soil P addition

After 15 days of rewatering, drought-stressed plants of both species increased the stem water potential and the gas exchanges to the level of plants under well-watered conditions, consistent with the literature (Pflug et al. 2018). Notably, gas parameter values, in agreement with the results of Jacobs et al. (2009) and Kebert et al. (2023), were higher in EO than RO, suggesting an interspecific difference irrespective of water supply.

P input contributed to EO recovery, showing the highest values of net photosynthesis among all EO and RO plants regardless of nutrient and water availability. The modulation of net photosynthesis by P has been reported in the literature: drought-stressed plants supplied by P addition generally recover their photosynthetic system more quickly than P-deficient plants (Radin and Eidenbock 1984; Singh et al. 1997; Burman et al. 2009). Although the net photosynthesis of RO was lower than EO, higher levels of C in leaves were found in the alien species after rewatering. This suggests that RO may have exhibited a more rapid turnover of primary carbon metabolites compared to EO following rewetting (Zang et al. 2014).

The chemical composition of leaves also pointed out an interspecific difference in plant nutrient demand. Even though P supply led to an increase in P leaves in both species, this allocation exhibited higher values in EO.

During the water relief and throughout the entire experiment, higher P content was consistently observed in the leaves of P-supplied plants. This supports the hypothesis mentioned previously Rolando et al. (2025) that RO species have a lower nutrient demand/higher nutrient use efficiency than EO.

Conclusions

In this study, the response of two *Quercus* spp. (EO and RO) to the combined effects of drought and P addition, was investigated. The findings provide novel insights into the interplay between nutrient availability and water stress in modulating metabolomic pathways. Under drought stress, EO was dependent on P, with significant upregulation of phenylpropanoid pathways and increased biosynthesis of antioxidant metabolites in P-fertilized plants. Conversely, P had a minimal influence on the metabolomic profile of RO under drought, highlighting the species' adaptability to drought conditions. The greater resilience of RO is likely related to its intrinsic ability to produce polyphenols and tannins, as well as to employ strategies for water uptake under dry conditions and optimize nutrient acquisition, maintaining a lower overall nutrient demand. This capacity allows RO to thrive in environments with limited water and nutrients, potentially giving it a competitive advantage over EO in drought-prone ecosystems.

However, P addition may enhance EO competitiveness, which could limit the spread of the alien species in favor of the native one in ecosystems increasingly affected by drought due to climate change.

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Author contributions All authors contributed to the study's conception and design. Conceptualization: Morena Rolando, Francesca Secchi, Luisella Celi. Data curation and visualization; Morena Rolando, Paola Ganugi, Luisella Celi, Francesca Secchi, Leilei Zhang. Formal analysis and investigation: Morena Rolando, Paola Ganugi, Francesca Secchi, Luisella Celi, Leilei Zhang, Luigi Lucini. Methodology: Morena Rolando, Luisella Celi, Francesca Secchi, Paola Ganugi, Luigi Lucini, Leilei Zhang. Supervision: Luisella Celi, Francesca Secchi. Project administration: Luisella Celi. Writing—original draft: Morena Rolando, Paola Ganugi, Leilei Zhang. Writing—review & editing: all authors.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests All the authors declare they have no financial interests.

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