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Drought resilience of Portuguese chestnut cultivars under Mediterranean rainfed conditions

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Abstract

Increasing summer drought and climatic irregularity threaten sweet chestnut production in Mediterranean regions. However, cultivar-specific drought resilience under field conditions remains insufficiently resolved because the relative roles of hydraulic status, gas exchange and biochemical adjustment are rarely evaluated together. This study aimed to characterise the drought response strategies of three traditional Portuguese chestnut cultivars, Judia, Longal and Martaínha, and the hybrid rootstock ColUTAD®, across four orchards representing a natural edaphoclimatic gradient during the 2023 growing season. Summer drought intensity was described using climatic descriptors combined with plant-based indicators of water status, particularly midday stem water potential (Ψ_{wmid}) and relative water content (RWC). Across cultivar-site combinations, Ψ_{wmid} ranged from −1.75 to −1.05 MPa, RWC from 78.9 to 91.9%, net photosynthesis from 4.5 to 15.0 µmol CO_2 m^{-2} s^{-1}, and transpiration from 1.1 to 3.5 mmol H_2O m^{-2} s^{-1}. Less negative Ψ_{wmid} values were associated with higher RWC, photosynthesis and transpiration, whereas electrolyte leakage increased as plant water status declined. Cultivar-dependent responses were particularly evident for transpiration, instantaneous water-use efficiency, soluble sugars, electrolyte leakage and antioxidant-related traits. Martaínha showed the most favourable integrated response under the 2023 climatic configuration, combining less negative Ψ_{wmid}, higher leaf hydration, sustained gas exchange and comparatively controlled biochemical activation. Longal displayed high physiological plasticity and strong antioxidant capacity, whereas Judia showed effective gas-exchange and biochemical responses but greater sensitivity to water limitation. In contrast, ColUTAD® exhibited pronounced hydraulic stress, elevated membrane damage and strong biochemical activation, indicating stress sensitivity rather than effective drought tolerance. Overall, the results indicate that drought resilience in chestnut is more closely associated with hydraulic stability and sustained physiological activity than with biochemical overactivation alone, and that cultivar performance should be interpreted in relation to year-specific climatic configuration.

Keywords: *Castanea sativa*; Drought stress; Mediterranean orchards; Plant water relations; Stress physiology

1. Introduction

Climate change is intensifying drought frequency, heat load and climatic irregularity in Mediterranean regions, posing a major threat to perennial crop systems. Among abiotic stresses, drought is especially critical because it restricts water availability, impairs carbon assimilation, disrupts plant metabolism and

promotes oxidative imbalance, ultimately compromising plant performance, productivity and survival (Farooq et al., 2012; Méndez-Vallejo et al., 2023; Miller et al., 2010; Zandalinas et al., 2021). In tree crops, these effects are particularly important because seasonal water deficits influence not only current physiological performance but also fruit development, reserve accumulation and long-term orchard sustainability.

Plants respond to drought through coordinated hydraulic, physiological and biochemical adjustments aimed at maintaining water balance and cellular homeostasis. These responses include stomatal regulation, reduced transpiration, osmotic adjustment through the accumulation of compatible solutes, and activation of antioxidant systems to mitigate reactive oxygen species (ROS) (Farooq et al., 2012; Krasensky and Jonak, 2012; Sharma et al., 2019). Soluble sugars, proline and phenolic compounds contribute to osmotic regulation, membrane stabilisation and redox buffering under water deficit, although their functional significance may vary depending on drought intensity, timing and genotype (Hajji et al., 2006; Heim et al., 2002; Krasensky and Jonak, 2012; Procházková et al., 2011). As a result, drought resilience is not determined by a single trait, but by the coordination of multiple response mechanisms linking water status, metabolism and gas exchange.

Sweet chestnut (*Castanea sativa* Mill.) is a temperate tree species of high ecological, cultural and economic importance in Mediterranean and Iberian regions. In chestnut orchards, the summer period coincides with critical phenological stages, including early fruit development, embryo growth and the initial phases of nut filling, all of which depend strongly on adequate water availability and sustained canopy activity. Summer drought and heat stress can therefore reduce stomatal conductance, limit carbon assimilation, impair assimilate translocation and increase fruit abortion, ultimately decreasing nut size and yield (Mota et al., 2018). At the national scale, Portuguese chestnut productivity shows marked interannual variability that closely follows summer climatic conditions, particularly precipitation availability, reinforcing the importance of climate-driven water stress as a major limitation to sustainable production.

Portuguese chestnut production includes numerous traditional cultivars, among which Judia, Longal and Martaínha are among the most relevant and commercially important in the country (Braga et al., 2023). Judia is valued for its large nuts and commercial relevance, with fruits typically around 50–80 fruits kg^{-1}, while available evidence indicates that its productive and physiological performance is strongly influenced by summer heat and water availability (Freitas et al., 2021; Gomes-Laranjo et al., 2006; Mota et al., 2018). Longal is one of the most widespread Portuguese cultivars and is widely regarded as an important variety for industrial use (Braga et al., 2023). Martaínha is likewise one of the main commercial cultivars and, together with Longal, is among the principal varieties of the Soutos da Lapa PDO (Braga et al., 2023). The contrasting agronomic profiles and territorial distribution of these cultivars provide a useful framework for investigating varietal differences in drought response under Mediterranean field conditions.

A previous field study conducted in the same orchard network and cultivar set during the 2022 growing season characterised drought-induced biochemical and antioxidant responses in Portuguese chestnut cultivars and showed that environmental conditions strongly modulated stress expression, with cultivars differing in their osmotic and antioxidant strategies (Marques et al., 2025). However, that study did not resolve whether those biochemical response patterns translate directly into whole-plant drought resilience when interpreted together with hydraulic regulation, leaf hydration, gas exchange and mineral

status under field conditions. This represents a key knowledge gap, because cultivar rankings based mainly on biochemical stress markers may not fully capture the mechanisms underlying functional drought performance across contrasting annual climatic scenarios.

We therefore hypothesized that cultivar-specific drought performance in chestnut is not fixed, but depends on the integration of hydraulic stability, sustained gas exchange and biochemical adjustment under the specific climatic configuration of each growing season. More specifically, we expected that cultivars showing stronger biochemical activation would not necessarily exhibit the highest whole-plant drought resilience, and that the relative prominence of each cultivar would depend on how seasonal water availability and summer stress were structured in the field.

Accordingly, the objectives of this study were to evaluate the drought response strategies of Judia, Longal, Martaínha and ColUTAD® during the 2023 growing season across four orchards representing a natural edaphoclimatic gradient in Portugal; to integrate soil properties, climatic drought descriptors, plant water status, gas exchange, biochemical markers and leaf mineral composition into a common interpretative framework; and to determine how these traits jointly explain cultivar-specific drought resilience under field conditions. By extending the 2022 assessment to a new climatic year and a broader analytical framework, this study aims to clarify how drought-response syndromes are expressed in chestnut under realistic Mediterranean conditions and to provide a stronger physiological basis for cultivar selection in drought-prone orchards.

2. Materials and Methods

2.1. Study locations and water regime

The study was conducted during the 2023 growing season in four commercial chestnut (*C. sativa* Mill.) orchards located in distinct chestnut-producing regions of Portugal, representing a natural gradient of climatic and edaphic conditions. The sites were located in Carrazedo de Montenegro, Parada, Penela da Beira, and Marvão. All four orchards belonged to the ClimCast demonstration network (Carneiro et al., 2022). The orchards differed in altitude, temperature, precipitation, and soil characteristics, allowing the assessment of cultivar responses under contrasting environmental conditions.

Water regime differed among sites and was explicitly considered in the experimental design. Carrazedo de Montenegro, Parada and Penela da Beira were managed under rainfed conditions. In contrast, the orchard located in Marvão was equipped with a drip irrigation system. According to ClimCast project records, maintenance irrigation was applied during July and August to support orchard establishment, although detailed information on irrigation dose and scheduling was not available for quantitative inclusion in this study (Carneiro et al., 2022). Site location, Protected Designation of Origin (PDO), geographic coordinates, altitude, and water regime are summarised in Table 1.

Table 1
Demonstrative orchard (DO), Protected Designation of Origin (PDO), coordinates, altitude (meters above sea level – m a.s.l.) and water regime used in the study.

DO	PDO	Coordinates	Altitude (m a.s.l.)	Water regime
Carrazedo de Montenegro	Padrela	41°33’41.76”N; 7°25’51.41’’W	765	Non-irrigated

Parada	Terra Fria	41º38'12.53"N; 6º42'42.94"W	740	Non-irrigated
Penela da Beira	Soutos da Lapa	41°01'38"N; 7°26´38"W	885	Non-irrigated
Porto da Espada	Marvão - Portalegre	39°21'19.5"N; 7°21'40.1"W	583	Irrigated

2.2. Plant material and experimental design

Four chestnut genotypes were evaluated, the traditional cultivars Judia, Longal and Martaínha, and the hybrid rootstock ColUTAD®. All trees were five years old at the time of sampling. Each genotype was represented by nine individual trees per orchard, resulting in a total of 36 experimental units (four genotypes × nine biological replicates) per site. At each location, fully developed and healthy trees were selected for physiological and biochemical measurements.

2.3. Soil chemical characterisation

Soil samples were collected from each orchard and analysed at the Soil and Plant Analysis Laboratory of the University of Trás-os-Montes and Alto Douro. Soil pH was measured potentiometrically in distilled water and in 1 mol L^{-1} KCl at a 1:5 soil-to-solution ratio, according to ISO 10390 (International Organization for Standardization, 2005). Available phosphorus and potassium were determined using the Egner-Riehm extraction method. Phosphorus was quantified as phosphate by molecular absorption spectrophotometry at 880 nm following Murphy and Riley, 1962, whereas potassium was determined by atomic absorption spectrophotometry. Results are expressed on an elemental basis. Soil chemical data were used to describe site-specific edaphic background and to support interpretation of cultivar performance under contrasting field conditions. The main soil properties of the four orchards are summarised in Table 2.

Table 2
Baseline soil chemical properties of Carrazedo de Montenegro, Parada, Penela da Beira and Marvão.

Local	**pH (H_2O)**	**pH (KCl)**	**OM**	**P**	**K**	**B**
Carrazedo de Montenegro	5.6	4.6	44	50	144	0.20
Parada	5.3	4.2	48	7	91	---
Penela da Beira	5.3	4.4	64	45	19	0.66
Marvão	5.7	4.6	53	27	100	1.46
	Ca	**Mg**	**K**	**Na**	**EA**	**CEC**
Carrazedo de Montenegro	1.24	0.21	0.34	0.17	0.02	1.98
Parada	2.20	0.25	0.19	0.03	0.69	3.37
Penela da Beira	2.80	0.29	0.05	0.10	0.63	3.86
Marvão	3.63	0.68	0.27	0.09	0.05	4.72

OM, organic matter (g kg^{-1}). **P**, **K** and **B** are expressed as mg kg^{-1}. **Ca, Mg, K,** and **Na** are exchangeable cations expressed as cmolc kg^{-1}. **EA**, exchangeable acidity (cmolc kg^{-1}). **CEC**, cation exchange capacity (cmolc kg^{-1}).

2.4. Climate and drought characterisation

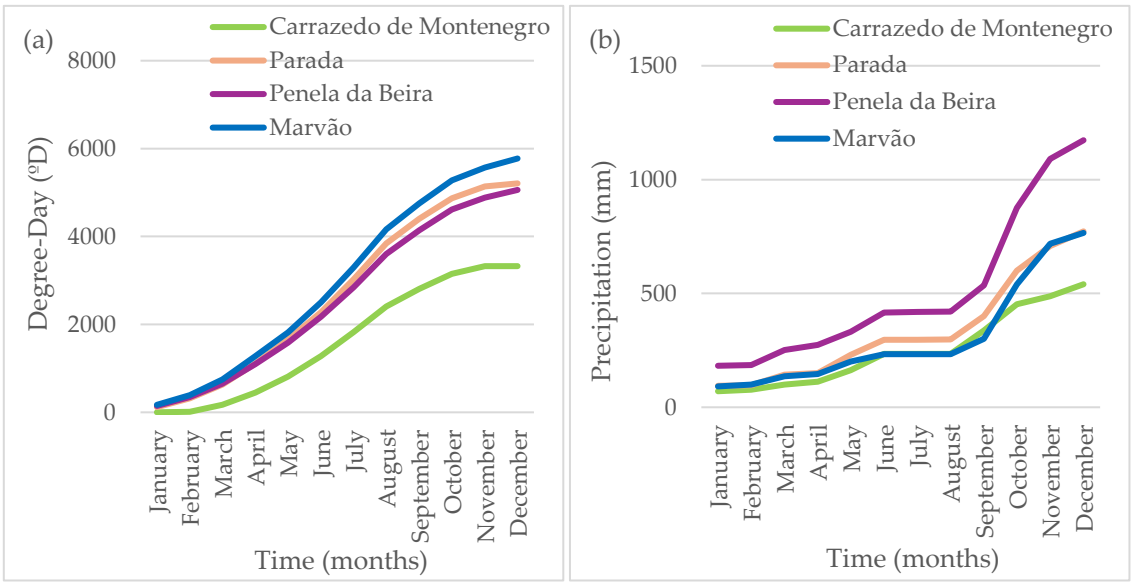
Climatic characterisation of each site was based on monthly precipitation and air temperature data retrieved from the IPMA platform for 2023 (Instituto Português do Mar e da Atmosfera, n.d.). Although all orchards belonged to the ClimCast demonstration network, IPMA data were used to compare site conditions

during the year of field measurements and to support interpretation of the results in relation to the 2022 field campaign previously reported by Marques et al., 2025.

Site climatic characterisation was based on cumulative precipitation, mean air temperature, degree-day accumulation, and xerothermic index, with particular emphasis on the summer drought period Fig. 1. These variables were used to describe the seasonal progression and intensity of drought typical of Mediterranean environments.

To characterise drought conditions during the field measurement period, climatic variables were complemented with plant-based indicators of water status. Midday stem water potential (Ψ_{wmid}) and leaf relative water content (RWC) were used to assess plant hydration and effective water availability experienced by the trees during summer drought. Midday stem water potential (Ψ_{wmid}) was used as the main plant-based reference variable for drought interpretation because it directly reflects plant water status under field conditions and provides an integrative physiological basis for relating hydraulic status to gas exchange, membrane integrity and biochemical responses

This combined climatic and physiological characterisation provided an integrated description of drought conditions across sites and supported the interpretation of cultivar-specific responses under contrasting water availability.



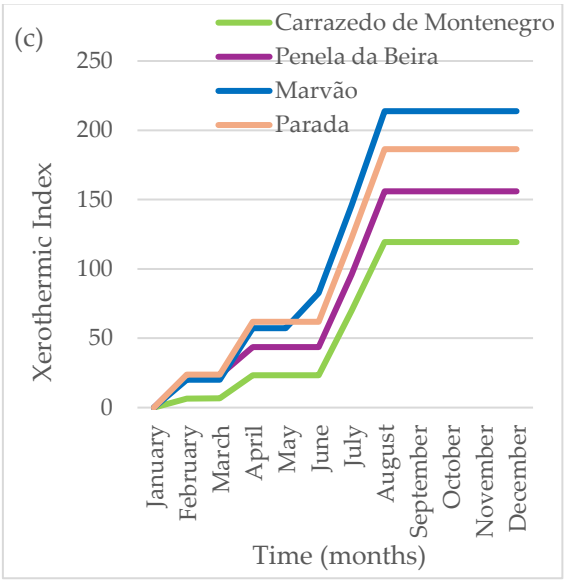


Fig. 1. Cumulative climatic profiles for the four study sites during 2023, based on IPMA data: (a) degree-day accumulation, (b) precipitation, and (c) xerothermic index from January to December for Carrazedo de Montenegro, Penela da Beira, Marvão, and Parada.

2.5. Timing and synchronization of field measurements and leaf sampling

Field work and leaf sampling were conducted between the second fortnight of July and the first fortnight of August 2023, corresponding to the core summer drought period in all study regions. For each orchard, all physiological measurements (Ψ_{wmid}, gas exchange and RWC) and leaf sampling for biochemical analyses were performed within a narrow temporal window of the same day or consecutive days, under stable meteorological conditions, in order to minimise short-term variability in drought intensity.

Fully expanded, sun-exposed leaves from the upper, outer canopy (northern side) were selected for sampling. For each cultivar at each location, six independent biological replicates (leaves collected from six different trees) were sampled. Leaf collection was carried out in the field immediately before physiological measurements.

Leaves were stored in an insulated container with cooling packs, ensuring low temperature conditions during transport to the laboratory. Upon arrival, samples were kept at 4 °C for 24 h prior to further processing, in order to stabilise carbohydrate pools, particularly starch reserves. On the following day, leaf material was processed and stored at −80 °C until biochemical analyses were performed.

Measurements across different orchards were necessarily conducted on different days due to logistical constraints. However, no significant rainfall events occurred during the overall measurement period, and drought conditions progressively intensified throughout the summer across all sites. This approach ensured internal consistency of cultivar comparisons within each orchard, while acknowledging that absolute drought intensity differed among sites due to climatic conditions and water regime.

2.6. Plant water status

Midday stem water potential (Ψ_{wmid}) was measured using a Scholander-type pressure chamber (PMS Instrument Company, Albany, NY, USA). Measurements were conducted between 12:00 and 14:00 h on fully expanded, healthy leaves that had been enclosed in aluminium foil bags for at least 30 min prior to measurement, allowing equilibration between leaf and stem water potential.

Leaf relative water content (RWC) was determined on freshly sampled leaves following standard protocols, using fresh weight, turgid weight after rehydration, and dry weight after oven-drying. Ψ_{wmid} and RWC were jointly used to characterise plant hydration status under field conditions.

2.7. Gas exchange

Gas exchange measurements were performed using an infrared gas analyser (IRGA, model LCA-2, Analytical Development Co., Hoddesdon, UK). Net photosynthetic rate (A, μmol CO_2 m^{-2} s^{-1}) and transpiration rate (E, mmol H_2O m^{-2} s^{-1}) were obtained directly from the instrument on fully expanded, sun-exposed leaves between 12:00 and 15:00 h. Instantaneous water-use efficiency (iWUE) was calculated as the ratio between net photosynthetic rate and transpiration rate (A/E) and expressed as mol CO_2 mol^{-1} H_2O. Measurements were conducted under saturating light conditions imposed in the chamber at a photosynthetic photon flux density of 1600 μmol m^{-2} s^{-1}, while CO_2 concentration, temperature and relative humidity followed ambient field conditions.

2.8. Soluble sugars and starch quantification

Soluble sugars and starch were quantified to assess the plant's carbohydrate metabolism and osmotic regulation mechanisms in response to heat stress. The determination of these parameters provides valuable information on the plant's energy status, carbon allocation, and ability to maintain cellular turgor pressure under stress conditions.

Total soluble sugars were extracted in 80% ethanol (*v/v*) using the colorimetric technique described by Irigoyen et al. (1992), with absorbance measured at 625 nm. Starch content was determined from the pellet obtained after soluble sugar extraction, with quantification performed spectrophotometrically at 625 nm following the method of Rose et al. (1991). Glucose was used as a standard for both soluble sugars and starch quantification, with six replications per treatment (n = 6).

2.9. Electrolyte leakage percentage content

Electrolyte leakage (EL, %) was analysed to assess cellular damage under heat stress. EL (%) was assessed to gauge stress-induced damage to plant tissues and tolerance to stress, following adjustments to the technique described by Abu-Muriefah (2015). Leaf discs were shaken in double-distilled water for 24 hours at room temperature (25 °C), and conductivity measurements (Jenway 470 portable conductivity meter) were taken before and after autoclaving. Electrolyte leakage percentage was calculated as EL (%) = (C1/C2) x 100, with six replications per treatment (n = 6).

2.10. Proline quantification

Proline content was quantified following a colorimetric assay standardized with purified proline. Acid–ninhydrin reagent was prepared by dissolving 1.25 g of ninhydrin in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid under heating and stirring; the reagent remained stable for 24 h at 4 °C. Fresh leaf material (0.5 g FW) were homogenized in 3% sulfosalicylic acid and centrifuged. The supernatant was reacted with acid-ninhydrin and glacial acetic acid at 100°C for 1 h. The chromophore was extracted with

toluene and absorbance measured at 520 nm. Purified proline (Sigma-Aldrich) was used for the standard curve and expressed as mg g^{-1} DW (dry weight), with six replications per treatment (n = 6).

2.11. Quantification of soluble proteins content

Soluble protein content was quantified from fresh leaf extracts using the Bradford method (Bradford, 1976). Absorbance was measured spectrophotometrically, and bovine serum albumin was used as the calibration standard. Results were expressed on a dry weight basis (mg g^{-1} DW), with four biological replicates per treatment (n = 4).

2.12. Determination of total phenolic compounds

Leaf samples were prepared by punching out tree discs of 8 mm in diameter from each leaf, which were then macerated in 10 mL of 80% (*v/v*) acetone for pigment extraction. The total phenolic content was then evaluated at 795 nm using the protocol established by Singleton and Rossi (1965), and a gallic acid calibration curve was used for quantification. For analysis, 5 mL of extract was employed. A standard curve was constructed (concentration range: 0-100 µg/mL, $R^2 > 0.99$). Results are expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW), with fresh material used for extraction and dry weight determined separately for data normalization, with four replicates per treatment (n=4).

2.13. Total flavonoids content (TFC)

Total flavonoid content was quantified using a colorimetric assay (Javanmardi et al., 2003; Zhishen et al., 1999). A mixture was prepared by combining diluted extracts (1 mL), bi-distilled water (4 mL), and a 5% W/V $NaNO_2$ solution (Sigma-Aldrich, 0.3 mL). $AlCl_3$ solution (Sigma-Aldrich, 10% (*w/v*), 0.3 mL) was added and allowed to stand for 6 minutes after shaking and remaining at room temperature for 5 minutes. After that, 2 mL of NaOH (Sigma-Aldrich, 1 M) was introduced. Finally, volume was completed to 10 mL with bi-distilled water and thoroughly mixed. The absorbance of the pink mixture was measured at 510 nm.

Standard catechin (Sigma-Aldrich) solutions (0–10 mg mL–1) were also essayed and calibration curve was obtained from the equation: y = 0.029x + 0.188; R2 = 0.993. A blank essay, using bi-distilled water, was also prepared. Total flavonoid content was expressed as milligrams of catechin equivalents per gram of dry weight (mg CAT eq. g^{-1} DW) with tree replications per treatment (n = 3).

2.14. Ferric reducing antioxidant power (FRAP) activity

The FRAP assay was performed following Benzie and Strain (1996) with minor modifications using 96-well microplates. Fresh FRAP reagent was prepared daily by mixing sodium acetate buffer (300 mM, pH 3.6), 10 mM TPTZ (in 40 mM HCl), and 20 mM $FeCl_3$ in a 10:1:1 (*v/v/v*) ratio. In each well, 25 µL of extract was mixed with 275 µL of FRAP reagent and incubated in the dark at room temperature for 5 min. Absorbance was measured at 593 nm using a microplate reader. $FeSO_4$ was used as the calibration standard. Results were expressed as µmol $FeSO_4$ equivalents per gram of dry weight (µmol $FeSO_4$ eq. g^{-1} DW), with tree biological replicates per treatment (n = 3).

2.15. Leaf mineral composition

Fully expanded leaves were collected from each cultivar and site during the field campaign and used for mineral analysis. Leaf samples were analysed for macronutrient and micronutrient composition. Macronutrients included nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) (**Table 3**), whereas micronutrients included boron (B), iron (Fe), zinc (Zn), manganese (Mn) and copper (Cu) (**Table 4**). Leaf mineral data are presented as descriptive laboratory values and were used to characterise the nutritional background of each cultivar × site combination under field conditions, rather than as variables for inferential statistical testing.

Table 3
Macronutrient concentrations in leaves of four chestnut cultivars sampled across four Portuguese orchards. Values are descriptive laboratory determinations and are expressed as g kg^{-1} DW.

Location	Cultivar	N	P	K	Ca	Mg	S
Carrazedo de Montenegro	ColUTAD®	26.1	2.28	8.8	12.3	3.30	1.83
	Judia	38.2	5.37	11.1	14.3	4.22	2.68
	Longal	30.5	4.46	10.7	9.5	3.57	2.06
	Martaínha	31.0	4.09	6.8	11.5	4.32	2.51
Parada	ColUTAD®	25.6	1.52	11.3	8.5	2.69	1.66
	Judia	28.3	1.62	11.9	7.5	3.97	1.91
	Longal	25.1	2.59	12.6	8.2	2.35	1.71
	Martaínha	25.2	2.06	10.0	7.7	2.93	1.76
Penela da Beira	ColUTAD®	22.9	1.78	6.3	7.4	1.65	2.08
	Judia	30.1	4.98	10.2	8.8	1.32	2.20
	Longal	29.9	4.86	14.6	8.8	1.66	2.38
	Martaínha	26.0	3.34	11.0	11.1	2.25	2.13
Marvão	ColUTAD®	20.9	2.19	13.0	16.5	2.10	1.55
	Judia	31.6	3.50	14.0	16.8	2.69	2.60
	Longal	25.6	3.04	18.0	14.3	2.65	1.86
	Martaínha	28.8	3.47	15.2	17.4	2.32	2.11

Table 4
Micronutrient concentrations in leaves of four chestnut cultivars sampled across four Portuguese orchards. Values are descriptive laboratory determinations and are expressed as mg kg^{-1} DW.

Location	Cultivar	B	Fe	Zn	Mn	Cu
Carrazedo de Montenegro	ColUTAD®	41.6	107.6	61.7	538	8.18
	Judia	99.2	93.9	108.2	1220	16.52
	Longal	66.5	74.3	117.5	901	15.28
	Martaínha	106.8	82.4	99.0	983	15.15
Parada	ColUTAD®	9.3	190.2	29.6	1574	8.36
	Judia	12.8	226.3	31.7	1911	11.40
	Longal	13.5	161.0	36.0	1146	9.12
	Martaínha	8.6	207.0	27.9	1663	9.14
Penela da Beira	ColUTAD®	82.6	93.9	25.0	483	8.54
	Judia	234.6	129.4	40.5	1806	10.45
	Longal	244.5	115.7	51.0	422	14.96

	Martaínha	107.9	72.2	26.5	526	12.51
Marvão	ColUTAD®	513.1	140.8	40.1	1661	6.88
	Judia	380.9	88.6	34.9	1042	11.27
	Longal	386.4	115.7	34.0	875	9.87
	Martaínha	417.5	128.6	42.3	1228	12.12

2.16. Statistical analyses

Statistical significance of differences among cultivars within each location was assessed using one-way analysis of variance (ANOVA), followed by Tukey's HSD test at $p < 0.05$. Different letters indicate statistically significant differences among means. Linear regression analyses were performed to examine relationships between midday stem water potential (Ψ_{wmid}) and the physiological and biochemical traits evaluated. To test whether these relationships differed among cultivars, analysis of covariance (ANCOVA) was applied using Ψ_{wmid} as a covariate, cultivar as a fixed factor, and the Ψ_{wmid} × cultivar interaction term. In addition, an integrated heatmap was constructed using standardized mean values (z-scores) for all physiological and biochemical variables, grouped by cultivar and water status class according to the proposed threshold, $\Psi_{wmid} > -1.2$ MPa and $\Psi_{wmid} \leq -1.2$ MPa. Red tones indicate above-average values for a given trait, whereas blue tones indicate below-average values.

3. Results

3.1. Water relations

Midday stem water potential (Ψ_{wmid}) ranged from −1.75 MPa in Martaínha at Carrazedo de Montenegro to −1.05 MPa in Martaínha at Penela da Beira (Fig. 2a). Across locations, ColUTAD® consistently presented the most negative Ψ_{wmid} values, whereas Martaínha and Judia generally showed less negative values, depending on site conditions. Two-way ANOVA showed significant effects of location, cultivar and their interaction on Ψ_{wmid} (all $p < 0.001$), indicating that cultivar responses depended on site conditions.

At Carrazedo de Montenegro, ColUTAD® and Martaínha showed more negative Ψ_{wmid} values (−1.69 and −1.75 MPa, respectively) than Judia and Longal (−1.46 and −1.38 MPa). At Marvão, Ψ_{wmid} values were less negative overall, ranging from −1.23 MPa in Longal to −1.11 MPa in Martaínha, and no significant differences were detected among cultivars. At Parada, Judia showed the most negative Ψ_{wmid} (−1.53 MPa) and Longal the least negative (−1.37 MPa), whereas ColUTAD® and Martaínha showed intermediate values close to −1.42 MPa and did not differ significantly from either cultivar. At Penela da Beira, Martaínha showed the least negative Ψ_{wmid} (−1.05 MPa), whereas ColUTAD®, Judia and Longal showed more negative values (−1.31, −1.18 and −1.29 MPa, respectively) and did not differ significantly from each other. Overall, most cultivar-site combinations remained below −1.20 MPa, with less negative values recorded mainly in Marvão and, to a lesser level, in Penela da Beira.

Relative water content (RWC) was positively related to midday stem water potential (Ψ_{wmid}), indicating that less negative Ψ_{wmid} values were associated with higher leaf hydration (Fig. 2b). Linear regression analysis showed a significant association between both variables, with Ψ_{wmid} explaining 20.9% of the variation in RWC ($R^2 = 0.209$, $p < 0.001$). When cultivar was included in the model, Ψ_{wmid} remained a significant predictor of RWC ($p = 0.001$), whereas neither cultivar nor the interaction between cultivar

and Ψ_{wmid} were significant ($p = 0.264$ and $p = 0.311$, respectively). These results indicate that the positive relationship between plant water status and leaf hydration was maintained across cultivars.

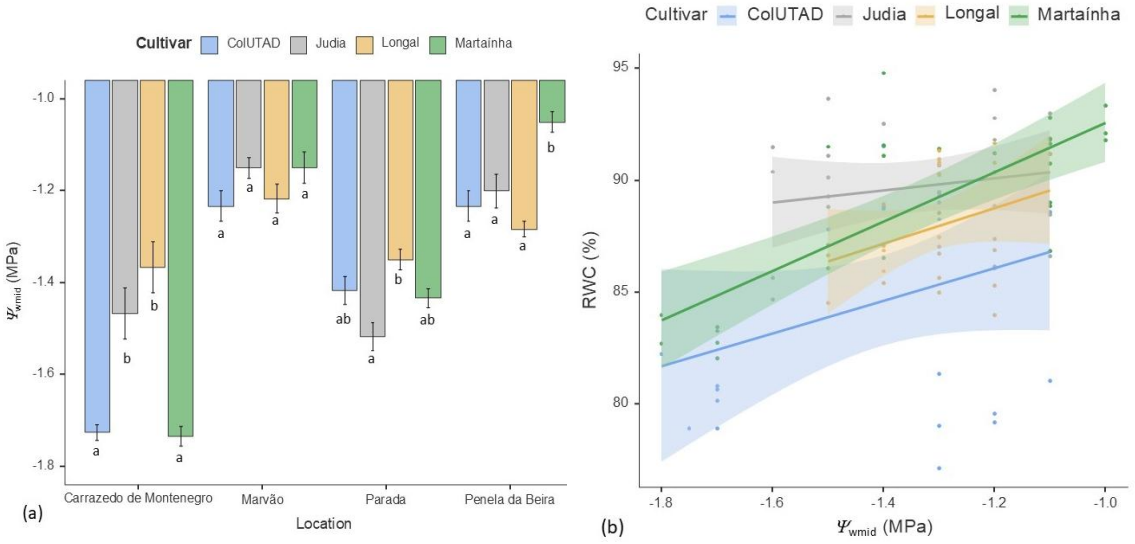


Fig. 2. Plant water status and its relationship with leaf hydration in four chestnut cultivars under field conditions. **(a)** Midday stem water potential (Ψ_{wmid}, MPa) of ColUTAD®, Judia, Longal and Martaínha evaluated at Carrazedo de Montenegro, Marvão, Parada and Penela da Beira. Bars represent mean ± SE ($n = 6$ biological replicates). Different letters indicate significant differences among cultivars within each location according to one-way ANOVA followed by Tukey's HSD test at $p < 0.05$. **(b)** Relationship between midday stem water potential (Ψ_{wmid}, MPa) and relative water content (RWC, %) across all observations. Each point represents one individual replicate. Solid lines represent the fitted linear regressions for each cultivar, and shaded areas indicate the 95% confidence intervals.

3.2. Gas exchange responses

Net photosynthesis (A) was positively related to midday stem water potential (Ψ_{wmid}), with less negative Ψ_{wmid} values associated with higher photosynthetic rates (Fig. 3a). Linear regression analysis showed a significant association between both variables, with Ψ_{wmid} explaining 14.8% of the variation in A ($R^2 = 0.148$, $p < 0.001$). When cultivar was included in the model, Ψ_{wmid} remained a significant predictor of A ($p = 0.003$), whereas neither cultivar nor the interaction between cultivar and Ψ_{wmid} were significant ($p = 0.266$ and $p = 0.461$, respectively). These results indicate that the positive relationship between plant water status and photosynthetic performance was maintained across cultivars.

Transpiration rate (E) was also positively related to Ψ_{wmid}, with less negative Ψ_{wmid} values associated with higher transpiration rates (Fig. 3b). This association was stronger for E than for A, as reflected by the higher proportion of explained variation ($R^2 = 0.404$, $p < 0.001$). When cultivar was included in the model, both Ψ_{wmid} and cultivar remained significant (both $p < 0.001$), and the interaction between cultivar and Ψwmid was also significant ($p < 0.001$). These results indicate that transpiration was strongly associated with plant water status, but that the magnitude of this response differed among cultivars. Overall, ColUTAD® tended to show the lowest transpiration values, Judia occupied an intermediate position, and Longal and Martaínha showed the highest fitted responses along the water status gradient.

Instantaneous water-use efficiency (iWUE) showed a significant negative relationship with Ψ_{wmid} when all observations were considered together ($R^2 = 0.145$, $p < 0.001$), indicating an overall tendency for higher iWUE values under more negative water potential conditions (Fig. 3c). However, when cultivar was included in the model, the main effect of Ψ_{wmid} was no longer significant ($p = 0.074$), whereas both cultivar and the interaction between cultivar and Ψ_{wmid} were significant (both $p < 0.001$). These results indicate that

the relationship between iWUE and plant water status was cultivar-dependent, consistent with differences in the coordination between carbon assimilation and transpiration along the water status gradient.

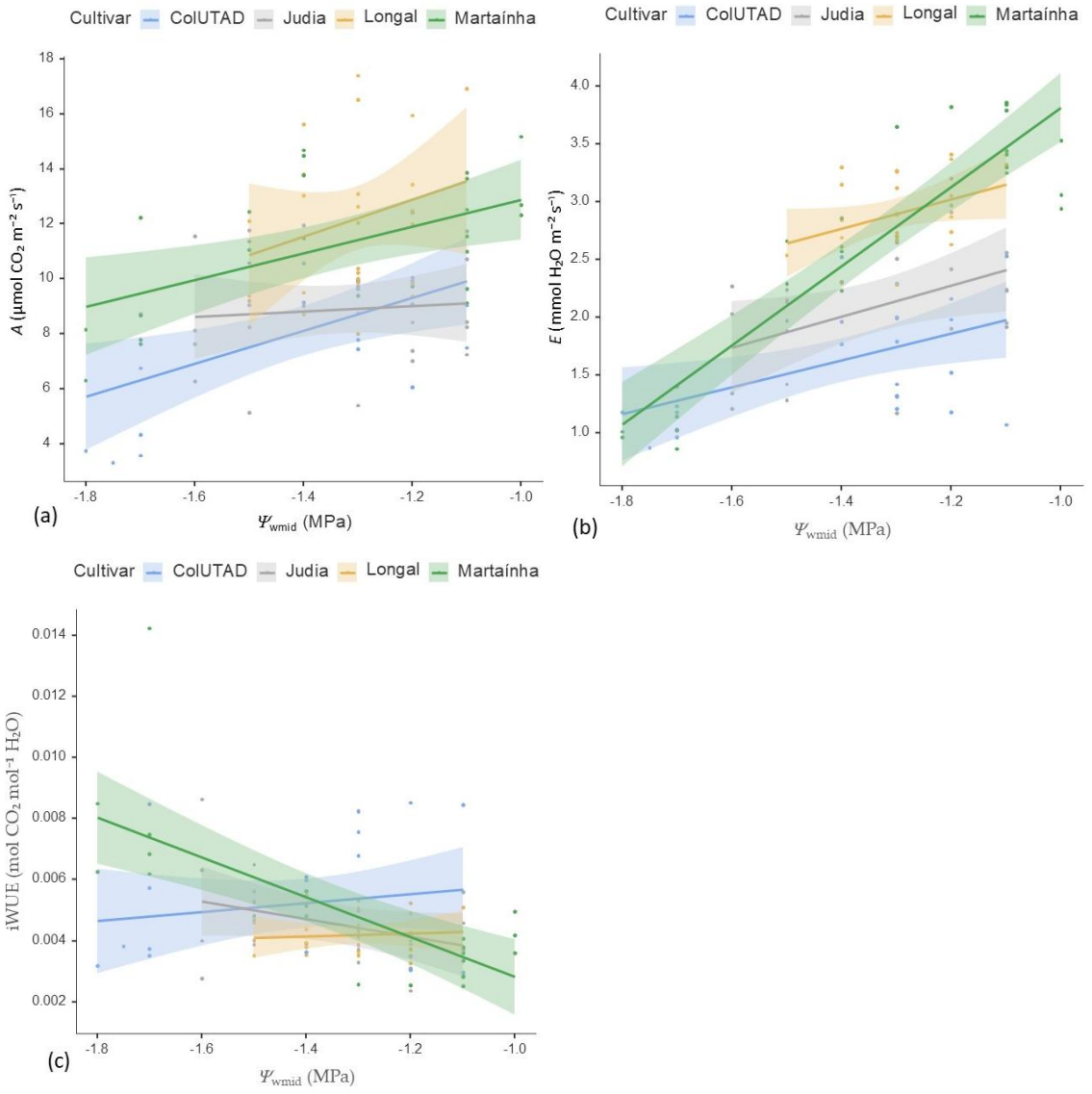


Fig. 3. Gas exchange responses in relation to midday stem water potential (Ψ_{wmid}) in four chestnut cultivars, ColUTAD®, Judia, Longal and Martaínha, across all observations. **(a)** Relationship between Ψ_{wmid} and net photosynthesis (A, μmol CO_2 m^{-2} s^{-1}). **(b)** Relationship between Ψ_{wmid} and transpiration rate (E, mmol H_2O m^{-2} s^{-1}). **(c)** Relationship between Ψ_{wmid} and instantaneous water-use efficiency (iWUE). Each point represents one biological replicate. Solid lines represent the fitted linear regressions for each cultivar, and shaded areas indicate the 95% confidence intervals.

3.3. Non-structural carbohydrate responses

Soluble sugar and starch contents showed contrasting patterns in relation to midday stem water potential (Ψ_{wmid}) (Fig. 4). Soluble sugar content showed no significant overall linear relationship with Ψ_{wmid} when all observations were analysed together ($R^2 = 0.001$, $p = 0.795$). However, when cultivar was included in the model, cultivar had a significant effect ($p = 0.013$), and the interaction between cultivar and Ψ_{wmid} was also significant ($p = 0.025$), whereas the main effect of Ψ_{wmid} remained non-significant ($p = 0.224$). These results indicate that soluble sugar responses to plant water status were cultivar-dependent and did not follow a common trend across the full dataset. In contrast, starch content showed no significant overall relationship with Ψ_{wmid} ($R^2 = 0.003$, $p = 0.595$), and, when cultivar was included in the model, neither Ψ_{wmid},

cultivar, nor the interaction between cultivar and Ψ_{wmid} were significant ($p = 0.183$, $p = 0.124$ and $p = 0.154$, respectively). Taken together, these results indicate that soluble sugars showed cultivar-specific adjustments along the water status gradient, whereas starch did not exhibit a consistent response across cultivars.

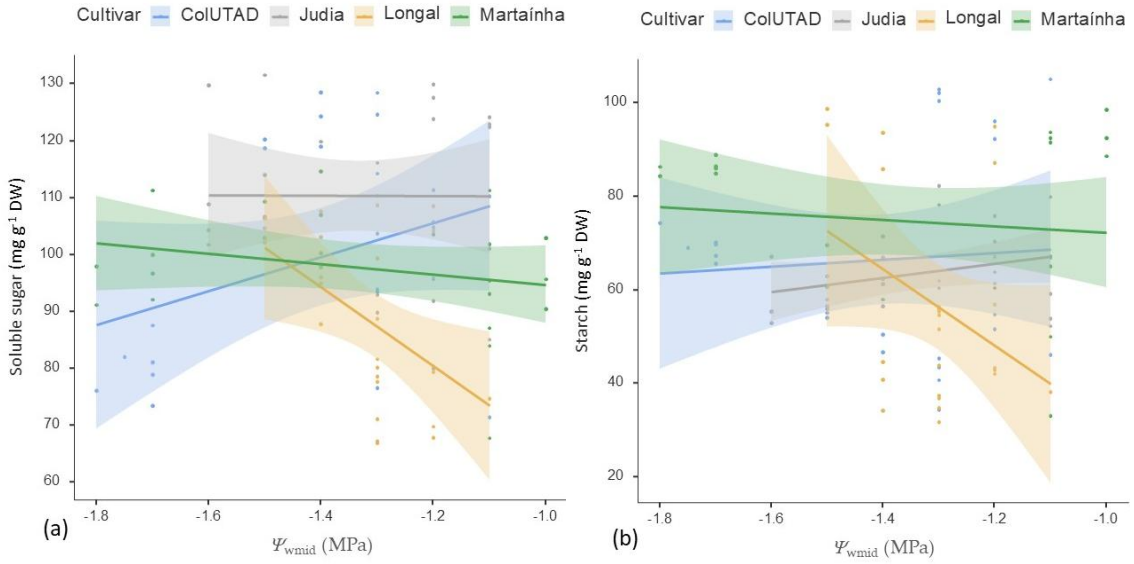


Fig. 4. Relationships between midday stem water potential (Ψ_{wmid}, MPa) and non-structural carbohydrate contents in four chestnut cultivars, ColUTAD®, Judia, Longal and Martaínha, across all observations. **(a)** Soluble sugar content in relation to Ψ_{wmid}. **(b)** Starch content in relation to Ψ_{wmid}. Each point represents one biological replicate. Solid lines represent the fitted linear regressions for each cultivar, and shaded areas indicate the 95% confidence intervals.

3.2. Oxidative stress responses

Electrolyte leakage (EL) showed a significant negative relationship with midday stem water potential (Ψ_{wmid}), indicating lower membrane leakage at less negative Ψ_{wmid} values (Fig. 5). Linear regression analysis showed that Ψ_{wmid} explained 11.8% of the variation in EL ($R^2 = 0.118$, $p < 0.001$). When cultivar was included in the model, Ψ_{wmid}, cultivar and the interaction between cultivar and Ψ_{wmid} were all significant ($p = 0.012$, $p = 0.033$ and $p = 0.004$, respectively), indicating that membrane damage was associated with plant water status in a cultivar-dependent manner. Overall, ColUTAD® tended to maintain higher EL values across the water status gradient, whereas Judia, Longal and Martaínha showed comparatively lower levels of membrane leakage.

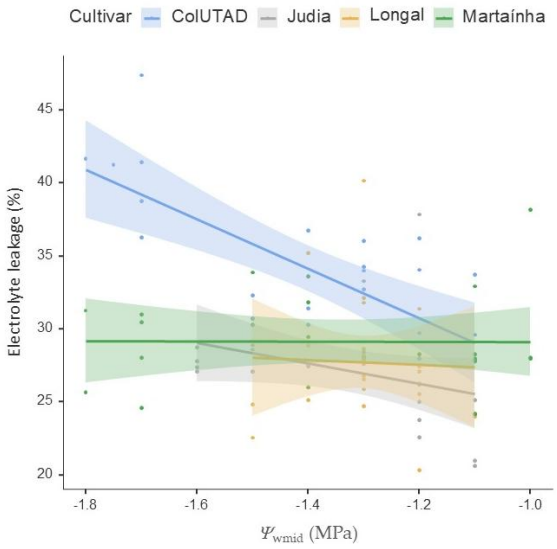


Fig. 5. Relationship between midday stem water potential (Ψ_{wmid}, MPa) and electrolyte leakage (EL) in four chestnut cultivars, ColUTAD®, Judia, Longal and Martaínha, across all observations. Each point represents one biological replicate. Solid lines represent the fitted linear regressions for each cultivar, and shaded areas indicate the 95% confidence intervals.

Proline and soluble protein contents were both negatively related to midday stem water potential (Ψ_{wmid}), with more negative Ψ_{wmid} values associated with higher accumulation of both compounds (Fig. 6). Proline showed a significant overall relationship with Ψ_{wmid}, with the latter explaining 8.9% of its variation ($R^2 = 0.089$, $p = 0.003$). When cultivar was included in the model, Ψ_{wmid} remained significant ($p < 0.001$), whereas neither cultivar nor the interaction between cultivar and Ψ_{wmid} were significant ($p = 0.161$ and $p = 0.176$, respectively), indicating that proline accumulation increased with declining plant water status and that this response was maintained across cultivars. Soluble protein content showed a similar pattern, with a significant negative relationship with Ψ_{wmid} ($R^2 = 0.175$, $p < 0.001$). When cultivar was included in the model, Ψ_{wmid} remained significant ($p = 0.027$), whereas neither cultivar nor the interaction between cultivar and Ψ_{wmid} were significant ($p = 0.446$ and $p = 0.578$, respectively). Taken together, these results indicate that both proline and soluble protein contents increased under declining water status, without significant cultivar-dependent differences in their responses along the Ψ_{wmid} gradient.

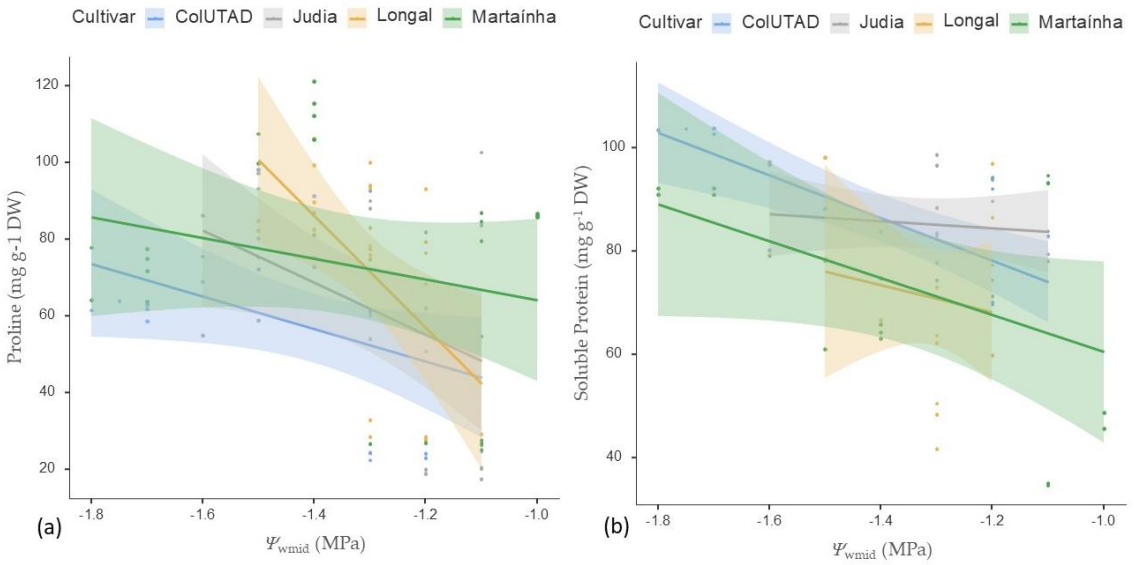


Fig. 6. Relationships between midday stem water potential (Ψ_{wmid}, MPa) and osmotic or metabolic adjustment traits in four chestnut cultivars, ColUTAD®, Judia, Longal and Martaínha, across all observations. **(a)** Proline content in relation to Ψ_{wmid}. **(b)** Soluble protein content in relation to Ψ_{wmid}. Each point represents one biological replicate. Solid lines represent the fitted linear regressions for each cultivar, and shaded areas indicate the 95% confidence intervals.

Phenolic compounds, flavonoids and FRAP showed antioxidant-related responses along the midday stem water potential (Ψ_{wmid}) gradient, although the magnitude and direction of these responses differed among cultivars (Fig. 7). Phenolic content showed a significant negative overall relationship with Ψ_{wmid}, with more negative Ψ_{wmid} values associated with higher phenolic accumulation ($R^2 = 0.168$, $p < 0.001$). However, when cultivar was included in the model, the main effect of Ψ_{wmid} was no longer significant ($p = 0.164$), whereas both cultivar and the interaction between cultivar and Ψ_{wmid} were significant ($p = 0.001$ and $p < 0.001$, respectively), indicating that phenolic responses to plant water status were cultivar-dependent. Across the fitted responses, Martaínha showed the highest phenolic levels under more negative Ψ_{wmid} values and the steepest decline along the water status gradient, whereas ColUTAD® tended to maintain more stable values. Judia and Longal showed intermediate patterns, with lower phenolic levels than Martaínha under the most negative water potential conditions.

Flavonoid content also showed a significant negative relationship with Ψ_{wmid}, with more negative Ψ_{wmid} values associated with higher flavonoid accumulation ($R^2 = 0.151$, $p = 0.006$). When cultivar was included in the model, Ψ_{wmid} remained significant ($p = 0.003$), whereas the main effect of cultivar was not significant ($p = 0.091$). However, the interaction between cultivar and Ψ_{wmid} was significant ($p = 0.023$), indicating that flavonoid responses to plant water status were likewise cultivar-dependent. Across the fitted responses, ColUTAD® showed the highest flavonoid levels under more negative Ψ_{wmid} values and the steepest decline along the water status gradient, whereas Judia and Longal also showed decreasing trends. In contrast, Martaínha maintained comparatively low and more stable flavonoid values across the Ψ_{wmid} range.

FRAP showed a similar overall negative relationship with Ψ_{wmid} ($R^2 = 0.098$, $p = 0.030$). In the ANCOVA, Ψ_{wmid}, cultivar and the interaction between cultivar and Ψ_{wmid} were all significant ($p < 0.001$, $p = 0.007$ and $p = 0.005$, respectively), indicating that antioxidant reducing capacity was also cultivar-dependent. Across the fitted responses, Longal showed the highest FRAP values under more negative Ψ_{wmid} conditions and a marked decline along the gradient, whereas Judia also showed a strong decrease.

ColUTAD® exhibited a more moderate decline, while Martaínha remained comparatively stable across the water status gradient. Taken together, these results indicate that antioxidant-related traits were strongly shaped by cultivar-specific adjustments along the Ψ_{wmid} gradient.

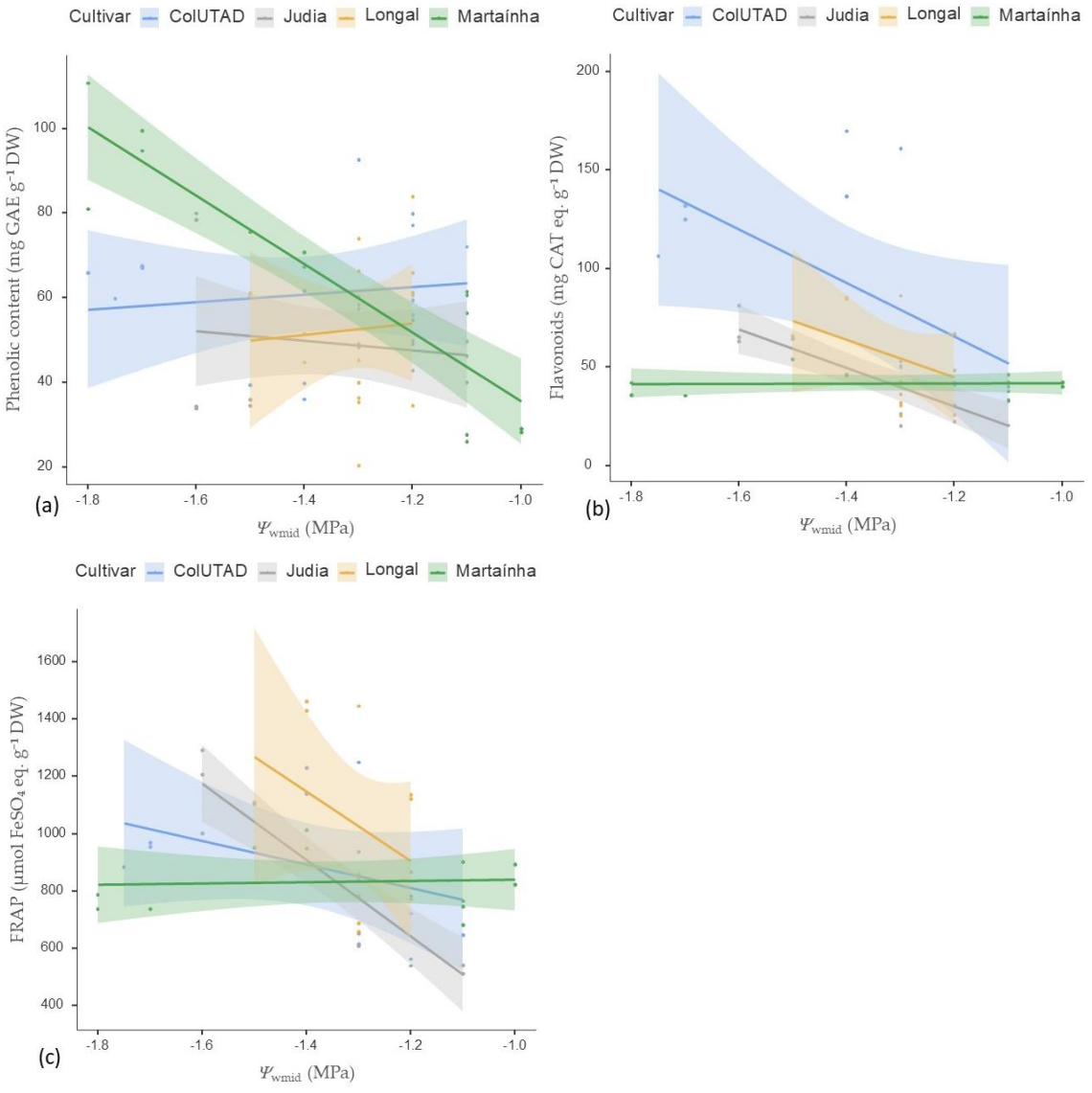


Fig. 7. Relationships between midday stem water potential (Ψ_{wmid}, MPa) and antioxidant-related traits in four chestnut cultivars, ColUTAD®, Judia, Longal and Martaínha, across all observations. **(a)** Phenolic compounds in relation to Ψ_{wmid}. **(b)** Flavonoid content in relation to Ψ_{wmid}. **(c)** Ferric reducing antioxidant power (FRAP) in relation to Ψ_{wmid}. Each point represents one biological replicate. Solid lines represent the fitted linear regressions for each cultivar, and shaded areas indicate the 95% confidence intervals.

3.3. Integrated heatmap of physiological and biochemical traits across the proposed midday stem water potential threshold

To provide an integrated overview of trait coordination across the proposed water status threshold, a heatmap based on standardized mean values was constructed for all physiological and biochemical variables grouped by cultivar and Ψ_{wmid} class (> -1.2 MPa and ≤ -1.2 MPa) (Fig. 8). Overall, the heatmap highlighted contrasting cultivar-specific response profiles rather than a single uniform shift below the threshold. ColUTAD® was characterised by consistently low RWC, *A* and *E* together with high electrolyte leakage and flavonoid levels, indicating a less favourable functional status across both Ψ_{wmid} classes. Judia maintained high RWC and soluble sugar levels, combined with comparatively low electrolyte leakage,

phenolic and flavonoid values. Longal showed the highest *A*, *E* and FRAP values, but also the lowest soluble sugar and starch levels, suggesting a response profile more strongly associated with gas exchange and antioxidant capacity than with carbon storage. Martaínha was distinguished by high starch under both Ψ_{wmid} classes and by marked increases in phenolics, proline and iWUE at $\Psi_{wmid} \leq$ -1.2 MPa, consistent with a stronger biochemical adjustment under lower water status. Taken together, this integrated view highlights that the reorganisation of physiological and biochemical traits around the proposed Ψwmid threshold was strongly cultivar-dependent.

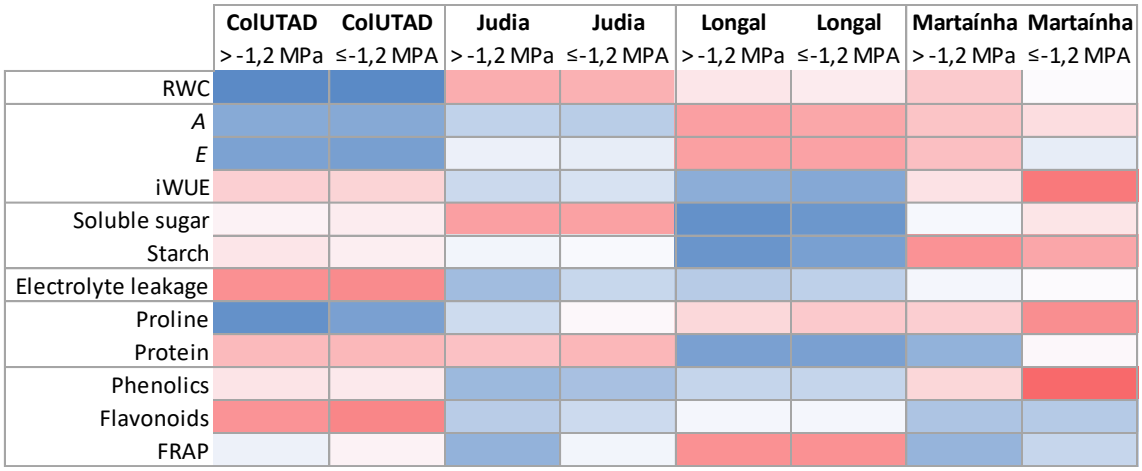


Fig. 8. Heatmap showing the relative variation of physiological and biochemical traits in four chestnut cultivars, ColUTAD®, Judia, Longal and Martaínha, grouped according to midday stem water potential threshold classes, > -1.2 MPa and ≤ -1.2 MPa. Values represent standardized mean values, z-scores, calculated for each variable within each cultivar × water status group. Red tones indicate above-average values for a given trait, whereas blue tones indicate below-average values.

4. Discussion

The present results should be interpreted in continuity with the 2022 field campaign previously reported by Marques et al., 2025, conducted in the same orchard network and with the same cultivar panel. However, the 2023 dataset adds an important new dimension by integrating plant water status, gas exchange, osmotic adjustment, oxidative markers and an overall threshold-based heatmap into the same interpretative framework. In this broader context, the cultivar responses observed in 2023 do not contradict those described for 2022. Rather, they reinforce the idea that drought resilience in chestnut is dynamic and year-dependent, with the relative prominence of each cultivar varying according to the seasonal configuration of water availability, heat load and drought progression. This interpretation is consistent with the growing view that sweet chestnut performance under Mediterranean conditions must be understood within a climate-sensitive and site-dependent framework, rather than as a fixed varietal ranking (Conedera et al., 2021; Freitas et al., 2021; Gomes-Laranjo et al., 2009).

A key difference between years lies in the climatic sequence preceding and accompanying summer drought. In 2022, summer conditions were generally hotter, which likely intensified thermal and metabolic stress. In 2023, by contrast, several orchards combined greater precipitation from winter to early summer with a sharper rainfall collapse during July and August. Thus, 2023 did not simply reproduce the 2022 drought scenario at a different intensity. Rather, it imposed a different seasonal progression of soil water recharge, drought onset and internal dehydration, likely altering the balance between hydraulic maintenance, gas exchange and biochemical activation (Chaves et al., 2010; Connor and Fereres, 2004).

Under Mediterranean conditions, chestnut responses are known to be strongly modulated by local climatic structure, elevation and seasonal temperature dynamics, which helps explain why cultivar performance may vary substantially across years and environments (Freitas et al., 2021; Gomes-Laranjo et al., 2009).

This study also differs from a controlled drought experiment because drought was not artificially imposed. Instead, cultivar responses were examined across a natural edaphoclimatic gradient, complemented by plant-based indicators of water status, particularly Ψ_{wmid} and RWC. This point is important for interpretation. Three orchards were rainfed, whereas Marvão received maintenance irrigation during summer, and absolute drought intensity therefore differed among sites. For this reason, the discussion is centred primarily on plant water status rather than on site identity alone. In field-grown chestnut, Ψ_{wmid} provides an integrative physiological reference because it reflects the actual degree of internal water limitation experienced by the tree and can therefore be used to interpret its consequences for gas exchange, membrane integrity and biochemical adjustment under realistic orchard conditions (Gomes-Laranjo et al., 2006; Martins et al., 2010; Mota et al., 2018).

4.1. Nutrient status and environmental background

Leaf mineral composition should be interpreted cautiously and mainly as contextual information. The descriptive macronutrient and micronutrient profiles indicate substantial edaphic heterogeneity among orchards and suggest that nutrient availability and uptake may have contributed to site-level performance differences. This is physiologically plausible, since nutrient mobility in soil and nutrient acquisition by roots are strongly influenced by soil moisture, rainfall distribution and root-zone activity during the growing season. In perennial tree crops, nutrient balance is therefore not independent of climatic context, but part of the broader environmental background that shapes drought responses (Connor and Fereres, 2004; Menéndez-Miguélez et al., 2015). At the same time, because mineral composition was treated descriptively in This study and not used as the main inferential basis of cultivar ranking, it is more appropriate to interpret it as supporting context than as a direct marker of drought tolerance.

Even so, the nutrient data help reinforce the view that field drought responses emerge from the interaction between genotype and environment. In orchards receiving greater precipitation before peak summer stress, plants likely entered the critical period with improved water recharge and potentially greater nutrient accessibility in the soil profile. Under these circumstances, cultivars better able to preserve nutrient balance while maintaining plant hydration may have gained a functional advantage. This interpretation remains cautious, but it is consistent with the broader literature showing that chestnut productivity and physiological functioning are shaped not only by genotype and climate, but also by altitude, soil conditions and nutrient availability (Freitas et al., 2021; Gomes-Laranjo et al., 2009; Menéndez-Miguélez et al., 2015).

4.2. Water relations as the primary constraint on physiological performance

Among all variables examined, plant water status emerged as the most consistent integrative dimension of drought response in 2023. Martaínha generally maintained less negative Ψ_{wmid} values and higher RWC, particularly at Penela da Beira and Marvão, whereas ColUTAD® consistently showed more negative Ψ_{wmid} values and lower leaf hydration. The positive relationship between Ψ_{wmid} and RWC was significant across the full dataset and remained stable across cultivars, indicating that declining plant water

status translated directly into reduced leaf hydration irrespective of genotype. This suggests that hydraulic status represented a common physiological constraint across cultivars, even though the absolute degree of stress differed among them.

This point is especially informative when compared with the 2022 campaign. In that earlier study, cultivar differentiation was more strongly expressed through osmotic and antioxidant traits. In 2023, by contrast, the clearest distinction among genotypes was associated with the ability to preserve tissue hydration and avoid severe internal stress. Such a shift is plausible under Mediterranean field conditions, where antecedent moisture availability and the seasonal timing of rainfall can strongly influence drought progression and tree water status (Connor and Fereres, 2004; Freitas et al., 2021). Chestnut eco-physiological studies have likewise shown that geographical context, altitude and environmental variability strongly affect photosynthetic behaviour and plant water relations, reinforcing the idea that hydraulic performance under field drought is highly context-dependent (Gomes-Laranjo et al., 2012, 2009, 2006).

Previous studies have identified Ψ_{wmid} values below approximately −1.2 MPa as physiologically relevant for chestnut, marking the onset of water limitation associated with declining stomatal conductance and carbon assimilation (Gomes-Laranjo et al., 2006; Mota et al., 2018). The present results support the relevance of this threshold, but they also suggest that the most pronounced functional decline occurred closer to approximately −1.4 MPa, when photosynthesis and transpiration decreased more sharply and electrolyte leakage became more evident. In this sense, Ψ_{wmid} around −1.2 MPa appears to represent a meaningful transition zone, whereas values approaching or exceeding −1.4 MPa indicate more severe impairment. This distinction is important because it avoids reducing drought response to a single absolute cut-off and instead recognizes a progressive deterioration in plant function along the water status gradient.

4.3. Gas exchange strategies under declining water status

Gas exchange responses added a functional layer to the interpretation of cultivar performance. Net photosynthesis and transpiration were both positively related to Ψ_{wmid}, confirming that less negative water potentials were associated with greater physiological activity. However, the two variables did not behave identically. Photosynthesis remained significantly related to Ψ_{wmid} without significant cultivar dependence, indicating that carbon assimilation responded to water status in a generally similar way across genotypes. Transpiration, by contrast, showed a stronger overall relationship with Ψ_{wmid} and a significant cultivar-dependent pattern, indicating that cultivars differed more clearly in how stomatal water loss was regulated along the water status gradient.

This distinction is biologically meaningful. It suggests that transpiration captured differences in water-use strategy more clearly than photosynthesis. Longal and Martaínha sustained the highest transpiration responses across the fitted gradient and also maintained comparatively high photosynthetic performance, indicating a more functionally active strategy under moderate water limitation. Judia occupied an intermediate position. ColUTAD®, in contrast, showed the lowest transpiration values and reduced carbon gain, indicating stronger restriction of gas exchange. Similar contrasts between active gas exchange maintenance and conservative water-use strategies have been described in Mediterranean woody species, where drought-tolerant performance depends not only on limiting water loss, but also on preserving

sufficient assimilation under progressive stress (Bacelar et al., 2007; Connor and Fereres, 2004; Schultz, 2003).

Instantaneous water-use efficiency further clarified these differences. When all observations were pooled, iWUE increased under more negative Ψ_{wmid} values. However, once cultivar was included in the model, the relationship became clearly cultivar-dependent. This indicates that higher iWUE under stress cannot be interpreted uniformly as a sign of superior performance. In ColUTAD®, elevated iWUE was associated with strong transpiration restriction and low assimilation, which reflects conservative water use at the expense of carbon gain rather than efficient maintenance of productivity. Similar trade-offs have been described in chestnut and other woody Mediterranean species, where apparent increases in efficiency may result from stronger stomatal limitation rather than from better physiological coordination (Bacelar et al., 2007; Martins et al., 2010; Schultz, 2003) In contrast, Martaínha and Longal appear to have maintained a more favourable coordination between water status and gas exchange.

Taken together, these results indicate that, under the 2023 climatic configuration, drought resilience was more closely associated with the capacity to sustain gas exchange under moderate stress than with conservative water use alone. This helps explain why Martaínha emerged as the most favourable integrated performer in 2023. Its advantage did not derive from the strongest biochemical overactivation, but from the maintenance of a more favourable balance between plant hydration and physiological activity as drought intensified.

4.4. Carbon metabolism, osmotic adjustment and membrane stability

Non-structural carbohydrates showed a more limited and less uniform relationship with instantaneous plant water status. Soluble sugars displayed no significant overall linear relationship with Ψ_{wmid}, yet their response became cultivar-dependent when genotype was included in the model. This indicates that soluble sugar accumulation did not follow a single common pattern across the dataset but instead reflected cultivar-specific adjustment. Starch was even less tightly coupled to Ψ_{wmid}, with neither the overall relationship nor the cultivar terms reaching significance. These findings suggest that carbohydrate pools in field-grown chestnut are regulated by multiple interacting processes, including genotype, seasonal carbon balance and the timing of remobilization, rather than by midday water potential alone. This interpretation is consistent with previous work showing that soluble sugars and starch may become partially decoupled from instantaneous water status under field conditions, especially in perennial species where carbon allocation reflects both short-term stress and longer-term metabolic regulation (Chaves et al., 2003; Ghosh et al., 2021; Krasensky and Jonak, 2012).

In contrast, electrolyte leakage responded more clearly to declining plant water status and did so in a cultivar-dependent manner. EL increased as Ψ_{wmid} became more negative, and ColUTAD® maintained the highest fitted values across the gradient. This is a key result because it directly links hydraulic deterioration with membrane destabilisation. Judia, Longal and Martaínha generally showed lower EL values, indicating better preservation of membrane integrity under stress. In this sense, EL was more informative than most biochemical markers for distinguishing between coordinated regulation and physiological failure. Similar use of membrane leakage as an indicator of stress injury has been reported in chestnut-related

ecophysiological studies and more broadly in woody plants exposed to abiotic stress (Abu-Muriefah, 2015; Carneiro-Carvalho et al., 2019; Wang et al., 2009).

Proline and soluble protein contents also increased under declining water status, but unlike EL they did not show significant cultivar-dependent responses. Their accumulation therefore appears to represent a more general stress-associated adjustment shared across genotypes rather than a strong discriminator of cultivar resilience. This is important for interpretation. It suggests that proline and soluble protein contents track drought intensity to some extent, but do not by themselves explain why some cultivars performed better than others in 2023. This agrees with the broader literature, where proline is often interpreted as a context-dependent marker of stress intensity and osmotic adjustment rather than a universal indicator of effective tolerance (Ghosh et al., 2021; Szabados and Savouré, 2010).

4.5. Antioxidant responses: effective regulation versus stress overactivation

The antioxidant-related traits revealed some of the clearest cultivar-dependent patterns in the study. Phenolics, flavonoids and FRAP all showed negative overall relationships with Ψ_{wmid}, but their fitted responses differed substantially among cultivars. This indicates that antioxidant activation was not a single generic response to drought, but part of genotype-specific regulatory strategies.

Phenolic compounds were especially informative in Martaínha, which showed the highest fitted phenolic levels under more negative Ψ_{wmid} values and the steepest decline along the gradient. This pattern suggests that phenolic activation in Martaínha was responsive and dynamic but not dissociated from hydraulic performance. Importantly, Martaínha combined this pattern with relatively low electrolyte leakage and better water status, which argues against interpreting phenolic accumulation in this genotype as a symptom of physiological collapse. The role of phenolics in stress buffering and redox regulation is well established, but their functional value depends on how they are integrated with the overall physiological response of the plant (Michalak, 2006; Sharma et al., 2019).

Flavonoids showed a different pattern. ColUTAD® displayed the highest flavonoid levels under more negative Ψ_{wmid} values and the steepest decline across the gradient, whereas Martaínha maintained lower and more stable flavonoid values. This contrast is highly informative. In ColUTAD®, strong flavonoid accumulation coincided with high electrolyte leakage and poor water status, suggesting that antioxidant upregulation reflected stress intensity and cellular injury rather than effective protection. Similar situations have been described in other woody crops, where strong biochemical activation accompanies damage rather than conferring true tolerance (Procházková et al., 2011; Romero et al., 2004; Schultz, 2003).

FRAP further supports a cultivar-dependent interpretation of antioxidant adjustment. Longal showed the highest FRAP values under more negative Ψ_{wmid} conditions and a marked decline along the gradient, whereas Judia also showed a strong decrease. ColUTAD® showed a more moderate decline, while Martaínha remained comparatively stable. This suggests that antioxidant reducing capacity was mobilized differently among cultivars and should not be interpreted in isolation. High FRAP may reflect strong inducible antioxidant capacity, as in Longal, but its physiological meaning depends on whether it is accompanied by preserved gas exchange and membrane stability, or by hydraulic deterioration and cellular damage. The same general principle has been reported in studies of oxidative balance under abiotic stress,

in which antioxidant capacity must be interpreted together with the magnitude of tissue injury and physiological dysfunction (Michalak, 2006; Sharma et al., 2019; Wang et al., 2009).

A central conclusion emerging from these patterns is that strong biochemical activation does not automatically equate to drought tolerance. ColUTAD® showed substantial antioxidant-related activation, especially for flavonoids, yet also showed the strongest membrane damage and the poorest hydraulic status. Martaínha, by contrast, did not exhibit the most extreme antioxidant signature across all variables, but combined lower membrane damage with better water status and sustained physiological activity. This supports the interpretation that drought tolerance in chestnut depends less on the magnitude of antioxidant induction per se and more on the coordination of biochemical responses with hydraulic stability and gas exchange (Michalak, 2006; Wang et al., 2009).

4.6. Integrated response syndromes and implications for cultivar evaluation

The integrated heatmap provides a useful synthesis of the response patterns described above. Rather than revealing a single uniform shift below the proposed Ψ_{wmid} threshold, it highlights contrasting cultivar-specific profiles. ColUTAD® was characterised by consistently low RWC, A and E together with high electrolyte leakage and flavonoid levels, indicating a less favourable functional status under both water-status classes. Judia maintained relatively high RWC and soluble sugar levels, together with comparatively low EL, phenolic and flavonoid values. Longal combined high A, E and FRAP with low soluble sugar and starch levels, suggesting a profile centred more on active physiological function and antioxidant capacity than on carbon storage. Martaínha was distinguished by high starch under both classes and by marked increases in phenolics, proline and iWUE at $\Psi_{wmid} \leq -1.2$ MPa, consistent with a more coordinated biochemical adjustment under lower water status.

This integrated view helps clarify the comparison between years. In 2022, Judia and Longal appeared comparatively more prominent because osmotic and antioxidant adjustment were especially conspicuous under that year's climatic structure. In 2023, Martaínha emerged as the strongest integrated performer because it combined less negative water potential, higher leaf hydration, sustained gas exchange and controlled biochemical activation. These are not contradictory outcomes. Rather, they show that cultivars differ not only in the intensity of stress they can tolerate, but also in the type of response syndrome they express under different seasonal drought configurations. Such plasticity is entirely plausible in chestnut, a species whose behaviour is strongly modulated by altitude, climate and environmental heterogeneity (Ciordia et al., 2012; Conedera et al., 2021; Gomes-Laranjo et al., 2009).

The present results therefore support a broader interpretation of drought resilience in chestnut. Resilience should not be identified with any single variable, whether hydraulic, gas-exchange or biochemical. Nor should it be inferred from strong antioxidant activation alone. Instead, drought resilience in chestnut appears to arise from coordinated trait syndromes in which hydraulic stability, maintenance of physiological activity and controlled biochemical adjustment are integrated at the whole-plant level. This conclusion is consistent with observations in other Mediterranean perennial crops, where tolerance likewise depends on coordination among water relations, gas exchange and oxidative regulation rather than on the overexpression of any isolated stress marker (Bacelar et al., 2007; Connor and Fereres, 2004; Girona et al., 1993; Romero et al., 2004; Schultz, 2003).

From an applied perspective, these findings suggest that cultivar evaluation under Mediterranean conditions should prioritize integrated physiological assessment, with particular emphasis on plant water status and its consequences for gas exchange, membrane integrity and stress-related metabolism. The proposed Ψ_{wmid} threshold around −1.2 MPa appears physiologically meaningful as a transition zone, while more negative values approaching −1.4 MPa are associated with sharper functional impairment. At the same time, caution remains necessary. The present interpretation is based on two contrasting field campaigns, includes one site with maintenance irrigation, and does not incorporate direct yield assessment. Broader agronomic extrapolation will therefore require validation across additional years, environments and production outcomes.

5. Conclusion

Under projected Mediterranean scenarios of increasing drought and heat, this study highlights clear genotype-dependent drought response strategies in *C. sativa* under field conditions. When interpreted together with the 2022 field campaign previously reported by Marques et al., 2025, the results show that cultivar performance cannot be reliably reduced to a fixed ranking based on a single season. Instead, drought resilience in chestnut should be understood as the capacity of each genotype to reorganize its physiological and biochemical responses according to the specific climatic configuration of each year.

Under the 2023 climatic configuration, Martaínha showed the most favourable integrated response, combining better hydraulic status, higher leaf hydration and sustained photosynthetic performance under restrictive conditions. Longal displayed high physiological plasticity and maintained favourable gas-exchange performance across the water status gradient, whereas Judia showed effective biochemical and gas-exchange responses but greater sensitivity to water limitation. In contrast, ColUTAD® exhibited strong biochemical activation associated with hydraulic failure and membrane damage, indicating stress sensitivity rather than effective drought tolerance. Taken together, the 2022 and 2023 campaigns indicate that drought resilience in Portuguese chestnut cultivars emerges from coordinated trait syndromes rather than from any single physiological or biochemical marker. While the 2022 campaign emphasised the relevance of osmotic and antioxidant adjustment, the 2023 results placed greater weight on hydraulic stability, leaf hydration and the capacity to sustain physiological activity as drought intensified.

Overall, these findings support cultivar selection matched to local soil and climatic conditions as a low-input strategy to improve drought adaptation and enhance the long-term sustainability of chestnut orchards. However, broader agronomic recommendations should remain cautious until these response patterns are validated across additional years, environmental scenarios and, ideally, direct yield assessments.

Author Contributions

T.M. conceived and conducted the study. T.M., A.F.-P. and A.A. performed biochemical analyses. T.P. and J.G.-L. supervised the project. All authors reviewed and approved the final manuscript.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Data will also be deposited in a public repository (e.g., Figshare) following article acceptance, in accordance with the *Acta Physiologiae Plantarum ae* data sharing policy.

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Conflicts of Interest

The authors declare no conflict of interest.

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