

Article

Drought-Induced Antioxidant and Biochemical Responses in *Castanea sativa* Cultivars: A Mediterranean Case Study

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Abstract

Chestnut (*Castanea sativa* Mill.) is a key crop in Mediterranean regions increasingly threatened by recurrent drought stress. To investigate cultivar-specific tolerance mechanisms, we evaluated four Portuguese cultivars (Longal, Judia, Martainha, and ColUTAD[®]) across four orchards with contrasting water regimes. Physiological (midday stem water potential— Ψ_{wmid} , soluble sugars, electrolyte leakage and proline) and biochemical traits (phenolics, flavonoids, catalase, peroxidase, ascorbate peroxidase and ferric reducing antioxidant power) were quantified under a natural drought gradient. Results revealed that environmental factors had a stronger influence than genetic background. Longal showed robust osmotic adjustment with high proline and soluble sugar levels, alongside stable starch reserves; Judia relied on inducible antioxidant activity, particularly peroxidase and ascorbate peroxidase; and Martainha exhibited intermediate plasticity, whereas ColUTAD[®] was consistently stress-sensitive, with weaker defences and greater membrane damage. Clustering analysis confirmed that location effects outweighed cultivar differences, separating orchards into conservative strategies (better water balance, higher starch, stronger peroxidase activity) and stress-adaptive strategies (enhanced enzymatic antioxidants). Overall, resilience in chestnut is not determined by a single trait but by a synergistic network of osmotic regulation, membrane protection, and antioxidant activity. Traits such as proline accumulation, starch stability, and inducible enzyme activation emerged as reliable biochemical indicators of tolerance. These findings provide a physiological basis for selecting climate-resilient cultivars and designing site-specific management strategies, thereby supporting the sustainability of chestnut production under Mediterranean climate change scenarios.

Keywords: *Castanea sativa*; drought stress; antioxidant enzymes; osmotic adjustment; cultivar resilience; mediterranean agriculture



Academic Editor: Mercè Llugany

Received: 1 October 2025

Revised: 13 November 2025

Accepted: 17 November 2025

Published: 20 November 2025

Citation: Marques, T.; Ferreira-Pinto, A.; Fevereiro, P.; Pinto, T.; Gomes-Laranjo, J. Drought-Induced Antioxidant and Biochemical Responses in *Castanea sativa* Cultivars: A Mediterranean Case Study. *Agriculture* **2025**, *15*, 2401. <https://doi.org/10.3390/agriculture15222401>

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<https://doi.org/10.3390/agriculture15222401>

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1. Introduction

Climate change is increasing the frequency and intensity of drought events in Mediterranean regions, posing a major threat to agricultural systems [1]. Chestnut (*Castanea sativa* Mill.), a key species in these areas, is particularly vulnerable to summer drought, which is often accompanied by high temperatures, leading to combined stress conditions [2,3].

Under drought stress, plants activate conserved signalling pathways centred on abscisic acid (ABA), which triggers stomatal closure and the expression of genes involved in osmoprotection and antioxidant defence [4,5]. A major consequence of this is the accumulation of reactive oxygen species (ROS) [6].

Plants use a complex antioxidant system to protect against oxidative damage. This system includes enzymes like catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), as well as non-enzymatic compounds like phenolics and osmolytes (such as proline and soluble sugars) [7,8].

At the same time, heat stress causes a different but related response, which includes a rapid production of heat shock factors (HSFs) and heat shock proteins (HSPs) that act as molecular chaperones to keep proteins from denaturation [9]. Drought and heat can both damage membranes, reduce photosynthesis, and cause ROS to build up, which means that both antioxidant and protective responses are needed [6,9].

C. sativa is well-suited to temperate climates, yet it cannot thrive effectively when it lacks sufficient water. Its orchards are ecologically and economically vital in the Mediterranean, supporting rich biodiversity including endangered species [10]. Nonetheless, the antioxidant and biochemical reactions of Portuguese *C. sativa* cultivars to simultaneous drought and heat stress in practical field conditions are still poorly comprehended.

This research examines the physiological and biochemical adaptations of four Portuguese chestnut cultivars growing along a natural drought gradient. We aimed to (i) assess cultivar-specific strategies in water relations, osmotic adjustment, and membrane stability, and (ii) identify essential enzymatic and non-enzymatic antioxidant characteristics linked to resilience. Our results offer guidance for the selection of climate-resilient cultivars and the enhancement of chestnut orchard management in Mediterranean areas experiencing climate change.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The study was conducted during the 2022 growing season across four orchards in Portugal: three non-irrigated, the three locations situated in Trás-os-Montes (Parada, Carrazedo de Montenegro, and Penela da Beira) and one irrigated, located in Portalegre (Marvão) (Table 1) [11]. Each orchard contained the four distinct genotypes under study: the three Portuguese cultivars Judia, Longal, and Martaínha, plus the hybrid ColUTAD[®]. All trees were 4 years old at the time of sampling. Each of these four genotypes was represented by nine individual trees, resulting in a total of 36 experimental units (4 genotypes × 9 biological replicates) per orchard. The same trees were sampled throughout the study. Soils ranged from well-drained silty textures to loamy sands.

Table 1. Location, Protected Designation of Origin (PDO), coordinates, altitude (metres above sea level—m a.s.l.), and water regime of the four demonstration orchards (DO) used in the study.

DO	PDO	Coordinates	Altitude (m a.s.l.)	Water Regime
Carrazedo de Montenegro (CM)	Padrela	41°33′41.76″ N; 7°25′51.41″ W	765	Non-irrigated
Parada (PB)	Terra Fria	41°38′12.53″ N; 6°42′42.94″ W	740	Non-irrigated
Penela da Beira (PBP)	Soutos da Lapa	41°01′38″ N; 7°26′38″ W	885	Non-irrigated
Porto da Espada (MRV)	Marvão–Portalegre	39°21′19.5″ N 7°21′40.1″ W	583	Irrigated

2.2. Climatic Data

Climatic information was obtained from IPMA and SNIRH meteorological stations for 2022. Ombrothermic diagrams were constructed following Gaussen's method (Figure 1) to characterise seasonal drought and humidity patterns across the four sites.

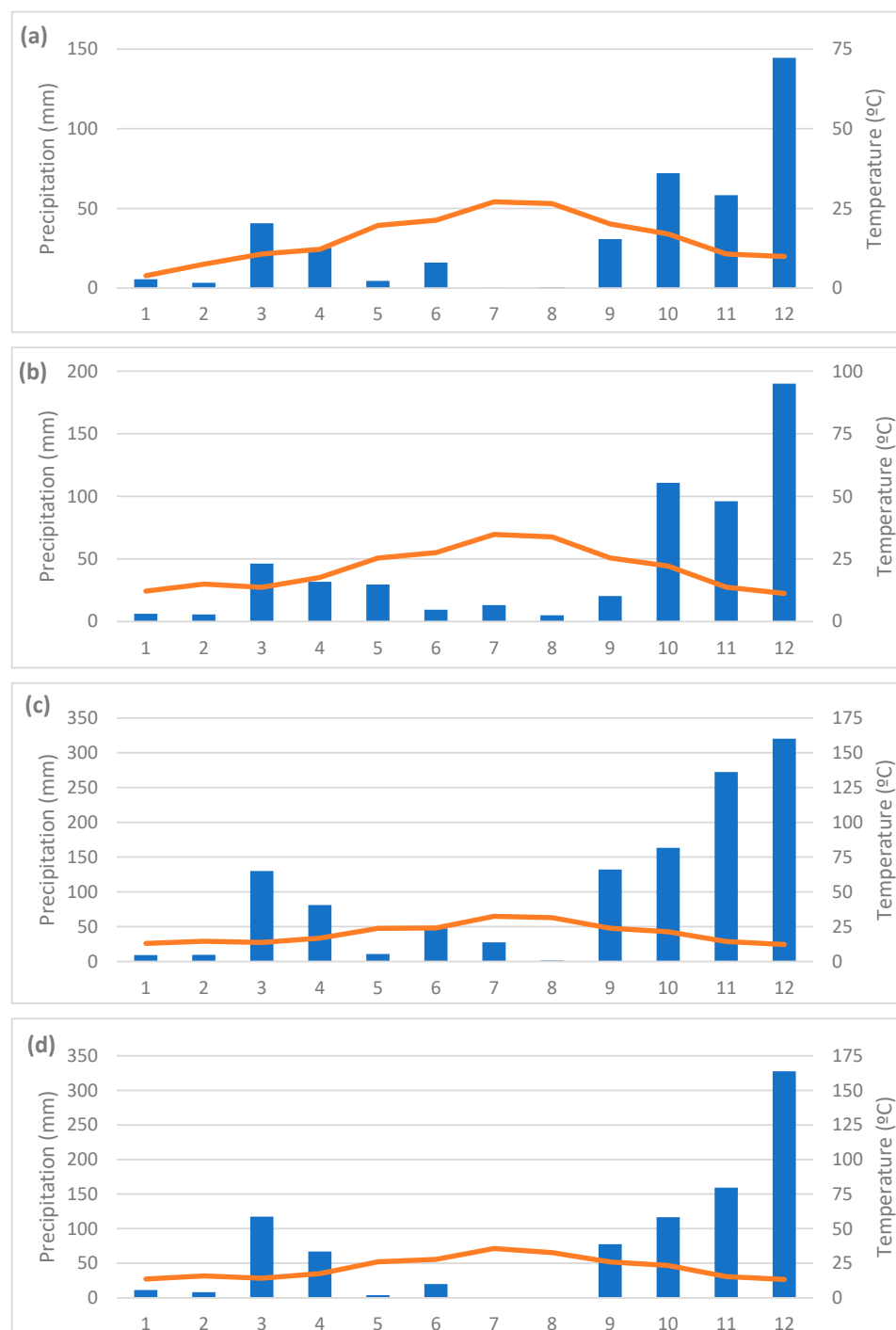


Figure 1. Ombrothermic diagrams (Gaussen's method) for the four demonstration orchards in 2022: (a) Carrazedo de Montenegro, (b) Parada, (c) Penela da Beira, (d) Porto da Espada (Marvão). The charts depict monthly precipitation (bars) and mean temperature (line). Biologically dry periods, indicative of drought stress, occur when the precipitation curve falls below twice the temperature curve (2 T).

2.3. Leaf Sampling Protocol

To ensure standardisation, leaf samples for all biochemical analyses were collected on clear days between 10:00 and 12:00 local time. Fully expanded, sun-exposed leaves from the upper, outer canopy (northern side) were selected. For each cultivar at each location, six independent biological replicates (leaves from six different trees) were collected, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

2.4. Stem Water Potential

Midday stem water potential (Ψ_{wmid}) was measured in August 2022 using a Scholander pressure chamber (PMS Instrument Company, Albany, NY, USA). For each cultivar and location, nine leaves (3 leaves in 3 trees) were bagged in reflective plastic for at least 20 min prior to excision to achieve equilibrium.

All spectrophotometric assays were performed with six biological replicates ($n = 6$), and each measurement was conducted in technical duplicate.

2.5. Determination of Total Phenolic Compounds

To assess the antioxidant potential of phenolic compounds in plants under drought stress, total phenolics were analysed using the Folin–Ciocalteu spectrophotometric method. Gallic acid served as the standard, chosen for its close structural similarity to typical phenolic substances found in plants.

Leaf samples were prepared by punching out four discs of 8 mm in diameter from each, followed by extraction in 10 mL of 80% acetone (v/v). The total phenolic content was then evaluated at 795 nm using the protocol established by [12], with a standard curve derived from gallic acid. For quantification, 5 mL of the acetone extract was used. A standard curve was constructed (concentration range: 0–100 $\mu\text{g/mL}$, $R^2 > 0.99$). Results are expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW).

2.6. Determination of Soluble Sugars and Starch

Soluble sugars and starch were quantified to assess the plant's carbohydrate metabolism and osmotic regulation mechanisms in response to drought 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 using the colorimetric technique described by Irigoyen et al. [13] in 80% ethanol (v/v), with absorbance measured at 625 nm. The 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. [14]. Glucose was used as a standard for both soluble sugars and starch quantification.

2.7. Electrolyte Leakage

Electrolyte leakage content were analysed to evaluate cellular damage under drought stress. This parameter is widely used as indicator of membrane integrity [15].

Electrolyte leakage was determined following a modified version of the method of [16]. Leaf discs were incubated in double-distilled water at 25°C for 24 h with continuous shaking, and conductivity was measured with a conductivity meter (Mettler Toledo AG8603 Schwerzenbach, Switzerland) before and after autoclaving. Electrolyte leakage (EL) was expressed as the ratio $C1/C2$, where $C1$ is the initial conductivity and $C2$ is the conductivity after autoclaving.

2.8. Quantification of Soluble Protein Content

The quantification of soluble proteins was carried out to evaluate changes in the plant proteome under drought stress conditions. Variations in soluble protein content can

indicate shifts in protein synthesis, turnover, and degradation, thereby providing valuable insights into the plant's stress adaptation strategies and metabolic adjustments.

The total soluble protein (SP) content was determined spectrophotometrically using bovine serum albumin as the standard, following the Bradford method [17].

2.9. Proline Quantification

Leaf samples from chestnut plants were collected for proline determination, which was performed according to the method of [18] with minor modifications. To prepare the acid-ninhydrin reagent, 1.25 g of ninhydrin was dissolved in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid by heating and stirring. When stored at 4 °C, the reagent remained stable for up to 24 h.

Briefly, leaf discs (0.5 g FW) were homogenised 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, Darmstadt, Germany) was used for the standard curve.

2.10. Enzymatic Activity Determination

Leaf discs (8 mm) were ground in liquid nitrogen and homogenised in 1–2 mL of 100 mM phosphate buffer (pH 7.0) containing 2% PVP. Extracts were centrifuged at $10,000\times g$ for 10 min at 4 °C and the supernatants were kept on ice and used for CAT, POD and APX determinations.

CAT activity was analysed according to [19] with small modifications. 200 μ L microplate reactions containing 100 mM phosphate buffer (pH 7.0), sample extract (10–50 μ L depending on activity) and H_2O_2 (10–20 μ L of a $10\times$ diluted stock). After a brief 1–2 min incubation with shaking, the decrease in absorbance at 240 nm was monitored. Activity was calculated from the rate of H_2O_2 decomposition and expressed as U mg^{-1} protein. No deviations from the standard procedure were required.

POD activity was measured in [20] 200 μ L reactions containing 100 mM phosphate buffer (pH 7.0), guaiacol (10 μ L of a 20 mM stock), H_2O_2 (10 μ L of a 10 mM stock) and 10–50 μ L of enzyme extract. Absorbance was recorded at 336 nm and 470 nm. One unit of POD was defined as the amount increasing absorbance by 0.100 units per minute. Minor adjustments included reaction miniaturisation to microplates and optimisation of sample volume.

APX activity was determined in 150 mM sodium phosphate buffer (pH 7.5) with 0.75 mM EDTA. The 200 μ L reaction contained sample extract (10–50 μ L), 5 μ L ascorbic acid (10 mM) and 10 μ L H_2O_2 (10 mM). The decrease in absorbance at 290 nm was monitored and activity calculated using $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. The method followed [21], with only the adaptation to microplate format.

2.11. Total Flavonoid Content

The total flavonoid content (TFC) was quantified using a colorimetric assay [22,23]. A mixture was prepared by combining diluted extracts (1 mL), bi-distilled water (4 mL), and a 5% *w/v* NaNO_2 solution (0.3 mL). AlCl_3 solution (10% *w/v*, 0.3 mL) was added and allowed to stand for 6 min after shaking and remaining at room temperature for 5 min. After that, 2 mL of NaOH (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, Darmstadt, Germany) solutions (0–10 mg mL^{-1}) were also assayed and calibration curve was obtained from the equation: $y = 0.029x + 0.188$; $R^2 = 0.993$. A blank assay, 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).

2.12. Ferric Reducing Antioxidant Power (FRAP) Activity

The FRAP assay was performed according to [24], with minor modifications for use in 96-well microplates. Fresh FRAP reagent was prepared daily by mixing sodium acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution (in 40 mM HCl), and 20 mM FeCl₃ solution in a 10:1:1 (v/v/v) ratio. In each well, 25 µL of extract was added to 275 µL of FRAP reagent. The microplates were incubated at room temperature in the dark for 5 min, and absorbance was measured at 593 nm using a microplate reader. FeSO₄ was used as the standard. Results are expressed as mean ± SE in µmol FeSO₄ equivalents per gram of dry weight (µmol FeSO₄ eq. g⁻¹ DW).

2.13. Statistical Analysis

Statistical significance of differences among cultivars within each location was assessed using one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Different letters indicate significant differences. Multivariate analysis (hierarchical clustering with Ward's linkage and Euclidean distance) was used to group cultivars and locations based on all standardised traits.

3. Results

A one-way ANOVA revealed significant effects of cultivar on all measured physiological and biochemical traits within each location ($p < 0.05$). Significant differences among cultivars at each site are indicated by different letters in Table 2.

Stem water potential (Ψ_{wmid}) varied significantly among locations, ranging from −0.59 MPa (PBP) to −1.56 MPa (PB) (Table 2), indicating greater soil water retention in PBP. Across sites, COL consistently showed the lowest values, while JUD, LON, and MRT displayed increases of up to 49% (Mv) relative to COL. Cultivars grafted onto the COL rootstock generally exhibited stronger declines in water potential under drought, particularly in PB and CM, with the COL rootstock itself being the most affected. This confirms distinct, cultivar-specific responses to water stress.

Total soluble sugars (TSS) varied significantly among locations with the highest concentration in PB (104.8 mg g⁻¹ DW) and the lowest in PBP and Mv (81.7 mg g⁻¹ DW) (Table 2). Among cultivars, TSS ranged from 83.2 mg g⁻¹ DW (LON) to 93.4 mg g⁻¹ DW (COL). A significant negative correlation was observed between TSS and stem water potential ($R^2 = 0.47$; Figure 2). The range of TSS values among cultivars widened with decreasing Ψ_{wmid} , from 19.1 mg g⁻¹ DW in PBP to 26.3 mg g⁻¹ DW in PB.

Total starch content (TSC) differed significantly among locations and cultivars (Table 2). The highest TSC was recorded in PBP (147.7 mg g⁻¹ DW) and the lowest in PB (39.0 mg g⁻¹ DW). Across cultivars, TSC ranged from 80.2 mg g⁻¹ DW (LON) to 101.8 mg g⁻¹ DW (MRT). The variation in TSC among cultivars was highest in PBP (66.6 mg g⁻¹ DW) and decreased with drought intensity to 11.1 mg g⁻¹ DW in PB. A positive correlation was observed between Ψ_{wmid} and TSC ($R^2 = 0.71$; Figure 3). The range of TSC values among cultivars narrowed with decreasing Ψ_{wmid} , from 66.6 mg g⁻¹ DW in PBP to 11.1 mg g⁻¹ DW in PB.

Table 2. Midday stem water potential (Ψ_{wmid}), metabolite content, oxidative stress markers, and antioxidant enzyme activities in four chestnut cultivars across four locations. Cultivar codes: COL (ColUTAD[®]), JUD (Judia), LON (Longal), MRT (Martaínha). Location codes: CM (Carrazedo de Montenegro), Mv (Marvão), PB (Parada), PBP (Penela da Beira). Values are means ($n = 6$). Different lowercase letters within a column and location indicate significant differences among cultivars (one-way ANOVA, Tukey's HSD test, $p < 0.05$).

Cultivar-Local	Ψ_{wmid} (MPa)	Total Phenolics (mg GAE g ⁻¹ DW)	Proline (mg g ⁻¹ DW)	Flavonoids (mg CAT eq. g ⁻¹ DW)	FRAP (μmol FeSO ₄ eq. g ⁻¹ DW)	Soluble Sugars (mg g ⁻¹ DW)	Starch (mg g ⁻¹ DW)	Electrolyte Leakage (%)	Soluble Proteins (mg g ⁻¹ DW)	CAT (U mg ⁻¹ Protein)	POD (U mg ⁻¹ Protein)	APX (U g ⁻¹ Protein)
COL_CM	−1.39 c	37.9 d	88.8 b	34.2 a	4447.3 a	100.9 a	73.2 b	27.5 a	71.3 b	26.2 b	34.8 a	39.0 a
JUD_CM	−1.24 b	59.9 a	93.9 a	22.2 d	416.9 a	95.4 ab	81.7 a	24.4 c	76.0 a	32.5 a	25.5 b	17.0 c
LON_CM	−1.17 a	40.9 c	81.4 c	31.0 b	644.4 a	85.5 b	65.5 c	22.6 d	62.6 d	35.7 a	33.2 a	24.0 a
MRT_CM	−1.14 a	42.6 b	75.0 c	24.9 c	533.3 a	75.7 c	81.0 a	24.9 b	67.6 c	17.9 c	16.9 c	20.8 b
Mean CM	−1.23	45.29	84.78	28.08	510.48	89.38	75.35	24.85	69.38	28.08	27.35	25.19
COL_Mv	−1.08 c	34.3 c	16.3 c	41.0 a	432.4 a	88.5 a	96.2 c	25.2 a	33.8 d	19.1 b	18.5 c	36.8 b
JUD_Mv	−0.61 a	37.8 b	25.0 b	31.8 b	504.0 a	84.4 b	93.8 c	23.7 b	46.1 c	4.3 c	26.1 b	31.3 c
LON_Mv	−0.71 b	39.6 a	38.1 a	25.6 c	461.1 a	82.1 b	119.5 a	21.7 c	54.2 b	21.7 a	37.3 a	48.2 a
MRT_Mv	−0.56 a	36.7 b	39.9 a	21.1 d	424.8 a	71.7 c	111.7 b	23.4 d	57.5 a	22.3 a	38.5 a	32.3 d
Mean Mv	−0.74	37.08	29.83	29.88	455.58	81.68	105.30	23.50	47.90	16.85	30.10	37.14
COL-PB	−1.57 b	42.1 c	136.9 a	32.5 b	403.7 a	94.2 c	37.0 b	29.2 a	74.4 b	36.7 c	7.8 b	60.8 c
JUD_PB	−1.70 c	59.6 a	124.1 b	36.6 a	501.8 a	92.1 c	43.0 a	25.5 c	78.2 a	44.7 b	4.8 c	60.2 a
LON_PB	−1.44 a	48.4 a	131.9 ab	33.0 b	563.9 a	118.4 a	31.9 c	25.0 d	76.4 a	46.3 b	7.0 b	40.3 c
MRT_PB	−1.53 b	44.9 b	134.4 a	37.7 a	571.4 a	114.5 b	44.0 a	25.9 b	73.7 b	74.3 a	9.1 a	43.6 b
Mean PB	−1.56	48.75	131.83	34.95	510.20	104.80	38.98	26.40	75.68	50.50	7.18	51.2
COL_PBP	−0.70 b	30.3 d	35.3 a	35.8 a	387.9 a	89.8 a	162.7 ab	20.0 a	32.6 c	34.0 a	29.4 c	19.5 a
JUD_PBP	−0.50 a	41.5 a	16.4 b	28.9 b	514.7 a	84.5 ab	153.6 b	19.4 b	46.8 b	32.9 a	44.6 a	9.8 c
LON_PBP	−0.49 a	39.4 b	12.9 c	27.6 b	559.4 a	81.9 b	104.0 c	17.9 c	48.4 b	31.3 a	35.1 b	19.5 a
MRT_PBP	−0.66 b	34.5 c	18.3 b	23.8 c	462.8 a	70.7 b	170.6 a	19.1 b	56.7 a	15.6 b	29.8 c	18.0 b
Mean PBP	−0.59	36.42	20.73	29.03	481.20	81.73	147.73	19.10	46.13	28.45	34.73	16.66
COL	−1.18	36.13	69.33	35.88	417.83	93.35	92.28	25.48	53.03	29.00	22.63	39.01
JUD	−1.01	49.68	64.85	29.88	484.35	89.10	93.03	23.25	61.78	28.60	25.25	29.57
LON	−0.95	42.05	66.08	29.30	557.20	91.98	80.23	21.80	60.40	33.75	28.15	32.98
MRT	−0.97	39.67	66.90	26.88	498.08	83.15	101.83	23.33	63.88	32.53	23.33	28.62

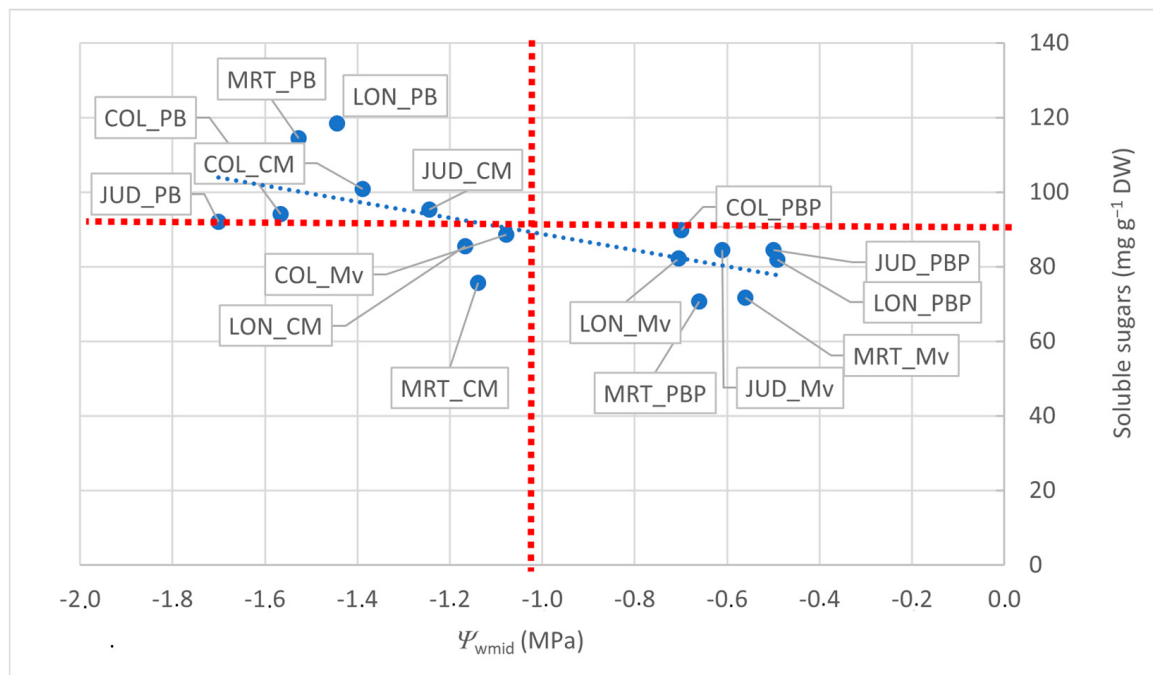


Figure 2. Relationship between midday stem water potential (Ψ_{wmid}) and total soluble sugar in leaves of four chestnut cultivars (COL, JUD, LON, MRT) grown across four locations. The dashed blue line represents the linear regression for all data points combined (Equation: $TSS = -21.755 \Psi_{wmid} + 66.98$ ($R^2 = 0.47$)). The dashed red line indicates the overall mean values of Ψ_{wmid} and total soluble sugar. Each data point represents the mean of six biological replicates.

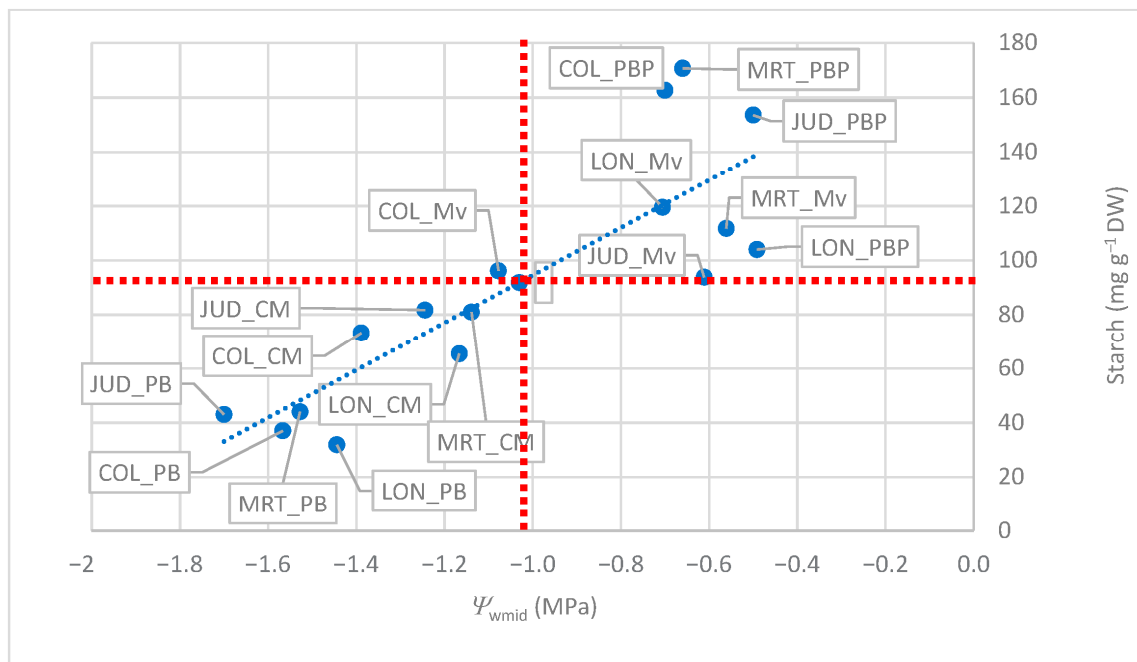


Figure 3. Relationship between midday stem water potential (Ψ_{wmid}) and total starch content in leaves of four chestnut cultivars (COL, JUD, LON, MRT) grown across four locations. The dashed blue line represents the linear regression for all data points combined (Equation: $Starch = 87.674 \Psi_{wmid} + 182.16$ ($R^2 = 0.71$)). The dashed red line indicates the overall mean values of Ψ_{wmid} and total starch content. Each data point represents the mean of six biological replicates.

Soluble protein content (SPC) differed significantly among locations and cultivars (Table 2). The highest concentration was observed in Mv ($78.3 \text{ mg g}^{-1} \text{ DW}$) and the lowest

in PBP (46.1 mg g⁻¹ DW). A negative correlation was found between SPC and stem water potential (Ψ_{wmid}) ($R^2 = 0.59$; Figure 4). Among cultivars, SPC ranged from 46.1 mg g⁻¹ DW (COL) to 78.3 mg g⁻¹ DW (LON). The variation in SPC among cultivars was greater at stressed sites, ranging from 8.5 mg g⁻¹ DW in Mv to 14.7 mg g⁻¹ DW in PB.

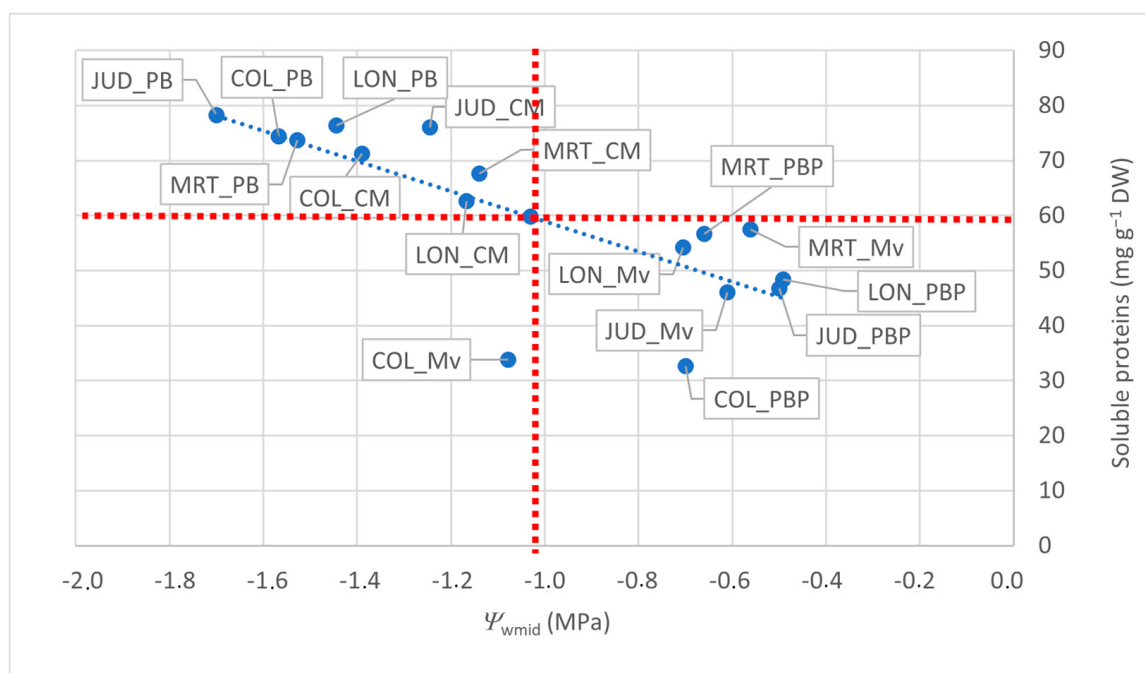


Figure 4. Relationship between midday stem water potential (Ψ_{wmid}) and total soluble proteins in leaves of four chestnut cultivars (COL, JUD, LON, MRT) grown across four locations. The dashed blue line represents the linear regression for all data points combined (Equation: Soluble proteins = $-27.391 \Psi_{wmid} + 31.537$ ($R^2 = 0.59$)). The dashed red line indicates the overall mean values of Ψ_{wmid} and total soluble proteins. Each data point represents the mean of six biological replicates.

Total phenolic content (TPC) varied significantly among locations and cultivars (Table 2), with the highest level recorded in JUD_CM (59.86 mg GAE g⁻¹ DW) and the lowest in COL_PBP (30.29 mg GAE g⁻¹ DW). TPC showed a negative correlation with stem water potential ($R^2 = 0.36$; Figure 5). The variation in TPC among cultivars increased under drought stress, ranging from 11.20 mg GAE g⁻¹ DW in PBP to 21.98 mg GAE g⁻¹ DW in CM. JUD consistently showed the highest TPC values across locations, while COL showed the lowest.

Electrolyte leakage percentage (ELP) differed significantly among locations and cultivars (Table 2). The highest ELP was recorded in PB (29.4%) and the lowest in Mv (21.1%). A strong negative correlation was observed between ELP and stem water potential ($R^2 = 0.68$; Figure 6). Among cultivars, ELP ranged from 21.1% (LON) to 29.4% (COL). The variation in ELP among cultivars increased under drought stress, ranging from 2.2% in PBP to 3.5% in PB.

Proline content differed significantly among locations and cultivars (Table 2). The highest levels were recorded in PB (131.9 mg g⁻¹ DW) and the lowest in PBP (16.3 mg g⁻¹ DW). A strong negative correlation was observed between proline content and stem water potential ($R^2 = 0.85$; Figure 7). Among cultivars, proline ranged from 16.3 mg g⁻¹ DW (COL) to 131.9 mg g⁻¹ DW (LON). The variation in proline content among cultivars increased under drought stress, ranging from 3.8 mg g⁻¹ DW in PBP to 19.1 mg g⁻¹ DW in CM.

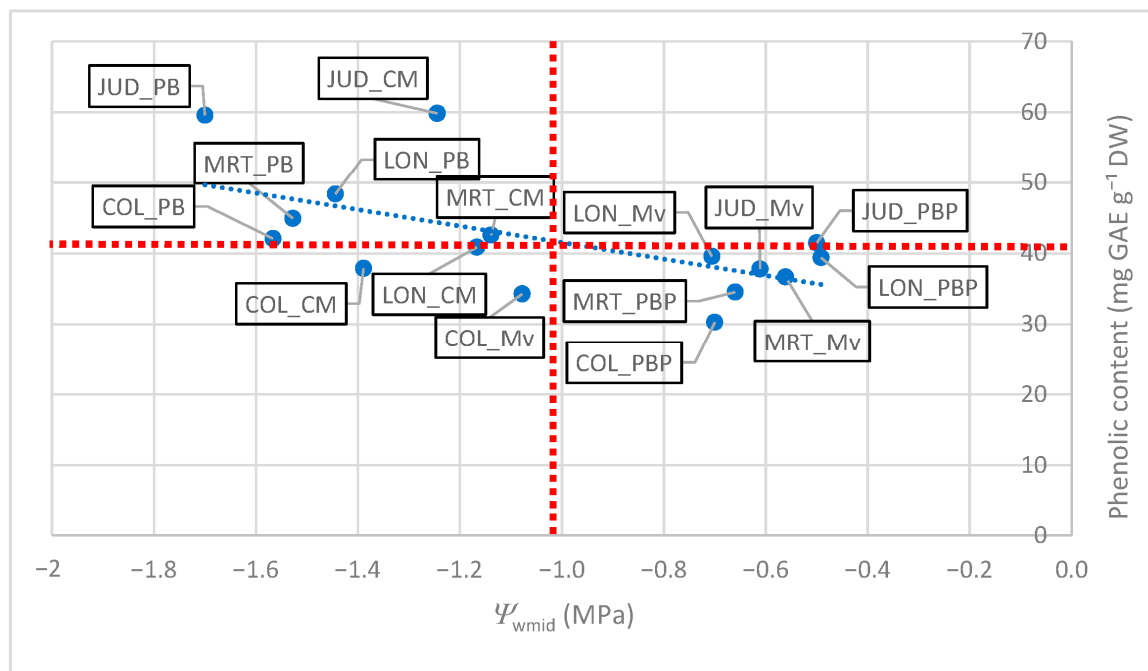


Figure 5. Relationship between midday stem water potential (Ψ_{wmid}) and total phenolic content in leaves of four chestnut cultivars (COL, JUD, LON, MRT) grown across four locations. The dashed blue line represents the linear regression for all data points combined (Equation: Total phenols = $-11.627 \Psi_{wmid} + 29.905$ ($R^2 = 0.36$)). The dashed red line indicates the overall mean values of Ψ_{wmid} and total phenolic content. Each data point represents the mean of six biological replicates.

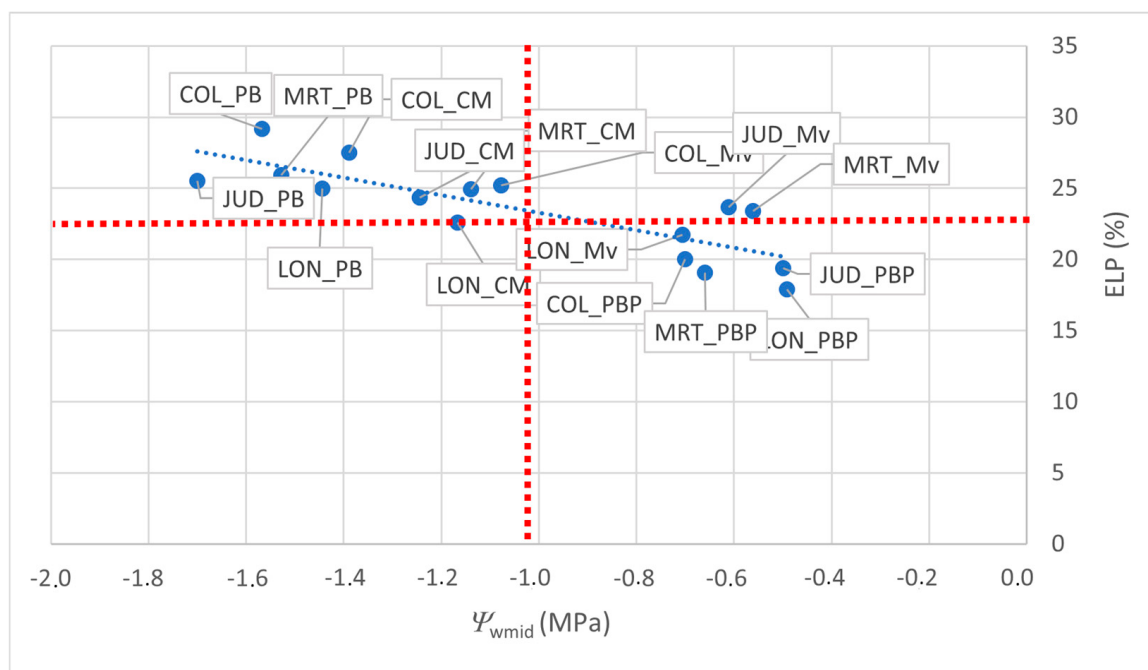


Figure 6. Relationship between midday stem water potential (Ψ_{wmid}) and electrolyte leakage percentage in leaves of four chestnut cultivars (COL, JUD, LON, MRT) grown across four locations. The dashed blue line represents the linear regression for all data points combined (Equation: Electrolyte leakage = $-6.164 \Psi_{wmid} + 17.104$ ($R^2 = 0.68$)). The dashed red line indicates the overall mean values of Ψ_{wmid} and electrolyte leakage percentage. Each data point represents the mean of six biological replicates.

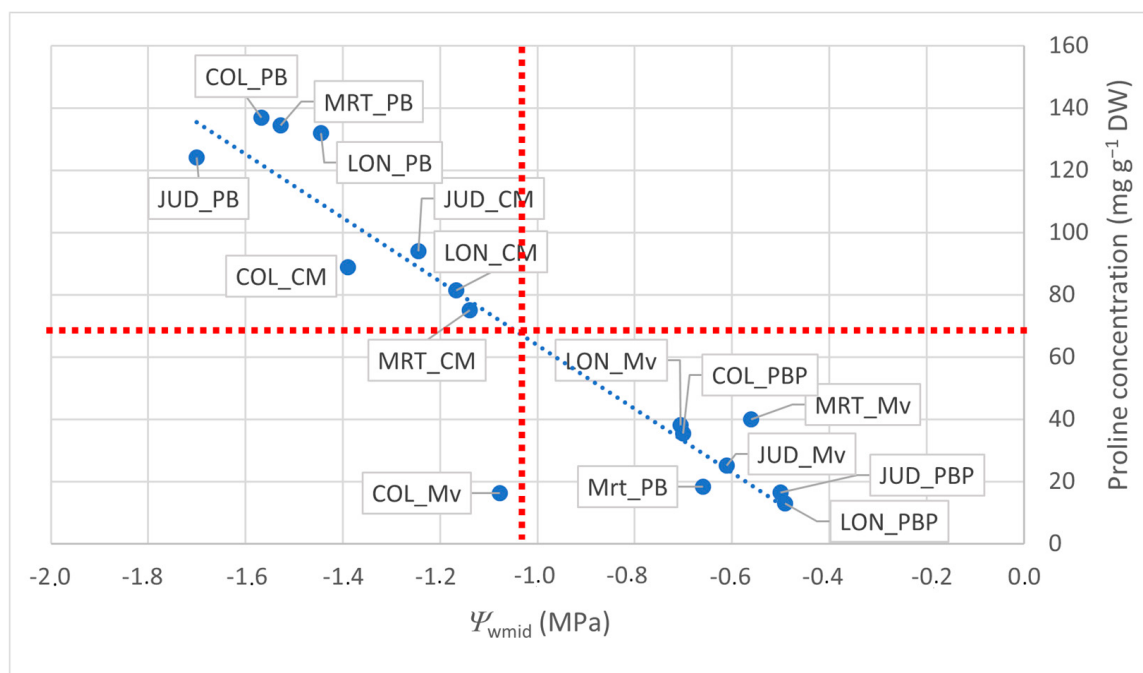


Figure 7. Relationship between midday stem water potential (Ψ_{wmid}) and proline content in leaves of four chestnut cultivars (COL, JUD, LON, MRT) grown across four locations. The dashed blue line represents the linear regression for all data points combined (Equation: $\text{Proline} = -102.32 \Psi_{wmid} - 38.625$ ($R^2 = 0.85$)). The dashed red line indicates the overall mean values of Ψ_{wmid} and proline content. Each data point represents the mean of six biological replicates.

Total flavonoid content (TFC) differed significantly among cultivars (Table 2), with the highest concentration observed in JUD ($32.5 \text{ mg CAT eq. g}^{-1} \text{ DW}$) and the lowest in COL ($24.9 \text{ mg CAT eq. g}^{-1} \text{ DW}$). A weak negative correlation was found between TFC and stem water potential ($R^2 = 0.23$).

FRAP values differed significantly among cultivars (Table 2), with LON showing the highest values ($644.4 \text{ } \mu\text{mol FeSO}_4 \text{ eq. g}^{-1} \text{ DW}$) and COL the lowest ($387.9 \text{ } \mu\text{mol FeSO}_4 \text{ eq. g}^{-1} \text{ DW}$). No significant correlation was found between FRAP and stem water potential ($R^2 = 0.013$).

Catalase (CAT) activity varied significantly among cultivars (Table 2), with LON consistently showing the highest activity ($35.8 \text{ U mg}^{-1} \text{ protein}$) and COL the lowest ($26.2 \text{ U mg}^{-1} \text{ protein}$). A weak negative correlation was observed between CAT activity and stem water potential ($R^2 = 0.34$). Peroxidase (POD) activity differed significantly among cultivars (Table 2), with JUD showing the highest activity ($34.8 \text{ U mg}^{-1} \text{ protein}$) and COL the lowest ($21.2 \text{ U mg}^{-1} \text{ protein}$). A positive correlation was observed between POD activity and stem water potential ($R^2 = 0.64$; Figure 8). The variation in POD activity among cultivars was highest under moderate drought stress ($13.6 \text{ U mg}^{-1} \text{ protein}$ in CM) compared to severe stress ($5.1 \text{ U mg}^{-1} \text{ protein}$ in PB).

Ascorbate peroxidase (APX) activity differed significantly among cultivars (Table 2), with LON showing the highest activity ($48.2 \text{ U g}^{-1} \text{ protein}$) and COL the lowest ($19.5 \text{ U g}^{-1} \text{ protein}$). A negative correlation was observed between APX activity and stem water potential ($R^2 = 0.42$; Figure 9). The variation in APX activity among cultivars increased with drought severity, ranging from $6.6 \text{ U g}^{-1} \text{ protein}$ in PBP to $8.5 \text{ U g}^{-1} \text{ protein}$ in CM.

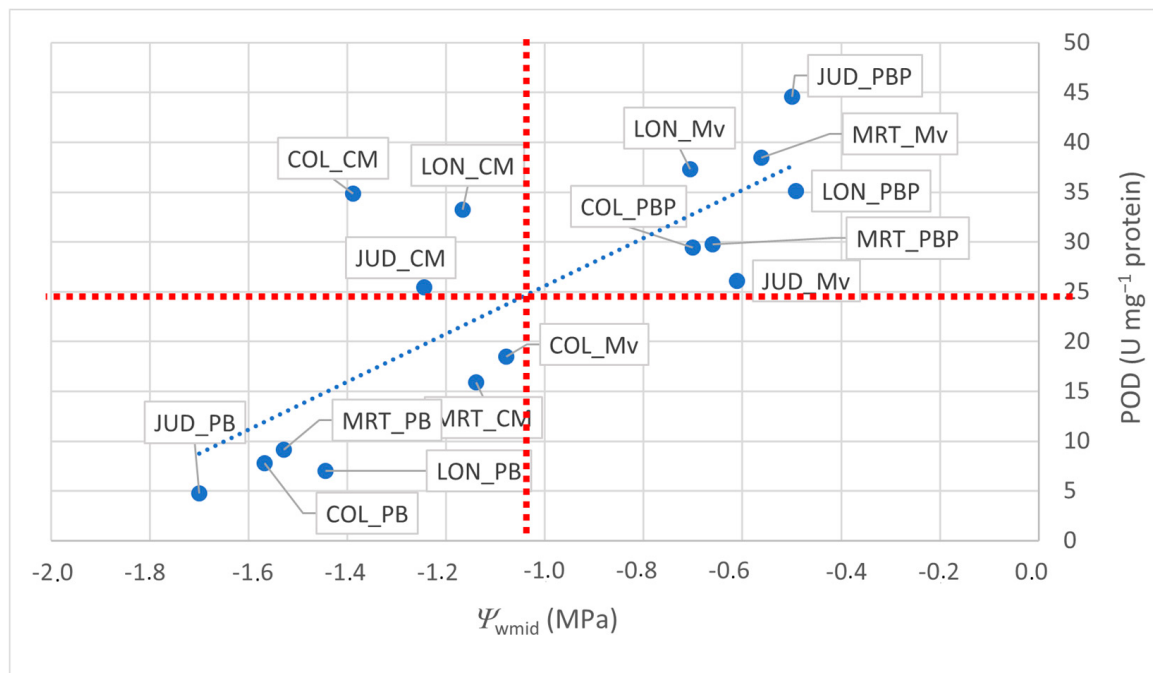


Figure 8. Relationship between midday stem water potential (Ψ_{wmid}) and peroxidase activity in leaves of four chestnut cultivars (COL, JUD, LON, MRT) grown across four locations. The dashed blue line represents the linear regression for all data points combined (Equation: $POD = 24.068 \Psi_{wmid} + 49.634$ ($R^2 = 0.64$)). The dashed red line indicates the overall mean values of Ψ_{wmid} and peroxidase activity. Each data point represents the mean of six biological replicates.

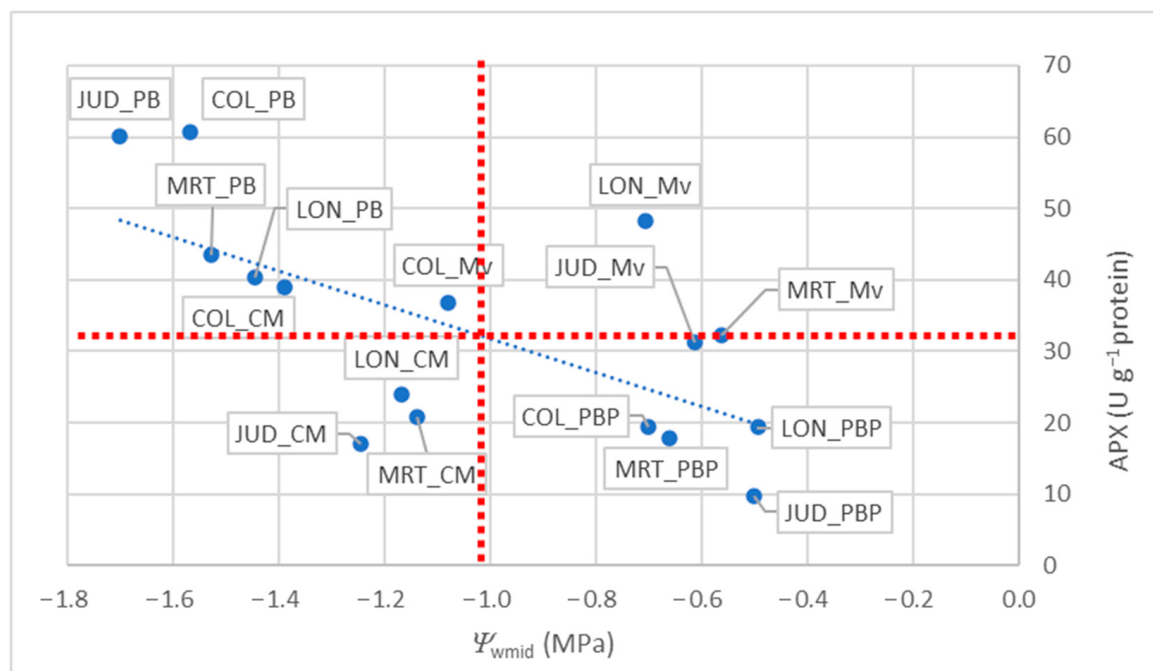


Figure 9. Relationship between midday stem water potential (Ψ_{wmid}) and ascorbate peroxidase activity in leaves of four chestnut cultivars (COL, JUD, LON, MRT) grown across four locations. The dashed blue line represents the linear regression for all data points combined (Equation: $APX = -23.684 \Psi_{wmid} + 8.144$ ($R^2 = 0.42$)). The dashed red line indicates the overall mean values of Ψ_{wmid} and ascorbate peroxidase activity. Each data point represents the mean of six biological replicates.

Hierarchical clustering of locations revealed two main clusters (Figure 10). Cluster 1 comprised Mv and PBP, while Cluster 2 contained CM and PB. The silhouette score was 0.52 and the centroid distance was 117.38. Clustering of parameters also formed two main groups. Group A included peroxidase activity, stem water potential, and starch content. Group B included proline, electrolyte leakage, soluble proteins, total phenolics, and FRAP.

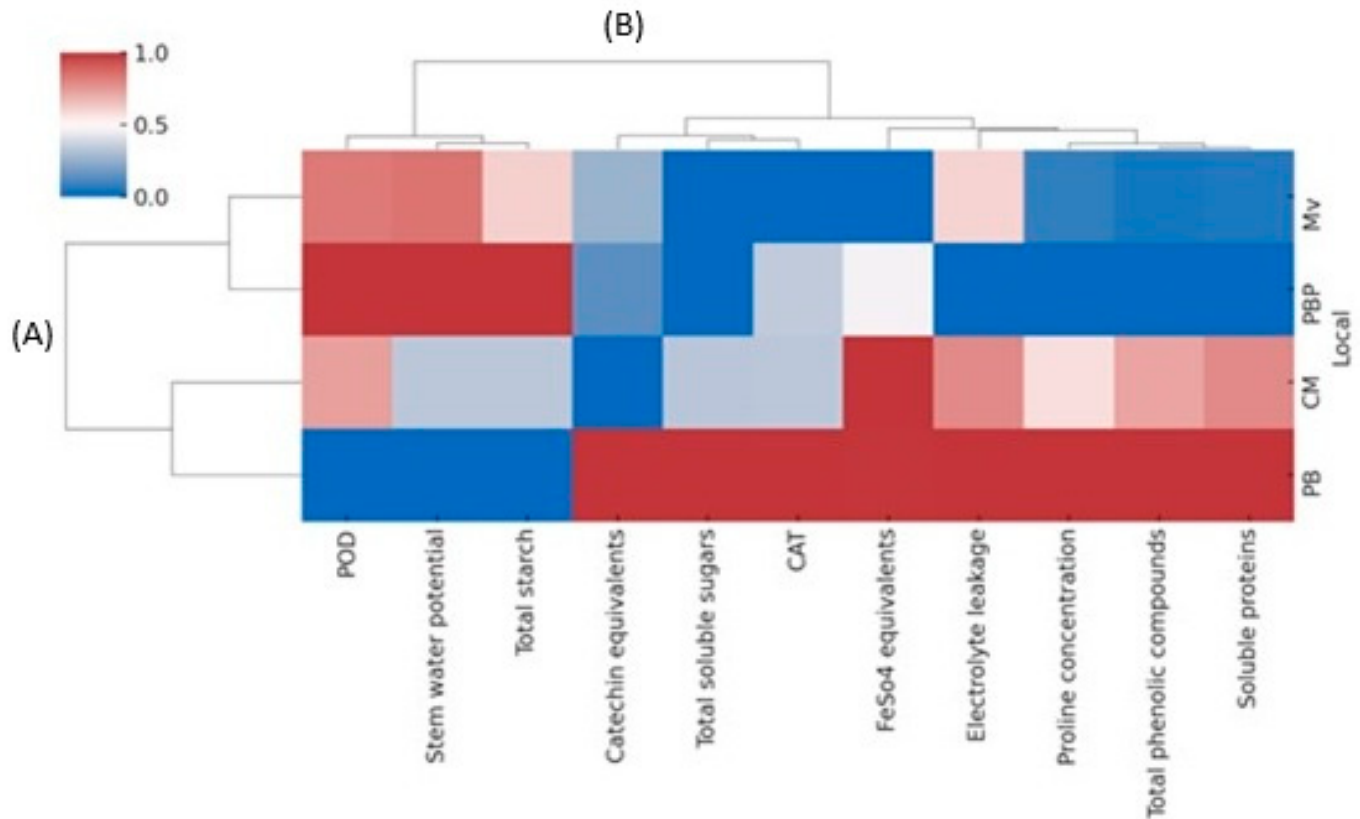


Figure 10. Hierarchical clustering and heatmap analysis based on standardised physiological and biochemical traits. (A) Clustering of the four study locations (CM, Mv, PB, PBP). (B) Clustering of the measured parameters. The heatmap depicts z-scores for each parameter (red: above mean, blue: below mean). The analysis clearly separates the less stressed locations (Mv, PBP) from the more stressed ones (CM, PB), and groups parameters into an “energy-stress balance” cluster (e.g., Starch, Ψ_{wmid}) and a “stress-response” cluster (e.g., Proline, Electrolyte Leakage).

Hierarchical clustering of cultivar-location combinations revealed two main clusters (Figure 11). Cluster 1 contained all genotypes from Mv and PBP, while Cluster 2 contained all genotypes from CM and PB. Within Cluster 1, two subclusters were formed: Subcluster 1.1 (COL_PBP, JUD_PBP, MRT_PBP) and Subcluster 1.2 (COL_Mv, JUD_Mv, LON_Mv, MRT_Mv, LON_PBP). Within Cluster 2, the subclusters were Subcluster 2.1 (COL_PB, JUD_PB, LON_PB, MRT_PB) and Subcluster 2.2 (COL_CM, JUD_CM, LON_CM, MRT_CM).

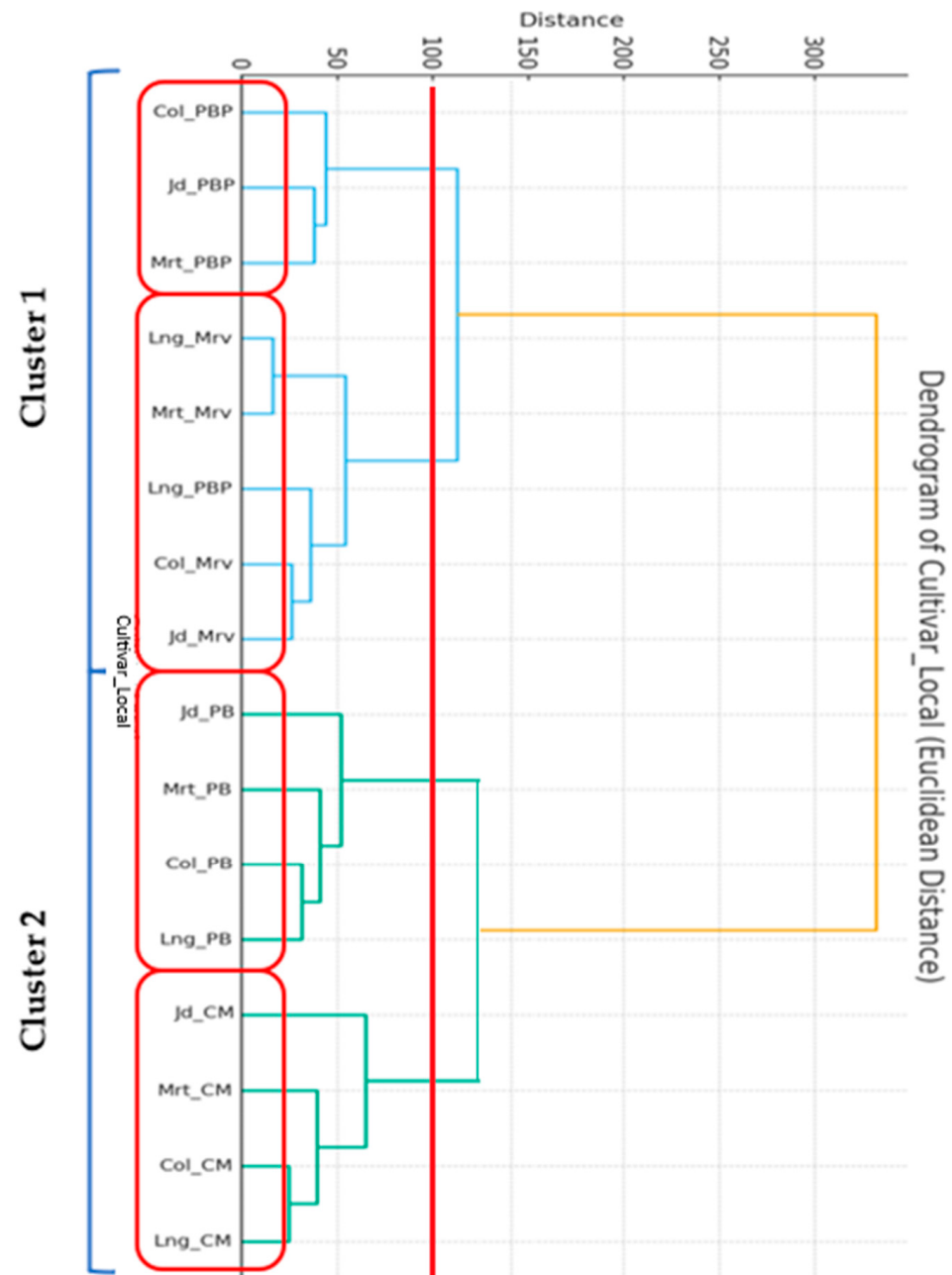


Figure 11. Hierarchical clustering of chestnut cultivar-location combinations based on all measured physiological and biochemical traits. The dendrogram was generated using between-groups linkage and Euclidean distance. The primary split separates all genotypes from the less stressed locations (Mv and PBP, blue branches; Cluster 1) from those in the more stressed locations (CM and PB green branches; Cluster 2), demonstrating that the environment is a stronger grouping factor than the genetic cultivar. The red boxes mark the groups defined at the Euclidean distance threshold of 100. Subclusters within these groups reflect finer-scale genotypic adaptations.

4. Discussion

The four orchards successfully established a natural drought gradient, confirming midday stem water potential (Ψ_{wmid}) as a reliable indicator of site-specific water status. Environmental conditions exerted a stronger influence on physiological and biochemical profiles than genetic background, a pattern also reported in other Mediterranean tree crops such as olive [25]. Despite irrigation, Mv experienced substantial atmospheric drought and therefore clustered with the cooler, rainfed PBP as the least stressed sites, whereas PB clearly represented the most severe stress environment.

The COL rootstock emerged as the most drought-sensitive genotype, consistently showing the lowest Ψ_{wmid} , highest electrolyte leakage and the weakest antioxidant activation. This highlights the importance of scion choice, as grafted cultivars such as LON and MRT sustained superior water status—a trend consistent with reported benefits of grafting in improving drought tolerance [24,26].

4.1. Osmotic Adjustment and Carbon Metabolism

Osmotic adjustment was a keyway to protect against drought. The significant buildup of proline in LON, especially at PB, shows that it is a key osmo-protectant that can stabilise proteins and membranes and directly eliminate the production of ROS [14]. This response is part of the ABA-driven signalling pathway that causes stomata to close and increases the production of ROS in chloroplasts and mitochondria. In this context, the buildup of proline helps keep turgor and protects against redox damage [27].

Simultaneously, the drought-induced remobilisation of carbon—reflected in starch degradation and the increased accumulation of soluble sugars—is a well-known way to help maintain osmotic balance and keep energy metabolism going when photosynthesis is limited [12]. The increase in soluble proteins in JUD and MRT indicates a proteomic reconfiguration aimed at stress defence, possibly involving LEA proteins or molecular chaperones [28,29]. These responses show how tolerant cultivars manage osmolyte accumulation, carbon redistribution, and protein-level changes to keep cells stable during drought [30].

4.2. Membrane Stability and the Antioxidant System

Membrane integrity, inferred from electrolyte leakage, proved to be a strong marker of cultivar resilience. The superior ability of LON to maintain membrane stability likely results from the combined effects of osmotic adjustment and efficient antioxidant defence, thereby limiting drought-induced oxidative damage—a relationship documented in other species facing abiotic stress [31].

The antioxidant strategies of the cultivars were clearly distinct. JUD relied on a strong inducible enzymatic response, with the highest POD activity and a marked induction of APX. Given that stomatal closure under ABA signalling increases intracellular H_2O_2 production, the activation of APX is consistent with its essential role in detoxifying ROS in chloroplasts and the cytosol [18].

Regarding non-enzymatic antioxidants, total phenolics were very sensitive to drought, especially in JUD. On the other hand, FRAP values seemed to be mostly constant. This indicates that the overall antioxidant capacity is significantly influenced by genotype, whereas certain phenolic compounds may be preferentially synthesised in response to stress [23]. The weak response of flavonoids to water deficit alone suggests that their biosynthesis in *C. sativa* is probably regulated by other factors as well, like high irradiance or UV exposure [32].

4.3. Genotype-by-Environment Interaction and Resilience

The hierarchical clustering analysis helps synthesise these mechanisms. The clear separation of sites into two main groups confirms the predominance of environmental pressure (Figure 10). Trait clustering revealed two functional axes: an “energy–stress balance” group (starch, Ψ_{wmid} , POD) and a “stress-response complex” (proline, electrolyte leakage, soluble proteins, phenolics).

Cultivar clustering within these environments highlights strong genotype-by-environment interactions (Figure 11). Two strategic syndromes became evident: (i) a conservative strategy (Mv, PBP), defined by better water status, higher starch reserves and moderate

antioxidant activity; (ii) a stress-responsive strategy (CM, PB), characterised by strong osmotic adjustment (proline) and activation of enzymatic antioxidants (APX).

Within these contexts, LON and JUD deployed these strategies most effectively, whereas COL consistently showed a limited capacity to mount an adequate defence [33].

5. Limitation Statement

The limitation of this study is the absence of complementary gene expression or metabolomic data, which would facilitate a more profound comprehension of the signalling pathways and molecular mechanisms driving the observed physiological responses.

6. Conclusions

This study demonstrates that drought resilience in Portuguese chestnut cultivars is governed by a synergistic network of physiological and biochemical defences rather than a single trait. To our knowledge, this provides the first integrated comparison of Portuguese cultivars under real-field drought gradients, identifying proline accumulation, starch stability, and inducible APX/POD activities as key tolerance markers.

We identified distinct, complementary resilience strategies: LON excels in osmotic adjustment, JUD in inducible antioxidant enzymes, and MRT in overall plasticity, while COL proved highly sensitive. Clustering confirmed that environment is the primary driver of physiological responses, separating orchards into conservative (Mv, PBP) and stress-adaptive (CM, PB) strategies.

These findings provide a physiological basis for selecting resilient cultivars and adapting orchard management to specific sites. Future work should focus on the molecular mechanisms underlying these traits and validate their utility in long-term breeding programmes aimed at enhancing climate resilience in Mediterranean chestnut agroecosystems.

Author Contributions: All authors contributed significantly to the writing, review, and editing. Conceptualization: T.M., A.F.-P., P.F., T.P. and J.G.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This work is supported by National Funds by FCT—Portuguese Foundation for Science and Technology, under the projects UID/04033/2025: Centre for the Research and Technology of Agro-Environmental and Biological Sciences and LA/P/0126/2020 (<https://doi.org/10.54499/LA/P/0126/2020>).

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors would like to thank the CITAB-Inov4Agro and FCT—Portuguese Foundation for Science and Technology, for their financial support.

Conflicts of Interest: The authors declare no conflicts of interest.

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