Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Physiological and biochemical responses of chestnut cultivars to drought and rewatering

Tiago Marques

tiagofcmarques@gmail.com

Centro de Investigação e de Tecnologias Agro-Ambientais e Biologicas

Andrea Ferreira-Pinto

University of Trás-os-Montes and Alto Douro

Teresa Pinto

Centro de Investigação e de Tecnologias Agro-Ambientais e Biologicas

José Gomes-Laranjo

Centro de Investigação e de Tecnologias Agro-Ambientais e Biologicas

Research Article

Keywords: Chestnut, Drought stress, Rewatering, Carbon metabolism, Antioxidant enzymes, Membrane stability, Cultivar resilience

Posted Date: November 12th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11340609/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Physiological and biochemical responses of chestnut cultivars to drought and rewatering

Tiago Marques [1,*], Andrea Ferreira-Pinto [2], Teresa Pinto [1] and José Laranjo [1]

[1] Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Inov4Agro, Institute for Innovation, Capacity Building and Sustainability of Agri-Food Production, University of Trás-os-Montes e Alto Douro, Quinta de Prados, 5000-801 Vila Real, Portugal; tiagofcmarques@gmail.com; tpinto@utad.pt; jlaranjo@utad.pt.

[2] School of Life and Environmental Sciences, University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5000-801 Vila Real, Portugal; al54555@utad.pt.

*Correspondence: tiagofcmarques@gmail.com

Abstract

Drought is an increasingly important constraint for chestnut cultivation in Mediterranean regions, where recurrent summer water deficits can restrict plant performance and compromise recovery after stress release. In Castanea sativa Mill., drought resilience depends on the coordinated adjustment of physiological and biochemical traits rather than on the behaviour of a single marker. This study evaluated three Portuguese chestnut cultivars, Judia, Longal, and Martaínha, grafted onto CA-90 rootstock, together with non-grafted CA-90 plants, under controlled drought and rewatering conditions. Plants were assessed at the initial irrigated phase (IP), after 7 days of irrigation withdrawal (NIP), and after 30 days of rewatering (RIP). Net photosynthesis, transpiration, leaf temperature, soluble sugars, starch, soluble proteins, electrolyte leakage, catalase (CAT), ascorbate peroxidase (APX), total phenolic compounds, total flavonoids, and ferric reducing antioxidant power (FRAP) were analysed. Drought reduced gas exchange in all plant materials and increased leaf temperature, indicating marked functional impairment under water deficit. Longal and Martaínha maintained comparatively higher physiological performance during drought and showed the strongest recovery after rewatering, whereas CA-90 displayed the lowest gas-exchange rates, greater starch depletion, and higher membrane damage. Soluble sugars, soluble proteins, CAT, and APX increased during drought, while starch declined in all cultivars. In contrast, phenolics, flavonoids, and FRAP decreased under water deficit and recovered partially or substantially after rewatering. Overall, the results show that drought resilience in chestnut is a dynamic trait shaped by the integration of gas exchange regulation, carbon metabolism, membrane stability, and

antioxidant adjustment, with Longal showing the most balanced response among the evaluated materials.

Keywords: Chestnut; Drought stress; Rewatering; Carbon metabolism; Antioxidant enzymes; Membrane stability; Cultivar resilience

---

1. Introduction

Drought is widely recognized as one of the most important environmental constraints limiting the performance of perennial crops, particularly in Mediterranean-type regions, where rising temperatures, increased evaporative demand, and shifts in the seasonal distribution of rainfall are expected to intensify water limitation under climate change scenarios (Farooq et al., 2009; Lelieveld et al., 2012; Tramblay et al., 2020). In woody perennial species, the effects of drought are especially relevant because plant survival and productivity depend not only on short-term stress resistance, but also on the capacity to preserve hydraulic and metabolic functionality across successive growing seasons and to recover after stress release (Gessler et al., 2020; Ruehr et al., 2019). For this reason, understanding both drought tolerance and post-stress recovery has become a central objective in the study of Mediterranean tree crops (Freitas et al., 2021; Gessler et al., 2020).

Among these species, European chestnut (*Castanea sativa* Mill.) has major ecological, economic, and cultural importance in southern Europe, where it remains closely associated with traditional production systems, rural landscapes, and multifunctional forest management (Aglietti et al., 2022; Freitas et al., 2021). However, chestnut is also increasingly exposed to climatic constraints, particularly in areas where hotter summers and longer dry periods are becoming more frequent, raising concerns about its future suitability under global change conditions (Conedera et al., 2021; Freitas et al., 2021). Water availability during the vegetative period is particularly important for this species, and insufficient water supply may negatively affect vigour, fruit development, and overall plant functioning (Mota et al., 2018). Experimental studies in chestnut have further shown that drought leads to reductions in stomatal conductance, transpiration, and net photosynthesis, while promoting the accumulation of stress-related metabolites such as proline, confirming the sensitivity of this species to water deficit (Beyazyüz & Kulaç, 2023). In this context, identifying the mechanisms involved not only in stress endurance but also in recovery is essential for a more complete understanding of drought resilience in chestnut.

Plant responses to water deficit depend on a coordinated set of physiological and biochemical adjustments. One of the earliest responses is stomatal closure, which restricts carbon dioxide (CO_2) entry into the leaf and thereby limits photosynthetic carbon assimilation (Chaves et al., 2009; Farooq et al., 2009). As drought progresses, the imbalance between energy capture and carbon metabolism may impair the photosynthetic machinery and stimulate the overproduction of reactive oxygen species (ROS), particularly in chloroplasts and other metabolically active compartments. When ROS accumulation exceeds the detoxification capacity of the cell, oxidative stress develops and may result in membrane destabilization, pigment loss, lipid peroxidation, and oxidative modification of proteins and other cellular components (Gill & Tuteja, 2010; Hasanuzzaman & Fujita, 2013). The severity of this damage is particularly relevant because it can constrain not only plant functioning during drought, but also the efficiency with which metabolic activity is restored after rewatering (Ruehr et al., 2019; Xu et al., 2010).

To mitigate these effects, plants activate an extensive biochemical defence network. Enzymatic antioxidants such as catalase (CAT) and ascorbate peroxidases (APX) are central to ROS scavenging, while non-enzymatic metabolites, including phenolic compounds and flavonoids, also contribute to redox regulation and stress protection (Gill & Tuteja, 2010; Sharma et al., 2019). In parallel, osmotic adjustment is a key component of drought acclimation, since the accumulation of compatible solutes such as proline and soluble sugars helps maintain cell turgor, stabilize proteins and membranes, and support metabolic activity under dehydration (Farooq et al., 2009; Szabados & Savouré, 2010). Changes in non-structural carbohydrates, especially starch and soluble sugars, are also highly informative, as they reflect shifts in carbon allocation, reserve mobilization, and the interaction between metabolism, hydraulic function, and stress recovery in woody plants (Sardans et al., 2020; Tomasella et al., 2019). Likewise, soluble proteins may vary under drought as a consequence of metabolic adjustment, protective protein synthesis, and oxidative damage, thereby providing additional information on the balance between defence and recovery processes (Farooq et al., 2009; Gill & Tuteja, 2010).

In woody plants, drought resilience does not depend on a single trait, but rather on the integration of multiple protective and restorative mechanisms acting across different levels of organization (Gessler et al., 2020; Ruehr et al., 2019). For this reason, the analysis of biochemical responses should not be restricted to the stress period itself. The recovery phase after rewatering is equally informative because it reveals the extent to which plants are able to reverse oxidative and metabolic damage, re-establish cellular homeostasis, and resume functional activity (Gessler et al., 2020; Xu et al., 2010). In this sense, genotypic

differences may become evident not only in the magnitude of drought-induced disruption, but also in the speed and effectiveness of post-stress recovery, which is a critical yet still insufficiently explored component of resilience in perennial crops (Gessler et al., 2020; Ruehr et al., 2019).

The present study aimed to characterize the biochemical responses of 4 chestnut cultivars (CA-90, Judia, Longal and Martaínha) subjected to severe drought induced by irrigation withdrawal, followed by a recovery phase after rewatering under controlled conditions. Particular attention was given to osmotic adjustment, membrane injury, carbon-related metabolism, and enzymatic and non-enzymatic antioxidant defence, as these processes are consistently identified as central components of plant drought response and recovery (Farooq et al., 2009; Gill & Tuteja, 2010; Sharma et al., 2019; Tomasella et al., 2019). We hypothesized that the cultivars would differ both in the extent of biochemical disruption during drought and in their capacity to restore metabolic homeostasis after rehydration, with the most resilient materials expected to show stronger osmotic regulation, more effective antioxidant activation, lower cellular damage, and a faster recovery of metabolic balance.

2. Materials and Methods

2.1. Plant material and experimental design

The experiment was conducted in 2023 at the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal (41°16'54.1" N, 7°44'38.8" W) (Figure 1), from mid-April to mid-September. The study evaluated the biochemical responses of two-year-old plants of three widely cultivated Portuguese chestnut cultivars, Judia, Longal, and Martaínha, grafted onto the Ferosacre rootstock CA-90, a hybrid genotype of *C. sativa* × *C. crenata*. Non-grafted CA-90 plants were also included for comparative purposes.

All experimental plants were commercially obtained from SERVIRURI – Prestação de Serviços Técnico-Agrícolas, Lda., in Ribeira de Machados, Parada de Cunhos, Vila Real, Portugal (41°17'38.50" N, 7°45'42.55" W). The plant material was accompanied by official European Union plant passports, bearing the professional operator registration number PT-1-1030, ensuring its identification and phytosanitary traceability.

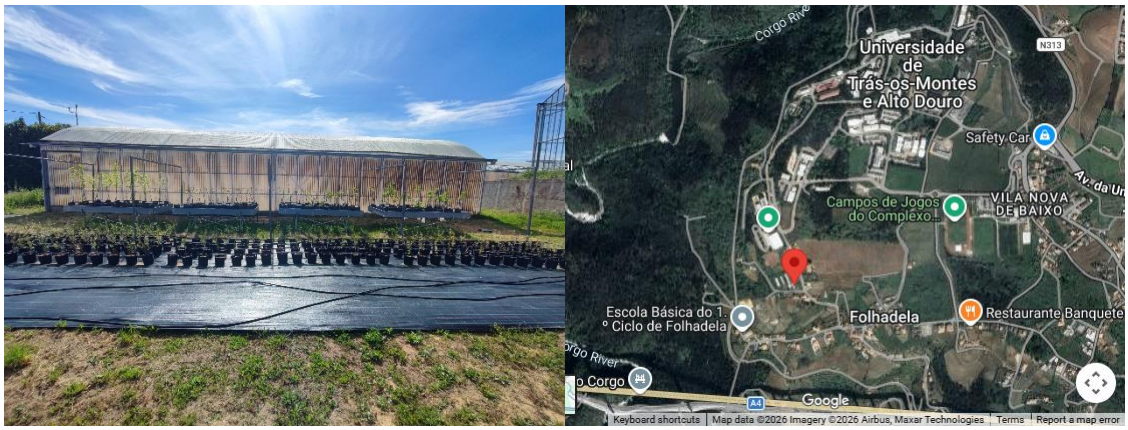


Figure 1. Location of the experimental site at the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal, and general view of the outdoor bench experiment used to evaluate the biochemical responses of chestnut genotypes subjected to drought-rewatering cycles in 2023.

Plants were grown in 5-L plastic pots filled with a substrate composed of 70% turf and 30% perlite and remained in the same pots throughout the experimental period. The experiment included four plant groups, corresponding to the three grafted cultivars and the non-grafted CA-90 plants, with 15 replicates per group. Pots were maintained outdoors on an experimental bench under natural environmental conditions. A drip irrigation system (4 L $drip^{-1}$ $plant^{-1}$) was installed to ensure water supply during the irrigation periods. During these phases, irrigation was applied daily for 15 min.

Most of the experiment took place during the summer, when temperatures were highest and weather conditions were dry. Under these conditions, plants did not receive effective rainfall inputs during the stress period. To ensure consistency under conditions of maximum atmospheric demand, plant evaluations were performed between 10:00 h and 15:00 h.

The experimental design was established to simulate summer drought conditions representative of the dry season in Portugal. Plants were first maintained under full irrigation for 30 days to allow acclimation, after which the initial phase (IP) was assessed. Irrigation was then withheld for 7 days to impose drought stress, corresponding to the non-irrigation phase (NIP). After this period, irrigation was restored and plants were maintained under rewatering conditions for 30 days, after which the recovery phase (RIP) was evaluated.

This experimental approach enabled the assessment of genotype-dependent biochemical responses during both severe drought imposition and post-stress recovery under a controlled and repeatable stress framework.

2.2. Physiological Measurements

2.2.1. Gas Exchange Measurements

Leaf gas exchange was evaluated using an infrared gas analyser (IRGA) during the experimental phases in order to assess the physiological impact of drought and recovery. Measurements were performed using an infrared gas analyser (IRGA, model LCA-2, Analytical Development Co., Hoddesdon, UK). Measurements were performed during the experimental phases on leaves selected from the middle crown. Net photosynthetic rate [*A*, μmol m^{-2} s^{-1}] and transpiration rate [*E*, mmol(H_2O) m^{-2} s^{-1}] were recorded between 10:00 h and 12:00 h under a photosynthetic photon flux density (PPFD) of 1,600 μmol m^{-2} s^{-1}.

2.2.2. Infrared Thermometry

Leaf surface temperature was measured using an infrared thermometer (Telatemp, model DT1100 Infrared Thermometer, Telatemp Corporation, Fullerton, CA, USA) during the main experimental phases associated with drought and recovery. Measurements were taken on sun-exposed leaves at an approximate distance of 20 cm and used as a complementary indicator of plant thermal status under water deficit and after rewatering. For data interpretation, leaf temperature measured under sun exposure was considered the most informative variable, as it better reflected the interaction between radiation load, plant water status, and transpirational cooling capacity.

2.3. Leaf Sampling and Sample Handling

Leaf sampling for biochemical analyses was performed at each experimental phase, namely the initial phase (IP), the non-irrigation phase (NIP) and the recovery phase (RIP). To ensure standardisation, fully expanded, sun-exposed leaves were collected from each plant under comparable environmental conditions. For each genotype and phase, six independent biological replicates were collected.

After sampling, leaves were placed in an insulated container with cooling packs to maintain low-temperature conditions during transport to the laboratory. Upon arrival, samples were kept at 4 °C in the dark 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 analysis. All spectrophotometric determinations were carried out using six biological replicates, with each measurement performed in technical duplicate.

2.4. Biochemical analyses

2.4.1. Determination of Soluble Sugars and Starch

Total soluble sugars were extracted in 80% ethanol (*v/v*) and quantified by the colorimetric method of Irigoyen et al., 1992, with absorbance measured at 625 nm. Starch

content was determined from the pellet remaining after soluble sugar extraction, following the method of Rose et al., 1991, also with spectrophotometric reading at 625 nm. Glucose was used as the standard for the quantification of both soluble sugars and starch.

2.4.2. Quantification of Soluble Protein Content

Total soluble protein content was determined spectrophotometrically according to the Bradford, 1976 method, using bovine serum albumin as the standard. Results were expressed on a dry weight basis and used to support the interpretation of metabolic adjustment under drought stress and recovery.

2.5. Membrane stability

2.5.1. Electrolyte Leakage

Electrolyte leakage was analysed as an indicator of membrane integrity, following a modified version of the method described in the biochemical reference study. Leaf discs were incubated in double-distilled water at 25 °C for 24 h under continuous shaking, and conductivity was measured before and after autoclaving using a conductivity meter. Electrolyte leakage was expressed as the ratio C1/C2, where C1 is the initial conductivity and C2 is the conductivity measured after autoclaving.

2.6. Antioxidant enzyme activities

For the determination of antioxidant enzyme activities, leaf discs of 8 mm diameter were ground in liquid nitrogen and homogenised in 1-2 mL of 100 mM phosphate buffer (pH 7.0) containing 2% polyvinylpyrrolidone (PVP). Extracts were centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatants were kept on ice and used immediately for catalase (CAT) and ascorbate peroxidase (APX).

2.6.1. Catalase

CAT activity was determined according to Aebi, 1984 with minor modifications. Reactions were performed in 200 µL microplate wells containing 100 mM phosphate buffer (pH 7.0), sample extract, and H_2O_2. After brief 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.

2.6.2. Ascorbate Peroxidase

APX activity was determined in 150 mM sodium phosphate buffer (pH 7.5) with 0.75mM EDTA. The 200 µL reaction contained sample extract (10-50 µL), 5 µL ascorbic acid (10 mM) and 10 µL H_2O_2 (10mM). The decrease in absorbance at 290 nm was monitored and

activity calculated using ε = 2.8 mM^{-1} cm^{-1} and expressed as U mg^{-1} protein. The method followed Nakano & Asada, 1981, with only the adaptation to microplate format.

2.7. Non-enzymatic antioxidant compounds and total antioxidant capacity

2.7.1. Determination of Total Phenolic Compounds

Total phenolic compounds were determined using the Folin-Ciocalteu spectrophotometric method. Leaf samples were prepared by punching four discs of 8 mm diameter from each sample and extracting them in 10 mL of 80% acetone (*v/v*). For quantification, 5 mL of the extract was used, and absorbance was measured at 795 nm. Gallic acid was used as the standard, and a calibration curve was prepared in the concentration range of 0-100 µg mL^{-1}. Results were expressed as mg gallic acid equivalents (GAE) g^{-1} dry weight (DW).

2.7.2. Determination of Total Flavonoids

Total flavonoid content was determined using a colorimetric assay. Diluted extracts were mixed with bi-distilled water and 5% $NaNO_2$ solution, followed by the addition of 10% $AlCl_3$. After incubation, 1 M NaOH was added, the volume was completed with bi-distilled water, and absorbance of the pink mixture was measured at 510 nm. Catechin was used as the standard, and results were expressed as mg catechin equivalents g^{-1} dry weight.

2.7.3. Ferric Reducing Antioxidant Power

Ferric reducing antioxidant power (FRAP) was used to estimate the total antioxidant capacity of leaf extracts according to the method of Benzie & Strain, 1996. Results were expressed as µmol $FeSO_4$ equivalents g^{-1} dry weight.

2.8. Statistical Analysis

Results are presented as mean ± standard error. Statistical analysis was performed using ANOVA-based procedures. When assumptions were met, a two-way ANOVA considering cultivar and experimental phase as main factors was applied, followed by Tukey's HSD post hoc test at $p < 0.05$. This structure is consistent with the analytical approach reported in the related controlled study, where ANOVA and Tukey post hoc tests were used for treatment comparisons.

3. Results

3.1. Gas exchange and leaf surface temperature

3.1.1. Gas exchange responses

Net photosynthetic rate (*A*, µmol m^{-2} s^{-1}) varied markedly across the experimental phases and among chestnut cultivars (Figure 2). In all genotypes, A decreased from the

initial phase, IP (Day 30), to the non-irrigation phase, NIP (Day 37), followed by an increase after rewatering in the recovery phase, RIP (Day 67).

At IP (Day 30), the highest mean *A* values were recorded in Longal and Martaínha, with 13.87 and 13.34 μmol m^{-2} s^{-1}, respectively, whereas Judia showed an intermediate value of 11.59 μmol m^{-2} s^{-1}. Ca-90 displayed the lowest photosynthetic rate at this stage, with a mean value of 5.80 μmol m^{-2} s^{-1}.

At NIP (Day 37), *A* declined in all cultivars. Ca-90 showed the lowest value under drought conditions, decreasing to 2.56 μmol m^{-2} s^{-1}. Judia also exhibited a pronounced reduction, reaching 4.87 μmol m^{-2} s^{-1}. Longal and Martaínha maintained comparatively higher values during this phase, with mean rates of 7.38 and 6.76 μmol m^{-2} s^{-1}, respectively.

At RIP (Day 67), *A* increased in all plant materials. Martaínha reached the highest value in the recovery phase, with a mean *A* of 15.48 μmol m^{-2} s^{-1}, followed by Longal with 13.36 μmol m^{-2} s^{-1}. Judia recovered to 9.47 μmol m^{-2} s^{-1}, while Ca-90 increased to 6.67 μmol m^{-2} s^{-1}. In Longal, the value at RIP (Day 67) remained close to that recorded at IP (Day 30), whereas in Martaínha the value at RIP (Day 67) exceeded that observed at IP (Day 30). Although Judia and Ca-90 also showed recovery after rewatering, their values remained below those recorded at IP (Day 30).

Overall, the temporal pattern of *A* was characterised by a marked reduction under irrigation withdrawal and a subsequent increase following the reintroduction of water supply, with distinct magnitudes of decline and recovery among cultivars.

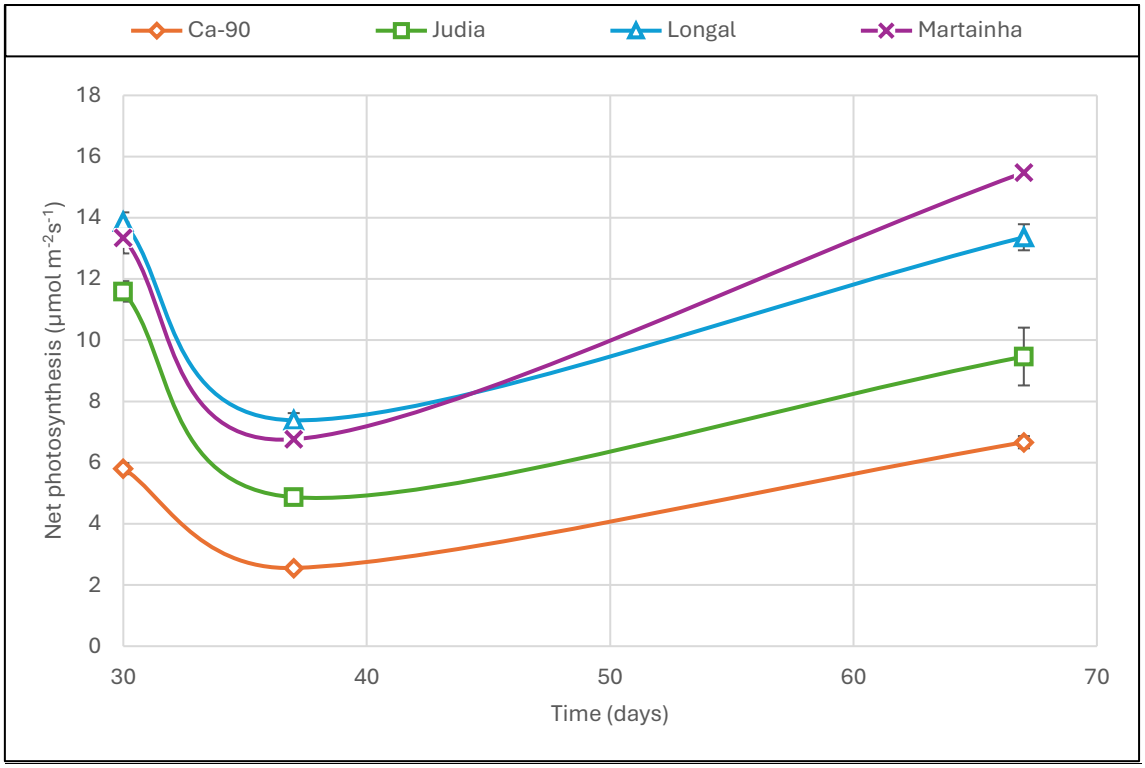


Figure 2. Net photosynthetic rate (*A*) of *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). Values are presented as mean ± standard error (SE).

Transpiration rate (*E*, mmol(H_2O) m^{-2} s^{-1}) varied across the experimental phases and among chestnut cultivars (Figure 3). In all genotypes, E decreased from the initial phase, IP (Day 30), to the non-irrigation phase, NIP (Day 37), and increased again after rewatering in the recovery phase, RIP (Day 67).

At IP (Day 30), the lowest mean *E* value was recorded in Ca-90, with 1.18 mmol(H_2O) m^{-2} s^{-1}. Judia showed an intermediate value of 1.92 mmol(H_2O) m^{-2} s^{-1}, whereas Longal and Martaínha presented the highest *E* at this stage, with 2.22 and 2.28 mmol(H_2O) m^{-2} s^{-1}, respectively.

At NIP (Day 37), *E* declined in all cultivars. Ca-90 showed the lowest value under drought, decreasing to 0.70 mmol(H_2O) m^{-2} s^{-1}. Judia also decreased to 1.22 mmol(H_2O) m^{-2} s^{-1}, while Longal maintained the highest value during this phase, with 1.88 mmol(H_2O) m^{-2} s^{-1}. Martaínha reached 1.50 mmol(H_2O) m^{-2} s^{-1}.

At RIP (Day 67), *E* increased in all plant materials. Martaínha showed the highest mean *E* value, reaching 2.87 mmol(H_2O) m^{-2} s^{-1}, followed by Longal with 2.57 mmol(H_2O) m^{-2} s^{-1}. Judia recovered to 1.78 mmol(H_2O) m^{-2} s^{-1}, while Ca-90 increased to 1.60 mmol(H_2O) m^{-2} s^{-1}. In Longal and Martaínha, *E* values at RIP (Day 67) exceeded those recorded at IP (Day 30), whereas in Judia the recovery value remained slightly below the

initial one. Ca-90 also showed recovery after rewatering, with a value higher than that observed at IP (Day 30).

Overall, the temporal pattern of *E* was characterised by a decline under irrigation withdrawal and a subsequent increase after rewatering, with differences among cultivars in both the magnitude of reduction during drought and the extent of recovery.

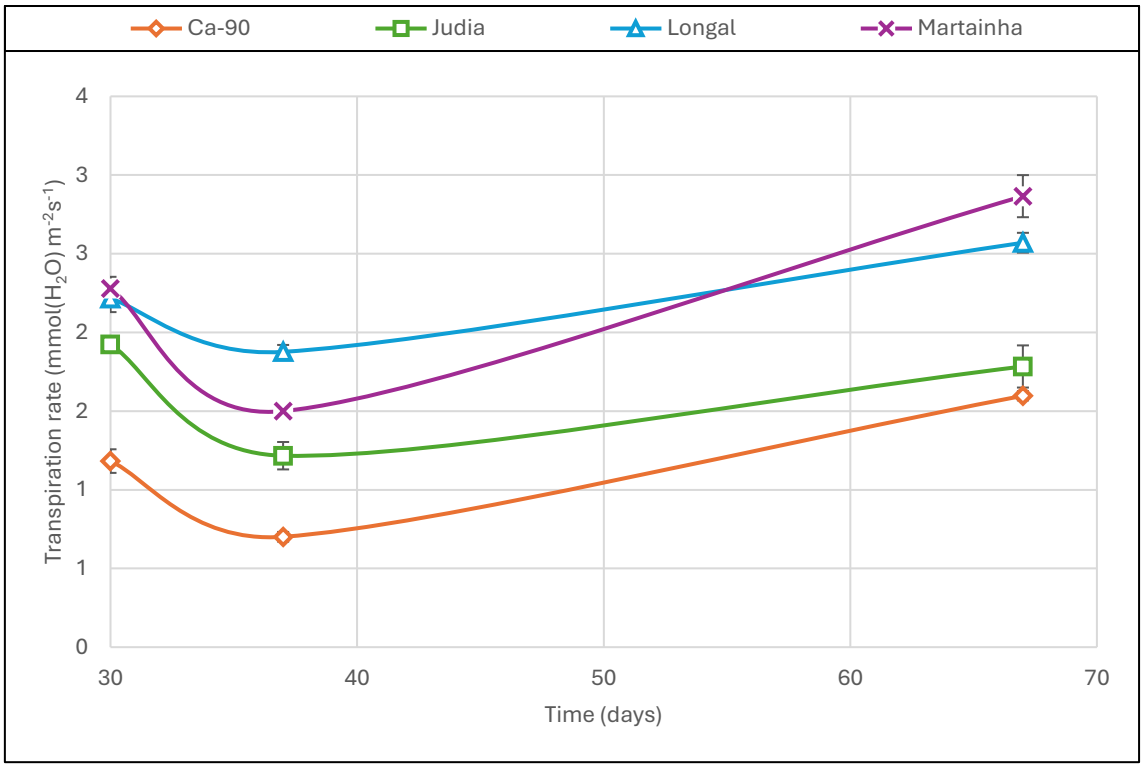


Figure 3. Transpiration rate (*E*) of *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). Values are presented as mean ± standard error (SE).

3.1.2. Leaf surface temperature

Sun-exposed leaf temperature varied across the experimental phases and among chestnut cultivars (Figure 4). In all genotypes, leaf temperature increased from the initial phase, IP (Day 30), to the non-irrigation phase, NIP (Day 37), and decreased after rewatering in the recovery phase, RIP (Day 67).

At IP (Day 30), Ca-90 showed the highest mean leaf temperature, reaching 34.5 °C. Longal presented an intermediate value of 28.9 °C, whereas Judia and Martaínha displayed lower temperatures, with 26.8 and 25.4 °C, respectively.

At NIP (Day 37), leaf temperature increased in all cultivars. Ca-90 maintained the highest value, rising to 39.4 °C. Martaínha also showed a marked increase, reaching 34.4 °C, followed by Longal with 33.8 °C and Judia with 32.3 °C.

At RIP (Day 67), leaf temperature declined in all plant materials. Ca-90 remained the warmest material, with a mean value of 28.7 °C, while Judia, Longal, and Martaínha

decreased to 26.0, 25.8, and 24.6 °C, respectively. In Judia, Longal, and Martaínha, the values recorded at RIP (Day 67) were similar to, or slightly lower than, those observed at IP (Day 30), whereas Ca-90, although cooler than under drought conditions, still remained above its initial value.

Overall, the temporal pattern of sun-exposed leaf temperature was characterised by an increase during irrigation withdrawal and a subsequent decrease after rewatering, with Ca-90 showing consistently higher values than the remaining cultivars throughout the experimental period.

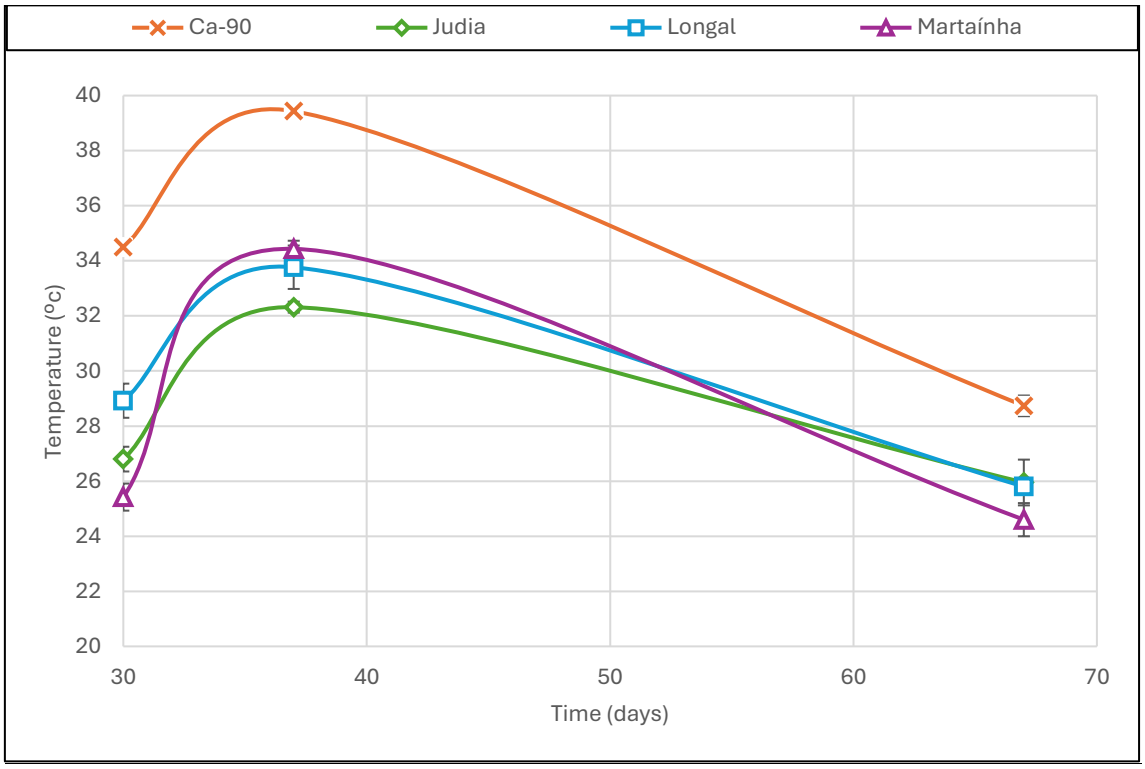


Figure 4. Sun-exposed leaf temperature of *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). Values are presented as mean ± standard error (SE).

3.2. Osmotic adjustment and carbon-related metabolism

Soluble sugar content (mg g $^{-1}$ DW) varied across the experimental phases and among chestnut cultivars (Figure 5; Table 1). In general, the drought period was associated with increased soluble sugar accumulation in Judia, Longal, and Martaínha, whereas Ca-90 showed a slight decrease from its initial value.

At the initial phase, IP (Day 30), Longal showed the highest mean soluble sugar content, reaching 135.57 mg g^{-1} DW, followed by Judia with 124.82 mg g^{-1} DW and Martaínha with 109.39 mg g^{-1} DW. Ca-90 displayed the lowest value, with 59.80 mg g^{-1} DW. At the non-irrigation phase, NIP (Day 37), soluble sugar content increased in Judia, Longal,

and Martaínha to 147.75, 160.72, and 117.06 mg g^{-1} DW, respectively, while Ca-90 decreased to 55.79 mg g^{-1} DW. At the recovery phase, RIP (Day 67), soluble sugars remained high in Longal and Judia, with values of 160.21 and 144.63 mg g^{-1} DW, respectively, and continued to increase slightly in Martaínha, reaching 120.94 mg g^{-1} DW. In Ca-90, soluble sugar content rose to 63.73 mg g^{-1} DW after rewatering.

Relative to IP (Day 30), the strongest increases during drought were observed in Longal and Judia, which showed increases of 18.55% and 18.37%, respectively (Table 1). Martaínha exhibited a more moderate increase of 7.01%, whereas Ca-90 showed a decrease of 6.71%. During recovery, soluble sugar content remained relatively stable in Longal and Judia, while Martaínha showed a slight additional increase. In contrast, Ca-90 exhibited the strongest relative increase after rewatering, although from the lowest drought value.

Overall, the temporal pattern of soluble sugars indicated distinct cultivar-dependent dynamics in carbon-related adjustment during drought imposition and subsequent recovery.

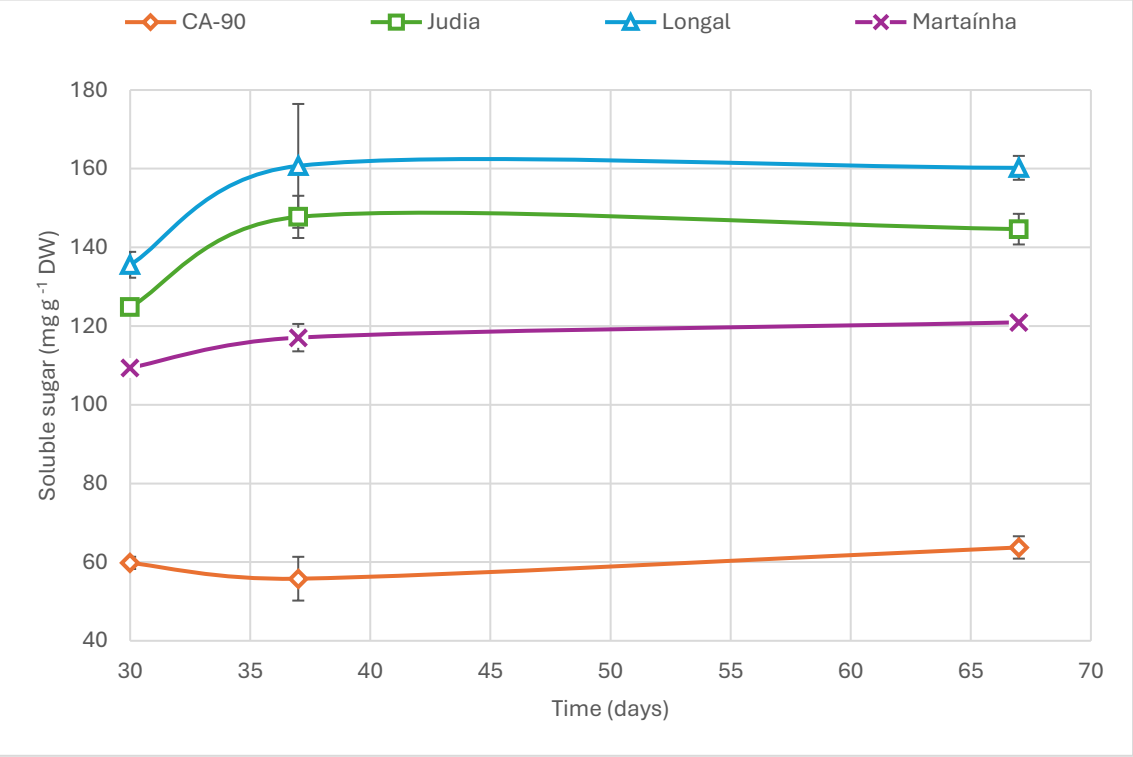


Figure 5 Soluble sugar content (mg g $^{-1}$ DW) of *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). Values are presented as mean ± standard error (SE).

Starch content (mg g^{-1} DW) varied across the experimental phases and among chestnut cultivars (Figure 6; Table 1). In all genotypes, starch concentration decreased from

the initial phase, IP (Day 30), to the non-irrigation phase, NIP (Day 37), and increased again after rewatering in the recovery phase, RIP (Day 67).

At IP (Day 30), Longal showed the highest mean starch content, reaching 96.24 mg g^{-1} DW, followed by Martaínha with 88.79 mg g^{-1} DW and Judia with 78.65 mg g^{-1} DW. Ca-90 displayed the lowest initial value, with 70.36 mg g^{-1} DW. At NIP (Day 37), starch content declined in all cultivars, reaching 85.62 mg g^{-1} DW in Longal, 72.68 mg g^{-1} DW in Judia, 70.28 mg g^{-1} DW in Martaínha, and 51.01 mg g^{-1} DW in Ca-90. At RIP (Day 67), starch concentration increased in all plant materials, with values of 98.75, 86.37, 76.26, and 60.51 mg g^{-1} DW in Longal, Judia, Martaínha, and Ca-90, respectively.

Relative to IP (Day 30), the strongest reduction during drought was observed in Ca-90, which showed a decrease of 27.51% (Table 1). Martaínha also exhibited a marked decline of 20.84%, whereas Longal and Judia showed more moderate decreases of 11.03% and 7.60%, respectively. During recovery, starch content increased in all cultivars. The highest relative increases from NIP (Day 37) to RIP (Day 67) were recorded in Judia and Ca-90, with increases of 18.84% and 18.62%, respectively, followed by Longal with 15.34% and Martaínha with 8.51% (Table 1). Despite this recovery, the extent of starch restoration differed among cultivars. Longal slightly exceeded its initial starch content at RIP (Day 67), whereas Judia also recovered to a value above that recorded at IP (Day 30). In contrast, Ca-90 and Martaínha remained below their respective initial values after rewatering.

Overall, the temporal pattern of starch content was characterised by depletion under drought and partial or complete recovery after irrigation was resumed, with distinct cultivar-dependent dynamics.

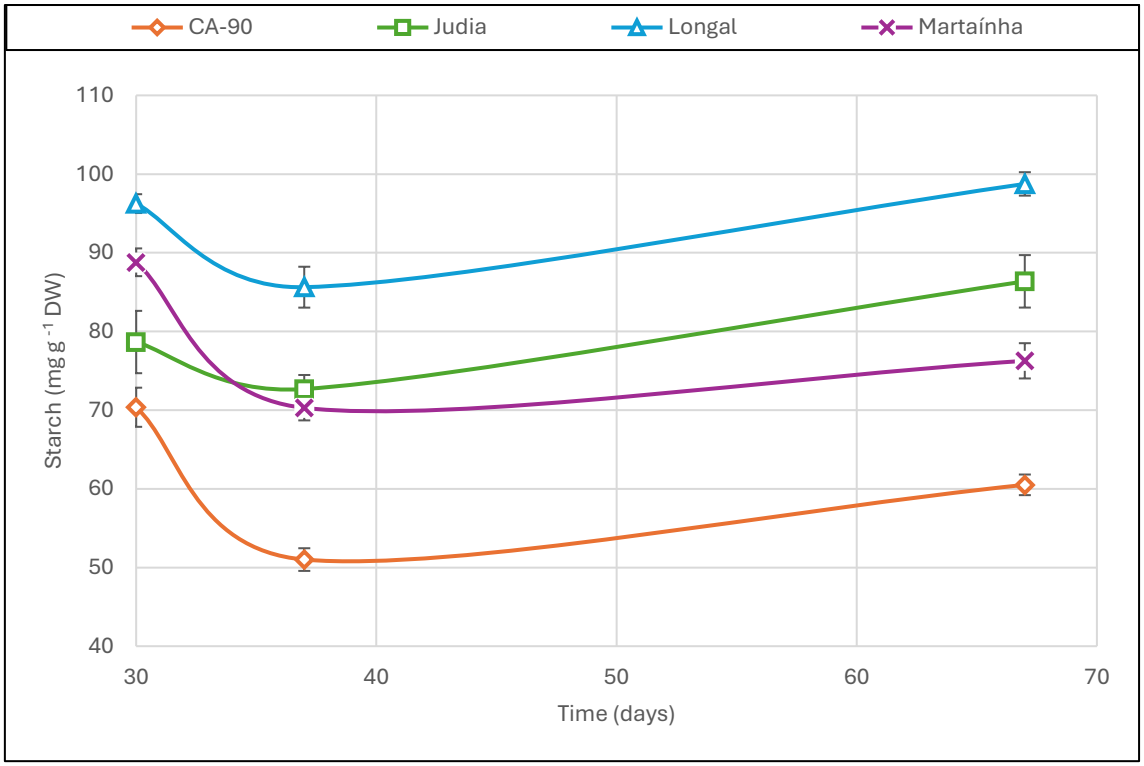


Figure 6. Starch content (mg g^{-1} DW) of *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). Values are presented as mean ± standard error (SE).

Table 1. Relative variation (%Δ) and slope of soluble sugar and starch contents in *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the drought phase, from the initial phase, IP (Day 30), to the non-irrigation phase, NIP (Day 37), and during the recovery phase, from NIP (Day 37) to the recovery phase, RIP (Day 67).

Cultivar	Soluble sugar %Δ (day 30→day 37)	Soluble sugar %Δ (day 37→day 67)	Starch %Δ (day 30→day 37)	Starch %Δ (day 37→day 67)
CA-90	-6.72	14.24	-27.51	18.62
Judia	18.37	-2.11	-7.60	18.83
Longal	18.55	-0.31	-11.03	15.33
Martaínha	7.01	3.32	-20.84	8.51

Soluble protein content (mg g^{-1} DW) varied across the experimental phases and among chestnut cultivars (Figure 7). In all genotypes, protein concentration increased from the initial phase, IP (Day 30), to the non-irrigation phase, NIP (Day 37), although the magnitude of this increase differed among cultivars. After rewatering, distinct patterns were observed among plant materials.

At IP (Day 30), Longal showed the highest mean soluble protein content, reaching 260.43 mg g^{-1} DW, followed by Martaínha with 251.76 mg g^{-1} DW and Judia with 240.34 mg g^{-1} DW. Ca-90 displayed the lowest value, with 109.30 mg g^{-1} DW. At NIP (Day 37), soluble protein content increased in all cultivars, reaching 313.46 mg g^{-1} DW in Longal, 279.33 mg g^{-1} DW in Martaínha, 260.74 mg g^{-1} DW in Judia, and 111.49 mg g^{-1} DW in Ca-90. At RIP (Day

67), soluble protein content remained high or increased further, with values of 310.94, 311.71, 283.25, and 139.70 mg g^{-1} DW in Longal, Judia, Martaínha, and Ca-90, respectively. Longal showed the highest protein content during both IP and NIP, whereas at RIP (Day 67) the highest value was recorded in Judia, closely followed by Longal. Ca-90 consistently displayed the lowest soluble protein content throughout the experimental period. Although all cultivars showed higher values at RIP (Day 67) than at IP (Day 30), the extent of increase differed among genotypes, with a more marked rise in Judia and Ca-90 after rewatering.

Overall, the temporal pattern of soluble protein content was characterised by an increase under drought conditions and the maintenance of elevated values, or further accumulation, after rewatering, with clear cultivar-dependent differences across the experimental phases.

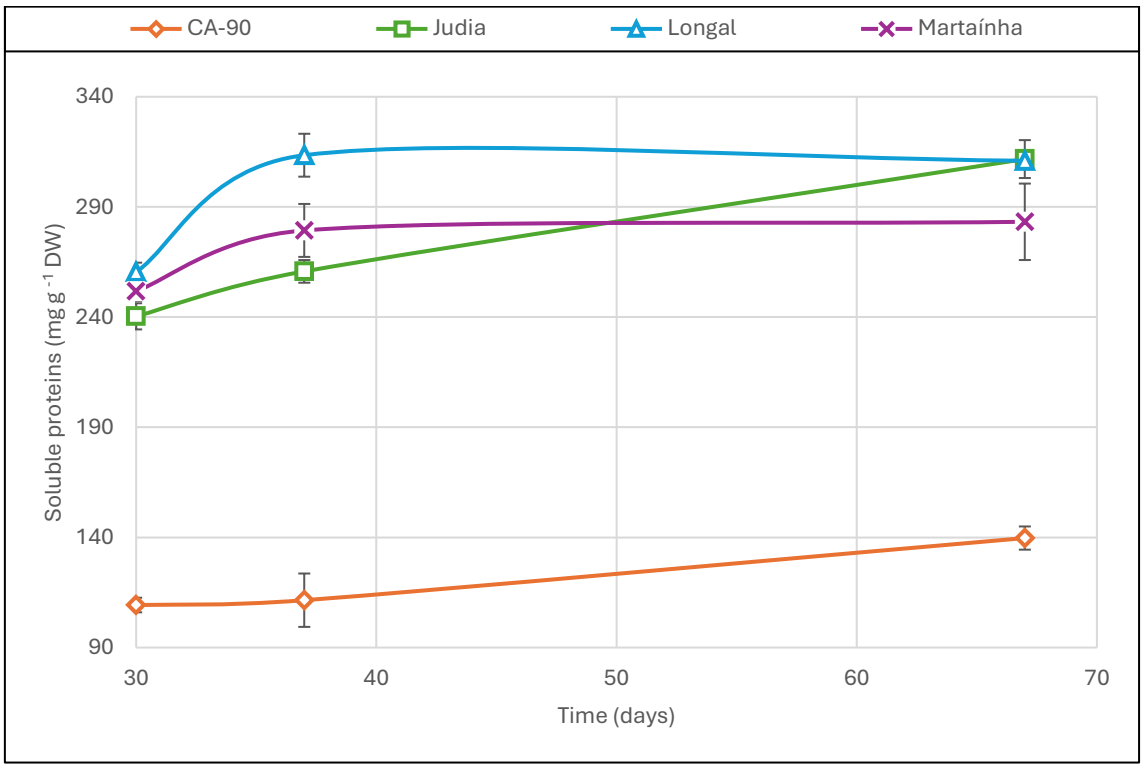


Figure 7. Soluble protein content (mg g^{-1} DW) of *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). Values are presented as mean ± standard error (SE).

3.3. Membrane injury under drought and recovery

Electrolyte leakage (%) varied across the experimental phases and among chestnut cultivars (Figure 8). Distinct temporal patterns were observed among genotypes, particularly during the recovery phase.

At the initial phase, IP (Day 30), Longal showed the highest mean electrolyte leakage, with 23.75%, followed by Ca-90 with 22.52%. Lower values were recorded in Martaínha and Judia, with 21.56% and 19.79%, respectively. At the non-irrigation phase, NIP (Day 37),

electrolyte leakage increased in Ca-90 and Judia, reaching 27.36% and 21.92%, respectively. In contrast, Longal and Martaínha showed values similar to those recorded at IP (Day 30), with 23.58% and 21.19%, respectively.

At the recovery phase, RIP (Day 67), electrolyte leakage decreased in Ca-90 and Judia relative to NIP (Day 37), reaching 24.88% and 18.21%, respectively. However, in Ca-90 the value remained above that recorded at IP (Day 30), whereas in Judia it fell below the initial level. A more pronounced reduction was observed in Longal and Martaínha, which reached 16.96% and 13.04%, respectively.

Overall, the temporal pattern of electrolyte leakage revealed cultivar-dependent differences in membrane response across drought and recovery phases. Ca-90 showed the highest value under drought and maintained comparatively elevated leakage after rewatering, whereas Longal and Martaínha displayed the lowest values at RIP (Day 67).

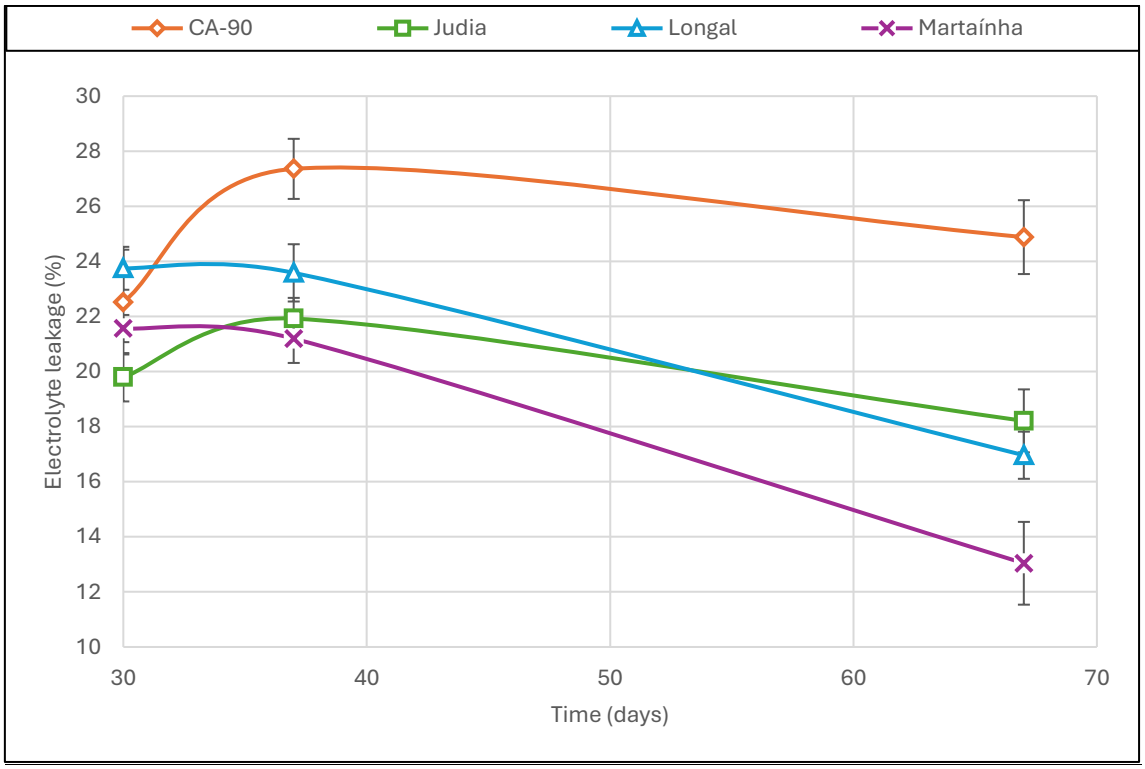


Figure 8. Electrolyte leakage of Castanea sativa cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). Values are presented as mean ± standard error (SE).

3.4. Enzymatic antioxidant responses

CAT and APX activities varied across the experimental phases and among chestnut cultivars (Figure 9). Although both enzymes responded to drought and rewatering, their temporal patterns differed.

CAT activity increased markedly from the initial phase, IP (Day 30), to the non-irrigation phase, NIP (Day 37), in all cultivars (Figure 9a). At IP, CAT values ranged from 33.21 U mg^{-1} protein in Judia to 37.22 U mg^{-1} protein in Longal, while CA-90 and Martaínha showed similar intermediate values of 36.19 and 36.13 U mg^{-1} protein, respectively. Under drought, CAT activity rose sharply in all plant materials, reaching its highest values at NIP, with Longal and Martaínha showing the greatest activities, 83.15 and 81.16 U mg^{-1} protein, respectively. Lower values were recorded in CA-90 and Judia, with 77.37 and 74.15 U mg^{-1} protein. After rewatering, CAT activity declined in all cultivars but remained above the initial levels. At RIP (Day 67), Longal maintained the highest CAT activity, followed by Martaínha, CA-90, and Judia.

APX activity showed a similar overall pattern, with low values at IP, a marked increase at NIP, and a subsequent decline after rewatering (Figure 9b). At IP, Martaínha showed the highest APX activity, with 26.88 U mg^{-1} protein, whereas Judia displayed the lowest value, at 21.35 U mg^{-1} protein. CA-90 and Longal showed intermediate activities of 23.35 and 22.23 U mg^{-1} protein, respectively. At NIP (Day 37), APX increased substantially in all cultivars, reaching 71.20 U mg^{-1} protein in Martaínha, 69.91 U mg^{-1} protein in Longal, 68.11 U mg^{-1} protein in Judia, and 59.28 U mg^{-1} protein in CA-90. After rewatering, APX activity declined markedly in all plant materials, with values at RIP (Day 67) ranging from 25.91 U mg^{-1} protein in Judia to 30.89 U mg^{-1} protein in Longal. CA-90 and Martaínha reached 28.27 and 29.76 U mg^{-1} protein, respectively.

Taken together, both CAT and APX displayed a pronounced increase during drought, followed by a decline after rewatering, although APX showed a more uniform response among cultivars at the recovery phase.

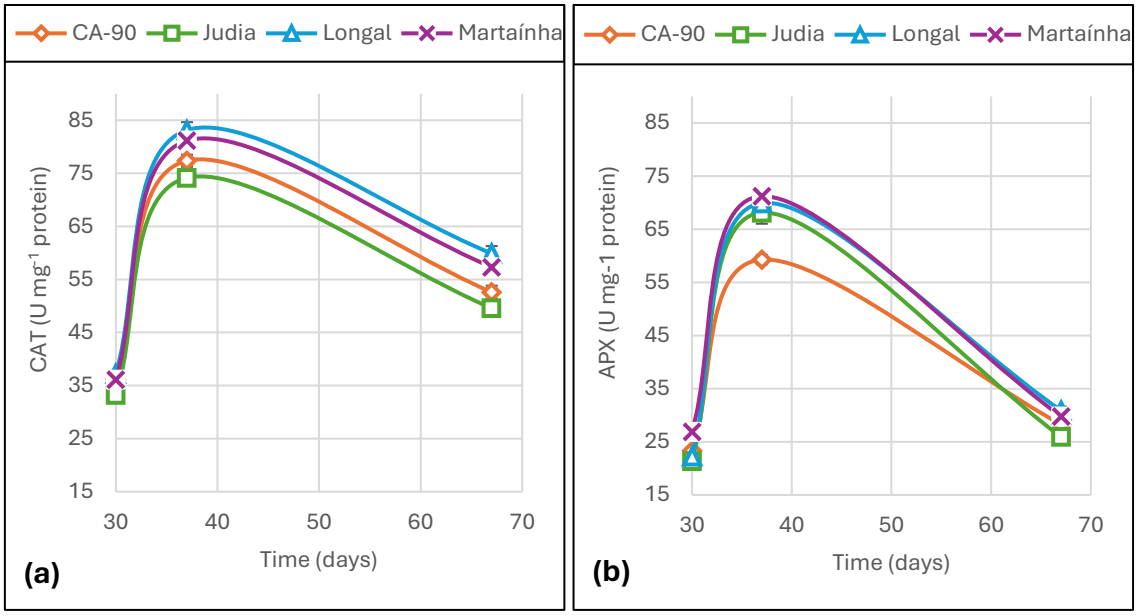


Figure 9. Antioxidant enzyme activities of *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). (a) Catalase (CAT) activity and (b) ascorbate peroxidase (APX) activity, both expressed as U mg^{-1} protein. Values are presented as mean ± standard error (SE).

3.5. Non-enzymatic antioxidant responses and antioxidant capacity

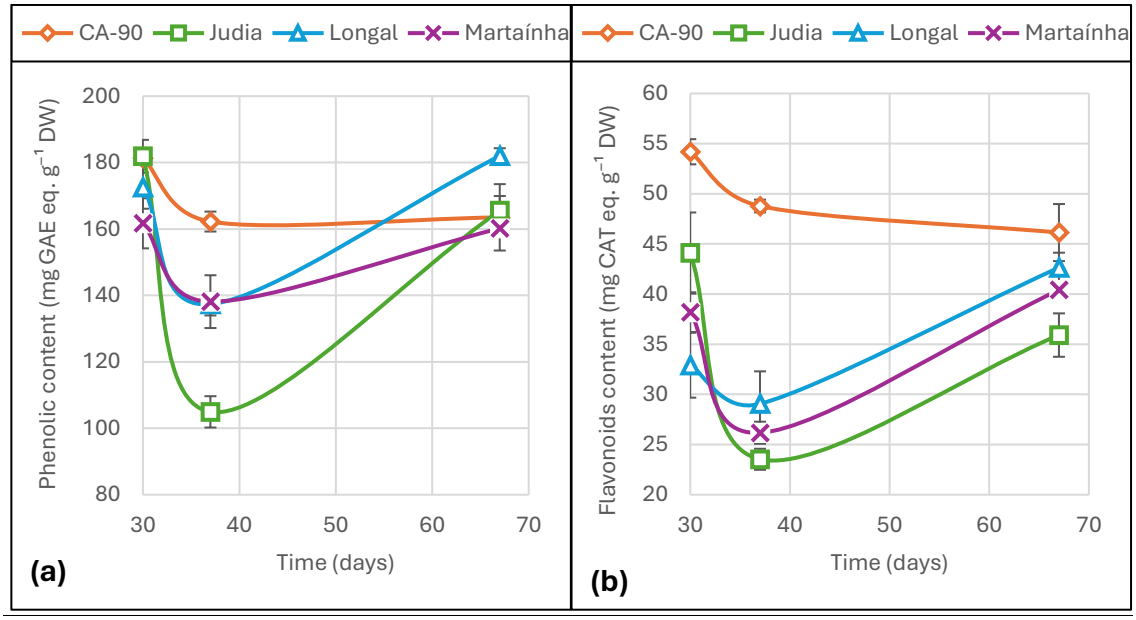
Non-enzymatic antioxidant responses varied across the experimental phases and among chestnut cultivars (Figure 10). Distinct patterns were observed for total phenolic compounds, total flavonoids, and ferric reducing antioxidant power (FRAP), particularly during the drought phase.

Total phenolic compounds decreased from the initial phase, IP (Day 30), to the non-irrigation phase, NIP (Day 37), in all cultivars, although with different magnitudes (Figure 10a). At IP (Day 30), the highest mean values were recorded in Judia and CA-90, with 181.90 and 181.32 mg GAE g^{-1} DW, respectively, followed by Longal with 172.59 mg GAE g^{-1} DW and Martaínha with 161.72 mg GAE g^{-1} DW. At NIP (Day 37), the lowest value was observed in Judia, which declined markedly to 104.94 mg GAE g^{-1} DW, while CA-90 maintained the highest value at 162.24 mg GAE g^{-1} DW. Longal and Martaínha showed intermediate values of 137.38 and 138.13 mg GAE g^{-1} DW, respectively. At RIP (Day 67), phenolic content recovered in Judia and Longal, reaching 165.51 and 182.01 mg GAE g^{-1} DW, respectively, whereas CA-90 and Martaínha remained close to their initial levels, with 163.51 and 160.22 mg GAE g^{-1} DW.

A similar phase-dependent response was observed for total flavonoids (Figure 10b). At IP (Day 30), CA-90 showed the highest flavonoid content, with 54.19 mg CAT eq. g^{-1} DW, followed by Judia with 44.09 mg CAT eq. g^{-1} DW, Martaínha with 38.20 mg CAT eq. g^{-1} DW, and Longal with 32.91 mg CAT eq. g^{-1} DW. At NIP (Day 37), flavonoid levels decreased in all cultivars, with the strongest reduction observed in Judia, which reached 23.53 mg CAT eq. g^{-1} DW. CA-90 maintained the highest value during drought, with 48.78 mg CAT eq. g^{-1} DW, whereas Longal and Martaínha reached 29.06 and 26.18 mg CAT eq. g^{-1} DW, respectively. At RIP (Day 67), flavonoid content increased in all cultivars relative to NIP (Day 37), reaching 46.14 mg CAT eq. g^{-1} DW in CA-90, 35.92 mg CAT eq. g^{-1} DW in Judia, 42.65 mg CAT eq. g^{-1} DW in Longal, and 40.42 mg CAT eq. g^{-1} DW in Martaínha.

FRAP values also varied across the experimental phases (Figure 10c). At IP (Day 30), Judia showed the highest ferric reducing antioxidant power, with 929.53 µmol $FeSO_4$ eq. g^{-1} DW, followed by Longal with 813.03 µmol $FeSO_4$ eq. g^{-1} DW, Martaínha with 788.03 µmol $FeSO_4$ eq. g^{-1} DW, and CA-90 with 773.53 µmol $FeSO_4$ eq. g^{-1} DW. At NIP (Day 37), FRAP decreased in all cultivars, with the lowest value again recorded in Judia, at 570.83 µmol $FeSO_4$ eq. g^{-1} DW. CA-90 showed the highest value during this phase, with 744.23 µmol $FeSO_4$ eq. g^{-1} DW, while Longal and Martaínha reached 705.73 and 656.13 µmol $FeSO_4$ eq. g^{-1}

DW, respectively. At RIP (Day 67), FRAP increased in Judia, Longal, and Martaínha, reaching 789.38, 850.33, and 751.28 µmol $FeSO_4$ eq. g^{-1} DW, respectively, whereas CA-90 declined slightly further to 696.03 µmol $FeSO_4$ eq. g^{-1} DW. Overall, the non-enzymatic antioxidant parameters showed a general reduction during drought, followed by partial or substantial recovery after rewatering, with distinct cultivar-dependent patterns across the experimental phases.



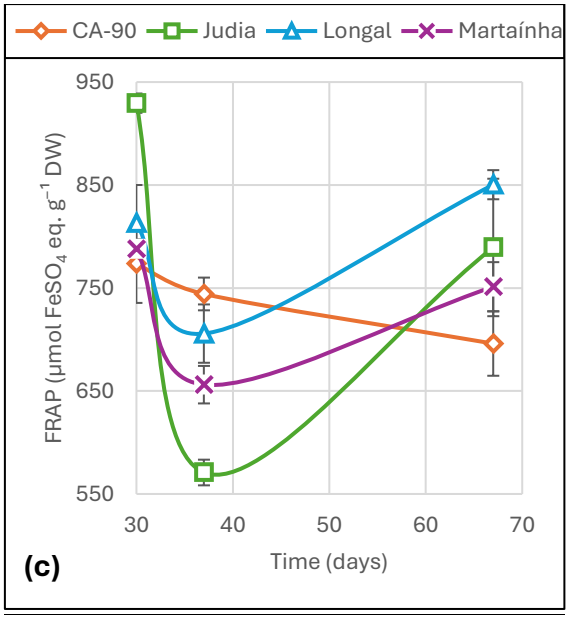


Figure 10. Non-enzymatic antioxidant responses of *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants during the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). **(a)** Total phenolic compounds, expressed as mg gallic acid equivalents (GAE) g^{-1} dry weight (DW); **(b)** total flavonoids, expressed as mg catechin equivalents (CAT eq.) g^{-1} DW; and **(c)** ferric reducing antioxidant power (FRAP), expressed as µmol $FeSO_4$ equivalents g^{-1} DW. Values are presented as mean ± standard error (SE).

3.6. Integrated cultivar responses to drought and rewatering

Correlation analysis revealed a coherent network of associations among the physiological and biochemical traits evaluated across cultivars and experimental phases (Table 2). *A* was strongly and positively associated with *E*, while both variables were negatively related to leaf temperature. Carbon-related metabolites followed a similar pattern, with soluble sugars showing positive relationships with *A*, *E*, starch, and especially soluble proteins, and starch also being positively associated with gas exchange and protein content. By contrast, electrolyte leakage showed the opposite trend, being negatively correlated with *A*, *E*, sugars, starch, and proteins, and positively associated with leaf temperature. Among the non-enzymatic antioxidant variables, total phenolic compounds were strongly correlated with FRAP and total flavonoids, whereas FRAP also maintained a positive association with flavonoids. CAT and APX were strongly and positively correlated with each other and showed positive relationships with leaf temperature, while both were negatively associated with *A*, *E*, phenolics, flavonoids, and FRAP.

Overall, the correlation matrix highlighted a close linkage between physiological performance, thermal status, carbon-related metabolism, membrane integrity, and both enzymatic and non-enzymatic antioxidant responses.

Table 2. Pearson correlation coefficients among physiological and biochemical variables measured in *C. sativa* cultivars grafted onto CA-90 and non-grafted CA-90 plants across the initial phase, IP (Day 30), the non-irrigation phase, NIP (Day 37), and the recovery phase, RIP (Day 67). Variables included net photosynthetic rate (*A*), transpiration rate (*E*), sun-exposed leaf temperature (Leaf_temp), soluble sugars (Sugar), starch, soluble proteins (Proteins), electrolyte leakage (EL), total phenolic compounds (Phenolics), total flavonoids (Flavonoids), ferric reducing antioxidant power (FRAP), Catalase (CAT) and ascorbate peroxidase (APX).

	A	*E*	Leaf_temp	Sugar	Starch	Proteins	EL	Phenolics	Flavonoids	FRAP	CAT	APX
A	1											
E	0,92	1										
Leaf_temp	-0,85	-0,89	1									
Sugar	0,51	0,42	-0,43	1								
Starch	0,83	0,66	-0,64	0,78	1							
Proteins	0,58	0,54	-0,52	0,95	0,75	1						
EL	-0,60	-0,78	0,72	-0,49	-0,46	-0,61	1					
Phenolics	0,45	0,38	-0,34	-0,29	0,22	-0,26	-0,16	1				
Flavonoids	-0,07	-0,02	0,01	-0,70	-0,32	-0,71	0,08	0,78	1			
FRAP	0,63	0,47	-0,47	0,06	0,44	0,05	-0,27	0,88	0,53	1		
CAT	-0,57	-0,45	0,57	0,18	-0,34	0,18	0,18	-0,70	-0,48	-0,68	1	
APX	-0,62	-0,58	0,68	0,16	-0,35	0,13	0,33	-0,82	-0,59	-0,74	0,93	1

4. Discussion

The present results show that drought response in chestnut was not defined by a single variable, but by the coordination between gas exchange, leaf thermal behaviour, carbon-related metabolism, membrane stability, and non-enzymatic antioxidant responses. Across all data, irrigation withdrawal caused a marked reduction in *A* and *E*, together with an increase in sun-exposed leaf temperature. This combination is consistent with the typical physiological response of woody plants to water deficit, in which stomatal limitation reduces transpiration and carbon assimilation, while diminished evaporative cooling promotes leaf warming (Gallé et al., 2007; Ruehr et al., 2019). The strong negative correlations between leaf temperature and both *A* and *E*, together with the positive correlation between *A* and *E*, reinforce this interpretation and indicate that thermal and gas-exchange traits were tightly coupled in the present experimental system.

Differences among cultivars became especially evident when drought (NIP) and recovery (RIP) were considered together. Longal and Martaínha maintained comparatively higher photosynthetic performance during drought (NIP) and showed the strongest recovery after rewatering (RIP), whereas CA-90 consistently displayed the lowest *A* and *E* values and the highest leaf temperatures. Judia showed a stronger decline during drought (NIP), but also a clear rebound after irrigation resumed (RIP). This distinction between stress tolerance and recovery capacity is important, because resilience in woody plants depends not only on the ability to withstand water deficit, but also on the capacity to restore functional activity after stress relief. Post-drought recovery has increasingly been recognised as a critical component of drought adaptation, and it may differ substantially among genotypes even when drought effects during the stress phase appear similar (Ruehr et al., 2019).

The carbohydrate data provide further insight into the mechanisms underlying these differences. Soluble sugars increased during drought (NIP) in Judia, Longal, and Martaínha, whereas starch declined in all cultivars, particularly in CA-90 and Martaínha. This pattern is consistent with drought-induced reorganisation of carbon metabolism, in which reserve mobilisation contributes to soluble sugar accumulation under limited photosynthetic input. In woody plants, the conversion of starch into soluble sugars under drought has often been interpreted as part of osmotic adjustment and short-term metabolic maintenance, while starch depletion reflects the use of stored carbon under reduced assimilation (Xiong et al., 2022). In the present study, Longal and Judia showed the strongest relative increase in soluble sugars during the drought phase (NIP), whereas CA-90 failed to increase sugar

content despite showing the lowest photosynthetic performance and one of the strongest reductions in starch. This suggests that CA-90 was less effective in maintaining a favourable balance between carbon consumption and carbon reallocation under severe water deficit.

The recovery phase (RIP) further clarified the meaning of these carbon shifts. Longal and Judia restored starch to values at or above the initial phase (IP), whereas CA-90 and Martaínha remained below their respective starting levels. This indicates that post-drought recovery (RIP) was not limited to a simple resumption of gas exchange but involved cultivar-dependent differences in reserve restoration. In tree species, rewatering after drought often triggers competition between immediate physiological repair, resumed growth, osmotic readjustment, and rebuilding of non-structural carbon reserves. As a result, reserve recovery may be incomplete even when gas exchange partially recovers (Ruehr et al., 2019). In this context, Longal appears to have combined relatively good drought performance with efficient metabolic restoration, while CA-90 remained functionally and metabolically constrained.

The pattern observed for soluble proteins supports this interpretation. Protein content increased during drought (NIP) in all cultivars and remained high, or increased further, after rewatering (RIP). This is in line with reports from other woody species, particularly oaks, where drought and rehydration induced coordinated changes in soluble sugars and soluble proteins, suggesting that both groups of compounds participate in osmotic regulation, metabolic adjustment, and recovery processes (Xiong et al., 2022). The very strong positive correlation between sugars and proteins in the present study suggests that these two variables formed part of a common acclimation response rather than representing independent processes. The fact that protein levels remained high after rewatering (RIP) may indicate that recovery involved not only the reversal of stress symptoms, but also active metabolic reorganisation.

Electrolyte leakage further differentiated the cultivars in terms of membrane stability. CA-90 showed the highest EL under drought (NIP) and remained above its initial value after recovery (RIP), whereas Judia showed a smaller drought increase and a decline below the initial level after rewatering. Longal and Martaínha changed little from IP to NIP, but both showed a marked reduction at RIP. Since higher EL is generally interpreted as a sign of greater membrane disruption, these results suggest that CA-90 suffered stronger structural impairment during drought (NIP) and recovered less completely than the grafted cultivars. This interpretation is supported by the correlation matrix, in which EL was negatively associated with *A*, *E*, sugars, starch, and proteins, and positively associated with leaf temperature. Relationships of this kind have been widely linked to drought-induced loss of

membrane integrity and reduced stress tolerance in plants, and membrane failure has been proposed as a strong indicator of severe drought damage in woody species (Guadagno et al., 2017). In the present study, EL therefore complements the gas-exchange and temperature data by indicating that the lower performance of CA-90 was accompanied by greater cellular instability.

The enzymatic antioxidant responses further refine this interpretation. Both CAT and APX increased markedly during drought (NIP) and declined after rewatering (RIP), although activities remained above the initial phase in all cultivars. This pattern is consistent with the role of both enzymes in the detoxification of hydrogen peroxide under water deficit, where CAT acts mainly in peroxisomes and APX operates in several cellular compartments through the ascorbate-glutathione cycle (Sofo et al., 2015). In the present study, Longal and Martaínha showed the highest CAT activities under drought, while APX also reached its highest values in these cultivars, with Judia showing a similar but slightly less pronounced response and CA-90 remaining comparatively lower, particularly for APX. This suggests that the cultivars that better maintained physiological function under drought were also able to reinforce the enzymatic scavenging of H_2O_2 more effectively. Similar drought-induced increases in CAT and APX have been described in woody and perennial species, including coffee and olive, where enhanced enzymatic antioxidant activity was associated with protection against oxidative stress during water deficit (Denaxa et al., 2020; Pinheiro et al., 2004). The decline of both enzymes after rewatering, while still remaining above initial values, is also biologically consistent with the progressive relaxation of oxidative pressure during recovery, as reported for APX and other components of the ascorbate-glutathione cycle in droughted and rewatered *Prunus* hybrids (Sofo et al., 2005). Within this framework, the stronger CAT and APX induction observed in Longal and Martaínha supports the idea that a more efficient enzymatic antioxidant adjustment contributed to their better overall drought performance and recovery.

The behaviour of the non-enzymatic antioxidant variables adds another important dimension. Total phenolic compounds, total flavonoids, and FRAP all declined during drought (NIP) and recovered partially or substantially after rewatering (RIP), although the magnitude of change varied among cultivars. Judia showed the strongest decline during NIP, followed by a marked recovery in RIP. Longal also showed a clear rebound, especially in phenolics and FRAP, whereas CA-90 remained comparatively stable in phenolics and flavonoids but showed a further decline in FRAP after rewatering (RIP). This suggests that drought did not induce a uniform accumulation of non-enzymatic antioxidants in this system. Instead, the stress phase may have involved depletion of pre-existing antioxidant

649 pools, reduced synthesis, or both, with subsequent restoration after rewatering (RIP).
650 Similar cultivar-dependent patterns have been reported in olive, where water deficit altered
651 phenolic composition and antioxidant activity in contrasting ways depending on genotype
652 and stress intensity (Petridis et al., 2012). The strong positive correlations between
653 phenolics and FRAP, and between phenolics and flavonoids, indicate that phenolic
654 metabolism contributed substantially to the antioxidant capacity measured here.

655 Taken together, the correlation analysis provides an integrative view of the coordinated
656 responses underlying drought tolerance and recovery in chestnut. The strong association
657 between *A* and *E*, together with their negative relationship with leaf temperature, is
658 consistent with the well-established coupling between stomatal regulation, transpirational
659 cooling, and photosynthetic performance under drought in woody plants (Gallé et al., 2007;
660 Ruehr et al., 2019). The positive association of sugars, starch, and proteins with gas-
661 exchange traits further suggests that cultivars maintaining higher physiological activity also
662 preserved a more favourable carbon and metabolic status, in agreement with studies
663 showing that carbohydrate dynamics are closely linked to drought adjustment and post-
664 stress recovery in trees (Guo et al., 2021; Kapazoglou et al., 2025). By contrast, the positive
665 relationship between leaf temperature and electrolyte leakage, together with the negative
666 association of EL with A, E, and carbon-related metabolites, supports the interpretation that
667 membrane destabilisation accompanied functional decline under water deficit, as
668 described in studies linking membrane failure to drought injury and reduced survival or
669 recovery capacity (Guadagno et al., 2017; dos Santos et al., 2022). In parallel, the close
670 relationship between phenolics, flavonoids, and FRAP indicates that phenolic metabolism
671 contributed substantially to antioxidant capacity, whereas the drought-induced rise in CAT
672 and APX suggests that enzymatic H_2O_2 scavenging formed an additional layer of protection
673 during the stress phase, as also reported in other drought-exposed woody plants and
674 perennial species (Denaxa et al., 2020; Sofo et al., 2015). Rather than identifying a single
675 dominant drought marker, these associations suggest that resilience depended on the
676 maintenance of coordination among leaf cooling, carbon assimilation, reserve
677 management, membrane protection, and both enzymatic and non-enzymatic antioxidant
678 balance.

679 From this perspective, Longal appears to be the most balanced drought-resilient
680 cultivar in the present experiment. It combined comparatively high A and E under drought,
681 substantial sugar accumulation, moderate starch depletion, strong reserve restoration
682 after rewatering, and low EL at recovery. This overall behaviour was also accompanied by
683 the strongest induction of CAT and APX during drought, reinforcing the view that efficient

enzymatic antioxidant adjustment formed part of the resilience strategy of these cultivars. Martaínha also showed strong physiological recovery and low EL at RIP, although its starch content remained below the initial value. Judia displayed greater suppression during drought, particularly in the non-enzymatic antioxidant variables, but also showed substantial post-drought recovery, suggesting a more reactive response pattern. By contrast, CA-90 showed the clearest signs of sensitivity, with lower gas-exchange performance, higher leaf temperature, stronger starch depletion, and persistently elevated EL after rewatering. These differences highlight that drought adaptation in chestnut should be assessed not only during the stress period, but also through the capacity to recover metabolic and physiological function once water becomes available again, as also emphasised in broader analyses of drought recovery in woody species (Gallé et al., 2007; Ruehr et al., 2019).

Overall, the present study shows that the recovery phase was essential for resolving cultivar behaviour. If the analysis had stopped at the drought phase, part of the metabolic plasticity and functional resilience of the cultivars would have remained hidden. By integrating gas exchange, leaf temperature, carbon-related metabolites, membrane integrity, and non-enzymatic antioxidant responses, the results support the view that drought resilience in chestnut is a dynamic trait emerging from coordinated physiological and biochemical regulation rather than from isolated stress markers. This framework may be particularly useful for screening chestnut germplasm under Mediterranean scenarios, where severe summer drought is increasingly followed by episodic rewatering events and the capacity to recover rapidly may be as important as tolerance to stress itself (Ruehr et al., 2019; Xiong et al., 2022).

5. Limitation Statement

A limitation of the present study is that the enzymatic antioxidant dataset was restricted to CAT and APX, while other important components of the antioxidant system, such as POD and SOD, were not included in the final integrative analysis. In addition, the experiment was conducted under controlled conditions and focused on a defined drought-rewatering sequence, which allowed a clear comparison among cultivars but may not fully capture the complexity of field environments, where stress intensity, duration, and recovery dynamics are more variable. Finally, although the recovery phase provided important insight into post-stress behaviour, the study was limited to a single recovery period and therefore does not resolve longer-term acclimation or repeated stress memory effects.

6. Conclusion

The present study demonstrates that drought response in chestnut results from the integration of multiple physiological and biochemical processes rather than from the isolated behaviour of a single trait. Water deficit reduced gas exchange and increased leaf temperature in all plant materials, while simultaneously altering carbon-related metabolism, membrane stability, antioxidant status, and enzymatic defence. The recovery phase proved essential for resolving cultivar behaviour, showing that drought resilience depended not only on tolerance during stress imposition, but also on the capacity to restore function and metabolic balance after rewatering.

Among the evaluated materials, Longal showed the most balanced response, combining comparatively higher photosynthetic performance under drought, effective recovery after rewatering, favourable carbohydrate dynamics, low membrane damage at recovery, and strong antioxidant adjustment. Martaínha also displayed a robust recovery pattern, particularly in physiological performance and antioxidant responses, although reserve restoration was less complete. Judia showed greater sensitivity during drought, especially in non-enzymatic antioxidant depletion, but also revealed substantial recovery capacity after rewatering. In contrast, CA-90 showed the clearest signs of stress susceptibility, with lower physiological performance, higher leaf temperature, stronger reserve depletion, and persistently elevated membrane damage.

Overall, these results highlight that drought resilience in chestnut is a dynamic and coordinated trait involving the interaction of gas exchange regulation, thermal balance, carbon allocation, membrane protection, and antioxidant responses. This integrated framework provides a useful basis for identifying cultivar-specific strategies of drought tolerance and recovery and may contribute to the selection of more resilient chestnut germplasm for Mediterranean environments increasingly exposed to severe and recurrent summer drought.

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

Funding: This work is supported by National Funds by FCT–Portuguese Foundation for Science and Technology, under the projects UI/BD/150726/2020: 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).

Permissions to collect plant material: All experimental plants were commercially obtained from SERVIRURI, a registered nursery in Portugal, and were accompanied by official European Union plant passports. No plant material was collected from wild populations or third-party properties. Therefore, no specific collection permits were required for the use of these commercially acquired plants in the present study.

Ethics and consent to participate: The study was conducted using cultivated chestnut plants obtained from a registered commercial nursery. All experimental procedures involving plant material were carried out in accordance with applicable local and national regulations and guidelines. No wild plants or protected species were collected, and no specific ethical approval was required for this study.

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.

7. References

Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology*, *105*, 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

Aglietti, C., Cappelli, A., & Andreani, A. (2022). From Chestnut Tree (*Castanea sativa*) to Flour and Foods: A Systematic Review of the Main Criticalities and Control Strategies towards the Relaunch of Chestnut Production Chain. *Sustainability*, *14*(19), 12181. https://doi.org/10.3390/SU141912181

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, *239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292

Beyazyüz, F., & Kulaç, Ş. (2023). Effects of Drought Stress on Leaf Gas Exchange Parameters and Proline Accumulation in Chestnut (*Castanea sativa* Mill.) Saplings. *Turkish Journal of Agriculture - Food Science and Technology*, *11*(7), 1231–1237. https://doi.org/10.24925/TURJAF.V11I7.1231-1237.6087

780 Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram
781 quantities of protein utilizing the principle of protein-dye binding. *Analytical*
782 *Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

783 Chaves, M. M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought and salt
784 stress: regulation mechanisms from whole plant to cell. *Annals of Botany*, *103*(4), 551–
785 560. https://doi.org/10.1093/aob/mcn125

786 Conedera, M., Krebs, P., Gehring, E., Wunder, J., Hülsmann, L., Abegg, M., & Maringer, J.
787 (2021). How future-proof is Sweet chestnut (*Castanea sativa*) in a global change
788 context? *Forest Ecology and Management*, *494*, 119320.
789 https://doi.org/10.1016/J.FORECO.2021.119320

790 Denaxa, N. K., Damvakaris, T., & Roussos, P. A. (2020). Antioxidant defense system in young
791 olive plants against drought stress and mitigation of adverse effects through external
792 application of alleviating products. *Scientia Horticulturae*, *259*(2), 108812.
793 https://doi.org/10.1016/j.scienta.2019.108812

794 Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., & Basra, S. M. A. (2009). Plant drought
795 stress: Effects, mechanisms and management. *Sustainable Agriculture*, *29*, 153–188.
796 https://doi.org/10.1007/978-90-481-2666-8_12/FIGURES/9_12

797 Freitas, T. R., Santos, J. A., Silva, A. P., & Fraga, H. (2021). Influence of Climate Change on
798 Chestnut Trees: A Review. *Plants*, *10*(7), 1463.
799 https://doi.org/10.3390/PLANTS10071463

800 Gallé, A., Haldimann, P., & Feller, U. (2007). Photosynthetic performance and water
801 relations in young pubescent oak (*Quercus pubescens*) trees during drought stress
802 and recovery. *New Phytologist*, *174*(4), 799–810. https://doi.org/10.1111/j.1469-
803 8137.2007.02047.x

804 Gessler, A., Bottero, A., Marshall, J., & Arend, M. (2020). The way back: recovery of trees from
805 drought and its implication for acclimation. *New Phytologist*, *228*(6), 1704–1709.
806 https://doi.org/https://doi.org/10.1111/nph.16703

807 Gill, S. S., & Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic
808 stress tolerance in crop plants. *Plant Physiology and Biochemistry*, *48*(12), 909–930.
809 https://doi.org/10.1016/j.plaphy.2010.08.016

Guadagno, C. R., Ewers, B. E., Speckman, H. N., Aston, T. L., Huhn, B. J., Devore, S. B., Ladwig, J. T., Strawn, R. N., & Weinig, C. (2017). Dead or Alive? Using Membrane Failure and Chlorophyll a Fluorescence to Predict Plant Mortality from Drought. *Plant Physiology*, *175*(1), 223–234. https://doi.org/10.1104/pp.16.00581

Hasanuzzaman, M., & Fujita, M. (2013). Exogenous sodium nitroprusside alleviates arsenic-induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by enhancing antioxidant defense and glyoxalase system. *Ecotoxicology*, *22*(3), 584–596. https://doi.org/10.1007/S10646-013-1050-4

Irigoyen, J. J., Einerich, D. W., & Sánchez-Díaz, M. (1992). Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiologia Plantarum*, *84*(1), 55–60. https://doi.org/10.1111/j.1399-3054.1992.tb08764.x

Lelieveld, J., Hadjinicolaou, P., Kostopoulou, E., Chenoweth, J., El Maayar, M., Giannakopoulos, C., Hannides, C., Lange, M. A., Tanarhte, M., Tyrlis, E., & Xoplaki, E. (2012). Climate change and impacts in the Eastern Mediterranean and the Middle East. *Climatic Change*, *114*(3), 667–687. https://doi.org/10.1007/s10584-012-0418-4

Mota, M., Pinto, T., Vilela, A., Marques, T., Borges, A., Caço, J., Ferreira-Cardoso, J., Raimundo, F., & Gomes-Laranjo, J. (2018). Irrigation positively affects the chestnut's quality: The chemical composition, fruit size and sensory attributes. *Scientia Horticulturae*, *238*, 177–186. https://doi.org/10.1016/J.SCIENTA.2018.04.047

Nakano, Y., & Asada, K. (1981). Hydrogen Peroxide is Scavenged by Ascorbate-specific Peroxidase in Spinach Chloroplasts. *Plant and Cell Physiology*, *22*(5), 867–880. https://doi.org/10.1093/OXFORDJOURNALS.PCP.A076232

Petridis, A., Therios, I., Samouris, G., Koundouras, S., & Giannakoula, A. (2012). Effect of water deficit on leaf phenolic composition, gas exchange, oxidative damage and antioxidant activity of four Greek olive (*Olea europaea* L.) cultivars. *Plant Physiology and Biochemistry*, *60*, 1–11. https://doi.org/10.1016/j.plaphy.2012.07.014

Pinheiro, H. A., DaMatta, F. M., Chaves, A. R. M., Fontes, E. P. B., & Loureiro, M. E. (2004). Drought tolerance in relation to protection against oxidative stress in clones of *Coffea canephora* subjected to long-term drought. *Plant Science*, *167*(6), 1307–1314. https://doi.org/10.1016/j.plantsci.2004.06.027

Rose, R., Rose, C. L., Omi, S. K., Forry, K. R., Durall, D. M., & Bigg, W. L. (1991). Starch Determination by Perchloric Acid vs Enzymes: Evaluating the Accuracy and Precision of Six Colorimetric Methods. *Journal of Agricultural and Food Chemistry*, *39*(1), 2–11. https://doi.org/10.1021/jf00001a001

Ruehr, N. K., Grote, R., Mayr, S., & Arneth, A. (2019). Beyond the extreme: recovery of carbon and water relations in woody plants following heat and drought stress. *Tree Physiology*, *39*(8), 1285–1299. https://doi.org/10.1093/treephys/tpz032

Sardans, J., Gargallo-Garriga, A., Urban, O., Klem, K., Walker, T. W. N., Holub, P., Janssens, I. A., & Peñuelas, J. (2020). Ecometabolomics for a Better Understanding of Plant Responses and Acclimation to Abiotic Factors Linked to Global Change. *Metabolites*, *10*(6), 239. https://doi.org/10.3390/metabo10060239

Sharma, A., Shahzad, B., Rehman, A., Bhardwaj, R., Landi, M., & Zheng, B. (2019). Response of Phenylpropanoid Pathway and the Role of Polyphenols in Plants under Abiotic Stress. *Molecules*, *24*(13), 2452. https://doi.org/10.3390/molecules24132452

Sofo, A., Scopa, A., Nuzzaci, M., & Vitti, A. (2015). Ascorbate Peroxidase and Catalase Activities and Their Genetic Regulation in Plants Subjected to Drought and Salinity Stresses. *International Journal of Molecular Sciences*, *16*(6), 13561–13578. https://doi.org/10.3390/ijms160613561

Sofo, A., Tuzio, A. C., Dichio, B., & Xiloyannis, C. (2005). Influence of water deficit and rewatering on the components of the ascorbate–glutathione cycle in four interspecific Prunus hybrids. *Plant Science*, *169*(2), 403–412. https://doi.org/10.1016/j.plantsci.2005.04.004

Szabados, L., & Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends in Plant Science*, *15*(2), 89–97. https://doi.org/10.1016/j.tplants.2009.11.009

Tomasella, M., Petrussa, E., Petruzzellis, F., Nardini, A., & Casolo, V. (2019). The Possible Role of Non-Structural Carbohydrates in the Regulation of Tree Hydraulics. *International Journal of Molecular Sciences*, *21*(1), 144. https://doi.org/10.3390/ijms21010144

Tramblay, Y., Koutroulis, A., Samaniego, L., Vicente-Serrano, S. M., Volaire, F., Boone, A., Le Page, M., Llasat, M. C., Albergel, C., Burak, S., Cailleret, M., Kalin, K. C., Davi, H., Dupuy, J. L., Greve, P., Grillakis, M., Hanich, L., Jarlan, L., Martin-StPaul, N., … Polcher,

J. (2020). Challenges for drought assessment in the Mediterranean region under future climate scenarios. *Earth-Science Reviews*, *210*(1), 103348. https://doi.org/10.1016/j.earscirev.2020.103348

Xiong, S., Wang, Y., Chen, Y., Gao, M., Zhao, Y., & Wu, L. (2022). Effects of Drought Stress and Rehydration on Physiological and Biochemical Properties of Four Oak Species in China. *Plants*, *11*(5), 679. https://doi.org/10.3390/PLANTS11050679/S1

Xu, Z., Zhou, G., & Shimizu, H. (2010). Plant responses to drought and rewatering. *Plant Signaling and Behavior*, *5*(6), 649–654. https://doi.org/10.4161/psb.5.6.11398