Climate change-induced effects on *Castanea sativa* Miller – can mycorrhization be an effective strategy?

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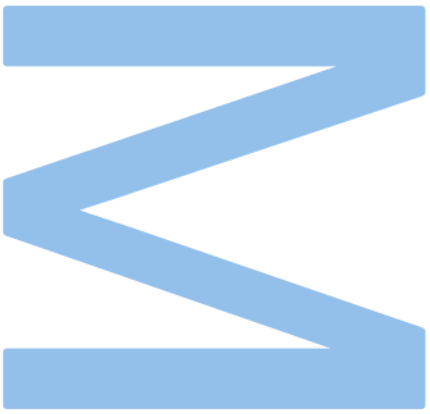

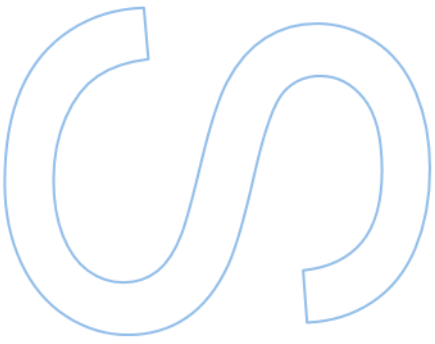

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Resumo

Num contexto de alterações climáticas, espera-se um aumento, em frequência e intensidade, de episódios de elevadas temperaturas e períodos de escassez de água na região do Mediterrâneo. O território português tem sido regularmente afetado por graves perturbações climáticas, cada vez mais comuns, que condicionam o crescimento e desenvolvimento de plantas jovens de castanheiro (*Castanea sativa* Miller), especialmente nos meses de verão. Apesar das respostas de *C. sativa* a stresses individuais estarem relativamente bem caracterizadas, o impacto combinado de diferentes fatores de stresse pode ter consequências distintas, pelo que o seu estudo é fundamental. Para além disso, no sentido de garantir a sustentabilidade e a competitividade económica da produção da castanha em Portugal, é necessário explorar estratégias sustentáveis, como a micorrização, para garantir a resiliência do castanheiro aos efeitos induzidos pelas alterações climáticas. Desta forma, o principal objetivo deste trabalhou foi perceber se a resposta fisiológica de *C. sativa* à combinação de défice hídrico e elevadas temperaturas era diferencialmente modulada pela presença de associações com fungos micorrízicos. Para tal, plantas jovens de castanheiro (cv. Marsol) não micorrizadas (NMR) e micorrizadas (MR) foram expostas a situações individuais e combinadas de calor (42 ºC; 4 h/dia) e secura (paragem da irrigação até se atingir uma capacidade de retenção de água no solo de 25%) durante 21 dias, para avaliação da resposta fisiológica e bioquímica. Os resultados revelaram que o tratamento por calor, individualmente, não afetou negativamente o crescimento, atividade fotossintética nem induziu stresse oxidativo tanto nas plantas MR como nas NMR. Por outro lado, a secura, tanto em situações de exposição singular e co-exposição, reduziu de forma acentuada o crescimento das plantas NMR, avaliado através do conteúdo relativo de água, produção de novas folhas e área foliar. A associação com ectomicorrizas (ECM) mitigou de forma eficiente parte destes efeitos, com as plantas MR expostas ao calor e à secura a apresentarem padrões de crescimento semelhantes aos das plantas controlo. Além disso, mesmo aquando da interação dos stresses, as plantas MR foram menos suscetíveis do que as NMR, com menores inibições de crescimento. Corroborando as evidências macroscópicas, o estudo do metabolismo fotossintético revelou que as trocas gasosas das plantas NMR foram severamente afetadas pela escassez de água, o que resultou numa redução da assimilação do CO_2. Contrariamente, a presença das ECM assegurou a manutenção das trocas gasosas de castanheiros expostos a situações de défice de água, a um nível semelhante ao das plantas controlo, e a um aumento da assimilação de carbono e uso eficiente de água em situações de stresse. Com o objetivo de relacionar os efeitos observados com possíveis danos oxidativos, o metabolismo redox foi avaliado nas raízes e folhas de plantas MR e NMR expostas a ambos os fatores de stresse. De uma forma geral, comparativamente às raízes, as folhas das plantas NMR e MR foram mais suscetíveis ao dano oxidativo. A secura, a título individual ou em combinação com o

calor, induziu stresse oxidativo nas plantas NMR, onde foi detetado um aumento dos níveis de peroxidação lipídica e de peróxido de hidrogénio (H_2O_2). Por outro lado, as plantas MR expostas ao stress combinado foram capazes de impedir a ocorrência de dano oxidativo, ao ativar mecanismos de defesa, através da estimulação de diversos metabolitos antioxidantes como a prolina, a glutationa e o ascorbato. Em suma, os resultados deste trabalho demonstraram, de forma clara, que a secura é o fator de stress que mais compromete o crescimento e o desenvolvimento de plantas jovens de castanheiro, registando-se uma amplificação destes efeitos aquando da co-exposição a elevadas temperaturas. Contudo, a associação com ECM permitiu aliviar parte desses efeitos ao promover o crescimento, relações hídricas, atividade fotossintética e ao estimular diferentes mecanismos internos de defesa. Pela primeira vez, este estudo caracterizou o impacto combinado do calor e secura em *C. sativa*, dando também um passo no estudo do potencial da micorrização como estratégia futura para aumentar a resiliência do castanheiro aos desafios impostos pelas alterações climáticas.

Palavras-chave:

Aquecimento global, castanheiro, ectomicorrizas, secura, calor, stress combinado, stress oxidativo, aquecimento global, performance fisiológica.

Abstract

In the context of climate change, episodes of high temperatures and periods of drought are expected to rise in frequency and intensity in the Mediterranean region. These drastic climatic shifts, which have been already affecting the Portuguese territory over the past years, can be major conditioners to the growth and development of chestnut seedlings (*Castanea sativa* Miller), especially during summer months. Still, while C. *sativa* responses to single stresses are relatively well characterized, the combined impact of these stressors may yield different outcomes, warranting further research efforts. Moreover, to ensure the sustainability and profitability of chestnut production in Portugal, it is essential to explore sustainable strategies, such as mycorrhization, to enhance young chestnut plants' tolerance to climate change-induced impacts. Therefore, the main goal of the present work was to understand whether the physiological responses of *C. sativa* to the combination of drought and heat was differentially modulated by the presence of mycorrhizal associations. For this purpose, non-mycorrhizal (NMR) and mycorrhizal (MR) hybrid chestnut plants (cv. Marsol) were exposed to the single and combined action of heat (42 ºC; 4h/day) and drought (withstand irrigation until reaching 25% of soil's water holding capacity) for 21 days for the evaluation of their physiological, biochemical, and photosynthetic performance. Results revealed that heat stress did not negatively affect the growth, photosynthetic performance nor induced oxidative stress in both MR and NMR plants. On the other hand, drought alone or in combination with high temperatures severely reduced the growth of NMR in terms of relative water content, production of new leaves and foliar area. The ectomycorrhizal (ECM) association efficiently mitigated some of these effects, by sustaining the growth of chestnut plants under heat and drought similar to control conditions. Furthermore, even under the co-exposure scenario, MR plants were less susceptible than NMR plants, with lower growth inhibitions. The gas-exchange relations of NMR were also negatively affected by drought stresses, which translated into a reduced CO_2 assimilation. In contrast, and supporting the macroscopic effects, the presence of ECM allowed drought-exposed plants to ensure gas-exchange relations comparable to non-stressed plants, with a higher carbon assimilation and water use efficiency under stress conditions. Attempting to link the observed effects to redox disorders, a thorough assessment of the oxidative metabolism was performed. Generally, leaves of both NMR and MR plants were much more susceptible to the imposed stress than roots. Drought alone, or in combination with heat, induced oxidative stress in NMR plants, where rises of lipid peroxidation and H_2O_2 were found. Contrariwise, MR plants were able to activate defence mechanisms, with the accumulation of antioxidant metabolites (proline, glutathione and ascorbate), effectively preventing oxidative damage, particularly under the co-exposure scenario. Overall, results from the present work clearly show that drought is the stress that mostly affects chestnut plants' growth, with the co-presence of heat further exacerbating some of these impacts. Still, ECM association was able to alleviate part of such effects by improving

growth and water relations, photosynthetic performance and activating several internal defence mechanisms. For the first time, this study unravelled the impact of combined heat and drought in *C. sativa* and demonstrated the potential of mycorrhization as a future strategy to increase chestnut plants' resilience to challenging conditions drove by climate change.

Keywords

Global warming, chestnut plant, ectomycorrhiza, physiological performance, combined stress, oxidative stress, heat, drought.

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Abbreviations, acronyms and symbols

ε extinction molar coefficient

$^1O_2$ singlet oxygen

3PGA 3-phosphoglycerate

A CO_2 assimilation rate

ABA abscisic acid

Abs absorbance

AM arbuscular mycorrhiza

AOX antioxidant

APX ascorbate peroxidase

AsA ascorbic acid

ATP adenosine triphosphate

BIP 4,4'-bipyridyl

Car carotenoids

CAT catalase

CC Climate change

Chl chlorophyll

Chl *a* chlorophyll a

Chl *b* chlorophyll b

CTL Control

d.m. dry mass

DHA dehydroascorbate

DHAR dehydroascorbate reductase

DTNB 5,5'-dithiobis-(2-nitrobenzoic acid)

E transpiration rate

ECM ectomycorrhiza

EDTA ethylenediaminetetraacetic acid

ETC electron transporter chain

ETR electron transport rate

F_0 minimum fluorescence

F_m maximum fluorescence

FM fresh mass

F_s fluorescence yield

F_v variable fluorescence

F_v/F_m maximum photochemical efficiency of PSII

GAP glyceraldehyde 3-phosphate

GPOX guaiacol peroxidase

GSH glutathione

GSSG oxidized glutathione

GST glutathione S-transferase

H_2O_2 hydrogen peroxide

H_3PO_4 phosphoric acid

HSP heat shock proteins

IRGA infrared gas analyser

LP lipid peroxidation

MDA malondialdehyde

MDHA monodehydroascorbate

MDHAR monodehydroascorbate reductase

MR mycorrhizal

NADPH nicotinamide adenine dinucleotide phosphate

NMR non-mycorrhizal

NPQ non-photochemical quenching

$O_2^{\cdot-}$ superoxide anion

OH· hydroxyl radical

PAM Pulse Amplitude Modulation

PAR photoshyntetic active radiation

PCA principal component analysis

PCD programmed cell death

PSI photosystem I

PSII photosystem II

PK potassium phosphate

PPFD photosynthetic photon flux density

Pro proline

PVPP polyvinylpolypyrrolidone

ROS reactive oxygen species

RuBisCO ribulose-1,5-bisphosphate carboxylase-oxygenase

RuBP ribulose-1,5-bisphosphate

RWC relative water content

SN supernatant

SOD superoxide dismutase

TBA thiobarbituric acid

TCA trichloroacetic acid

TM turgid mass

WHC whater holding capacity

WHC_{max} maximum water hold capacity

WUE water use efficiency

WUE_i intrinsic water use efficiency

Φ_{PSII} effective quantum efficiency of PSII

1 Introduction

1.1 Climate change and the Mediterranean region

Climate change (CC) is defined as the shift in climate patterns, assessed by the average values of meteorological elements such as precipitation and temperatures (Malhi et al., 2021). The Earth's climate has continuously evolved over time, but in recent decades, significant changes have been observed due to enhanced human activities related to energy production, industrial activity, forestry, and land-use modifications. These activities have resulted in increased greenhouse gas emissions, which are responsible for altering the composition of the global atmosphere, and unequivocally causing global warming (Fawzy et al., 2020; Malhi et al., 2021; Yue & Gao, 2018). Global surface temperature has risen more rapidly since 1970 than during any other 50-year span in the past 2000 years (IPCC, 2023). In fact, recent projections estimate that global temperatures may rise by 1.5 ºC between 2030 and 2052 (IPCC, 2023). Anthropic-caused CC strongly impacts weather and climate extremes worldwide, although these effects can differ in various regions. Notably, in the Mediterranean region, CC is outpacing global trends for most variables, with annual temperatures being already 1.4 ºC higher than those in late-1900s. Forecasts indicate that the region's warming is expected to surpass global rates by 25%, with summer temperatures anticipated to increase at a pace 40% higher than the global average (Cramer et al., 2018; Lionello & Scarascia, 2018). Alongside the rise in heatwaves, the Mediterranean basin (Portugal, Spain, Greece, Turkey, Italy) is currently experiencing a significant decrease in annual rainfall, although paradoxically increases of sudden extreme precipitation events are also occurring. For instance, during the summer months, there could be reductions in precipitation of up to 30% (Cramer et al., 2018; Soares et al., 2015; Zittis et al., 2021). The effects of CC are extensive, and its vast influence on the agricultural sector is now readily apparent (Arora, 2019). Year-to-year fluctuations in precipitation and temperature, along with the occurrence of extreme climatic events during crucial phenological stages, are responsible for unforeseen losses caused by crop diseases, decreased yields, and heightened yield variability. Consequently, the expected CC in the Mediterranean basin endangers agricultural production and food security (Aguilera et al., 2020; Cramer et al., 2018).

1.2 Chestnut tree – economic importance, growth habits, and current threats

Chestnut (*Castanea sativa* Miller) is a highly-valued crop in the Mediterranean region, serving as an essential resource for its wood and fruit. The chestnut fruit is widely used in the preparation of various recipes due to its significant nutritional value. In addition to its direct uses, the cultivation of chestnut stands has given rise to another lucrative income stream through the collection and commercialization of wild edible mushrooms (Baptista et al., 2010; Conedera et al., 2021). Beyond its economic value, sweet chestnut (*C*. *sativa*) is a key landscape element, providing important ecosystems services. Forests and managed coppices of this tree support diverse faunal species, reduce wildfire risks, enhance water conservation and soil stabilization, and serve as crucial habitats for pollinators (Clark et al., 2023). On top on that, there is potential in using young chestnut plantations as mitigators off CC, by capturing and accumulating carbon, which helps reducing the concentration of greenhouse gases (Menéndez-Miguélez et al., 2023).

Portugal is the fifth major exporter of chestnuts in shell worldwide (FAO, 2021). In Europe, it ranks as the country with the largest *C. sativa* production area, summing up a total of 50,370 ha, which resulted in a production of almost 37,200 tons in 2021 (FAO, 2021). This ancestral species can be found across the entire Portuguese territory where the production areas are organized into four Protected Denomination of Origin (PDO) regions. The main producing one, accounting for more than 80% of the total distribution area, is found in the northeast Portugal, namely in Trás-os-Montes, where chestnut production is one of the most profitable activities for rural populations (INE 2019; Freitas et al., 2022).

Described as a mesophilic species, moderate temperatures and humidity from warm temperate climates are the preferential conditions for chestnut growth (Freitas et al., 2021). *C. sativa* preferentially develops in areas with annual rainfall ranging from 600-700 mm to 1500-1600 mm, annual mean temperatures between 8 ºC and 15 ºC and can be found at elevations up to 1800 m (Freitas et al., 2022). Temperate climate trees, like chestnuts, usually require cool winters for proper physiological and phenological development, including budburst, flowering, fruit set, and maturation (Santos et al., 2017). Accumulated exposure to low temperatures enables plants to properly set inflorescence production when warmer temperatures arise in spring. Within the face of the current climate instability, chestnut plants encounter many growth limitations, due to pest attacks and diseases, but also by drastic abiotic shifts (Gomes-Laranjo et al., 2012).

CC can have implications for yields and fruit quality, altering the physiological and reproductive cycle of species, including the anticipation or delay of phenological timing (Freitas et al., 2021). For instance, warmer temperatures favour a quicker vegetative activity, which accelerates the progression of phenological stages and increases the occurrence of diseases and pests (Freitas et al., 2021). Thus, nowadays, *C. sativa* must deal with significant challenges from destructive biotic stresses, specifically ink disease caused by *Phytophthora cinnamomi* Rands. This disease is the most widespread and damaging among the various threats to chestnut trees, and it becomes more severe in areas impacted with heatwaves, low relative humidity, and dry summers (Dorado et al., 2023; Fernandes et al., 2022).

Although European chestnut is characterized by a relatively high adaptability to different abiotic stresses, due to their rich genetic background, this acclimation potential will not be enough to face new and stronger challenges (Ciordia et al., 2012; Freitas et al., 2021). Along with the rest of the Mediterranean basin, the Portuguese chestnut producing regions, mainly the PDO *Terra Fria Transmontana*, in Trás-os-Montes, located within the Montesinho Natural Park (MNP), can be significantly impacted by CC. Therefore, the adaptation of *C. sativa* to adverse environmental conditions is of utmost importance for chestnut production (Fernandes et al., 2022; Pérez-Girón et al., 2020). In fact, due to the above-mentioned climacteric projections for the Mediterranean/Iberian Peninsula, the production and quality of chestnut fruit and timber are at serious risk, leading to significant losses of goods and ecosystem services (Fernandes et al., 2022; Pereira et al., 2021). Indeed, current projections indicate that days with maximum temperatures exceeding 30 ºC and 40 ºC will become more common (20 to 50 days/year) in the IP in the coming years (Pereira et al., 2021). These temperatures alone are already damaging for most crops (Luo, 2011), including chestnut, which tolerates temperatures of only up to 27-31 ºC (Gomes-Laranjo et al., 2008, 2009). Moreover, chestnut plants – especially in the first years of growth – are particularly sensitive to summer droughts (Conedera et al., 2021). Additionally, while the physiological adjustments (*e.g.* mineral nutrition and photosynthesis) of *C. sativa* to single stress are relatively well characterized (Camisón et al., 2020; Carneiro-Carvalho et al., 2021; Gomes-Laranjo et al., 2004, 2006), not much is known regarding its responses to the simultaneous exposure to drought and heat. This aspect is particularly important since plants respond to multiple stresses differently from how they react to individual ones (Atkinson & Urwin, 2012).

1.3 Drought and heat stresses

Water scarcity and heat are two of the major abiotic stresses threatening global crop productivity and food security, especially considering the present and future worrisome impacts of global warming and CC (Lamaoui et al., 2018). On top of that, heat waves and drought are intrinsically linked, as high temperatures exacerbate soil drying, thus increasing drought severity (Stéfanon et al., 2014; Teskey et al., 2015).

Drought is caused by scarcity or absence of water and occurs when the plant's water requirement cannot be fully satisfied (Zargar et al., 2017). Generally, drought takes place when soil and atmospheric humidity are low and ambient temperature is high, causing a continuous loss of water by transpiration and evaporation (Jaleel et al., 2009). High evaporative demand, lack of precipitation or irrigation, failing groundwater levels and water retention by soil particles are some variables that can result in plant water deficit (dos Santos et al., 2022). The imbalance between water absorption and water losses can interfere with several crucial process, including nutrient metabolism, ion transport, solute translocation, several metabolic reactions (photosynthesis, respiration, protein synthesis) and cellular development (Zargar et al., 2017). The response of plants to drought varies among species, development stage, and age and depends on the duration and severity of water scarcity (dos Santos et al., 2022).

On its turn, heat stress is defined as the rise in temperature beyond a threshold level for a minimum amount of time, enough to induce harmful effects on plant growth and development (Wahid et al., 2007). Plants are susceptible to heat stress when: i) the air temperature is high and plants receive energy through sensible energy transfer, and ii) solar radiation incident on the soil raises its temperature (dos Santos et al., 2022). An increase in temperature, usually up to 15 ºC above ambient temperature, is considered a heat shock or stress. However, it is a complex function of temperature (in degrees) duration, and the rate of increase (Wahid et al., 2007) that primary determines plant response to heat. Similarly to drought, high temperature stress can affect plants throughout all of their developmental stages, although some phenological periods are more sensitive than others. Furthermore, there are tremendous variation in what concerns heat tolerance both between and within species (Jagadish et al., 2021; Wahid et al., 2007), suggesting that plant responses to high temperatures is a very complex phenomenon.

Interlinked and interdependent, dehydration is a typical side consequence of both drought and heat stress. These stresses can differentially affect plant homeostasis,

inducing both general and specific stress responses in plant metabolism. While drought mainly interferes with nutrient uptake, water relations, and assimilate portioning, high temperatures can cause severe damage to proteins, disturb their synthesis, inactive major enzymes, and damage membranes (Fahad et al., 2017). Additionally, the combined exposure to these stressors can portray distinct impacts on plant cellular processes compared to the effects of any stressor applied alone (Priya et al., 2019).

Plants handle abiotic stresses at the morphological, biochemical, and molecular levels (Huber & Bauerle, 2016). For instance, tolerant plants to heat and/or drought usually exhibit avoidance mechanisms, which comprise a set of morphological and physiological adjustments (dos Santos et al., 2022). These adjustments may include an enlarged root system, decreased stomatal conductance and stomatal density, decreased leaf area, increased leaf thickness, and leaf folding or rolling to minimize evapotranspiration (Goufo et al., 2017). Moreover, to maintain a state of optimal hydration, even in conditions of water deprivation, plants have also developed sophisticated biochemical and molecular responses. One of these key mechanisms is the accumulation of compatible solutes, commonly known as osmolytes (Ashraf & Foolad, 2007). These compounds are highly soluble organic metabolites of low molecular mass, that are usually nontoxic at high cellular concentrations and include soluble carbohydrates (*e.g.* glucose and fructose), amino acids (*e.g.* proline), quaternary ammonium compounds (*e.g.* glycine betaine), and specialized metabolites (*e.g.* lignin and flavonoids) (Wahid et al., 2007). Their accumulation ensures the maintenance of cell turgor, stabilization of cellular membrane as cells dehydrate, and protection of protein structure (Lamaoui et al., 2018). In parallel, phytohormones are also of high importance to drought, especially abscisic acid (ABA), which can be highlighted as a key regulator of plant stress responses, with heat and drought stresses usually triggering an increase in its levels (Lamaoui et al., 2018; Verma et al., 2016; Wahid et al., 2007). Under osmotic stress, ABA is known for inducing the restraint of leaf growth and short-term responses like stomatal closure, in order to maintain water balance. As a longer-term response, ABA regulates the expression of many stress-response genes including those related to osmoprotectants synthesis (Sah et al., 2016; Verma et al., 2016).

In addition, the expression of heat shock proteins (HSP) is a well-known response to cell water deficit and is an effective adaptive strategy under conditions of high temperature exposure. Through the regulation of heat transcript factors and HSP, ABA may confer thermotolerance (Li et al., 2021). The protective role of HSP is correlated

with stress tolerance, photosynthesis and water use efficiency, membrane stability, and maintenance of cellular hydration (Lamaoui et al., 2018).

Although heat and drought stresses have varying impacts on plant growth and physiology, they both disrupt photosynthesis and can induce, as a secondary effect, oxidative damage, which can compromise cell viability and, ultimately, induce cell and organ death. In the following sections, the effects of both heat and drought on the photosynthetic apparatus and oxidative stress, considering their varying degrees of impact, will be explored.

1.4 Photosynthesis

Photosynthesis is one of the essential biological processes that plays a crucial role in sustaining life on Earth. It converts light energy into chemical energy, fuelling various metabolic chains (Ashraf & Harris, 2013). Photoautotrophic organisms, such as higher plants, use solar energy to oxidize water, releasing oxygen (O_2), and to reduce CO_2, producing organic molecules [*e.g.* glucose ($C_6H_{12}O_6$), sugars, starch]. This process takes places within the chloroplasts where two key sequential and interdependent phases occur – the photochemical reactions and the Calvin-Benson cycle (or chemical reactions). The thylakoid membrane system hosts the photochemical reactions, leading to the production of nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP). These energy-rich compounds are then utilized in the carbon reactions, also known as the Calvin-Benson cycle, which takes place in the stroma, to fix atmospheric CO_2 into carbohydrates (Stirbet et al., 2020; Taiz et al., 2015). The photosynthetic process begins with the absorption of the energy from incoming photons and consequent excitation of photosynthetic pigments, chlorophyll (Chl) and carotenoids, associated with the two photosystems (PSI and PSII). The excitation energy of the photoreceptors can be transferred to other photoreceptors to drive photosynthetic reactions, while a small part is dissipated as heat or re-emitted as fluorescence (Nath et al., 2017; Stirbet et al., 2020; Taiz et al., 2015). The pigment-protein antenna complexes funnel the energy to the reaction centres (P680 in PSI and P700 in PSII), where it is converted into redox chemical energy very efficiently. Despite the physical and chemical distinctions between PSI and PSII, each one having its own antenna pigments and photochemical reaction centre, they are linked by an electron transporter chain (ETC). The light-dependent electron transfer reaction begins with the oxidation of water by PSII. The electrons follow a linear pathway from water to $NADP^+$ to form NADPH in the PSI. Simultaneously, a proton gradient across the thylakoid membrane is created, required to allow ATP synthase to produce ATP. Both NADPH and ATP are fundamental for CO_2

assimilation in the stroma, where they play a role in a series of reductions and phosphorylation of intermediate compounds within the Calvin-Benson cycle (Johnson, 2016; Taiz et al., 2015). The Calvin-Benson cycle comprises several enzymes, and the biochemical processes have been categorized into main three phases. The first is carboxylation, carried out by ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO; EC 4.1.1.39). At this stage, the CO_2 that diffuses from the atmosphere into the leaves through stomata is combined with ribulose-1,5-bisphosphate (RuBP) producing 3-phosphoglycerate (3PGA). In the second phase, the ATP and NADPH molecules, originated from the light-dependent reactions, are used to reduce 3PGA to glyceraldehyde 3-phosphate (GAP). The final step is the regeneration of RuBP, with the exportation of the cycle products to several biosynthetic pathways (isoprenoid, starch, sucrose, shikimate, thiamine, and nucleotide) (Clapero et al., 2023; Gurrieri et al., 2023; Raines, 2022).

Such as in any other metabolic event, unfavourable environmental conditions, such as drought and/or high temperatures, can limit plants photosynthetic efficiency in different phases and affect several of its components (Ashraf & Harris, 2013; Singh & Thakur, 2018).

Regarding photosynthetic pigments, plants exposed to drought exhibit a lower Chl content, and the exposure to high temperatures results in a reduction in the accumulation of Chl due to decreased biosynthesis and/or increased degradation (Fahad et al., 2017; Zahra et al., 2023; Zargar et al., 2017). Elevated temperatures lead to impaired Chl biosynthesis due to the abundance of heat-sensitive enzymes within the Chl biosynthesis pathway (Dutta et al., 2009; Fahad et al., 2017). The activity of chlorophyllase and Chl-degrading enzymes is also enhanced under heat stress conditions, resulting in a serious reduction in Chl levels (Wang 2018; Hu et al 2020). Additionally, heat and drought induce ultrastructural deformations in chloroplasts, including damage to thylakoids membranes (Razi et al., 2021; Zargar et al., 2017). As reviewed by Zahra and colleagues (2023) heat stress leads to a reduction in starch content, elevated plastoglobuli production, disrupted thylakoid stacking, distorted intergranular lamellar and stroma systems, and excessive swelling of outer chloroplast membranes. These alterations end up in several chloroplast malfunctions, though the subcellular responses to heat are species-specific (Zahra et al., 2023).

Besides affecting photosynthetic pigments, high temperatures and drought stresses are also known to induce damage to the PSI and PSII, and to diminish the electron transport rate. As a result, the production of ATP and NADPH is reduced,

consequently limiting the CO_2 reduction process (Sharma et al., 2019; Singh & Thakur, 2018). Notably, PSII is particularly vulnerable to high temperatures due to increased fluidity in thylakoid membranes and its reliance on intact electron transport. In fact, heat exposure may disrupt the water-oxidizing complex and compromise the structural and functional stability of both PSII reaction centre and light-harvesting complex (Hu et al., 2020; Muhammad et al., 2021). Additionally, heat stress induces an over-reduction of the ETC and triggers excessive reactive oxygen species (ROS) generation, leading to photoinhibition. These ROS can hinder the synthesis of the D1 protein, a crucial component of the PSII complex (Yamori, 2016; Zahra et al., 2023). The impact of this stress also extends to the cyclic electron flow around PSI, altering its activity and the thylakoid proton conductance (Sharma et al., 2019). On the other hand, drought stress negatively affects the quantum yield of PSII by influencing chlorophyll fluorescence kinetics and, thereby, the PSII reaction centre (Sharma et al., 2019). Like heat stress, water scarcity also induces an excessive reduction in the ETC and subsequent ROS overproduction, disrupting the photosynthetic machinery, including the D1 and D2 proteins of PSII and thylakoid membranes (Kaur & Asthir, 2017; Singh & Thakur, 2018). Broadly, both heat and water scarcity can reduce the maximum quantum efficiency and quantum yield of PSII, while simultaneously increasing non-photochemical quenching to dissipate excessive energy (Ashraf & Harris, 2013; Yamori, 2016; Zahra et al., 2023; Zlatev & Lidon, 2012).

Alongside, the activity of key photosynthetic enzymes and protein structure, as well as CO_2 assimilation rates, can also be greatly hampered by both water deficit and heat. Drought restricts gas-exchange by reducing stomatal conductance. While this promptly prevents transpiration and reduces water loss, this action hinders the intake of CO_2, consequently affecting photosynthesis and leading to oxidative damage. Under prolonged water scarcity, the decreased CO_2 concentration in the chloroplasts reduces RuDP regeneration capacity and deactivates RuBisCO (Muhammad et al., 2021; Singh & Thakur, 2018). Moreover, this photosynthetic enzyme, as well as others involved in the carbon reactions, are particularly sensitive to high temperatures. It is also known that thermal stress inactivates RuBisCO activase, an enzyme that regulates the activation state of RuBisCO (Yamori, 2016).

It is important to recognize that, while stress-induced stomatal limitations play a role in photosynthesis under water-limited conditions, they are not the sole limiting factor (Ashraf & Harris, 2013; Zlatev & Lidon, 2012). As stomatal conductance and transpiration rate are closely related to leaf temperature, plants' ability to maintain CO_2 assimilation

rate can serve as an indicator of its tolerance to heat stress. In this context, temperatures above optimal values can impact stomatal conductance, intracellular CO_2 concentration and leaf water status (Hassan et al., 2020; Sharma et al., 2019). As stated by Urban et al. (2017), a wide range of stomata behaviour in response to increased temperatures have been reported including: stomatal opening, closure and no significant changes among.

1.5 Oxidative stress and plant antioxidant (AOX) system

Aerobic cell metabolism continuously generates ROS, which are partially reduced or activated forms of O_2. The different ROS include molecules – hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) – and free radicals, such as the hydroxyl radical ($OH\cdot$) and the superoxide anion ($O_2^{\cdot-}$) (Mittler, 2017). Although firstly described only as highly harmful compounds, it is now widely accepted that ROS play a dual role in plant cells depending on their concentration. According to Foyer et al. (2017), at low levels, they serve as essential signalling agents and, at higher concentrations, they become toxic and can interact with a wide range of organic compounds, thus disturbing the redox homeostasis and imposing severe oxidative damage (Figure 1). Under adverse environmental conditions, plants often experience oxidative stress resulting from an unbalance between ROS production and elimination (Halliwell, 2006; Soares et al., 2019a). Once the cellular threshold is surpassed, ROS can induce lipid and protein peroxidation, membrane breakdown, enzyme inhibition, damage to nucleic acids, activation of programmed cell death (PCD) pathway, and, eventually, cell death (Farooq et al., 2019).

1.5.1 Unravelling main ROS in plants: characteristics, production sites and cellular effects

In plant cells, ROS are ubiquitously produced as by-products of several metabolic processes in multiple cell compartments and locations such as: chloroplast, mitochondria, peroxisomes, plasma membrane, cell wall and apoplast (Hasanuzzaman et al., 2021; Sharma et al., 2012). From a general perspective, given the higher availability of O_2, chloroplasts and peroxisomes are the primary sources of ROS in the presence of light, whereas mitochondria are the main source in the absence of light (Das & Roychoudhury, 2014).

The primary cell-generated ROS, as a result from the monovalent reduction of O_2, is the superoxide anion ($O_2^{\cdot-}$). Its generation is mainly associated with the ETC, being the chloroplast PSI and II and the mitochondria complex I and III its major intracellular sources (Hasanuzzaman et al., 2021; Soares et al., 2019a). $O_2^{\cdot-}$ production can also take

place at glyoxysomes, peroxisomes and even cell wall by the action of NADPH oxidases (EC 1.6.3.1) (Tripathy & Oelmüller, 2012). When compared to other ROS, $O_2^{\cdot-}$ is moderately reactive and has low mobility, given its very short half-life (1-4 µs) and negative charge, reasons why it is unable to cross biological membranes (Demidchik, 2015; Mittler, 2017). Nevertheless, it is also generally accepted that $O_2^{\cdot-}$ does not directly interact with organic macromolecules, hence being less toxic than other ROS. Still, $O_2^{\cdot-}$ can trigger a cascade of reactions including the formation of secondary ROS, either directly or via enzymatic and metal-catalysed processes, depending on the cell type or cellular compartment (Dumanović et al., 2021; Sharma et al., 2012). In fact, due to its potent reducing ability, $O_2^{\cdot-}$ can change the redox state of transient metals, such as Fe^{3+} to Fe^{2+}, arising the production of more toxic and reactive ROS, such as the hydroxyl radical ($OH\cdot$) through a reaction globally known as Heiber-Weiss (Das & Roychoudhury, 2014; Dumanović et al., 2021; Sharma et al., 2012; Soares et al., 2019a).

One of the most widely studied ROS, given its particular features, is hydrogen peroxide (H_2O_2). Similar to $O_2^{\cdot-}$, this moderately reactive ROS is generally produced as a consequence of the ETCs in mitochondria, chloroplast, endoplasmic reticulum, and plasma membrane. In addition to these sources, the β-oxidation of fatty acids and photorespiration (Das & Roychoudhury, 2014), processes typically occurring in peroxisomes, are also major sources of H_2O_2 in plant cells. Furthermore, H_2O_2 can be generated through the action of superoxide dismutase (SOD; EC 1.15.1.1), as well as different reactions involving photo-oxidation by NADPH oxidases and via $O_2^{\cdot-}$, through a two-electron reduction of O_2 facilitated by various oxidases, such as glycolate oxidase located within peroxisomes (Noctor et al., 2018). Regarded as a primary ROS, H_2O_2 plays a dual role, by participating in signal transduction at low concentrations while also having harmful effects on cells at high doses (Dumanović et al., 2021). Since H_2O_2 does not contain unpaired electrons, it presents a higher chemical stability, being able to induce a more severe oxidative damage than $O_2^{\cdot-}$ (Soares et al., 2019a). In fact, compared with other ROS, H_2O_2 has a significantly longer half-life (1 ms or more) and is able to cross biological membranes trough aquaporins and diffuse across long distances. Consequently, H_2O_2 can evoke its functions far from its site of production either causing extensive oxidative damage or activating different cell signalling cascades (Bhattacharjee, 2019; Farooq et al., 2019). As signal molecule H_2O_2, can induce tolerance responses against several environmental stresses and regulates essential physiological processes such as senescence, photosynthesis, photorespiration, stomatal movement, cell cycle and growth, and development (Gill & Tuteja, 2010).

Among the ROS family, the most reactive and toxic form is the hydroxyl radical (OH·). This ROS is produced from the interaction between H_2O_2 and $O_2^{\cdot-}$ in the presence of iron (Fe) or copper (Cu) ions (Dumanović et al., 2021). Due to its short half-life (1 ns) (Mittler, 2017), strong redox potential and high affinity for biomolecules, OH· can cause damage in different cellular organelles by inducing LP, protein damage and genetic instability by injuring both pyrimidine and purine bases (Bhattacharjee, 2019; Roldán-Arjona & Ariza, 2009). Owning to the non-existence of an enzymatic system responsible for its scavenging, situations favouring OH· accumulation can ultimately end up in cell death (Bhattacharjee, 2019).

Lastly, the most peculiar ROS is probably $^1O_2$, an excited state of O_2 with two unpaired electrons, since it is generated under very specific conditions, namely when the chlorophyll (Chl) triplet state in the antenna system reacts with O_2, reducing it, rather than channelling the electrons to the ETC (Das & Roychoudhury, 2014). It can also be produced under low CO_2 availability conditions and/or strong light intensity, threatening the entire photosynthetic machinery and causing severe damage to both PSI and PSII (Gill & Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019a). Interestingly, stomatal closure, induced by environmental stresses like salinity, drought, and heavy metals, can indirectly favour the formation of this ROS, by lowering the levels of CO_2 in the leaf mesophyll (Das & Roychoudhury, 2014). Despite having a short half-life (1-4 µs), a portion of $^1O_2$ is able to diffuse considerable distances (100 nm) in cellular systems (Das & Roychoudhury, 2014; Gill & Tuteja, 2010). As a ROS,$^1O_2$ can transfer its excitation energy to other biological molecules and/or cause lipid peroxidation (LP) (Bhattacharjee, 2019).

1.5.2 Impact of ROS on cellular oxidative balance

Despite being continuously produced as by-products of the normal cell metabolism, the generation of ROS can be greatly boosted under unfavourable environmental conditions such as extreme temperatures and drought (Choudhury et al., 2017). In such conditions, the balance between the accumulation and elimination of ROS can be jeopardized, giving rise to oxidative stress (Figure 1). This complex phenomenon refers to ROS-induced damage on lipids, proteins, carbohydrates, DNA and RNA (Gill & Tuteja, 2010; Hasanuzzaman et al., 2021; Mittler, 2017).

The LP results from a series of biochemical processes, in which the unsaturated fatty acids of cellular and organelle membranes are oxidized by ROS and/or ROS-induced signals. As one of the earliest and most detrimental process, LP impairs cell membrane integrity, fluidity, causes electron leakage and deactivation of enzymes and

receptors, while also giving rise to various lipid radicals that can intensify oxidative harm by interacting and injuring proteins and DNA (Alché, 2019; Ayala et al., 2014). The degree of LP can be experimentally estimated by the quantification of its sub-products, namely malondialdehyde (MDA), responsible for cellular damage (Sharma et al., 2012; Soares et al., 2019a).

Besides lipids, excessive ROS levels can also cause diverse alterations in proteins through direct and indirect mechanisms. These modifications can be reversible or irreversible, impacting protein structure and function (Jacques et al., 2013; Sharma et al., 2012). Protein function can be directly altered by ROS through nitrosylation, carbonylation, disulphide bond formation, and glutathionylation, while proteins can also be structurally modified through conjugation with metabolites generated from fatty acid peroxidation (Sharma et al., 2012). These modifications primarily affect specific amino acid residues, namely cysteines, increasing protein vulnerability to proteolytic degradation. In fact, the vulnerability of amino acid residues to oxidative modification varies considerably, being cysteine, histidine, methionine, tyrosine and tryptophan the most susceptible ones (Farooq et al., 2019).

Besides affecting protein and lipids, the accumulation of excessive ROS can also cause oxidative damage to nuclear, mitochondrial, and chloroplastic DNA. While nuclear DNA is somewhat protected by the presence of histones and other proteins, the absence of this protection, coupled with the proximity to ROS production sites, places mitochondrial and chloroplastic DNA at a higher risk of ROS-induced damage (Das & Roychoudhury, 2014; Sharma et al., 2012). ROS-mediated DNA damage encompasses modifications of nucleotide bases, oxidation of deoxyribose sugar residues, nucleotide exclusions, DNA strand breaks, and cross-linking of DNA with associated proteins (Roldán-Arjona & Ariza, 2009; Tripathi et al., 2023). All of this results in cell membrane damage and impacts vital cellular processes such as replication and protein synthesis (Gill & Tuteja, 2010; Sharma et al., 2012). Despite plants having proper DNA damage sensing and repair mechanism, failure in this pathway and the accumulation of DNA lesions may lead to plant cell death affecting the whole organism development (Das & Roychoudhury, 2014; Szurman-Zubrzycka et al., 2023).

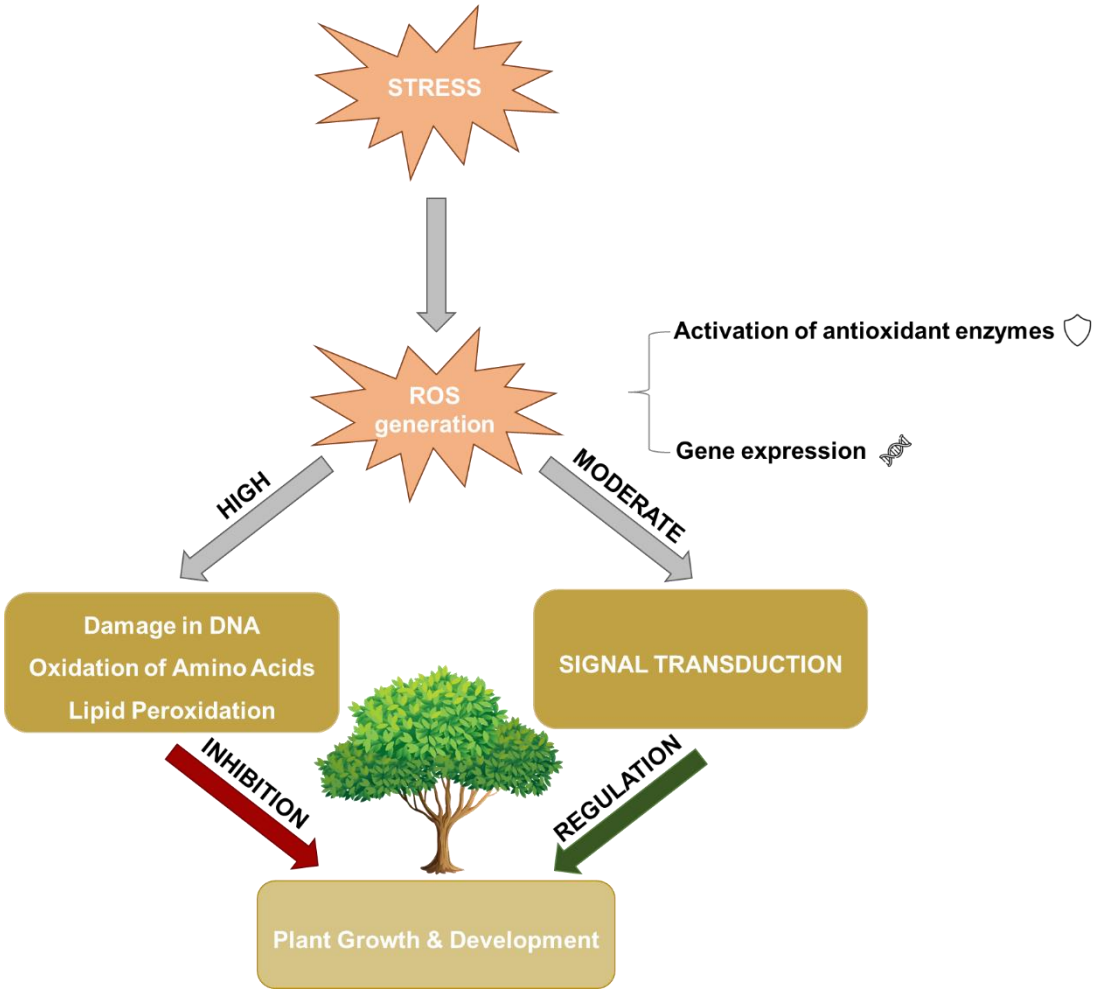


Figure 1: Roles of ROS in plant cell signalling and oxidative stress. Adapted from Sharma et al. (2017).

1.5.3 The antioxidant (AOX) orchestra: enzymatic and non-enzymatic components of the plant's defence network

In order to counteract ROS overaccumulation and prevent oxidative damage, plants have evolved an AOX system comprising both enzymatic and non-enzymatic mechanisms, that are involved in the detection, detoxification, elimination, and/or neutralization of ROS (Soares et al., 2019a). The non-enzymatic components of the AOX system include diverse low-mass metabolites, such as glutathione (GSH), ascorbic acid (AsA), phenolic compounds, carotenoids, and proline (Pro). These intracellular agents support plants' redox cellular equilibrium under stress by neutralising, removing, and/or transforming ROS, enabling the management and monitoring of ROS homeostasis (Soares et al., 2019a). Working alongside, AOX enzymes synergistically function to balance intracellular generation of ROS up to an optimal threshold, promoting plant

growth and development, facilitating cellular processes such as the cell cycle and hormone signaling, and fortifying defense against abiotic/biotic environmental stressors (Gupta et al., 2018). The effectiveness and response of the non-enzymatic AOX machinery to stress are influenced by distinct factors, including the type of stress, time-length of exposure and its intensity, plant species and their genotypes, the organ or tissue involved, among other variables (Soares et al., 2019a).

Glutathione (GSH) is the major non-protein thiol in plants undertaking several crucial roles in different aspects of plant physiology. Given its importance, it can be found in all cell compartments like cytosol, endoplasmatic reticulum, vacuole, mitochondria, chloroplast, peroxisomes and the apoplast. It acts as radical scavenger by chemically reacting with $O_2^{\cdot-}$, $OH\cdot$ and H_2O_2 (Soares et al., 2019a). Additionally, it serves as a transporter and storage molecule of sulphur (S), participates in the conjugation of several metabolites, namely xenobiotics through the activity of glutathione S-transferase (GST; EC 2.5.1.18), and it is the precursor of several chelating agents, such as phytochelatins and metallothioneins. Thus, GSH also protects protein thiol groups and aids in the detoxification of metals and xenobiotics, such as herbicides (Gong et al., 2018). Moreover, it regulates the redox status of cells, facilitates the progression of the cell cycle, influences processes such as cell death and senescence, while also modulating gene expression patterns (Noctor et al., 2012; Orabi et al., 2018) .

Ascorbic acid (AsA), frequently referred to as vitamin C or ascorbate, holds a significant position as the most abundant water-soluble AOX in plant cells (Ishikawa et al., 2018). Besides its ability to act as an AOX metabolite, AsA plays other crucial roles in plants. The AsA-hormone crosstalk actively participates in the regulation of key biochemical and physiological processes like mitosis, cellular elongation, seed germination, determination of flowering timing, fruit expansion, senescence, and cell death (Akram et al., 2017; Ishikawa et al., 2018). Additionally, AsA serves as an essential cofactor necessary for the biosynthesis of gibberellins, ethylene, and ABA, suggesting a strict link of this AOX with phytohormones directly involved in plant development and growth (Fàbregas & Fernie, 2021). Specifically recalling its AOX potential, AsA is known to be a potent neutralizer of ROS, such as $OH\cdot$, $O_2^{\cdot-}$ and $^1O_2$, while also serving as one the substrates of ascorbate peroxidase (APX; EC 1.11.1.11), one of the H_2O_2-neutralizing enzymes (Pandey et al., 2017). As an AOX molecule, AsA is one of the main agents involved in the AsA-GSH cycle (further described), which is an effective metabolic pathway for excessive energy and H_2O_2 dissipation. The levels of this metabolite vary

depending on the tissue type, with photosynthetic tissues generally exhibiting higher concentrations of AsA compared to roots and other tissues (Gill & Tuteja, 2010).

Proline (Pro) stands out among the proteinogenic amino acids due to its cycle structure and a distinct secondary amino group (the α-amino group), making it an effective osmoprotectant (Hosseinifard et al., 2022). Besides its essential role in primary metabolism, Pro has been identified to play specific roles in plants cells, particularly during abiotic stresses (Spormann et al., 2023). Accumulation of Pro has been strongly linked with improved stress tolerance in plants (Hosseinifard et al., 2022). Pro is recognized as a powerful osmolyte, accumulating in the cytosol without causing harm to cellular structures. Its homeostatic levels are contingent on the equilibrium between its production and utilization for protein synthesis, along with its translocation to the mitochondria (Forlani et al., 2019). Furthermore, Pro acts as a metal chelator, an inhibitor of PCD, and improves the integrity of proteins and membranes by binding to hydrogen bounds (Hosseinifard et al., 2022). In what concerns its AOX potential, Pro acts as a redox buffer, serves as an electron sink since Pro synthesis requires NADPH, and prevents the occurrence of LP (Forlani et al., 2019). On top of that, Pro is a $^1O_2$ quencher and directly scavenges the most toxic ROS, OH· (Rejeb et al., 2014). Apart from the mentioned functions, Pro has been shown to serve as a signalling agent for the activation of specific gene functions, crucial for stress recovery (Raza et al., 2023; Spormann et al., 2023).

Carotenoids, such as zeaxanthin, tocopherol, and β-carotene, are lipophilic AOXs vital to different plant processes. They function as light receptors and protect the photosystems by extinguishing and scavenging the triple state chlorophyll and $^1O_2$, dissipating excess of light energy as heat during stress conditions and stabilizing membranes, thereby preventing LP (Uarrota et al., 2018).

Acting simultaneously and synergistically with the non-enzymatic AOX players, the enzymatic component of plants' AOX system also offers an efficient defensive mechanism to ensure ROS homeostasis. This AOX component is composed of a set of enzymes including superoxide dismutase (SOD; EC 1.15.1.1), the only enzymatic mechanism to eliminate $O_2^{\cdot-}$, together with a set of H_2O_2-degrading enzymes, such as catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (GPOX; EC 1.11.1.7), and APX. Other enzymes, as those involved in the AsA-GSH cycle, such as glutathione reductase (GR, EC: EC: 1.6.4.2), dehydroascorbate reductase (DHAR; EC 1.5.8.1) and monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), are also worth mentioning, mostly due to their role in regenerating important AOX players. Nonetheless, the

effectiveness of the AOX machinery depends on a variety of variables, including the type of stress, the duration, and intensity of exposure, the plant species, and their genotypes, or even the organ or tissue (Soares et al., 2019a).

The first line of defence against oxidative stress is SOD. This family of metalloenzymes catalyses the dismutation or disproportionation of $O_2^{\cdot-}$ into molecular oxygen and H_2O_2. By regulating $O_2^{\cdot-}$ levels, SOD efficiently prevents the subsequent generation of OH·, facilitated by the Haber-Weiss reaction (Gill & Tuteja, 2010). In plants, depending on the prosthetic metals in their active sites, there are three groups of SODs, differing by their metal co-factors, that can be found in distinct subcellular compartments: manganese (Mn-SOD), usually found in mitochondria; copper and zinc (Cu,Zn-SOD), mostly accumulated in cytosol, chloroplast and peroxisomes; and iron (Fe-SOD), exclusively from chloroplasts (Río et al., 2018).

Upon H_2O_2 production through the activity of SOD or by any other metabolic process, CAT is responsible for the neutralization of H_2O_2 into H_2O and O_2 and plays a relevant role not only in plant defence and metabolism, but also in signal perception (Anjum et al., 2016). Despite the reports of its occurrence in different subcellular compartments, CAT is mainly localized in peroxisomes, where many plant oxidases in the matrix and membrane generate H_2O_2 (Leung, 2018; Soares et al., 2019a). Although plants present different types of H_2O_2-degrading enzymes, CATs are unique as they do not require any reducing power (Sharma et al., 2012). Moreover, CAT stands out with one of the most remarkable turn-over rates among AOX enzymes, capable of reducing 6 million H_2O_2 molecules per min (Sharma & Ahmad, 2014). Nonetheless, due to its high specificity for H_2O_2, CAT is only able to sense H_2O_2 at high concentrations, unlike other enzymes, such as APX (Mittler, 2002; Soares et al., 2019a).

Belonging to the family of heme-containing peroxidases, APX is a key AOX enzyme that catalyses the reduction of H_2O_2 into H_2O, using AsA as an electron donor, with the production of monodehydroascorbate (MDHA) (Pandey et al., 2017). In plants, different APX isoforms, that have distinct structural and kinetic properties, have been classified based on their subcellular localization (Pandey et al., 2017). For further reading, consult Maruta & Ishikawa (2018) and Anjum et al. (2016). Given its broad locations inside the plant cell, H_2O_2 produced in different organelles is efficiently scavenged by APX. The higher affinity of APX to H_2O_2 enables it to effectively perform its functions even when H_2O_2 levels are low. For this reason, APX is primarily responsible for the fine-tuning detoxification of H_2O_2 which is crucial for its signalling function. In contrast, CAT's main role is to protect the cell from H_2O_2-induced cellular damage by

efficiently removing its higher levels during stressful conditions (Mittler, 2002). APX requires AsA as a substrate, making the regeneration of this metabolite crucial to ensure APX performance (Pandey et al., 2017). This role is mostly ensured by the AsA-GSH cycle. In general terms, to maintain a proper AsA availability, MDHA regenerates back to AsA through MDHAR activity. Alternatively, MDHA can be spontaneously converted into dehydroascorbate (DHA), which is later reduced back to AsA by DHAR using GSH as a substrate, leading to the oxidation of GSH to GSSG. To restore the GSH levels, GR utilizes NADPH as an electron donor to regenerate GSH from GSSG (Hasanuzzaman et al., 2019). This AsA-GSH cycle, also known as Foyer-Halliwell-Asada cycle, operates in various cellular compartments including the cytosol, mitochondria, chloroplast, and peroxisomes and is one of the most important metabolic cycles playing a vital role in ROS detoxification and protecting plants from various abiotic stress-induced damage (Dumanović et al., 2021; Pandey et al., 2017).

1.6 Thriving under pressure: investigating single *vs* combined stresses in plants

On a global scale, the environmental change is a multifactorial phenomenon. The CC-driven fluctuating and extreme weather events (*e.g.* heat waves and/or drought episodes), combined with poor soil conditions (saline, basic and/or acidic), low water quality, environmental contamination (metals, microplastics, pesticides) and limited nutrient availability, give rise a to widespread uncertainty in prediction effects and pose a unique challenge to plants (Rillig et al., 2019; Zandalinas et al., 2021). Across the past decades, plant research has heavily focused on studying the effects of single abiotic stresses on different aspects of plant physiology. These studies provided valuable knowledge at the physiological and molecular level of plant responses to several environmental cues such as drought, heat, salt, and cold (Sewelam et al., 2020). However, as sessile organisms, plants are frequently and simultaneously exposed to different stress factors under field conditions. The physiological, metabolic, and molecular response of plants to a particular combination of stresses can be different than that of single stresses and not always is merely the sum of the effects recorded under single exposure (Mittler, 2006; Sánchez-Bermúdez et al., 2022). The adaptation of plants to stress combinations comprises both “unique” and “shared” responses that need to be unravelled. In this way, different stress combinations will require the activation of unique metabolic and signalling cascades, differentially modulating essential processes, such as photosynthesis, redox mechanism and osmolyte synthesis (Zandalinas et al., 2018). Indeed, plants interpret and combine signals from multiple stresses in distinct ways

based on the specific nature of the combination, causing interference or even generating opposing responses when compared to how the plant reacts to individual stresses. Moreover, certain stress combinations can trigger diverse signalling pathways and the interaction between them can either be neutral, additive, synergetic or may lead to novel unpredictable adjustments (Atkinson & Urwin, 2012; Pandey et al., 2015; Zandalinas et al., 2020; Zhang & Sonnewald, 2017). Recently, several reports and reviews have summarized the physiological and molecular effects of different crop models grown under heat and drought combination. Generally, co-exposure to these stresses results in a much more detrimental effect in comparison with the single factors (Cohen et al., 2021; Zandalinas et al., 2018). Typically, the response of different plant species to water scarcity and high temperatures includes the induction of ROS detoxification enzymes, upregulation of various antioxidants and inhibition of photosynthesis (Pandey et al., 2015; Priya et al., 2019; Zandalinas et al., 2018). For instance, Raja et al. (2020), when studying the effects of isolated and simultaneous heat (45 ºC for 7 days) and drought (no irrigation for 10 days) exposure, found out that tomato plants (*Solanum lycopersicum* L.) were affected in a larger way when both treatments were combined, especially in what concerns the photosynthetic activity (evaluated in terms of PSII efficiency and quantum yield). An equivalent output was also observed in four wheat (*Triticum aestivum* L.) genotypes (Urban et al., 2018), lentil (*Lens culinaris* Medikus) varieties (Sehgal et al., 2017) and in *Brassica napus* L (Elferjani & Soolanayakanahally, 2018). Nevertheless, factors such as stress severity, age of plant, the genotype, its susceptibility or tolerance to any of the individual stress, among other factors, takes a huge role in the combined stresses interactions (Ma et al., 2020; Tommasino et al., 2018). Overall, it is important to focus current and future research understanding plant responses to stress combinations in both laboratory and field conditions. Only by digging into the intricate mechanisms governing these responses, we may pave the way for the development of climate-resilient agricultural crops (Rivero et al., 2022).

1.7 Mycorrhization as a tool to boost chestnut tolerance to CC

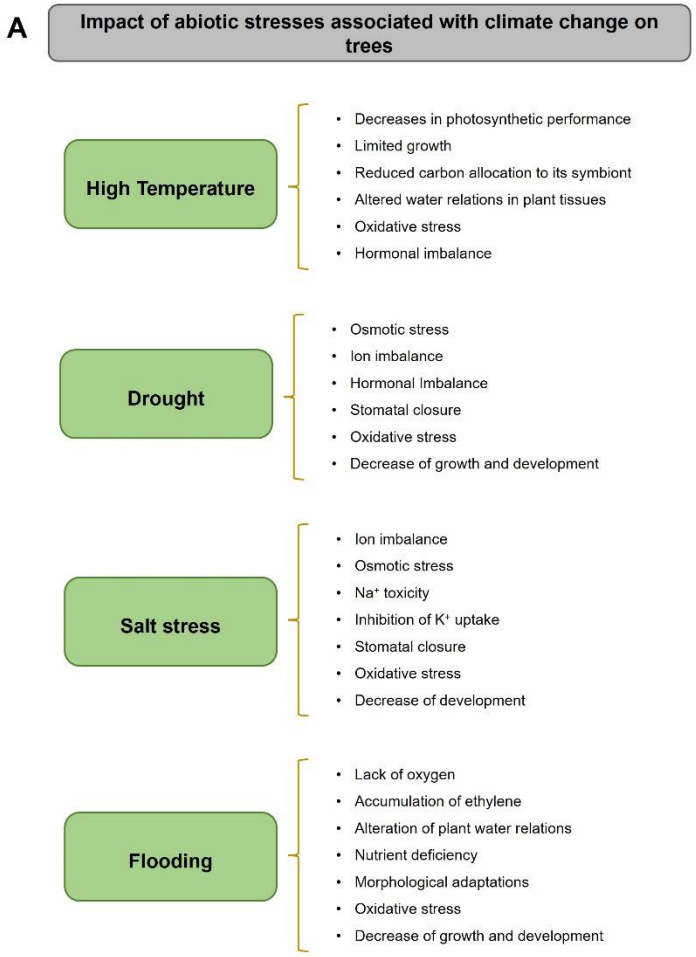
Most plants live in close collaboration with a diversity of soil organisms. Mycorrhizae form symbiotic associations with plant roots, aiding in their development and safeguarding them from numerous environmental challenges (Figure 2) (Siddiqui et al., 2008). Four primary mycorrhizal types, namely arbuscular mycorrhiza (AM), ectomycorrhiza (ECM), orchid mycorrhiza and ericoid mycorrhiza, have been classified according to their structural, functional, anatomical, and evolutionary characteristics, showcasing unique differences in their associations (Tedersoo & Bahram, 2019). From this diversity, AM and

ECM fungi are the most widespread and abundant groups of mycorrhizae (Siddiqui et al., 2008). In fact, AM fungi, belonging to the Glomeromycota phylum, are recognized for creating intracellular structures known as "arbuscules", and are able to establish mutualistic relationships with 80% of terrestrial plants species (Lenoir et al., 2016; Smith & Read, 2008). Similarly, ECM from Basidiomycota and Ascomycota phylla can form symbiotic relationships with around 6,000 tree species of different taxonomic families, including economically relevant species such as pines, oaks and eucalyptus that are spread across extensive regions of boreal and temperate forests in both globe hemispheres (Futai et al., 2008; Martin et al., 2016). Despite the phenotypic diversity of ECM fungi, the vast majority establish associations by forming a “sheathing mantle”. This structure engulfs fine root tips with hyphae that further develop inwards between epidermal cells and cortical tissue making the Hartig net. Additionally, extrametrical hyphae from the “sheathing mantle” explore the rhizosphere and nearby soil niches, increasing the surface area to absorb nutrients and water (Genre et al., 2020; Martin et al., 2016). The uptake of physiologically important nutrients by plants, such as nitrogen (N) and phosphorus (P), can be considerably improved by the interaction with ECM fungi, through a favourable exchange of photosynthetic assimilates (Liu et al., 2020). Besides increasing the uptake of macro and micronutrients, the mycelium network of ECM fungi can increase the water supply, by extending the volume of land accessible to plants, as the hyphae are able to penetrate to narrower soil pores (Domínguez-Núñez et al., 2019; Guerrero-Galán et al., 2019). Additionally, ECM can significantly improve seedlings establishment while also providing tolerance to numerous abiotic stresses, such as metal toxicity and salinity, as well as improving plant resistance to biotic challenges, including pests, pathogens, and diseases (Becquer et al., 2019; Chot & Reddy, 2022; Gonthier et al., 2019; Guerrero-Galán et al., 2019; Laliberté et al., 2015; Vishwanathan et al., 2020). Despite all these beneficial effects, it should be stressed out that the observed outcomes are often variable and dependent on the fungi and plant species, as well as the environmental context (van der Heijden et al., 2015).

ECM have also been reported to increase host plant tolerance to drought (Kebert et al., 2023a; Y. Li et al., 2021; Sebastiana et al., 2018; J. Wang et al., 2021; Yin et al., 2018). As highlighted by Sebastiana et al. (2019), the mechanisms behind ECM-mediated alleviation of drought stress in plants encompasses various aspects. These include the increase in water uptake via fungal hyphae, leading to enhanced root conductance and subsequent stomatal opening, which in turn promotes carbon assimilation. Moreover, the adequate mineral nutrition acquisition can improve the

photosynthetic machinery efficiency. Another mechanism is the activation of plant AOX system, allowing a better management of ROS overproduced under water-deficit conditions. However, the mechanisms involved are far from being completely understood with some reports showing no effect of ECM or even negative impacts on plants' water relations in comparison with non-mycorrhizal plants (Kilpeläinen et al., 2020; Lehto & Zwiazek, 2011).

While there is an abundance of reports of mycorrhizal plants under drought stress, the number of studies exploring the potential of mycorrhizae to improve plant stress tolerance to heat remains limited (Duc et al., 2018), probably because heat stress is often associated with damage to the aerial parts of the plant, rather than roots. Yet, Maya & Matsubara (2013) reported an increased biomass and heat damage alleviation in mycorrhizal plants. Similarly, Jumrani et al. (2022) showed that AM-infected soybean plants presented better growth characteristics (leaf area, dry weights, and length of shoots and roots) than those non-inoculated under high-temperature exposure. More recently, Kebert and colleagues (2022) showed that the presence of ECM (*Scleroderma citrinum*) was capable of modulating heat stress responses in pedunculate oaks (*Quercus robur* L.). Despite being widely accepted that mycorrhization can alleviate various stresses, including individual exposure to drought and high temperatures (Begum et al., 2019), there is a lack of studies on mycorrhizal plants grown under combined drought and heat. As a matter of fact, the studies available on this topic (Duc et al., 2018; Haddidi et al., 2020) only focus on tomato plants. In tree species, to the best of our knowledge, there is not a single report exploring the potential of mycorrhiza to enhance plant tolerance to combined stress conditions. Regarding this issue, it is known that *C. sativa* is able to establish associations with several ECM fungi, in order to boost mineral nutrients availability, growth, productivity, and protection against root pathogens (Baptista et al., 2010). The use of beneficial mycorrhiza has also been shown to increase the survival and growth of micropropagated chestnut plants, facilitating acclimation to *ex vitro* conditions (Martins, 2008). Moreover, Aryal et al. (2021) recently observed that ECM colonization can help chestnut trees in their early growth and stress tolerance, since inoculated seedlings showed a faster recovery from water scarcity stress. Considering the well-known benefits of mycorrhization in enhancing plant growth and performance under abiotic stress situations (Begum et al., 2019), knowledge around the potential of this strategy to increase the resilience of forest tree species to CC is a matter of special interest that needs to be addressed.


A
Impact of abiotic stresses associated with climate change on trees
High Temperature
Decreases in photosynthetic performance
Limited growth
Reduced carbon allocation to its symbiont
Altered water relations in plant tissues
Oxidative stress
Hormonal imbalance
Drought
Osmotic stress
Ion imbalance
Hormonal Imbalance
Stomatal closure
Oxidative stress
Decrease of growth and development
Salt stress
Ion imbalance
Osmotic stress
Na+ toxicity
Inhibition of K+ uptake
Stomatal closure
Oxidative stress
Decrease of development
Flooding
Lack of oxygen
Accumulation of ethylene
Alteration of plant water relations
Nutrient deficiency
Morphological adaptations
Oxidative stress
Decrease of growth and development

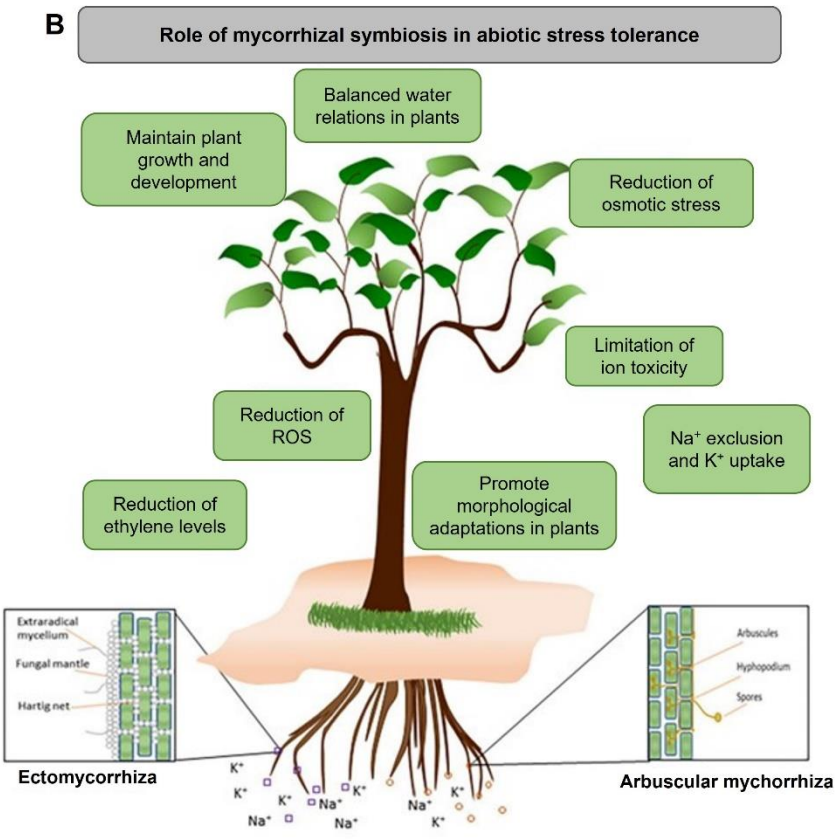

B
Role of mycorrhizal symbiosis in abiotic stress tolerance
Balanced water relations in plants
Maintain plant growth and development
Reduction of osmotic stress
Limitation of ion toxicity
Reduction of ROS
Na+ exclusion and K+ uptake
Reduction of ethylene levels
Promote morphological adaptations in plants
Extraradical mycelium
Fungal mantle
Hartig net
Ectomycorrhiza
Arbuscules
Hyphopodium
Spores
Arbuscular mychorrhiza
K+
Na+

Figure 2: Mycorrhizal fungi alleviate climate change-linked abiotic stress by affecting tree growth in temperate and boreal forests. (**A**) Trees are affected by increasing abiotic stress linked to climate change such as high temperatures, drought, salt stress, and flooding. (**B**) However, tree-associated mycorrhizal (ECM, ectomycorrhizal and AM, arbuscular mycorrhizal) fungi improve plant water and mineral nutrition (among them potassium K+) and help to adapt to stressful environmental conditions (among them elevated sodium Na+ in saline conditions). Adapted from Usman et al. (2021).

1.8 Aims

Over the recent years, the production of chestnut in Portugal has been suffering a significant decline, either due to the appearance of emerging diseases (*e.g.* ink disease) or to CC-related effects, namely the increasing frequency and intensity of extreme weather conditions, such as heat waves and longer periods of drought (Freitas et al., 2021). Given the importance of chestnut production in Portugal, it is necessary to ensure the adaptability of *C. sativa* to an ever-changing environment. These challenges are particularly relevant for Marsol, a common *C. sativa* variety used in Portugal, both as a rootstock and direct producer. This is hybrid chestnut clone resulting from the cross between *Castanea crenata* and *Castanea sativa* was developed to be resistant to ink disease. Aligned with the overall goal of the recent project CC&NUTS (MTS/SAS/0103/2020), funded by a cooperation between *BPI & Fundação La Caixa* and *Fundação para a Ciência e Tecnologia* (FCT), this work addresses the potential of beneficial mycorrhiza associations to boost *C. sativa* tolerance to the single and combined action of high temperatures and drought stresses.

To achieve that, the following set of objectives were pursued:

- Evaluate the morphophysiological responses of mycorrhizal (MR) and non-mycorrhizal (NMR) chestnut plants to the single and combined action of heat and drought;
- Assess the photosynthetic performance of MR and NMR Marsol hybrid plants upon exposure to single and combined stresses;
- Unravel the modulation of the plants' redox status, by focusing on the evaluation of oxidative stress markers and AOX mechanisms in response to both stresses;

As studies exploring the interaction between mycorrhization and combined stresses are scarce, the results collected will be pioneer in unravelling the role of mycorrhization in counteracting the joint effects of two of the most damaging conditions towards crop growth (high temperatures and water scarcity), with the ultimate goal of obtaining chestnut plants more resilient to CC.

2 Material & Methods

2.1 Plant material and experimental setup

Three months-old micro-propagated plantlets (≈ 15 cm height) of non-mycorrhizal (NMR) and mycorrhizal (MR) Marsol hybrid chestnut plants, potted in a commercial substrate (undisclosed due to Deifil Biotechnology Lda© policy) and previously grown at Deifil Biotechnology Lda© (Póvoa de Lanhoso, Braga, Portugal), were transported to the Department of Biology of the Faculty of Sciences of University of Porto (FCUP). Upon arrival, plants were placed in a growth chamber under controlled conditions similar to those available at the company [temperature – 25 ºC, photoperiod – 16 h light/8 h dark, and photosynthetic photon flux density (PPFD) – 120 µmol m^{-2} s^{-1}]. Then, the number of leaves of each plant was recorded. After one week of acclimatization, both plant sets (MR and NMR) were randomly divided into different experimental groups to unravel the single and combined effects of heat and drought. In accordance with the current projections for the Mediterranean region (Pereira et al., 2021), the heat stress was induced by a 4 h-daily exposure at 42 ºC between 12 h and 16 h. On its turn, drought was simulated by suspending the watering of plants, as previously tested by (Alcaide et al. (2019) and Camisón et al. (2020), until reaching a field capacity of 25%.

At the beginning of the acclimatization week, and after assessing the substrate maximum water hold capacity (WHC_{max}; ISO, 2023), substrate samples from each potted plant were taken and dried at 106 ºC for 24 h to determine the exact amount of tap water needed to adjust the WHC of each pot to 70%. Throughout the assay (7 d acclimatization + 21 d of stress conditions), for both plant sets (MR and NMR), the WHC of pots from control and heat-treated groups was maintained at 70%, whereas for drought-exposed plants, no irrigation was performed until the WHC reached 25%. Every alternate day, each pot was weighted on a digital electronic balance and the proper WHC was re-established by slowly adding the required amount of water to the surface of the substrate, ensuring that there was no water leakage. The following experimental groups were considered:

- **Control (CTL):** MR or NMR plants grown at 25 ºC and irrigated with tap water;
- **Drought:** MR or NMR plants grown at 25 ºC without irrigation, until a 25% WHC was reached, for 21 d;
- **Heat**: MR or NMR plants grown at 25 ºC, but daily exposed to 42 ºC for 4 h, in a twin growth chamber, and irrigated with tap water;

- **Combined**: MR or NMR plants, grown at 25 ºC, but daily exposed to 42 ºC for 4 h, in a twin growth chamber, without irrigation, until a 25% WHC was reached, for 21 d.

For each experimental group (CTL, Drought, Heat, and Combined) of each set of plants (NMR and MR), at least 8 biological replicates (each one consisting of a potted plant) were considered. Four independent assays were carried out to obtain enough biomass to perform all the biochemical parameters and to validate the results.

After the stress exposure (21 d), gas-exchange measurements and chlorophyll fluorescence analyses were carried out *in vivo* in fully expanded leaves (2^{nd} and 3^{rd} from the apex) in, at least, four MR and NMR plants from all treatments. Moreover, the number of leaves of each plant was also counted. Afterwards, plant material from every replicate was collected and separated into roots and leaves. Part of the plant material from each replicate of all experimental groups was immediately used for the estimation of the relative water content (RWC) and $O_2^{\cdot-}$ levels, while the remainder was frozen in liquid nitrogen (N_2) and stored at -80 ºC to be used in further biochemical techniques.

2.2 Relative water content (RWC)

The RWC was determined following the protocol described by Sade et al. (2015). At harvesting, root segments (≈ 3 cm) and leaf disks (≈ 1.5 cm diameter) from fully opened leaves were taken from at least 6 plants of every experimental group from NMR and MR plant sets. Then, after recording the leaf area and fresh mass (FM) of each sample, these were immersed in distilled water overnight and re-weighted to measure the turgid mass (TM). Subsequently, the samples were placed at 60 ºC for two weeks to record the dry mass (DM). The relative water content was expressed according to the formula:

$$RWC\ (\%) = (FM\text{-}DM/TM\text{-}DM) * 100\%$$

2.3 Chlorophyll fluorescence analysis

Chlorophyll fluorescence analysis was performed on the 2^{nd} and 3^{rd} young fully expanded leaves of chestnut plants (MR and NMR) growing in different experimental conditions, by a Pulse Amplitude Modulation Fluorometer (mini-PAM, Photosynthesis Yield Analyzer; Walz, Effeltrich, Germany), using two scripts, as described in Bernardo et al. (2022). Briefly, in the first script, measurements were done on light-exposed leaves. This involved a 35 s exposure to actinic light (1,450 µmol m^{-2} s^{-1}) to determine the light-adapted steady-state fluorescence yield (F_s). Subsequently, a saturating pulse light (6,000 µmol m^{-2} s^{-1}) for 0.6 s was provided to establish F'_m. A 5-s shading period with

far-red light source was then employed to determine F'_0. Based on these measurements, several fluorescence attributes were calculated (Bilger & Schreiber, 1986; Genty et al., 1989): non-photochemical quenching [$NPQ = (F_m - F'_m)/F_m'$] and efficiency of electron transport, as a measure of the effective quantum efficiency of PSII [$\Phi_{PSII} = \Delta F/F'_m = (F'_m - F_s)/F'_m$]. The photosynthetic electron transport rate (ETR) was estimated as PAR x ΦPSII x 0.5 x 0.84, in which the factor 0.5 assumes equal excitations of the two photosystems (Rascher et al., 2000); the ETR correction factor of 0.84 considers that only a fraction of incident light is absorbed by photosystems, being this the mean value of absorbance for green leaves (Rascher et al., 2000). In the second script, the same leaf portion used in the first script was immediately dark acclimated for 30-45 min utilizing the dark leaf clip (DLC-8). Afterwards, the maximum photochemical efficiency of PSII was calculated by $F_v/F_m = (F_m - F_0)/F_m$, where F_0 corresponds to the minimum fluorescence level excited by the very low intensity of measuring light to keep PSII reaction centres open, F_v represents the variable fluorescence level and F_m corresponds to the maximum fluorescence level elicited by a pulse of saturating light (6,000 μmol m^{-2} s^{-1}) that closes all PSII reaction centres (Bilger & Schreiber, 1986).

2.4 Leaf gas-exchange measurements

Like chlorophyll fluorescence measurements, gas-exchange determinations were performed *in vivo* on the 2nd and 3rd young fully expanded leaves of chestnut plants (MR and NMR), with an infrared portable gas exchange analyser (IRGA; LC pro-T, ADC, Hoddersdon, UK) coupled with a broad light source. The measurements occurred under simulated greenhouse conditions (atmospheric CO_2 concentration, PPFD of 174 μmol m^{-2} s^{-1} and the temperature was 25 ºC). Furthermore, all measurements were carried out in four replicates for each experimental situation. Net CO_2 assimilation rate (A; μmol mol^{-1}), transpiration rate (E; mmol m^{-2} s^{-1}) and stomatal conductance (g_s mmol m^{-2} s^{-1}) were estimated according to the equations developed by von Caemmerer & Farquhar, (1981). Additionally, the intrinsic water use efficiency ($WUE_i = A/g_s$) was determined.

2.5 Biochemical parameters

2.5.1 Extraction and quantification of photosynthetic pigments

The evaluation of photosynthetic pigments was performed based on the method described by Lichtenthaler (1987). For each situation, frozen aliquots of leaves (ca. 200 mg) were homogenized in 80% (v/v) acetone and centrifuged for 10 min at 3,500 *g*. Until complete decolorization of the sediment, centrifugations were repeated upon the addition of 80% (v/v) acetone and vigorously mixing. Afterwards, the supernatant (SN) was

collected and the absorbance (Abs) at 470, 647 and 663 nm was recorded, using 80% (v/v) acetone as blank. The concentration of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Car) was calculated according to the following equations (Lichtenhaler 1987):

- Chl *a* (mg L^{-1}) = (12.25 x $Abs_{663\ nm}$) - (2.79 x $Abs_{647\ nm}$)
- Chl *b* (mg L^{-1}) = (21.50 x $Abs_{647\ nm}$) - (5.10 x $Abs_{663\ nm}$)
- Car (mg L^{-1}) = [(1000 x $Abs_{470\ nm}$) - (1.82 x Chl *a*) - (85.02 x Chl *b*)] / 198

The results were expressed in mg g^{-1} dry mass (d.m.).

2.5.2 Determination of ROS

2.5.2.1 $O_2^{\cdot-}$ content

The production of $O_2^{\cdot-}$ was estimated by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\cdot-}$, as described by Sharma et al. (2017). Freshly cut root and leaf tissues (ca. 200 mg) were homogenized, on ice, with potassium phosphate (PK) buffer (65 mM, pH 7.8) containing 1% (m/v) polyvinylpolypyrrolidone (PVPP) (1.5 mL for shoots and 1.2 mL for roots). The homogenates were then centrifuged for 20 min (5,000 *g*; 4 ºC). Upon being collected, 45 µL of the SN were mixed with 45 µL PK buffer (65 mM, pH 7.8) and with 10 µL of 10 mM hydroxylamine hydrochloride in a 96-well microplate. Following an incubation at 25 ºC for 20 min, 100 µL of 58 mM 3-aminobenzenesulphonic acid and 100 µL of 7 mM 1-naphtylamine were added to the mixture and further kept at 25 ºC for 30 min. Finally, the Abs at 530 nm was recorded using the microplate reader Multiskan GO (Thermo Fisher Scientific). In order to calculate the $O_2^{\cdot-}$ levels, a standard curve of sodium nitrite was prepared. Results were expressed in µmol g^{-1} d.m.

2.5.2.2 H_2O_2 content

The quantification of H_2O_2 was spectrophotometrically performed according to Alexieva et al. (2001). Frozen aliquots (ca. 200 mg) of leaves and roots were homogenized, respectively, in 1.5 mL and 1.2 mL of 0.1 % (m/v) trichloroacetic acid (TCA) under cold conditions using a bead miller (Bead Ruptor 12 Homogenizer, Omni International Inc.). Three sequential cycles of 15 s at 5 m s^{-1} were performed, with an incubation on ice for 1 min between them. Following a 15 min centrifugation (4 ºC; 16,000 *g*), 50 µL of SN were added to 50 µL of PK buffer (100 mM, pH 7) and 250 µL of 1 M potassium iodide (KI), in a 96-well UV microplate. After a brief shake, the microplate was left in dark conditions for 1 h and, at the end, the Abs was measured at 390 nm. The amount of H_2O_2

was calculated using a standard curve prepared with known concentrations of H_2O_2 and results were expressed in µmol H_2O_2 g^{-1} d.m.

2.5.3 Determination of lipid peroxidation (LP)

The membrane damage was evaluated in terms of LP, based on the determination of malondialdehyde (MDA) content in accordance with Heath & Packer (1968). Here, the same extraction procedure described for the quantification of H_2O_2 was followed. After the centrifugation (15 min; 4ºC; 16,000 *g*), 1 mL of 0.5% (m/v) thiobarbituric acid (TBA) in 20% (m/v) TCA was added to 250 µL of SN. The reaction mixtures were heated for 30 min at 95 ºC and subsequently cooled in ice before a final centrifugation (10,000 *g*; 7 min). Afterwards, the Abs of each sample was recorded at 532 and 600 nm, with the last being subtracted to the former to minimize unspecific turbidity effects. MDA levels, expressed as MDA g^{-1} d.m., were determined using the extinction molar coefficient (ε) of 155 mM^{-1} cm^{-1}.

2.5.4 Extraction and quantification of AOX metabolites

2.5.4.1 Proline (Pro)

As described by Bates et al. (1973), the colorimetric detection of Pro-ninhydring complex was used to determine Pro levels. Accordingly, frozen aliquots from leaves and roots (ca. 200 mg) were homogenized with a series of homogenization cycles (5 m s^{-1}; 15 s) in the bead miller (Bead Ruptor 12 Homogenizer, Omni International Inc.), with 1 mL of 3% (m/v) sulfosalicylic acid and centrifuged for 20 min at 16,000 *g*. Following this step, 200 µL of each sample SN was combined with 200 µL of glacial acetic acid and 200 µL of acid ninhydrin. All the reaction mixtures were incubated for 1 h at 96 ºC, followed by a brief cooling period in ice. Then, 1 mL of toluene was added to each tube, accompanied by vigorous mixing in the vortex for 15 s, in order to extract the ninhydrin-Pro complex. Upon the complete separation of the aqueous (pinkish; top) and organic phases (whiteish; bottom), the upper one was collected, and its Abs read at 520 nm, using toluene as blank. Pro content was estimated through a calibration curve, prepared with different solutions of increasing Pro concentrations. Results expressed in µg g^{-1} d.m.

2.5.4.2 Glutathione (GSH)

The levels of GSH were quantified by following the protocol optimized by Soares et al. (2019b), using the same SN as for the Pro assay. Briefly, upon centrifugation, 50 µL of SN were added to 200 µL of H_2O and 750 µL of a reaction mixture containing 100 mM PK buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.043 mg mL^{-1} 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's Reagent). After an incubation period

of 10 min, to allow the reaction between GSH and DTNB, GSH levels were measured spectrophotometrically at 412 nm. The concentrations of this metabolite were calculated based on a standard curve prepared with solutions of known GSH concentrations. The results were expressed in nmol GSH g^{-1} d.m.

2.5.4.3 Ascorbate-reduced form (AsA)

The quantification of reduced ascorbate was conducted based on the methodology outlined in Gillespie & Ainsworth (2007). This method relies on the AsA-mediated conversion of ferric ion (Fe^{3+}) to ferreous ion (Fe^{2+}), subsequently producing a by-product with 2-2'-bipyridyl, detectable at 525 nm. Briefly, frozen aliquots of leaf tissue and roots (ca. 200 mg) were, respectively, homogenised under cold condition in 1.5 and 1.2 mL of 6% (m/v) TCA, in the bead miller (Bead Ruptor 12 Homogenizer, Omni International Inc.) with a series of homogenization cycles (5 m s^{-1}; 15 s). The homogenates were then centrifuged for 10 min (15,000 *g* at 4 ºC) and the SN was collected. Following this, 25 µL of SN were mixed with 12.5 µL of 75 mM PK buffer (pH 7.0), 25 µL off ddH_2O and 187.5 µL of a reaction mixture containing 10% (m/v) of TCA, 200 µL of 43 % (v/v) phosphoric acid (H_3PO_4), 200 µL of 4 % (m/v) 4,4'-bipyridyl (BIP) and 100 µL of 3 % (m/v) iron (III) chloride ($FeCl_3$) in a 96 well- UV microplate. Finally, after 1 h incubation at 37 ºC the Abs was read at 525 nm. Results were calculated and expressed as µmol AsA g^{-1} d.m, after preparing a calibration curve with known AsA concentration.

2.6 Statistical analysis

All biometric and biochemical parameters for both MR and NMR plants were assessed in at least three biological replicates, with three technical replicates per assay. The results were expressed as mean ± standard error of the mean (SEM). Following the verification of the homogeneity and normality assumption, a two-way ANOVA was performed in order to check significant differences between the fixed factors "stress type" (three levels – drought, heat and combination) and "mycorrhization" (two levels – MR and NMR). To identify differences between MR and NMR plants, means were compared by Sidak *post-hoc* test assuming a significance level (α) of 0.05, while Tukey's test was used to discriminate differences for the stress factor. In cases where a significant interaction was found, a correction for simple main effects was carried out. In parallel, a principal component analysis (PCA) was carried out to evaluate the resemblances among conditions and identify the primary relationships between variables contributing to the observed differences. To achieve this, the mean values for each assessed parameter and the first two components were used to make biplots. All statistical data was generated by GraphPad® Prism 9 (GraphPad Software Inc., USA). Additionally, a

clustered heatmap was produced through the web-based heatmap2 tool (Galaxy Version 3.0.1).

3 Results

3.1 Biometric parameters – foliar area, production of new leaves and relative water content (RWC)

After 21 d of stress exposure, plant growth, evaluated in terms of foliar area, percentage of new leaves produced and RWC, was significantly modulated by both factors – MYCORRHIZATION (MYCO) and STRESS (Drought, Heat or Combined) – and their interaction, as can be seen in Tables S1 and S2 and illustrated in Figure 3. Under control conditions, leaves from NMR plants exhibited a higher area than those of MR ones. However, the foliar area of both NMR and MR plants was significantly reduced in response to all stresses, when compared with the respective CTL. In particular, heat and drought, resulted in a significant reduction of 51% and 39% in NMR and 22% and 35% in MR, respectively, (Figure 3a). The combination of both stressors imposed a more severe decrease, though the inhibition degree was higher in NMR (about 84%) than in MR plants (54%), whose foliar area was significantly higher than that of NMR ones under such treatment (Figure 3a).

In what regards the production of new leaves, expressed on a percentage basis in relation to the control groups, only NMR plants co-exposed to heat and drought underwent a reduction of this parameter, with inhibition values around 70% in relation to the CTL (Figure 3b). In spite of that, MR and NMR did not present any differences between each other under any stress conditions, though a tendency for MR to show and increased production rate of leaves can be noticed (Figure 3b).

Following a similar pattern, in comparison with the CTL, the RWC of NMR plants significantly decreased around 21% and 28% under drought and combined stresses, respectively (Figure 3c); in the MR plants, only drought induced a significant decrease of this parameter (19%), in comparison with non-stressed MR plants. Once again, mycorrhization led to an augmented RWC, specially under the heat treatments (single and combined), with MR presenting up to a 1.5-fold increase when compared with NMR plants (Figure 3c).

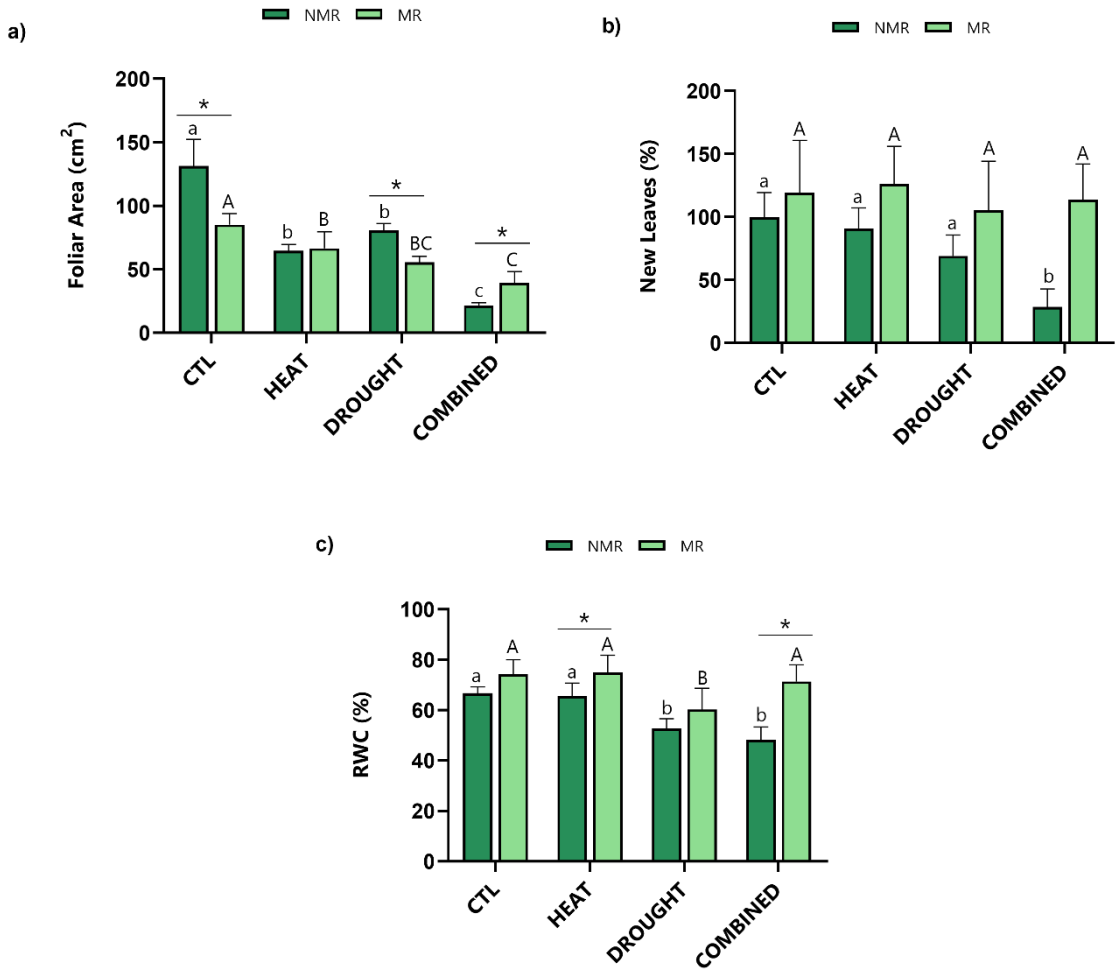


Figure 3 Foliar area (**a**), percentage of new leaves (**b**) and relative water content (RWC) (**c**) of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4 h d^{-1}); DROUGHT – drought-exposed plants (no irrigation until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. Results are expressed as mean ± SD (n ≥ 3). Different letters above bars demonstrate differences between treatments for each plant set (lowercase letters – NMR plants; uppercase letters – MR plants), based on Tukey's post-hoc test at $p \leq 0.05$). * Above bars indicate differences between MR and NMR plants for each treatment (Sidak: $p \leq 0.05$).

3.2 Carbon assimilation efficiency — gas-exchange parameters

Regarding gas-exchange measurements, all parameters (transpiration rate, stomatal conductance, CO_2 assimilation rate and intrinsic water use efficiency) were significantly changed in response to both MYCO and STRESS factors, and their interaction (Table S1).

From what can be observed in Figure 4 (a,b), NMR plants generally presented a higher transpiration rate (*E*) and stomatal conductance (g_s) than MR ones. Still, both set of plants responded similarly to the different stress treatments (Figure 4a,b). In fact, the transpiration rate of both NMR and MR only significantly decreased under single exposure to water scarcity (36% for NMR and 26% for MR) in comparison with their

respective CTLs (Figure 4a). Regarding g_s, in NMR plants, it was significantly decreased under drought (44%) and combined (22%) stresses, while no differences were recorded among treatments for MR plants (Figure 4b).

On its turn, stress-induced effects on carbon assimilation (*A*) varied between NMR and MR plants (Figure 4c). In general, MR plants under stress conditions presented enhanced assimilation rates than NMR (Figure 4c). Moreover, while in NMR a significant decrease was registered in response to drought, either single (33%) or combined (43%), MR plants did not present any significant inhibition and even enhanced their carbon assimilation under HEAT, with almost a 2-fold increase, in comparison with the respective CTL (Figure 4c). Lastly, the evaluation of the water use efficiency (WUE_i) revealed that MR plants presented increased values than their NMR counterparts in all experimental groups. Additionally, as can be seen in Figure 4d, in NMR plants, WUE_i did not change across the different stresses in relation to the CTL (Figure 4d); in the MR plants, this parameter significantly increased under individual stresses (1.64- and 1.16-fold in response to heat and drought, in relation to the CTL) (Figure 4d).

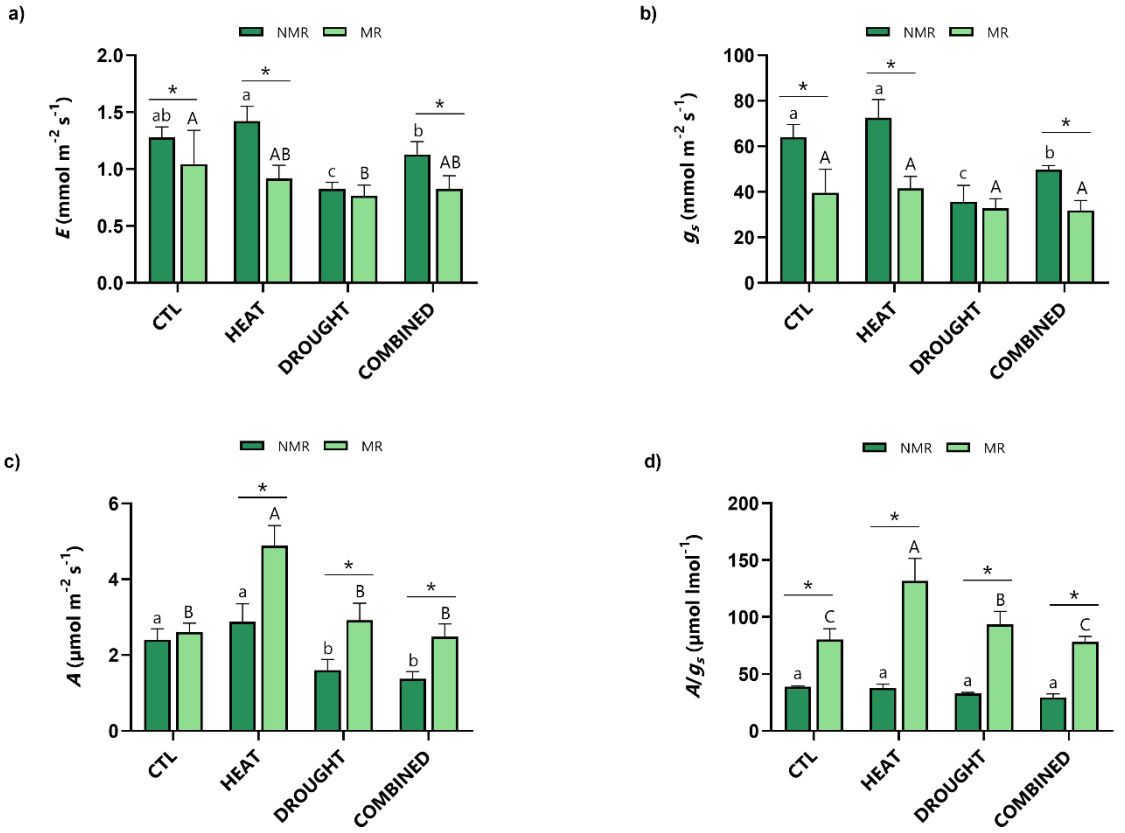


Figure 4 Transpiration rate (*E*) (**a**), stomatal conductance (g_s) (**b**), carbon assimilation (*A*) (**c**) and intrinsic water use efficiency ($WUE_i = A/g_s$) (**d**) of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d^{-1}); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. Results are expressed as mean ± SD (n ≥ 3). Different

letters above bars demonstrate differences between treatments for each plant set (lowercase letters – NMR plants; uppercase letters – MR plants), based on Tukey's post-hoc test at $p \leq 0.05$). * Above bars indicate differences between MR and NMR plants for each treatment (Sidak: $p \leq 0.05$).

3.3 Photochemical reactions – chlorophyll fluorescence analysis

According to the ANOVA results, only the interaction of both factors induced a significant effect on the maximum photochemical efficiency of PSII (F_v/F_m) and non-photochemical quenching (NPQ) (Table S1). In contrast, both electron transport rate (ETR) and effective yield of photosystem II (ΦPSII) were significantly changed in response to MYCO and the interaction of both factors (Table S1).

In general, under control conditions, NMR and MR plants showed identical values for all evaluated parameters (Figure 5); on the contrary, the presence of mycorrhization modulated stress responses differently, with a high variability found. For instance, while ETR and ΦPSII were higher in heat-exposed NMR plants, NPQ and F_v/F_m did not change between mycorrhizal and non-mycorrhizal plant sets under high temperature exposure (Figure 5). Regarding the response of each plant set, MR plants did not show any changes any of the studied indicators among stress groups (Figure 5a,b,c,d). Regarding NMR plants, when compared with their respective CTL, increases in F_v/F_m (1.15-fold), ETR (1.8-fold) and ΦPSII (1.6-fold) were found under single heat (Figure 5a,c,d); additionally, NMR plants exposed to single stresses did not increase the energy dissipation from non-photochemical mechanisms, however a significant rise of NPQ (1.4-fold) was recorded under the stress combination (Figure 5b).

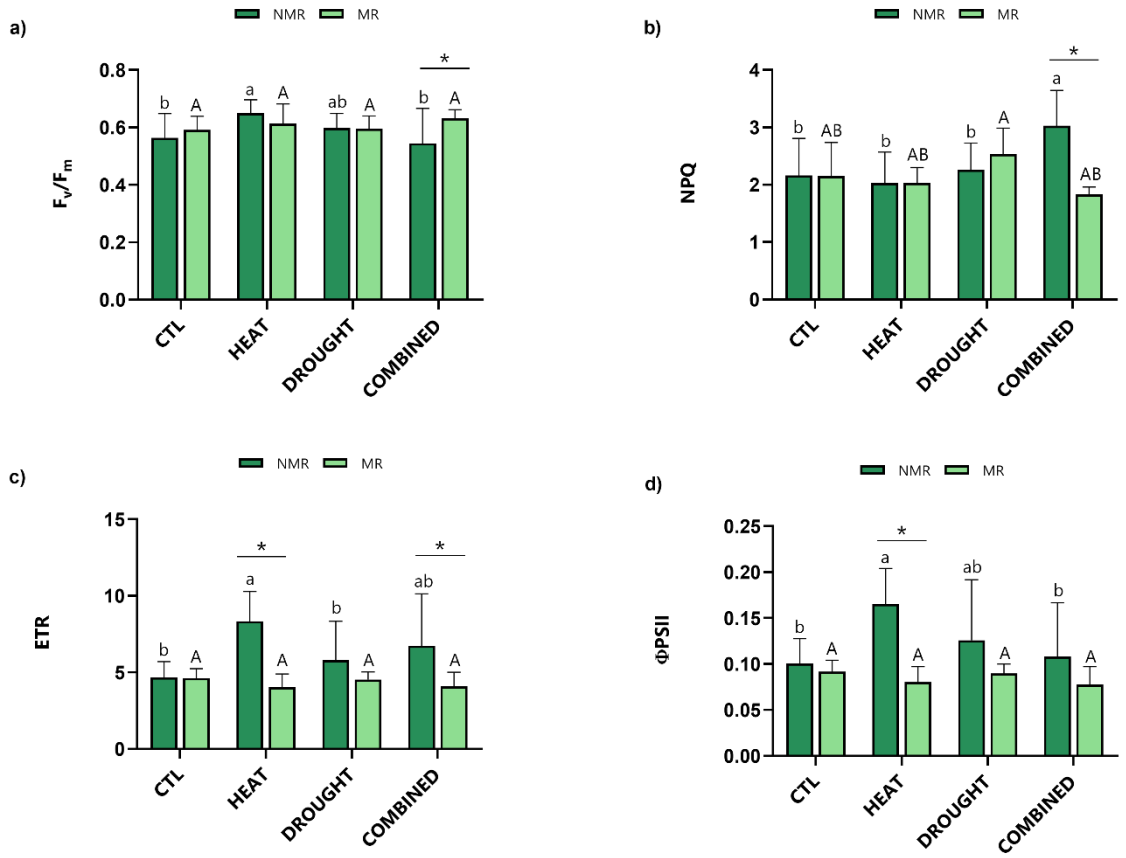


Figure 5 Maximum photochemical efficiency of PSII (Fv/Fm) (**a**), non-photochemical quenching (NPQ) (**b**), electron transporter rate (ETR) (**c**) and effective quantum efficiency of PSII (ΦPSII) (**d)** of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d^{-1}); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. Results are expressed as mean ± SD (n ≥ 3). Different letters above bars demonstrate differences between treatments for each plant set (lowercase letters – NMR plants; uppercase letters – MR plants), based on Tukey's post-hoc test at $p \leq 0.05$). * Above bars indicate differences between MR and NMR plants for each treatment (Sidak: $p \leq 0.05$).

3.4 Photosynthetic pigments

Data revealed that the levels of total chlorophylls (Chl a + b) and carotenoids were only significantly changed by the MYCO factor (Figure 6a,b). As can be seen, under control and heat single stress, the levels of photosynthetic pigments were higher in MR plants than NMR ones (control – car: > 1.5-fold; heat – chl: >1.7-fold; car: > 1.5-fold) (Figure 6).

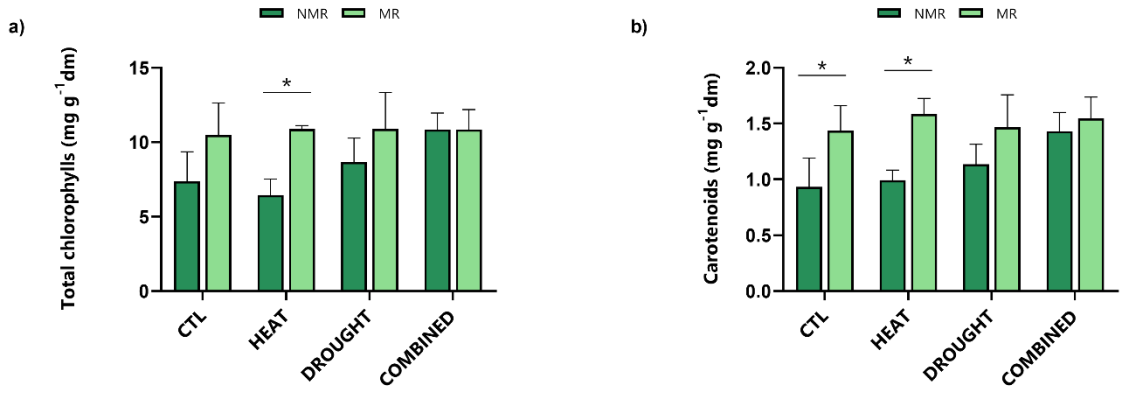


Figure 6 Total chlorophylls (Chl a + b) (**a**) and carotenoids (**b**) of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d^{-1}); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. Results are expressed as mean ± SD (n ≥ 3). Different letters above bars demonstrate differences between treatments for each plant set (lowercase letters – NMR plants; uppercase letters – MR plants), based on Tukey's post-hoc test at $p \leq 0.05$). * Above bars indicate differences between MR and NMR plants for each treatment (Sidak: $p \leq 0.05$).

3.5 Redox status – H_2O_2, $O_2^{\cdot-}$ and LP

The levels of H_2O_2 are represented in Figure 7 (a,b). According to the ANOVA results, both factors (MYCO and STRESS) and their interaction had a significant effect in H_2O_2 accumulation in leaves of chestnut plants (Table S1); at the root level, differences were found for the STRESS factor and its interaction with MYCO (Table S2). As shown in Figure 7a, under control conditions, NMR showed increased and decreased values of this ROS in leaves and roots, respectively, when compared to the MR set. Moreover, the exposure of NMR pants to the individual stresses induced a significant increase of H_2O_2 in leaves (1.5-fold in heat and 1.3-fold in drought), over the CTL. In contrast, H_2O_2 levels in roots significantly decreased with all stress treatments (78% in heat, 61% in drought and 81% in combined) (Figure 7b). Regarding MR plants, for both leaves and roots, only the combined treatment induced a significant reduction in this ROS, decreasing by 25% in leaves (Figure 7a) and by 73% in roots (Figure 7b), in comparison with the respective CTL.

When it comes to $O_2^{\cdot-}$ levels (Figure 7c,d), data indicated that the STRESS and its interaction with MYCO influenced this ROS levels in leaves, while in roots, differences for both factors and their interaction were found (Tables S1 and S2). Generally, NMR and MR plants accumulated $O_2^{\cdot-}$ at the same extent, exhibiting nearly identical values in almost experimental groups in both organs, except for HEAT stress in leaves and DROUGHT stress in roots. NMR plants exposed to the stress treatments presented a tendency to decrease the accumulation of this ROS in leaves, reaching statistical significance upon the co-exposure to both factors, where a 32% reduction was registered

(Figure 7c). Following the same pattern, in roots of NMR plants, the levels of this ROS significantly decreased in all stress treatments, over the CTL (34% in heat, 31% in drought and 48% in combined) (Figure 7d). In parallel, leaves (Figure 7c) and roots (Figure 7d) of MR plants exposed to the individual or combined stresses exhibited lower $O_2^{\cdot-}$ levels (up to 54 and 64%, respectively), when compared to the respective CTL.

Concerning the LP degree, the two-way ANOVA indicated significant differences for both factors and their interaction in both leaves and roots (Tables S1 and S2). Under control conditions, the MDA content was identical between MR and NMR plants (Figure 7e,f). In what regards the response of NMR and MR plants to stresses, it was possible to observe that the levels of LP of NMR plants were differently affected depending on the organ (Figure 7e,f). In leaves, drought, either alone (1.6-fold) or combined (1.4- fold), induced a significantly higher LP degree, in comparison with the CTL (Figure 7e). Contrarily, concerning the MR set, LP did not significantly change among groups in leaves. In roots, compared to the CTL, the MDA content diminished up to 50% under all stress condition in NMR plants, while, in MR ones, it was markedly lower (52%) under the combined treatment (Figure 7f).

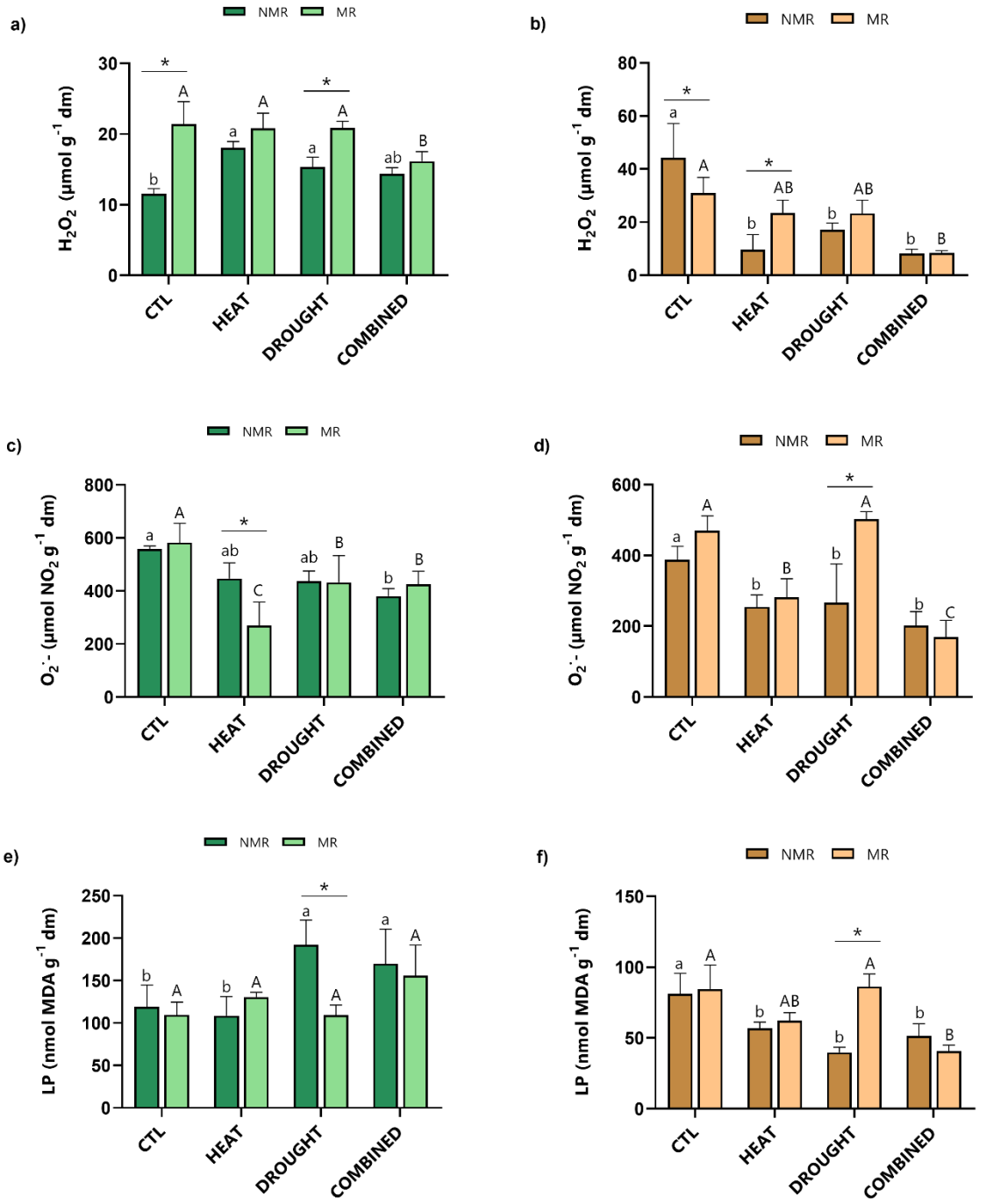


Figure 7 Levels of oxidative stress markers: H_2O_2 (**a,b**), $O_2^{\cdot-}$ (**c,d**) and lipid peroxidation (LP) (**e,f**) in leaves (green bars) and roots (brown bars) of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d^{-1}); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. Results are expressed as mean ± SD (n ≥ 3). Different letters above bars demonstrate differences between treatments for each plant set (lowercase letters – NMR plants; uppercase letters – MR plants), based on Tukey's post-hoc test at $p \leq 0.05$). * Above bars indicate differences between MR and NMR plants for each treatment (Sidak: $p \leq 0.05$).

3.6 Non-enzymatic AOX system- proline, GSH and reduced AsA

Starting with Pro levels, ANOVA results indicated that both factors and their interaction significantly affected this biomarker in leaves and roots of chestnut plants (Tables S1 and S2). It is noticeable that ECM association had a positive effect on Pro accumulation across all experimental groups in both plant organs, but especially in leaves, where Pro levels were always higher than in NMR plants (Figure 8a,b). Additionally, leaves and roots of NMR plants exhibited similar Pro levels independently of the applied treatment, as no significant changes between groups were detected (Figure 8a,b). Concerning the MR plants, when compared to their respective CTL, those exposed to single stresses exhibited lower Pro accumulation in leaves (30% and 20% in heat and drought, respectively) (Figure 8a), while no significant differences were recorded in roots (Figure 8b). In contrast, MR plants subjected to the combined treatment accumulated Pro to a much higher extent, with the levels of this AOX metabolite increasing by 1.8-fold in leaves (Figure 8a) and 3.6-fold in roots (Figure 8b).

Regarding GSH content in leaves, and following a significant effect for the STRESS factor and its interaction with MYCO (Table S1), it can be observed that only MR plants under the combined treatment stimulated the accumulation of this AOX, compared to the respective CTL (Figure 8c). On the other hand, GSH content of roots was significantly modulated in response to both factors and their interaction (Table S2). While in NMR plants the GSH content was similar across the different groups, in MR plants the individual and combined stresses induced a significant reduction of this AOX metabolite. More precisely, in comparison with the CTL, heat and drought alone inhibited GSH levels by 43% and 33%, respectively, with their combination further aggravating this response (73%) (Figure 8d). Lastly, it is evident that ECM association enhanced GSH content across all treatments in roots, while in leaves no changes were found (Figure 8c,d).

With respect to AsA, the ANOVA analysis revealed significant changes in response to MYCO in leaves, and in response to STRESS in roots, with the interaction of both factors influencing AsA levels in both organs (Tables S1 and S2). First, it can be observed that, under CTL conditions, AsA levels were similar between the two sets of plants (Figure 8 e,f). As Figure 8e suggests, AsA levels in leaves of NMR plants did not change significantly, although there was a tendency for decreased values in the combined treatment. In the roots, NMR plants exhibited reduced AsA levels with decreases of 60% and 44% in drought alone or combined with heat, respectively and in relation to the respective CTL (Figure 8f). In MR plants, no changes were found for the

single stresses in both organs; however, the combined treatment induced contrasting responses between organs in this group of plants: while in leaves (Figure 8e), the AsA content increased by 1.4-fold, in roots (Figure 8f) a 76% decrease was recorded, over the respective CTL.

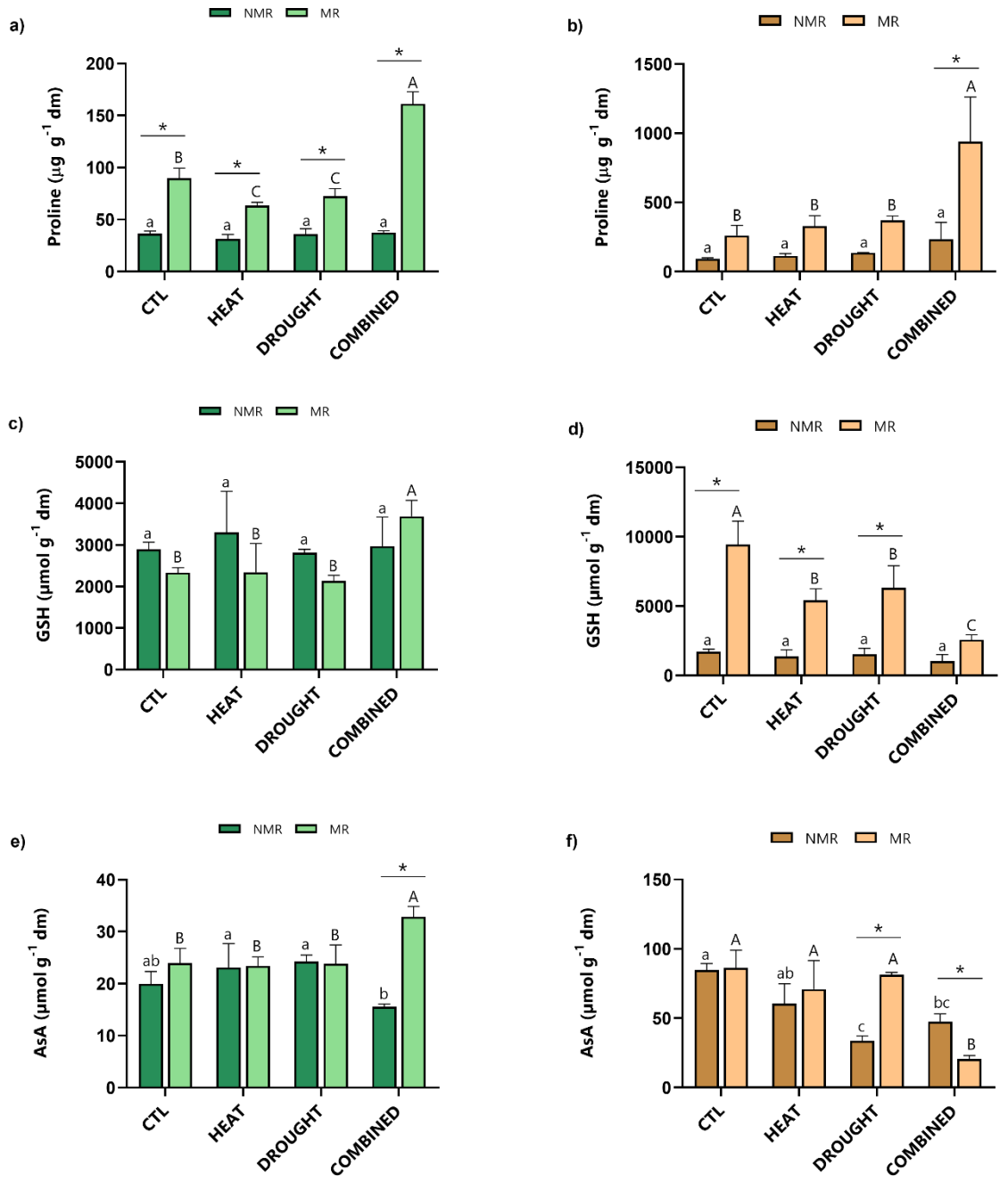


Figure 8 Content of proline (**a,b**), GSH (**c,d**) and AsA (**e,f**) in leaves (green bars) and roots (brown bars) of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d^{-1}); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. Results are expressed as mean ± SD (n ≥ 3). Different letters above bars demonstrate differences between treatments for each plant set (lowercase letters – NMR plants; uppercase letters – MR plants), based on Tukey's post-hoc test at $p \leq 0.05$). * Above bars indicate differences between MR and NMR plants for each treatment (Sidak: $p \leq 0.05$).

3.7 Principal Component Analysis (PCA) and heatmap

To gain a comprehensive understanding of treatment effects and the relationships between the studied variables, as well as to infer correlations among all tested parameters, a PCA was conducted. On top of that, an heatmap analysis was also carried out for a broad insight on the overall response of both NMR and MR plants under the imposed stress conditions. The results obtained showed that the first component (PC1) explained 27.13% of total variance while the second (PC2) explained 22.73%. Overall, Figure 9 shows that chestnut plants were differently affected by the stress type and the presence of beneficial mycorrhiza. Indeed, four clear cluster are noticeable. All NMR plants are located in the first and fourth quadrants: in the first quadrant, there is a cluster of NMR CTL/HEAT, while in the fourth quadrant, there is a close association of NMR DROUGHT/COMBINED plants. In the second quadrant, control and MR plants exposed to single stresses form a clear cluster, while MR COMBINED plants stand out in the third quadrant as a distinct group. The same identified clusters are also evident in the heatmap (Figure 10). Altogether, this indicates a very pronounced effect of mycorrhization in modulating the plants' physiological status in both control and stressful conditions. Moreover, the higher similarity between the individual stresses and the control in the MR plants, compared to NMR plants, highlights the important role of this association in ameliorating the plants' response to stress. Indeed, by analysing Figure 10, it is recognizable that generally, the response of MR plants exposed to singles stresses (MR HEAT-DROUGHT) was similar to the ones of control conditions (MR CTL) across all evaluated parameters in terms of photosynthesis, ROS levels and AOX metabolites. Moreover, despite being clustered apart, MR COMBINED plants presented a close-to-control performance regarding growth and photosynthetic capacity, with the main differences being lower ROS and AOX accumulation, as well as increased osmolyte production, also indicating a beneficial plant response.

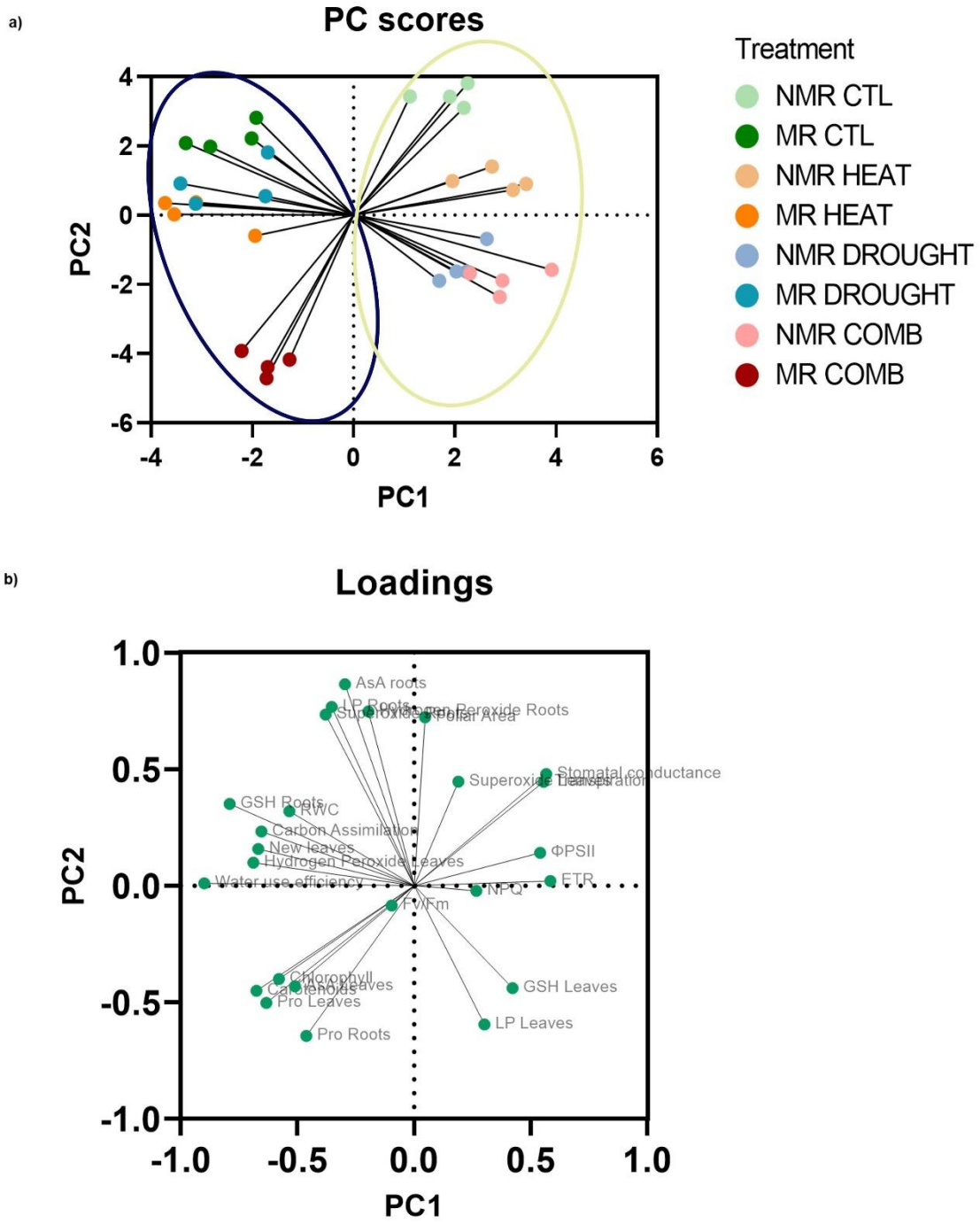


Figure 9 Score plot (**a**) and loading pot (**b**) of the first two PCA components showing the differential response of of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d^{-1}); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants.

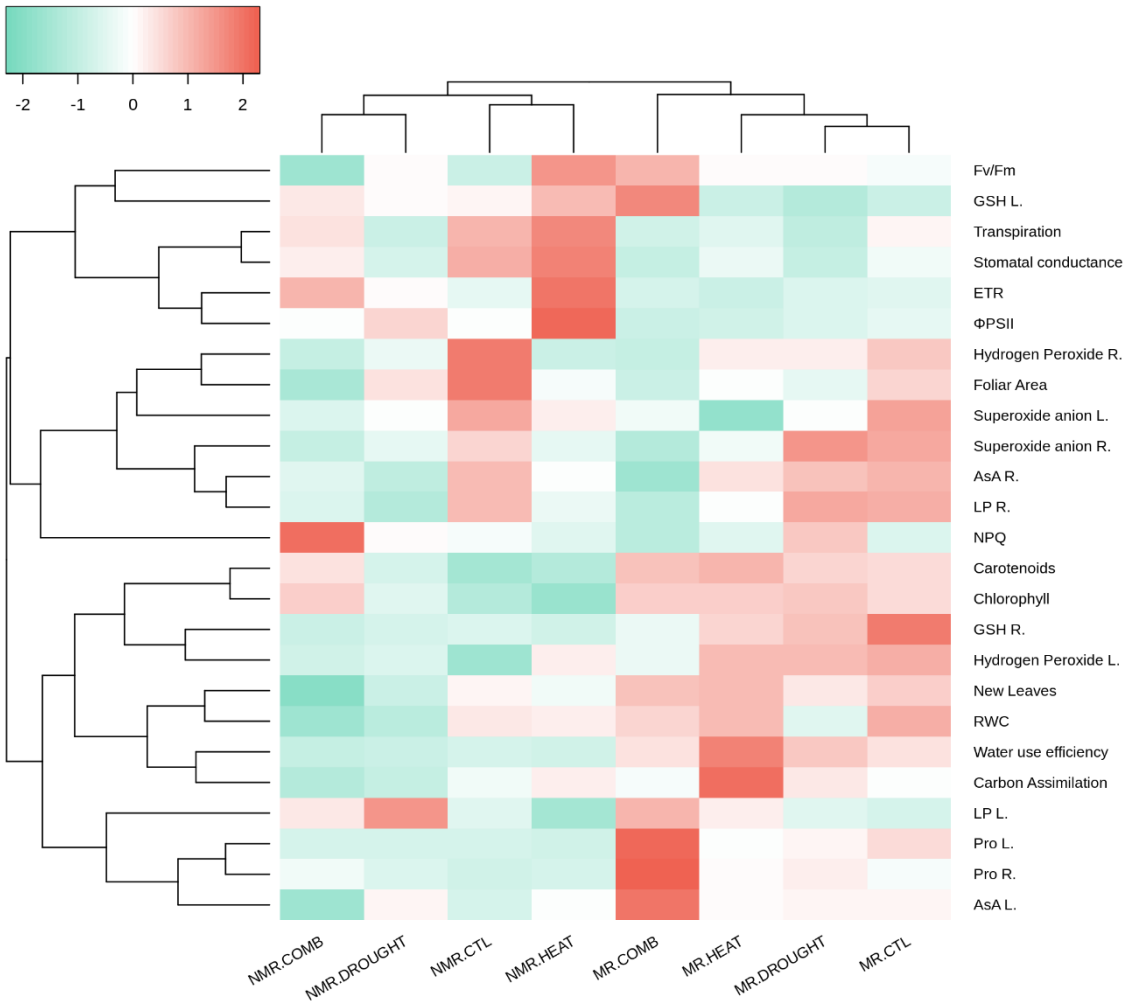


Figure 10 Clustered heatmap, elucidating the differential response of NMR and MR chestnut after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d^{-1}); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. The color scale displays the intensity of z-scores for the different parameters.

4 Discussion

High temperatures and prolonged periods of drought represent major environmental threats for chestnut tree productivity and yield (Fernandes et al., 2022). Yet, little attention has been given to the understanding of their combined impacts on chestnut physiological performance. Actually, to the best of our knowledge, this is the first assessment of heat and drought effects on the physiology and photosynthesis of chestnut seedlings (*Castanea sativa* cv. Marsol). Aside from that, a step forward on the obtention of climate-resilient chestnut plants was also foreseen in this work, given the current need to find sustainable strategies, such as mycorrhizal association, against

drastic climatic challenges. Therefore, in the present study, the beneficial role of a symbiotic association with *Paxillus involutus* in alleviating heat and drought co-exposure in the growth and physiological performance of *C. sativa* was also evaluated.

4.1 The combination of heat and drought majorly hampered plant growth, but mycorrhization came for the rescue

Plants can exhibit distinct responses when subjected to a combination of stresses in comparison to their individual counterparts (Zandalinas & Mittler, 2022). Here, to unravel how *C. sativa* could be affected by the simultaneous exposure to heat and water scarcity, plants were either single or co-exposed to both stress factors. Single treatment with heat, besides reducing the foliar area, barely affected the growth of NMR and MR plants, suggesting that they were able to deal with this stress, at least macroscopically. Heat stress is a multifaceted result of factors such as intensity (temperature degrees) and exposure duration (Wahid et al., 2007). In most cases, tree species can handle elevated ambient temperatures (> 40 °C) for brief periods, provided they have a sufficient water supply (Teskey et al., 2015). In this way, although the heat treatment was applied daily, the 4 h time-period may not have been enough to completely impair chestnut plants growth, as they were able to recover. In fact, the reduction of the foliar area under high temperatures is an avoidance mechanism to help plants maintain a higher water status, and it is often linked to heat tolerance traits (Khan et al., 2019). As a matter of fact, the relative water content (RWC) of both NMR and MR plants exposed to heat alone was similar to CTL plants, possibly due to the reduction of the foliar area. On the other hand, drought, either individually or combined, severely impaired the growth of chestnut plants, especially those non-mycorrhizal, by decreasing the foliar area and reducing plants' RWC. The growth inhibition was even more obvious under the co-exposure scenario, with a marked reduction of the production of new leaves in NMR plants. These results are aligned with the ones obtained for many plant species, where the combination of heat and drought appears to be more detrimental for plant growth than the individual ones (Xu et al., 2023; Zandalinas et al., 2018). Furthermore, Zhou et al. (2017) concluded that, when in combination, drought had a dominant effect over heat stress in three tomato (*Solanum lycopersicum* L.) cultivars. According to their data, leaf area and RWC of plants under combined stresses were similar to those exposed solely to drought. Aligned with that, in the present work, drought also had a predominant effect in relation to heat, regarding the growth of NMR chestnut plants. Still, in this case, the presence of heat further aggravated these consequences, hampering plant growth by inhibiting the production of new leaves. Thus, it seems that drought, by reducing roots' water

availability, somehow impaired the ability of plants to recover from the high temperature stress. This resulted in a further reduction of the foliar area and impaired the production of new leaves.

Under non-stress conditions, mycorrhization did not contribute to a higher growth performance of chestnut plants, thus, the variations in stress tolerance are not exclusively link to a positive effect on growth by the symbiotic association. Mycorrhizal association has been shown to improve several plant species growth under drought (Kebert et al., 2023; Meddich et al., 2015; Wang et al., 2021). Yet, for example, Sebastiana et al. (2018) observed that inoculation of *Quercus suber* L. with the ECM (*Pisolithus tinctorius*) did not result in a higher shoot growth (shoot heigh, diameter and weight, leaf area and number of leaves) under water stress. Comparable to that, in this study, leaf area was lower under water scarcity in MR plants. Still, they were able to sustain a production of new leaves similarly to non-stressed plants and in a higher number than NMR ones. Under drought alone, the ECM association did not result in a significantly higher RWC in relation to NMR plants and was lower than the respective CTL. Although surprising, this could be firstly interpreted as the ECM association not being significantly beneficial for chestnut plants to cope with individual water stress. In fact, while ECM fungi can influence root water transport of host plants, as stated by Kilpeläinen et al. (2020) negative or no effects of ECM in host water relations have also been reported. However, the inhibition degree found in MR was less evident than that for non-mycorrhizal plants. Moreover, the monitorization of the WHC of both NMR and MR potted plants, throughout 21 days of stress exposure (Figure S1) suggests that ECM association allowed chestnut plants to lose water at a lower rate than NMR ones during the stress period. Thus, the presence of beneficial microorganisms' associations can be an effective strategy to prevent greater water losses.

Interestingly, the presence of the beneficial mycorrhiza significantly boosted chestnut plants tolerance to the stress combination. This is evident, for instance, by the significant increase in foliar area in relation to NMR plants exposed to the same conditions. Furthermore, contrary to drought single exposure, MR plants were able to sustain a higher water balance in leaves in a similar level to non-stressed plants. The positive effect of the ECM in alleviating the severe combined stress effects on chestnut plants growth is also highlighted by the fact that, contrary to NMR plants, MR ones were able to produce leaves at levels comparable to non-stressed plants. Most likely, in clear opposition to NMR plants, the presence of beneficial mycorrhiza, by allowing chestnut

plants to absorb water more efficiently, allowed them to recover from the heat treatment, even when it was combined with drought.

4.2 Mycorrhization sustained CO_2 assimilation and improved water use efficiency under single and combined stress

Essential physiological processes in plants are mirrored in the interactions involving carbon metabolism and water relations, which are sensitive to elevated temperatures and drought (Ruehr et al., 2019). The interplay of carbon and water at the leaf level is closely connected through stomata, which are carefully controlled to uphold hydraulic stability while enhancing carbon uptake (Ruehr et al., 2019). Stomata regulate rates of CO_2 assimilation and water loss, optimizing photosynthesis and water use efficiency (WUE) (Wei et al., 2018). Usually, at high temperatures, stomata remain open to allow the evaporative cooling to prevent thermal damage (Urban et al., 2017). Nevertheless, closure and lack of significant response of the stomatal conductance (g_s) under high temperatures have also been reported (Marchin et al., 2022 and references within). In this study, gas-exchange of NMR chestnut plants was not particularly sensitive to high temperatures, as stomatal conductance (g_s) transpiration (E), CO_2 assimilation (A) and water use efficiency (A/g_s) was comparable to non-stressed plants. These results are partially supported by the data of Dorado et al. (2023). The authors evaluated the effects of heat stress (42.5 ºC for 6 h for 7 days) on 6 months-old chestnut seedlings, with origin from three wild populations in Spain, being one of them Puebla de Sanabria located in northern Spain, close to the Trás-os-Montes region of Portugal. In their study, the authors reported that, after the 7-d period, heat stress did not affect plant photosynthetic rate, E and g_s, but induced a significant decrease in WUE_i. Contrarily, on day one of high temperatures, photosynthetic rate and g_s decreased exponentially, while E values increased. Moreover, they also observed that only 10% of the chestnut plants exposed to heat presented leaf damage (brown margins, yellowing and browning of inner areas, and scorching), but all seedlings exhibited lower leaf, root and total biomass compared to control plants, which corroborates the outcomes of the present work. Dorado et al. (2023) discussed that the differences in gas-exchange parameters, at the first and seventh day of heat stress, suggests that the CO_2 assimilation is more sensitive to daily temperatures than to prolonged heat exposure, possibly indicating plant adaptations to elevated temperatures. In fact, as reviewed by Ruehr et al. (2019), hydraulic conductance of woody plant species might not be significantly impacted in response to high temperatures in circumstances of adequate water availability. In this sense, the gas-exchange parameters align with the hypothesis that chestnut plants were able to recover

from the heat treatment, since the growth-related parameters of NMR plants also mostly remained unaffected. Generally, stomatal closure to prevent water loss from transpiration is one of the first responses of plants to drought. As a consequence, gas-exchange ability becomes limited and CO_2 supply is reduced, ultimately affecting the overall photosynthetic performance (Pirasteh-Anosheh et al., 2016). Consistent with this, when exposed to drought alone, NMR chestnut plants possibly reduced g_s as a mechanism to prevent water loss, subsequently leading to a reduction in *E* and a decrease in *A*. In accordance to this, Camisón et al. (2020), when evaluating the effect of drought (5% of soil volumetric water content) on two year-old *C.sativa* seedlings for 60 d, reported that, 20-d after the stress application, g_s and *A* were similar and close to zero, and the RWC had also significantly decreased. In fact, the authors suggested that the decrease in g_s due to drought was strongly associated with the decline in RWC and that the stress-induced stomatal closure contributed for a reduction in *A*. They also suggest that the reduction in *A* could have been a consequence of Chl degradation and soluble sugar accumulation in leaves. Curiously, when heat and drought were imposed together, NMR plants showed a diminished stomatal conductance, though the transpiration rate remained relatively equal to the control plants. Still, carbon assimilation was greatly hampered, indicating a disruption in the chemical reactions of photosynthesis. These findings suggest that, in terms of g_s and *E,* neither stress factor prevailed over the other. However, in terms of carbon assimilation, the negative effects of drought prevailed. This aligns with the results of the growth-related parameters, suggesting that drought hindered the ability of NMR chestnut plants to recover from the heat treatment. Likewise, Birami et al. (2018) observed that *Pinus halepensis* seedlings during heat periods exhibited a delay on gas-exchange recovery under water-limiting conditions.

Mycorrhization can significantly influence stomatal conductance, with MR plants frequently displaying higher g_s in relation to NMR ones (Augé et al., 2015). In contrast, in the present study, apart from the individual drought stress, MR plants exhibited lower g_s and *E* values in relation to NMR plants. Yet, it should be emphasized that stomatal response to *P. involutus* colonization exhibits variability, depending on the host species (Danielsen & Polle, 2014). Despite of that, results also revealed that, under both individual and combined stresses, the ECM association helped to mitigate the impact of drought in gas-exchange parameters. Actually, stomatal conductance and transpiration rates did not vary between control plants and those co-exposed to both stress factors. Moreover, it is also worth noting that carbon assimilation was even boosted under single

exposure to high temperatures, along with an increase in water use efficiency, which was always higher than in NMR plants under stress conditions. Some studies suggest that MR plants exhibit higher rates of net photosynthesis likely due to a strong mycorrhizal demand for carbohydrates, which influences stomatal opening (Heinonsalo et al., 2015). This did not happen in this present study, as MR plants, particularly under heat and combined stresses, exhibited a significantly lower g_s coupled with a marked increase of *A* in relation to NMR ones. Thus, as hypothesized by Heinonsalo et al. (2015), under individual and combined stresses, MR chestnut plants may have used plant-assimilated carbon more efficiently while NMR plants lose carbon in the form of exudates. This is supported by the significantly lower *A* of NMR in relation to MR plants under the different stresses. Moreover, ECM can indirectly improve water relations through a more efficient nutrient uptake. Indeed, adequate nitrogen nutrition, by increasing the photosynthetic machinery efficiency, can increase WUE if transpiration remains at the same rate (Lehto & Zwiazek, 2011). An improved phosphorus nutrition can also result in increased WUE (Kulmann et al., 2023). Thus, altogether, WUE may have increased in MR plants indirectly by a better nutrient absorption and by enhancing *A* without changes in g_s and *E*. To confirm this hypothesis, the mineral status of the chestnut plants should be analysed in future studies.

A well-know effect of the ECM association is the improved and more efficient water absorption by the plant host roots, even under stress conditions (Smith & Read, 2008). In this sense, in contrast to the NMR set, MR plants were able to maintain a leaf water status comparable to that of non-stressed plants, when exposed to both heat and water scarcity. This ability to maintain a similar leaf water status enabled MR plants to preserve their gas-exchange capacity, particularly stomatal conductance, which is strongly influenced by leaf RWC (Buckley, 2019).

4.3 Photochemical reactions were not affected by stresses nor mycorrhization

Chlorophyl fluorescence measurements are a powerful technique, widely used to detect stress effects in the operation of the photosynthetic system (Guidi et al., 2019). Changes in maximum photochemical efficiency of PSII (F_v/F_m), under abiotic stress, reflect damage to PSII (Baker, 2008). In this study, exposure to heat and drought did not result in photoinhibition of both NMR and MR, as F_v/F_m and ΦPSII did not decrease in response to any of the stresses, even in combination. These results are distinct from the ones obtained by Duc et al. (2018), where the F_v/F_m of MR and NMR tomato plants (*Solanum*

lycopersicum var. MoneyMaker) decreased in response to heat, drought, and their combination. However, in their study, the heat treatment consisted in a 6 h treatment at 42 ºC prior to plant harvesting, a much more acute exposure than the one implemented in the present work. On the other hand, for example, the NPQ, F_v/F_m and ETR of *Populus simonii* growing under a greenhouse context, exposed for 3 and 6 h to high temperatures (42 ºC), was similar to CTL plants (Song et al., 2014). In this work, heat alone stimulated the photochemical reactions of NMR plants. This is evident by the fact these plants exhibited a higher F_v/F_m and an increased ETR through the PSII that translated into a higher ΦPSII. Abiotic stress, such as excessive-light, drought, and heat can result in excessive reduction of the ETC. To overcome this, plants have several mechanisms such as NPQ (Gururani et al., 2015). This is a photoprotection mechanism related to the dissipation of excessive energy as heat and, when increased, can reflect a decrease in photosynthetic efficiency (Pshybytko, 2023; Ruban, 2016). In this case, although NMR plants exposed to the combination of heat and drought exhibited a higher NPQ, the photosynthetic efficiency was similar to non-stressed plants. Furthermore, the quantification of photosynthetic pigment levels was consistent with the chlorophyll fluorescence measurements. In fact, total Chl levels and carotenoids did not change in response to heat and drought. Additionally, despite not statistically significant, carotenoids levels seemly increased in NMR plants exposed to the combined stress, behaviour that is consistent with the higher NPQ registered in these plants. In fact, NPQ is a complex mechanism comprised by several components: the energy-dependent (qE), zeaxanthin-dependent (qZ) and photoinhibitory quenching (qI) (Derks et al., 2015). Acidification of the thylakoid lumen arise by the electron transport, induced the protonation of PsbS protein (PSII subunit S) which then activates the xanthophyll cycle, where vioxanthin is converted to zeaxanthin (Zhao et al., 2017). Considering that xanthophylls belong to a group of carotenoids (Cazzonelli, 2011), they might have been responsible for the heat dissipation as NPQ of NMR plants.

The results also revealed that ECM association did not significantly affect the photochemical efficiency of chestnut plants. A similar result was obtained by Aryal et al. (2021), addressing the drought tolerance of chestnut seedlings colonized by ECM. In their study, the F_v/F_m of chestnut seedlings exposed to drought was identical among inoculated and non-inoculated plants. Furthermore, although MR plants exposed to heat alone exhibited higher levels of photosynthetic pigments than NMR ones, this did not result in higher F_v/F_m, ΦPSII or ETR.

Overall, the results reveal that drought, alone or combined, did not inhibit the photochemical reactions. Thus, the lower carbon assimilation may possibly be related to stomatal limitations, rather than damage to the photosystem apparatus. Moreover, the chlorophyll fluorescent measurements and the levels of photosynthetic pigments support the hypothesis that, under drought conditions, either alone or in combination, ECM association with chestnut plants allowed them to sustain photosynthetic performance similar to non-stressed plants by maintaining gas-exchange capacity rather than by directly influencing the photochemical reactions.

4.4 The redox status of chestnut plants varied according to the stress treatment and the presence of mycorrhizae

A typical consequence of heat and drought stresses is the excessive production of ROS, such as H_2O_2 and $O_2^{\cdot-}$**,** which can further translate into severe redox disorders with impacts on growth and development (Hasanuzzaman et al., 2021). Seeking to evaluate the impact of both stresses, individually or in combination, on the redox metabolism, the accumulation of H_2O_2 and $O_2^{\cdot-}$ was evaluated in roots and leaves of NMR and MR chestnut plants. This assessment was also complemented with the evaluation of LP, indicative of membrane damage (measured by the MDA content). Overall, neither water deficit nor high temperatures led to an evident situation of oxidative stress in roots of both sets of plants. This suggests that chestnut plants were able to employ defence mechanisms and/or that leaves were the most sensitive organ to the action of both stressors. Curiously, when considering ROS monitoring and LP degree, it was found that leaves of both NMR and MR plants were much more susceptible to the imposed stresses than roots.

Still, the response observed varied according to the plant set (NMR and MR) and the treatment, as discussed further. Interestingly, it would be expected that heat-treated NMR plants would present a higher production of $O_2^{\cdot-}$, given the recorded enhanced ETR rate. Indeed, in the chloroplasts, during electron transport, the reduction of O_2 produces $O_2^{\cdot-}$ (Khorobrykh et al., 2020). Yet, NMR plants under heat stress (which also exhibited a higher ΦPSII) did not have their $O_2^{\cdot-}$ levels increased, when compared to non-stressed plants. Still, H_2O_2 levels rose, suggesting that the possible generation of $O_2^{\cdot-}$ is being quickly managed by AOX defences, such as SOD, arising the production of H_2O_2, a much more stable ROS with signalling functions (Smirnoff & Arnaud, 2019), that can be inducing downstream physiological adjustments. Actually, despite this rise in H_2O_2, no evident signs of oxidative damage, in terms of LP, were recorded in NMR plants single

exposed to heat. Similarly, heat did not lead to ROS overproduction or membrane damage in the leaves of MR plants. Additionally, under high temperatures, the levels of the main non-enzymatic AOX metabolites (Pro, AsA and GSH) were not changed in both MR and NMR plant sets. Thus, overall, one can suggest that, under heat single exposure, leaves of chestnut plants did not experience severe oxidative damage, supporting the hypothesis that they were able to tolerate the high temperatures treatment. To the best of our knowledge, studies evaluating the redox status and antioxidant system of MR chestnut plants do not exist. Nevertheless, contrary to our study, Dorado et al. (2023) observed that *C.sativa* seedlings exposed to heat significantly accumulated Pro in leaves. Similarly, exposure of NMR and MR *Quercus robur* L. to heat stress (40 °C/30 °C day/night temperature) for 72 h, resulted in enhanced osmolytes levels, namely Pro, paired with an upsurge of LP (Kebert et al., 2022).

In contrast to heat, drought stress, single or combined with heat, induced a meaningful impact on NMR and MR plants redox status. Drought alone stimulated H_2O_2 production in NMR and, although $O_2^{\cdot-}$ levels did not increase, this resulted in substantial damage to the cellular membranes. In accordance, an upsurge in LP is one of the most common responses of plants facing water deprivation. In fact, under such conditions, as stomata close to prevent water loss, CO_2 assimilation decrease (Zargar et al., 2017). This creates an imbalance in utilization and consumption of absorbed light. Despite the excessive energy can be dissipated through NPQ, photorespiration and Mehler reaction, this can result in excessive ROS production and oxidative damage (Ma et al., 2015). Indeed, higher H_2O_2 levels under drought may be explained by the leakage of photosynthetic electrons to O_2 (Mehler reaction) (De Carvalho, 2008; Ma et al., 2015). Equivalent results were also found by Wang et al. (2018) and Ben Abdallah et al. (2018), working with apple-trees (*Malus domestica*) and olive (*Olea europaea* L.) grown under water deprivation conditions, respectively. Interestingly, the AOX metabolite levels remained unchanged, revealing that the AOX system failed to prevent the overaccumulation of ROS. This result, especially the absence of changes in Pro, is quite surprising since the production of compatible solutes is often considered a signature response of plants to drought (Ashraf & Foolad, 2007). Indeed, it is suggested that during abiotic stress Pro confers a degree of osmoprotection via stabilization of subcellular structures while also quenching $^1O_2$ and directly scavenging OH·, hence protecting DNA, proteins, and membranes (Filippou et al., 2011). In contrast to NMR plants, leaves of MR plants individually exposed to drought did not experience an enhanced ROS accumulation and no upsurges in LP were observed. Thus, the ECM association clearly

contributed for a better tolerance of chestnut plants to drought, as also reported by Aryal et al. (2021). These results are in accordance with several former works, suggesting that ECM association can improve the tolerance of several tree species such as *Nothofagus dombeyi* (Alvarez et al., 2009), *Pinus tabulaeformis* (Wang et al., 2021), *Quercus robur* L. (Kebert et al., 2023) to drought. Yet, the AOX metabolite levels of MR plants exposed to drought was not significantly distinct from MR non-stressed plants, revealing that the stimulation of the non-enzymatic defences in response to drought does not seem to be a mechanism of chestnut plants to endure water stress. In this way, the lack of oxidative damage and the maintenance of ROS homeostasis under drought stress is most certainly related to other mechanisms, possibly the activation of enzymatic defences. In the future, it would be interesting to understand how the enzymatic component of the AOX system is behaving in such conditions. Furthermore, as reviewed by Bueno et al. (2022), while arbuscular mycorrhizal fungi (AM) primary rely on strengthening physiological and biochemical plant strategies such as osmotic regulation and AOX enzyme activity to enhance tolerance to drought, ECM fungi usually boost plant tolerance by assisting with water use efficiency, an hypothesis previously raised in the present study. This is achieved through the development of a larger extraradical-mycelium, allowing the host plant to acquire water from greater distances compared to AM. Indeed, as concluded by Lehto and Zwiazek, (2011), ECM fungi have a higher potential to assist in host-plant-water relations, such as increased gas-exchange, altered hydraulic conductance of mycorrhizal roots and osmotic adjustments. The results seem to be aligned with those assumptions, since MR plants had gas-exchange relations comparable to non-stressed plants.

Besides differentially affecting the redox responses of MR and NMR plants, drought- and heat-combined effects were substantially different from those coming from their individual counterparts. Starting with the NMR plants, the co-exposure to heat and drought did not result in an enhanced ROS production, in relation to the individual stresses. Actually, while H_2O_2 remained unaltered, the $O_2^{\cdot-}$ levels were even lower under the stress combination. Still, LP degree remained higher than the CTL. This suggests that drought-mediated effects somehow prevailed under the combined treatment, and that other ROS, rather than $O_2^{\cdot-}$and H_2O_2, are behind the upsurge in LP. Indeed, it is known that during the Haber-Weiss process, $O_2^{\cdot-}$ can donate an electron to Fe^{3+} yielding Fe^{2+} which then can be oxidized by H_2O_2, rapidly generating $OH\cdot$ (Fenton reaction) (Gill & Tuteja, 2010). Thus, the increased LP degree may have arisen from that mechanism, as $OH\cdot$ is a highly reactive ROS that can injure cellular membranes (Bhattacharjee,

2019). Once again, plants not inoculated with the ECM did not change the levels of their AOX metabolites under the combined treatment, sustaining the previously raised hypothesis. Conversely to that, ROS accumulation in leaves of MR plants co-exposed to heat and drought were lower in relation to non-stressed plants. Furthermore Pro, GSH and AsA were all significantly increased in MR plants leaves in response to the combined stress, suggesting the stimulation of the non-enzymatic component of the AOX defence system. Indeed, the AOX metabolites were effective in scavenging and neutralizing ROS, preventing oxidative damage, as no signs of membrane damage under the stress combination were detected. On top of that, despite the resources investment by MR in the activation of the non-enzymatic components of the AOX system, their growth and development was not compromised under the combination of heat and drought.

Altogether, as evidenced in the PCA and the heatmap analysis, there is a clear separation between MR and NMR plant sets, revealing that the presence of ectomycorrhiza significantly modulated plant responses under both control and stress conditions. Furthermore, these multivariate analyses also highlighted the beneficial role of the associated ECM in ameliorating chestnut plants' responses to stress, since single drought and heat grouped together with control plants. Actually, though at a first glance, the segregation of MR plants exposed to combined stresses might seem a negative outcome, this separation mostly results from the activation of the non-enzymatic AOX, namely proline.

5 Main conclusions and future prospects

- **Growth performance**
  - NMR and MR were able to recover from the high temperature treatment, and growth was not compromised under individual heat stress;
  - Drought alone impaired the growth of NMR plants, and the combination with heat induced a more severe effect;
  - ECM association contributed for mitigating the negative effects of drought, alone or combined, in chestnut plants growth.
- **Photosynthetic metabolism**
  - The gas-exchange parameters of NMR plants remained unaffected by heat alone, but under drought, both individual and combined with heat, negative effects on carbon metabolism were observed;
  - MR plants were able to sustain higher gas-exchange parameters, under drought and combined stresses, similar to non-stressed plants;

- ECM association boosted *C. sativa* CO_2 assimilation and water-use efficiency under all stressful conditions;
- The ameliorative action of ECM association on photosynthesis was most likely related to an improvement in carbon reactions, rather than photochemical efficiency.

- **Redox status and non-enzymatic AOX defences**
  - No ROS overaccumulation or membrane damage was perceived in roots of NMR and MR chestnut among stress treatments;
  - Leaves of both NMR and MR plants exhibited variation in ROS levels (H_2O_2 and $O_2^{\cdot-}$) in response to the individual and combined stresses;
  - Heat alone, in general, did not induce oxidative damage nor activate the non-enzymatic AOX system in both NMR or MR plants;
  - Drought, alone or combined with heat, triggered oxidative stress in NMR plants;
  - The combination of heat and drought stresses stimulated the production of AOX metabolites (Pro, GSH and AsA) in MR plants, effectively scavenging ROS and preventing oxidative damage.

In summary, single and co-exposure to heat and drought differently affected chestnut plants, with mycorrhization playing a clear role in modulating these responses. In this study, both NMR and MR chestnut plants were able to recover from the imposed heat treatment, with the ECM presence further easing that process. Conversely, drought, whether individually or in combination, significantly impacted the physiological performance, in terms of growth and photosynthesis (gas-exchange), and disrupted the redox status of chestnut plants, hampering their recovery from heat treatment, especially in non-mycorrhizal plants. Indeed, the ECM association mitigated to some extent those effects, mainly by sustaining chestnut plants growth, water status, promoting gas-exchange features and the production of AOX metabolites. Overall, this work, besides providing a characterization of the impacts of heat and drought combined stresses in *C. sativa*, also demonstrated that mycorrhization can be a practical and cost-effect tool to boost chestnut tree tolerance to climate change.

Nevertheless, on a short term, it would be interesting to explore the following ideas:

- Asses the mineral status of NMR and MR chestnut plants in response to heat and drought to unravel the role of ECM association in modulating plant's mineral nutrition under different stresses;
- Evaluate the response of the enzymatic components of the AOX system of NMR and MR chestnut plants in response to heat and drought;
- Understand how ECM associations modulate the levels of different phytohormones under stress conditions, namely ABA and strigolactones, key plant growth regulators involved in water stress response and mychorrizal associations, respectively.

Furthermore, aligned with the main goals of the CC&NUTS project, to the following steps would most certainly focus on:

- Extending this knowledge to other important chestnut varieties in Portugal, such as Judia, Ca-90, Belle Epine among others, in order to select potentially tolerant genotypes;
- Evaluate the efficiency of stress priming, by activating plant memory, to develop chestnut plant varieties more tolerance to heat and drought stresses;
- Validating the combination of both strategies (stress priming and mychorrization) under field conditions.

6 Bibliography

Aguilera, E., Díaz-Gaona, C., García-Laureano, R., Reyes-Palomo, C., Guzmán, G. I., Ortolani, L., Sánchez-Rodríguez, M., & Rodríguez-Estévez, V. (2020). Agroecology for adaptation to climate change and resource depletion in the Mediterranean region. A review. *Agricultural Systems*, *181*, 102809. https://doi.org/10.1016/J.AGSY.2020.102809

Akram, N. A., Shafiq, F., & Ashraf, M. (2017). Ascorbic acid- a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Frontiers in Plant Science*, *8*, 238088. https://doi.org/10.3389/FPLS.2017.00613/BIBTEX

Alcaide, F., Solla, A., Mattioni, C., Castellana, S., & Martín, M. Á. (2019). Adaptive diversity and drought tolerance in *Castanea sativa* assessed through EST-SSR genic markers. *Forestry: An International Journal of Forest Research*, *92*(3), 287–296. https://doi.org/10.1093/FORESTRY/CPZ007

Alché, J. de D. (2019). A concise appraisal of lipid oxidation and lipoxidation in higher plants. *Redox Biology*, *23*, 101136. https://doi.org/10.1016/J.REDOX.2019.101136

Alexieva, V., Sergiev, I., Mapelli, S., & Karanov, E. (2001). The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell & Environment*, *24*(12), 1337–1344. https://doi.org/10.1046/J.1365-3040.2001.00778.X

Alvarez, M., Huygens, D., Fernandez, C., Gacita, Y., Olivares, E., Saavedra, I., Alberdi, M., & Valenzuela, E. (2009). Effect of ectomycorrhizal colonization and drought on reactive oxygen species metabolism of *Nothofagus dombeyi* roots. *Tree Physiology*, *29*(8), 1047–1057. https://doi.org/10.1093/TREEPHYS/TPP038

Anjum, N. A., Sharma, P., Gill, S. S., Hasanuzzaman, M., Khan, E. A., Kachhap, K., Mohamed, A. A., Thangavel, P., Devi, G. D., Vasudhevan, P., Sofo, A., Khan, N. A., Misra, A. N., Lukatkin, A. S., Singh, H. P., Pereira, E., & Tuteja, N. (2016). Catalase and ascorbate peroxidase—representative H_2O_2-detoxifying heme enzymes in plants. *Environmental Science and Pollution Research 2016 23:19*, *23*(19), 19002–19029. https://doi.org/10.1007/S11356-016-7309-6

Arora, N. K. (2019). Impact of climate change on agriculture production and its sustainable solutions. *Environmental Sustainability 2019 2:2*, *2*(2), 95–96. https://doi.org/10.1007/S42398-019-00078-W

Aryal, P., Meiners, S. J., & Carlsward, B. S. (2021). Ectomycorrhizae determine chestnut seedling growth and drought response. *Agroforestry Systems*, *95*(7), 1251–1260. https://doi.org/10.1007/S10457-020-00488-4/TABLES/1

Ashraf, M., & Foolad, M. R. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, *59*(2), 206–216. https://doi.org/10.1016/J.ENVEXPBOT.2005.12.006

Ashraf, M., & Harris, P. J. C. (2013). Photosynthesis under stressful environments: An overview. *Photosynthetica 2013 51:2*, *51*(2), 163–190. https://doi.org/10.1007/S11099-013-0021-6

Atkinson, N. J., & Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of Experimental Botany*, *63*(10), 3523–3544. https://doi.org/10.1093/JXB/ERS100

Augé, R. M., Toler, H. D., & Saxton, A. M. (2015). Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza*, *25*(1), 13–24. https://doi.org/10.1007/S00572-014-0585-4/FIGURES/6

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative Medicine and Cellular Longevity*, *2014*. https://doi.org/10.1155/2014/360438

Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annual Review of Plant Biology*, *59*, 89–113. https://doi.org/10.1146/ANNUREV.ARPLANT.59.032607.092759

Baptista, P., Martins, A., Tavares, R. M., & Lino-Neto, T. (2010). Diversity and fruiting pattern of macrofungi associated with chestnut (*Castanea sativa*) in the Trás-os-Montes region (Northeast Portugal). *Fungal Ecology*, *3*(1), 9–19. https://doi.org/10.1016/J.FUNECO.2009.06.002

Bates, L. S., Waldren, R. P., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil*, *39*(1), 205–207. https://doi.org/10.1007/BF00018060/METRICS

Becquer, A., Guerrero-Galán, C., Eibensteiner, J. L., Houdinet, G., Bücking, H., Zimmermann, S. D., & Garcia, K. (2019). The ectomycorrhizal contribution to tree nutrition. *Advances in Botanical Research*, *89*, 77–126. https://doi.org/10.1016/BS.ABR.2018.11.003

Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ashraf, M., Ahmed, N., & Zhang, L. (2019). Role of Arbuscular Mycorrhizal Fungi in Plant Growth Regulation: Implications in Abiotic Stress Tolerance. *Frontiers in Plant Science*, *10*, 1068. https://doi.org/10.3389/FPLS.2019.01068/BIBTEX

Ben Abdallah, M., Trupiano, D., Polzella, A., De Zio, E., Sassi, M., Scaloni, A., Zarrouk, M., Ben Youssef, N., & Scippa, G. S. (2018). Unraveling physiological, biochemical and molecular mechanisms involved in olive (*Olea europaea* L. cv. Chétoui) tolerance to drought and salt stresses. *Journal of Plant Physiology*, *220*, 83–95. https://doi.org/10.1016/J.JPLPH.2017.10.009

Ben Rejeb, K., Abdelly, C., & Savouré, A. (2014). How reactive oxygen species and proline face stress together. *Plant Physiology and Biochemistry*, *80*, 278–284. https://doi.org/10.1016/J.PLAPHY.2014.04.007

Bernardo, S., Rodrigo, M. J., Vives-Peris, V., Gómez-Cadenas, A., Zacarías, L., Machado, N., Moutinho-Pereira, J., & Dinis, L. T. (2022). Fine-tuning of grapevine xanthophyll-cycle and energy dissipation under Mediterranean conditions by kaolin particle-film. *Scientia Horticulturae*, *291*, 110584. https://doi.org/10.1016/J.SCIENTA.2021.110584

Bhattacharjee, S. (2019). ROS and Oxidative Stress: Origin and Implication. *Reactive Oxygen Species in Plant Biology*, 1–31. https://doi.org/10.1007/978-81-322-3941-3_1

Bilger, W., & Schreiber, U. (1986). Energy-dependent quenching of dark-level chlorophyll fluorescence in intact leaves. *Photosynthesis Research*, *10*(3), 303–308. https://doi.org/10.1007/BF00118295/METRICS

Birami, B., Gattmann, M., Heyer, A. G., Grote, R., Arneth, A., & Ruehr, N. K. (2018). Heat Waves Alter Carbon Allocation and Increase Mortality of Aleppo Pine Under Dry Conditions. *Frontiers in Forests and Global Change*, *1*, 418910. https://doi.org/10.3389/FFGC.2018.00008/BIBTEX

Buckley, T. N. (2019). How do stomata respond to water status? *New Phytologist*, *224*(1), 21–36. https://doi.org/10.1111/NPH.15899

Bueno, C. G., Meng, Y., & Neuenkamp, L. (2022). How can mycorrhizal symbiosis mediate multiple abiotic stresses in woody plants? *Flora*, *295*, 152146. https://doi.org/10.1016/J.FLORA.2022.152146

Camisón, Á., Ángela Martín, M., Dorado, F. J., Moreno, G., & Solla, A. (2020). Changes in carbohydrates induced by drought and waterlogging in *Castanea sativa*. *Trees - Structure and Function*, *34*(2), 579–591. https://doi.org/10.1007/S00468-019-01939-X

Carneiro-Carvalho, A., Anjos, R., Pinto, T., & Gomes-Laranjo, J. (2021). Stress Oxidative Evaluation on SiK®-Supplemented *Castanea sativa* Mill. Plants Growing Under High Temperature. *Journal of Soil Science and Plant Nutrition*, *21*(1), 415–425. https://doi.org/10.1007/S42729-020-00370-3/FIGURES/6

Cazzonelli, C. I. (2011). Carotenoids in nature: Insights from plants and beyond. *Functional Plant Biology*, *38*(11), 833–847. https://doi.org/10.1071/FP11192

Chot, E., & Reddy, M. S. (2022). Role of Ectomycorrhizal Symbiosis Behind the Host Plants Ameliorated Tolerance Against Heavy Metal Stress. *Frontiers in Microbiology*, *13*, 855473. https://doi.org/10.3389/FMICB.2022.855473/BIBTEX

Choudhury, F. K., Rivero, R. M., Blumwald, E., & Mittler, R. (2017). Reactive oxygen species, abiotic stress and stress combination. *The Plant Journal*, *90*(5), 856–867. https://doi.org/10.1111/TPJ.13299

Ciordia, M., Feito, I., Pereira-Lorenzo, S., Fernández, A., & Majada, J. (2012). Adaptive diversity in *Castanea sativa* Mill. half-sib progenies in response to drought stress. *Environmental and Experimental Botany*, *78*, 56–63. https://doi.org/10.1016/J.ENVEXPBOT.2011.12.018

Clapero, V., Arrivault, S., & Stitt, M. (2023). Natural variation in metabolism of the Calvin-Benson cycle. *Seminars in Cell & Developmental Biology*. https://doi.org/10.1016/J.SEMCDB.2023.02.015

Clark, S. L., Marcolin, E., Patrício, M. S., & Loewe-Muñoz, V. (2023). A silvicultural synthesis of sweet (*Castanea sativa*) and American (*C. dentata*) chestnuts. *Forest Ecology and Management*, *539*, 121041. https://doi.org/10.1016/J.FORECO.2023.121041

Cohen, I., Zandalinas, S. I., Huck, C., Fritschi, F. B., & Mittler, R. (2021). Meta-analysis of drought and heat stress combination impact on crop yield and yield components. *Physiologia Plantarum*, *171*(1), 66–76. https://doi.org/10.1111/PPL.13203

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

Cramer, W., Guiot, J., Fader, M., Garrabou, J., Gattuso, J. P., Iglesias, A., Lange, M. A., Lionello, P., Llasat, M. C., Paz, S., Peñuelas, J., Snoussi, M., Toreti, A., Tsimplis, M. N., & Xoplaki, E. (2018). Climate change and interconnected risks to sustainable development in the Mediterranean. *Nature Climate Change 2018 8:11*, *8*(11), 972–980. https://doi.org/10.1038/s41558-018-0299-2

Cruz De Carvalho, M. H. (2008). Drought stress and reactive oxygen species: Production, scavenging and signaling. *Plant Signaling & Behavior*, *3*(3), 156. https://doi.org/10.4161/PSB.3.3.5536

Danielsen, L., & Polle, A. (2014). Poplar nutrition under drought as affected by ectomycorrhizal colonization. *Environmental and Experimental Botany*, *108*, 89–98. https://doi.org/10.1016/J.ENVEXPBOT.2014.01.006

Das, K., & Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science*, *2*(DEC), 121942. https://doi.org/10.3389/FENVS.2014.00053/BIBTEX

del Río, L. A., Corpas, F. J., López-Huertas, E., & Palma, J. M. (2018). Plant superoxide dismutases: Function under abiotic stress conditions. *Antioxidants and Antioxidant Enzymes in Higher Plants*, 1–26. https://doi.org/10.1007/978-3-319-75088-0_1/COVER

Demidchik, V. (2015). Mechanisms of oxidative stress in plants: From classical chemistry to cell biology. *Environmental and Experimental Botany*, *109*, 212–228. https://doi.org/10.1016/J.ENVEXPBOT.2014.06.021

Derks, A., Schaven, K., & Bruce, D. (2015). Diverse mechanisms for photoprotection in photosynthesis. Dynamic regulation of photosystem II excitation in response to rapid environmental change. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1847*(4–5), 468–485. https://doi.org/10.1016/J.BBABIO.2015.02.008

Domínguez-Núñez, J. A., Albanesi, A. S., Domínguez-Núñez, J. A., & Albanesi, A. S. (2019). Ectomycorrhizal Fungi as Biofertilizers in Forestry. *Biostimulants in Plant Science*. https://doi.org/10.5772/INTECHOPEN.88585

Dorado, F. J., Alías, J. C., Chaves, N., & Solla, A. (2023). Warming Scenarios and *Phytophthora cinnamomi* Infection in Chestnut (*Castanea sativa* Mill.). *Plants*, *12*(3), 556. https://doi.org/10.3390/PLANTS12030556/S1

Dorado, J. F., Pinto, G., Monteiro, P., Chaves, N., Alías, J. C., Rodrigo, S., Camisón, Á., & Solla, A. (2023). Heat stress and recovery effects on the physiology and biochemistry of *Castanea sativa* Mill. *Frontiers in Forests and Global Change*, *5*, 1072661. https://doi.org/10.3389/FFGC.2022.1072661/BIBTEX

dos Santos, T. B., Ribas, A. F., de Souza, S. G. H., Budzinski, I. G. F., & Domingues, D. S. (2022). Physiological Responses to Drought, Salinity, and Heat Stress in Plants: A Review. *Stresses*, *2*(1), 113–135. https://doi.org/10.3390/STRESSES2010009

Duc, N. H., Csintalan, Z., & Posta, K. (2018). Arbuscular mycorrhizal fungi mitigate negative effects of combined drought and heat stress on tomato plants. *Plant Physiology and Biochemistry*, *132*, 297–307. https://doi.org/10.1016/J.PLAPHY.2018.09.011

Dumanović, J., Nepovimova, E., Natić, M., Kuča, K., & Jaćević, V. (2021). The Significance of Reactive Oxygen Species and Antioxidant Defense System in Plants: A Concise Overview. *Frontiers in Plant Science*, *11*, 552969. https://doi.org/10.3389/FPLS.2020.552969/BIBTEX

Dutta, S., Mohanty, S., & Tripathy, B. C. (2009). Role of Temperature Stress on Chloroplast Biogenesis and Protein Import in Pea. *Plant Physiology*, *150*(2), 1050–1061. https://doi.org/10.1104/PP.109.137265

Elferjani, R., & Soolanayakanahally, R. (2018). Canola responses to drought, heat, and combined stress: shared and specific effects on carbon assimilation, seed yield, and oil composition. *Frontiers in Plant Science*, *9*, 376655. https://doi.org/10.3389/FPLS.2018.01224/BIBTEX

Fàbregas, N., & Fernie, A. R. (2021). The interface of central metabolism with hormone signaling in plants. *Current Biology*, *31*(23), R1535–R1548. https://doi.org/10.1016/J.CUB.2021.09.070

Fahad, S., Bajwa, A. A., Nazir, U., Anjum, S. A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S., Saud, S., Ihsan, M. Z., Alharby, H., Wu, C., Wang, D., & Huang, J. (2017). Crop production under drought and heat stress: Plant responses and management options. *Frontiers in Plant Science*, *8*, 1147. https://doi.org/10.3389/FPLS.2017.01147/BIBTEX

FAOSTAT. Crops. Available online https://www.fao.org/faostat/en/#data/QCL (retrieved on January 9, 2023)

Farooq, M. A., Niazi, A. K., Akhtar, J., Saifullah, Farooq, M., Souri, Z., Karimi, N., & Rengel, Z. (2019). Acquiring control: The evolution of ROS-Induced oxidative stress

and redox signaling pathways in plant stress responses. *Plant Physiology and Biochemistry*, *141*, 353–369. https://doi.org/10.1016/J.PLAPHY.2019.04.039

Fawzy, S., Osman, A. I., Doran, J., & Rooney, D. W. (2020). Strategies for mitigation of climate change: a review. *Environmental Chemistry Letters 2020 18:6*, *18*(6), 2069–2094. https://doi.org/10.1007/S10311-020-01059-W

Fernandes, P., Colavolpe, M. B., Serrazina, S., & Costa, R. L. (2022). European and American chestnuts: An overview of the main threats and control efforts. *Frontiers in Plant Science*, *13*, 2999. https://doi.org/10.3389/FPLS.2022.951844/BIBTEX

Filippou, P., Antoniou, C., & Fotopoulos, V. (2011). Effect of drought and rewatering on the cellular status and antioxidant response of Medicago truncatula plants. *Plant Signaling & Behavior*, *6*(2), 270. https://doi.org/10.4161/PSB.6.2.14633

Forlani, G., Trovato, M., Funck, D., & Signorelli, S. (2019). Regulation of Proline Accumulation and Its Molecular and Physiological Functions in Stress Defence. *Osmoprotectant-Mediated Abiotic Stress Tolerance in Plants: Recent Advances and Future Perspectives*, 73–97. https://doi.org/10.1007/978-3-030-27423-8_3/COVER

Foyer, C. H., Ruban, A. V., & Noctor, G. (2017). Viewing oxidative stress through the lens of oxidative signalling rather than damage. *The Biochemical Journal*, *474*(6), 877–883. https://doi.org/10.1042/BCJ20160814

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

Freitas, T. R., Santos, J. A., Silva, A. P., Martins, J., & Fraga, H. (2022). Climate Change Projections for Bioclimatic Distribution of Castanea sativa in Portugal. *Agronomy 2022, Vol. 12, Page 1137*, *12*(5), 1137. https://doi.org/10.3390/AGRONOMY12051137

Futai, K., Taniguchi, T., & Kataoka, R. (2008). Ectomycorrhizae and their importance in forest ecosystems. *Mycorrhizae: Sustainable Agriculture and Forestry*, 241–285. https://doi.org/10.1007/978-1-4020-8770-7_11/COVER

Genre, A., Lanfranco, L., Perotto, S., & Bonfante, P. (2020). Unique and common traits in mycorrhizal symbioses. *Nature Reviews Microbiology 2020 18:11*, *18*(11), 649–660. https://doi.org/10.1038/s41579-020-0402-3

Genty, B., Briantais, J. M., & Baker, N. R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *990*(1), 87–92. https://doi.org/10.1016/S0304-4165(89)80016-9

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

Gillespie, K. M., & Ainsworth, E. A. (2007). Measurement of reduced, oxidized and total ascorbate content in plants. *Nature Protocols*, *2*(4), 871–874. https://doi.org/10.1038/NPROT.2007.101

Gomes-Laranjo, J., Almeida, P., Ferreira-Cardoso, J., & Peixoto, F. (2009). Ecophysiological characterization of *C. sativa* trees growing under different altitudes. *Acta Horticulturae*, *844*, 119–126. https://doi.org/10.17660/ACTAHORTIC.2009.844.16

Gomes-Laranjo, J., Araújo-Alves, J., Ferreira-Cardoso, J., Pimentel-Pereira, M., Abreu, C. G., & Torres-Pereira, J. (2004). Effect of Chestnut Ink Disease on Photosynthetic Performance. *Journal of Phytopathology*, *152*(3), 138–144. https://doi.org/10.1111/J.1439-0434.2004.00814.X

Gomes-Laranjo, J., Coutinho, J. P., & Peixoto, F. (2008). Ecophysiological characterization of C. saliva mill. *Acta Horticulturae*, *784*, 99–105. https://doi.org/10.17660/ACTAHORTIC.2008.784.14

Gomes-Laranjo, J., Dinis, L.-T., Martins, L., Portela, E., Pinto, T., Ara, M. C., Díaz, I. F., Majada, J., Peixoto, F., Lorenzo, S. P., Cabrer, A. M. R., Zhang, C., Martins, A., Costa, R., Gomes-Laranjo, J., Dinis, L.-T., Martins, L., Portela, E., Pinto, T., Costa, R. (2012). Characterization of Chestnut Behavior with Photosynthetic Traits. *Applied Photosynthesis*. https://doi.org/10.5772/26227

Gomes-Laranjo, J., Peixoto, F., Wong Fong Sang, H. W., & Torres-Pereira, J. (2006). Study of the temperature effect in three chestnut (*Castanea sativa* Mill.) cultivars' behaviour. *Journal of Plant Physiology*, *163*(9), 945–955. https://doi.org/10.1016/J.JPLPH.2005.06.020

Gong, B., Sun, S., Yan, Y., Jing, X., & Shi, Q. (2018). Glutathione metabolism and its function in higher plants adapting to stress. *Antioxidants and Antioxidant Enzymes in Higher Plants*, 181–205. https://doi.org/10.1007/978-3-319-75088-0_9/COVER

Gonthier, P., Giordano, L., Zampieri, E., Lione, G., Vizzini, A., Colpaert, J. V., & Balestrini, R. (2019). An ectomycorrhizal symbiosis differently affects host susceptibility to two congeneric fungal pathogens. *Fungal Ecology*, *39*, 250–256. https://doi.org/10.1016/J.FUNECO.2018.12.008

Goufo, P., Moutinho-Pereira, J. M., Jorge, T. F., Correia, C. M., Oliveira, M. R., Rosa, E. A. S., António, C., & Trindade, H. (2017). Cowpea (*Vigna unguiculata* L. Walp.) metabolomics: Osmoprotection as a physiological strategy for drought stress resistance and improved yield. *Frontiers in Plant Science*, *8*, 586. https://doi.org/10.3389/FPLS.2017.00586/BIBTEX

Guerrero-Galán, C., Calvo-Polanco, M., & Zimmermann, S. D. (2019). Ectomycorrhizal symbiosis helps plants to challenge salt stress conditions. *Mycorrhiza*, *29*(4), 291–301. https://doi.org/10.1007/S00572-019-00894-2/FIGURES/1

Guidi, L., Lo Piccolo, E., & Landi, M. (2019). Chlorophyll fluorescence, photoinhibition and abiotic stress: Does it make any difference the fact to be a C3 or C4 species?

Frontiers in Plant Science, *10*, 438548. https://doi.org/10.3389/FPLS.2019.00174/BIBTEX

Gupta, D. K., Palma, J. M., & Corpas, F. J. (2018). Antioxidants and antioxidant enzymes in higher plants. *Antioxidants and Antioxidant Enzymes in Higher Plants*, 1–300. https://doi.org/10.1007/978-3-319-75088-0/COVER

Gurrieri, L., Sparla, F., Zaffagnini, M., & Trost, P. (2023). Dark complexes of the Calvin-Benson cycle in a physiological perspective. *Seminars in Cell & Developmental Biology*. https://doi.org/10.1016/J.SEMCDB.2023.03.002

Gururani, M. A., Venkatesh, J., & Tran, L. S. P. (2015). Regulation of Photosynthesis during Abiotic Stress-Induced Photoinhibition. *Molecular Plant*, *8*(9), 1304–1320. https://doi.org/10.1016/J.MOLP.2015.05.005

Haddidi, I., Duc, N. H., Tonk, S., Rápó, E., & Posta, K. (2020). Defense Enzymes in Mycorrhizal Tomato Plants Exposed to Combined Drought and Heat Stresses. *Agronomy 2020, Vol. 10, Page 1657*, *10*(11), 1657. https://doi.org/10.3390/AGRONOMY10111657

Halliwell, B. (2006). Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. *Plant Physiology*, *141*(2), 312–322. https://doi.org/10.1104/PP.106.077073

Hasanuzzaman, M., Borhannuddin Bhuyan, M. H. M., Anee, T. I., Parvin, K., Nahar, K., Al Mahmud, J., & Fujita, M. (2019). Regulation of Ascorbate-Glutathione Pathway in Mitigating Oxidative Damage in Plants under Abiotic Stress. *Antioxidants (Basel, Switzerland)*, *8*(9). https://doi.org/10.3390/ANTIOX8090384

Hasanuzzaman, M., Raihan, Md. R. H., Masud, A. A. C., Rahman, K., Nowroz, F., Rahman, M., Nahar, K., & Fujita, M. (2021). Regulation of Reactive Oxygen Species and Antioxidant Defense in Plants under Salinity. *International Journal of Molecular Sciences 2021, Vol. 22, Page 9326*, *22*(17), 9326. https://doi.org/10.3390/IJMS22179326

Hassan, M. U., Chattha, M. U., Khan, I., Chattha, M. B., Barbanti, L., Aamer, M., Iqbal, M. M., Nawaz, M., Mahmood, A., Ali, A., & Aslam, M. T. (2020). Heat stress in cultivated plants: nature, impact, mechanisms, and mitigation strategies—a review. *Https://Doi.Org/10.1080/11263504.2020.1727987*, *155*(2), 211–234. https://doi.org/10.1080/11263504.2020.1727987

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: II. Role of electron transfer. *Archives of Biochemistry and Biophysics*, *125*(3), 850–857. https://doi.org/10.1016/0003-9861(68)90523-7

Heinonsalo, J., Juurola, E., Linden, A., & Pumpanen, J. (2015). Ectomycorrhizal fungi affect Scots pine photosynthesis through nitrogen and water economy, not only through increased carbon demand. *Environmental and Experimental Botany*, *109*, 103–112. https://doi.org/10.1016/J.ENVEXPBOT.2014.08.008

Hosseinifard, M., Stefaniak, S., Javid, M. G., Soltani, E., Wojtyla, Ł., & Garnczarska, M. (2022). Contribution of Exogenous Proline to Abiotic Stresses Tolerance in

Plants: A Review. *International Journal of Molecular Sciences 2022, Vol. 23, Page 5186*, *23*(9), 5186. https://doi.org/10.3390/IJMS23095186

Hu, S., Ding, Y., & Zhu, C. (2020). Sensitivity and Responses of Chloroplasts to Heat Stress in Plants. *Frontiers in Plant Science*, *11*, 520328. https://doi.org/10.3389/FPLS.2020.00375/BIBTEX

Huber, A. E., & Bauerle, T. L. (2016). Long-distance plant signaling pathways in response to multiple stressors: the gap in knowledge. *Journal of Experimental Botany*, *67*(7), 2063–2079. https://doi.org/10.1093/JXB/ERW099

Instituto Nacional de Estatística - Recenseamento Agrícola. Análise dos principais resultados: 2019. Lisboa: INE, 2021. DISBN 978-989-25-0562-6

IPCC, 2023. Climate Change 2022 – Impacts, Adaptation and Vulnerability: Working Group II Contribution to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. *Climate Change 2022 – Impacts, Adaptation and Vulnerability*. https://doi.org/10.1017/9781009325844

IPCC, 2023: Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, H. Lee and J. Romero (eds.)]. IPCC, Geneva, Switzerland, 184 pp., doi: 10.59327/IPCC/AR6-9789291691647.

ISO (2023) Soil quality: effects of pollutants on earthworms *(Eisenia fetida*). Part 2. Determination of effects on reproduction. Annex C. ISO/Dis 11268-2:2023. International Organization for Standardization, Geneva, Switzerland.

Ishikawa, T., Maruta, T., Yoshimura, K., & Smirnoff, N. (2018). Biosynthesis and regulation of ascorbic acid in plants. *Antioxidants and Antioxidant Enzymes in Higher Plants*, 163–179. https://doi.org/10.1007/978-3-319-75088-0_8/COVER

Jacques, S., Ghesquière, B., Van Breusegem, F., & Gevaert, K. (2013). Plant proteins under oxidative attack. *PROTEOMICS*, *13*(6), 932–940. https://doi.org/10.1002/PMIC.201200237

Jagadish, S. V. K., Way, D. A., & Sharkey, T. D. (2021). Plant heat stress: Concepts directing future research. *Plant, Cell & Environment*, *44*(7), 1992–2005. https://doi.org/10.1111/PCE.14050

Jaleel, C. A., Manivannan, P., Wahid, A., Farooq, M., Al-Juburi, H. J., Somasundaram, R., & Panneerselvam, R. (2009). Drought stress in plants: a review on morphological characteristics and pigments composition. *International Journal of Agriculture and Biology*, *11*(1), 100–105.

Johnson, M. P. (2016). Photosynthesis. *Essays in Biochemistry*, *60*(3), 255. https://doi.org/10.1042/EBC20160016

Jumrani, K., Bhatia, V. S., Kataria, S., Alamri, S. A., Siddiqui, M. H., & Rastogi, A. (2022). Inoculation with Arbuscular Mycorrhizal Fungi Alleviates the Adverse Effects of High Temperature in Soybean. *Plants 2022, Vol. 11, Page 2210*, *11*(17), 2210. https://doi.org/10.3390/PLANTS11172210

Kaur, G., & Asthir, B. (2017). Molecular responses to drought stress in plants. *Biologia Plantarum*, *61*(2), 201–209. https://doi.org/10.1007/S10535-016-0700-9/METRICS

Kebert, M., Kostić, S., Čapelja, E., Vuksanović, V., Stojnić, S., Markić, A. G., Zlatković, M., Milović, M., Galović, V., & Orlović, S. (2022). Ectomycorrhizal Fungi Modulate Pedunculate Oak´s Heat Stress Responses through the Alternation of Polyamines, Phenolics, and Osmotica Content. *Plants 2022, Vol. 11, Page 3360*, *11*(23), 3360. https://doi.org/10.3390/PLANTS11233360

Kebert, M., Kostić, S., Stojnić, S., Čapelja, E., Markić, A. G., Zorić, M., Kesić, L., & Flors, V. (2023a). A Fine-Tuning of the Plant Hormones, Polyamines and Osmolytes by Ectomycorrhizal Fungi Enhances Drought Tolerance in Pedunculate Oak. *International Journal of Molecular Sciences*, *24*(8), 7510. https://doi.org/10.3390/IJMS24087510/S1

Khan, S., Anwar, S., Ashraf, M. Y., Khaliq, B., Sun, M., Hussain, S., Gao, Z. Q., Noor, H., & Alam, S. (2019). Mechanisms and Adaptation Strategies to Improve Heat Tolerance in Rice. A Review. *Plants*, *8*(11). https://doi.org/10.3390/PLANTS8110508

Khorobrykh, S., Havurinne, V., Mattila, H., & Tyystjärvi, E. (2020). Oxygen and ROS in Photosynthesis. *Plants 2020, Vol. 9, Page 91*, *9*(1), 91. https://doi.org/10.3390/PLANTS9010091

Kilpeläinen, J., Aphalo, P. J., Barbero-López, A., Adamczyk, B., Nipu, S. A., & Lehto, T. (2020). Are arbuscular-mycorrhizal *Alnus incana* seedlings more resistant to drought than ectomycorrhizal and nonmycorrhizal ones? *Tree Physiology*, *40*(6), 782–795. https://doi.org/10.1093/TREEPHYS/TPAA035

Kulmann, M. S. de S., Aguilar, M. V. M., Tassinari, A., Schwalbert, R., Tabaldi, L. A., Araujo, M. M., Antoniolli, Z. I., Nicoloso, F. T., Brunetto, G., & Schumacher, M. V. (2023). Effects of increasing soil phosphorus and association with ectomycorrhizal fungi (*Pisolithus microcarpus*) on morphological, nutritional, biochemical, and physiological parameters of *Pinus taeda* L. *Forest Ecology and Management*, *544*, 121207. https://doi.org/10.1016/J.FORECO.2023.121207

Laliberté, E., Lambers, H., Burgess, T. I., & Wright, S. J. (2015). Phosphorus limitation, soil-borne pathogens and the coexistence of plant species in hyperdiverse forests and shrublands. *New Phytologist*, *206*(2), 507–521. https://doi.org/10.1111/NPH.13203

Lamaoui, M., Jemo, M., Datla, R., & Bekkaoui, F. (2018). Heat and drought stresses in crops and approaches for their mitigation. *Frontiers in Chemistry*, *6*, 26. https://doi.org/10.3389/FCHEM.2018.00026/BIBTEX

Lehto, T., & Zwiazek, J. J. (2011). Ectomycorrhizas and water relations of trees: A review. *Mycorrhiza*, *21*(2), 71–90. https://doi.org/10.1007/S00572-010-0348-9/FIGURES/3

Lenoir, I., Fontaine, J., & Lounès-Hadj Sahraoui, A. (2016). Arbuscular mycorrhizal fungal responses to abiotic stresses: A review. *Phytochemistry*, *123*, 4–15. https://doi.org/10.1016/J.PHYTOCHEM.2016.01.002

Leung, D. W. M. (2018). Studies of catalase in plants under abiotic stress. *Antioxidants and Antioxidant Enzymes in Higher Plants*, 27–39. https://doi.org/10.1007/978-3-319-75088-0_2/COVER

Li, N., Euring, D., Cha, J. Y., Lin, Z., Lu, M., Huang, L. J., & Kim, W. Y. (2021). Plant Hormone-Mediated Regulation of Heat Tolerance in Response to Global Climate Change. *Frontiers in Plant Science*, *11*, 627969. https://doi.org/10.3389/FPLS.2020.627969/BIBTEX

Li, Y., Zhang, T., Zhou, Y., Zou, X., Yin, Y., Li, H., Liu, L., & Zhang, S. (2021). Ectomycorrhizal symbioses increase soil calcium availability and water use efficiency of *Quercus acutissima* seedlings under drought stress. *European Journal of Forest Research*, *140*(5), 1039–1048. https://doi.org/10.1007/S10342-021-01383-Y/FIGURES/3

Lichtenthaler, H. K. (1987). [34] Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods in Enzymology*, *148*(C), 350–382. https://doi.org/10.1016/0076-6879(87)48036-1

Lionello, P., & Scarascia, L. (2018). The relation between climate change in the Mediterranean region and global warming. *Regional Environmental Change*, *18*(5), 1481–1493. https://doi.org/10.1007/S10113-018-1290-1/FIGURES/6

Liu, Y., Li, X., & Kou, Y. (2020). Ectomycorrhizal Fungi: Participation in Nutrient Turnover and Community Assembly Pattern in Forest Ecosystems. *Forests 2020, Vol. 11, Page 453*, *11*(4), 453. https://doi.org/10.3390/F11040453

Luo, Q. (2011). Temperature thresholds and crop production: A review. *Climatic Change*, *109*(3–4), 583–598. https://doi.org/10.1007/S10584-011-0028-6/METRICS

Ma, P., Bai, T. hui, & Ma, F. wang. (2015). Effects of progressive drought on photosynthesis and partitioning of absorbed light in apple trees. *Journal of Integrative Agriculture*, *14*(4), 681–690. https://doi.org/10.1016/S2095-3119(14)60871-6

Ma, Y., Dias, M. C., & Freitas, H. (2020). Drought and Salinity Stress Responses and Microbe-Induced Tolerance in Plants. *Frontiers in Plant Science*, *11*, 591911. https://doi.org/10.3389/FPLS.2020.591911/BIBTEX

Malhi, G. S., Kaur, M., & Kaushik, P. (2021). Impact of Climate Change on Agriculture and Its Mitigation Strategies: A Review. *Sustainability 2021, Vol. 13, Page 1318*, *13*(3), 1318. https://doi.org/10.3390/SU13031318

Marchin, R. M., Backes, D., Ossola, A., Leishman, M. R., Tjoelker, M. G., & Ellsworth, D. S. (2022). Extreme heat increases stomatal conductance and drought-induced mortality risk in vulnerable plant species. *Global Change Biology*, *28*(3), 1133–1146. https://doi.org/10.1111/GCB.15976

Martin, F., Kohler, A., Murat, C., Veneault-Fourrey, C., & Hibbett, D. S. (2016). Unearthing the roots of ectomycorrhizal symbioses. *Nature Reviews Microbiology 2016 14:12*, *14*(12), 760–773. https://doi.org/10.1038/nrmicro.2016.149

Martins, A. (2008). In vitro mycorrhization of micropropagated plants: Studies on *Castanea sativa* mill. *Mycorrhizae: Sustainable Agriculture and Forestry*, 321–336. https://doi.org/10.1007/978-1-4020-8770-7_14/COVER

Maruta, T., & Ishikawa, T. (2018). Ascorbate peroxidase functions in higher plants: The control of the balance between oxidative damage and signaling. *Antioxidants and Antioxidant Enzymes in Higher Plants*, 41–59. https://doi.org/10.1007/978-3-319-75088-0_3/COVER

Maya, M. A., & Matsubara, Y. ichi. (2013). Influence of arbuscular mycorrhiza on the growth and antioxidative activity in cyclamen under heat stress. *Mycorrhiza*, *23*(5), 381–390. https://doi.org/10.1007/S00572-013-0477-Z/FIGURES/7

Meddich, A., Jaiti, F., Bourzik, W., Asli, A. El, & Hafidi, M. (2015). Use of mycorrhizal fungi as a strategy for improving the drought tolerance in date palm (*Phoenix dactylifera*). *Scientia Horticulturae*, *192*, 468–474. https://doi.org/10.1016/J.SCIENTA.2015.06.024

Menéndez-Miguélez, M., Álvarez-Álvarez, P., Pardos, M., Madrigal, G., Ruiz-Peinado, R., López-Senespleda, E., Del Río, M., & Calama, R. (2023). Development of tools to estimate the contribution of young sweet chestnut plantations to climate-change mitigation. *Forest Ecology and Management*, *530*, 120761. https://doi.org/10.1016/J.FORECO.2022.120761

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, *7*(9), 405–410. https://doi.org/10.1016/S1360-1385(02)02312-9

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. *Trends in Plant Science*, *11*(1), 15–19. https://doi.org/10.1016/j.tplants.2005.11.002

Mittler, R. (2017). ROS Are Good. In *Trends in Plant Science* (Vol. 22, Issue 1, pp. 11–19). Elsevier Ltd. https://doi.org/10.1016/j.tplants.2016.08.002

Muhammad, I., Shalmani, A., Ali, M., Yang, Q. H., Ahmad, H., & Li, F. B. (2021). Mechanisms Regulating the Dynamics of Photosynthesis Under Abiotic Stresses. *Frontiers in Plant Science*, *11*, 615942. https://doi.org/10.3389/FPLS.2020.615942/BIBTEX

Nath, K., O'Donnell, J. P., & Lu, Y. (2017). Chlorophyll fluorescence for high-throughput screening of plants during abiotic stress, aging, and genetic perturbation. *Photosynthesis: Structures, Mechanisms, and Applications*, 261–273. https://doi.org/10.1007/978-3-319-48873-8_12/COVER

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y., Neukermans, J., Marquez-Garcia, B., Queval, G., & Foyer, C. H. (2012). Glutathione in plants: an integrated overview. *Plant, Cell & Environment*, *35*(2), 454–484. https://doi.org/10.1111/J.1365-3040.2011.02400.X

Noctor, G., Reichheld, J. P., & Foyer, C. H. (2018). ROS-related redox regulation and signaling in plants. *Seminars in Cell & Developmental Biology*, *80*, 3–12. https://doi.org/10.1016/J.SEMCDB.2017.07.013

Orabi, S. A., and Abou-Hussein, S. D. (2019). Antioxidant defense mechanisms enhance oxidative stress tolerance in plants. A review. Curr. Sci. Int. 8, 565–576.

Pandey, P., Ramegowda, V., & Senthil-Kumar, M. (2015). Shared and unique responses of plants to multiple individual stresses and stress combinations: Physiological and molecular mechanisms. *Frontiers in Plant Science*, *6*(September), 153997. https://doi.org/10.3389/FPLS.2015.00723/BIBTEX

Pandey, S., Fartyal, D., Agarwal, A., Shukla, T., James, D., Kaul, T., Negi, Y. K., Arora, S., & Reddy, M. K. (2017). Abiotic stress tolerance in plants: Myriad roles of ascorbate peroxidase. *Frontiers in Plant Science*, *8*, 215288. https://doi.org/10.3389/FPLS.2017.00581/BIBTEX

Pereira, S. C., Carvalho, D., & Rocha, A. (2021). Temperature and Precipitation Extremes over the Iberian Peninsula under Climate Change Scenarios: A Review. *Climate 2021, Vol. 9, Page 139*, *9*(9), 139. https://doi.org/10.3390/CLI9090139

Pérez-Girón, J. C., Álvarez-Álvarez, P., Díaz-Varela, E. R., & Mendes Lopes, D. M. (2020). Influence of climate variations on primary production indicators and on the resilience of forest ecosystems in a future scenario of climate change: Application to sweet chestnut agroforestry systems in the Iberian Peninsula. *Ecological Indicators*, *113*, 106199. https://doi.org/10.1016/J.ECOLIND.2020.106199

Pirasteh-Anosheh, H., Saed-Moucheshi, A., Pakniyat, H., & Pessarakli, M. (2016). Stomatal responses to drought stress. *Water Stress and Crop Plants: A Sustainable Approach*, *1–2*, 24–40. https://doi.org/10.1002/9781119054450.CH3

Priya, M., Dhanker, O. P., Siddique, K. H. M., HanumanthaRao, B., Nair, R. M., Pandey, S., Singh, S., Varshney, R. K., Prasad, P. V. V., & Nayyar, H. (2019). Drought and heat stress-related proteins: an update about their functional relevance in imparting stress tolerance in agricultural crops. *Theoretical and Applied Genetics 2019 132:6*, *132*(6), 1607–1638. https://doi.org/10.1007/S00122-019-03331-2

Pshybytko, N. L. (2023). Non-Photochemical Quenching of Chlorophyll a Fluorescence as an Indicator of the State of a Plant Photosynthetic Apparatus Under Abiotic Stress. *Journal of Applied Spectroscopy*, *90*(1), 60–65. https://doi.org/10.1007/S10812-023-01503-Z/METRICS

Raines, C. A. (2022). Improving plant productivity by re-tuning the regeneration of RuBP in the Calvin–Benson–Bassham cycle. *New Phytologist*, *236*(2), 350–356. https://doi.org/10.1111/NPH.18394

Raja, V., Qadir, S. U., Alyemeni, M. N., & Ahmad, P. (2020). Impact of drought and heat stress individually and in combination on physio-biochemical parameters, antioxidant responses, and gene expression in Solanum lycopersicum. *3 Biotech 2020 10:5*, *10*(5), 1–18. https://doi.org/10.1007/S13205-020-02206-4

Rascher, U., Liebig, M., & Lüttge, U. (2000). Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. *Plant, Cell & Environment*, *23*(12), 1397–1405. https://doi.org/10.1046/J.1365-3040.2000.00650.X

Raza, A., Charagh, S., Abbas, S., Hassan, M. U., Saeed, F., Haider, S., Sharif, R., Anand, A., Corpas, F. J., Jin, W., & Varshney, R. K. (2023). Assessment of proline function in higher plants under extreme temperatures. *Plant Biology*, *25*(3), 379–395. https://doi.org/10.1111/PLB.13510

Razi K, Muneer S (2021) Drought stress-induced physiological mechanisms, signaling pathways and molecular response of chloroplasts in common vegetable crops. Crit Rev Biotechnol. https://doi.org/10.1080/07388551.2021.1874280

Rillig, M. C., Ryo, M., Lehmann, A., Aguilar-Trigueros, C. A., Buchert, S., Wulf, A., Iwasaki, A., Roy, J., & Yang, G. (2019). The role of multiple global change factors in driving soil functions and microbial biodiversity. *Science*, *366*(6467), 886–890.

Rivero, R. M., Mittler, R., Blumwald, E., & Zandalinas, S. I. (2022). Developing climate-resilient crops: improving plant tolerance to stress combination. *The Plant Journal*, *109*(2), 373–389. https://doi.org/10.1111/TPJ.15483

Roldán-Arjona, T., & Ariza, R. R. (2009). Repair and tolerance of oxidative DNA damage in plants. *Mutation Research/Reviews in Mutation Research*, *681*(2–3), 169–179. https://doi.org/10.1016/J.MRREV.2008.07.003

Ruban, A. V. (2016). Nonphotochemical Chlorophyll Fluorescence Quenching: Mechanism and Effectiveness in Protecting Plants from Photodamage. *Plant Physiology*, *170*(4), 1903. https://doi.org/10.1104/PP.15.01935

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

Sade, N., Galkin, E., & Moshelion, M. (2015). Measuring Arabidopsis, Tomato and Barley Leaf Relative Water Content (RWC). *BIO-PROTOCOL*, *5*(8). https://doi.org/10.21769/BIOPROTOC.1451

Sah, S. K., Reddy, K. R., & Li, J. (2016). Abscisic acid and abiotic stress tolerance in crop plants. *Frontiers in Plant Science*, *7*(MAY2016), 190245. https://doi.org/10.3389/FPLS.2016.00571/BIBTEX

Sánchez-Bermúdez, M., del Pozo, J. C., & Pernas, M. (2022). Effects of Combined Abiotic Stresses Related to Climate Change on Root Growth in Crops. *Frontiers in Plant Science*, *13*, 918537. https://doi.org/10.3389/FPLS.2022.918537/BIBTEX

Santos, J. A., Costa, R., & Fraga, H. (2017). Climate change impacts on thermal growing conditions of main fruit species in Portugal. *Climatic Change*, *140*(2), 273–286. https://doi.org/10.1007/S10584-016-1835-6/FIGURES/5

Sebastiana, M., da Silva, A. B., Matos, A. R., Alcântara, A., Silvestre, S., & Malhó, R. (2018). Ectomycorrhizal inoculation with Pisolithus tinctorius reduces stress

induced by drought in cork oak. *Mycorrhiza*, *28*(3), 247–258. https://doi.org/10.1007/S00572-018-0823-2/FIGURES/6

Sebastiana, M., Duarte, B., Monteiro, F., Malhó, R., Caçador, I., & Matos, A. R. (2019). The leaf lipid composition of ectomycorrhizal oak plants shows a drought-tolerance signature. *Plant Physiology and Biochemistry*, *144*, 157–165. https://doi.org/10.1016/J.PLAPHY.2019.09.032

Sehgal, A., Sita, K., Kumar, J., Kumar, S., Singh, S., Siddique, K. H. M., & Nayyar, H. (2017). Effects of drought, heat and their interaction on the growth, yield and photosynthetic function of lentil (*Lens culinaris* medikus) genotypes varying in heat and drought sensitivity. *Frontiers in Plant Science*, *8*, 298024. https://doi.org/10.3389/FPLS.2017.01776/BIBTEX

Sewelam, N., Brilhaus, D., Bräutigam, A., Alseekh, S., Fernie, A. R., & Maurino, V. G. (2020). Molecular plant responses to combined abiotic stresses put a spotlight on unknown and abundant genes. *Journal of Experimental Botany*, *71*(16), 5098–5112. https://doi.org/10.1093/JXB/ERAA250

Sharma, A., Kumar, V., Shahzad, B., Ramakrishnan, M., Singh Sidhu, G. P., Bali, A. S., Handa, N., Kapoor, D., Yadav, P., Khanna, K., Bakshi, P., Rehman, A., Kohli, S. K., Khan, E. A., Parihar, R. D., Yuan, H., Thukral, A. K., Bhardwaj, R., & Zheng, B. (2019). Photosynthetic Response of Plants Under Different Abiotic Stresses: A Review. *Journal of Plant Growth Regulation 2019 39:2*, *39*(2), 509–531. https://doi.org/10.1007/S00344-019-10018-X

Sharma, A., Thakur, S., Kumar, V., Kesavan, A. K., Thukral, A. K., & Bhardwaj, R. (2017). 24-epibrassinolide stimulates imidacloprid detoxification by modulating the gene expression of *Brassica juncea* L. *BMC Plant Biology*, *17*(1), 1–10. https://doi.org/10.1186/S12870-017-1003-9/TABLES/5

Sharma, I., & Ahmad, P. (2014). Catalase: A Versatile Antioxidant in Plants. *Oxidative Damage to Plants: Antioxidant Networks and Signaling*, 131–148. https://doi.org/10.1016/B978-0-12-799963-0.00004-6

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany*, *2012*, 1–26. https://doi.org/10.1155/2012/217037

Sharma, S., Kiran, U., Sopory, S.K., (2017). Developing stress-tolerant plants by manipulating components involved in oxidative stress. In: Abdin, M.Z., Kiran, U., Kamaluddin, Ali, A. (Eds.), Plant Biotechnology: Principles and Applications.

Springer Singapore, pp. 233–248. https://doi.org/10.1007/978-981-10-2961-5_9

Siddiqui, Z. A., Akhtar, M. S., & Futai, K. (2008). Mycorrhizae: Sustainable agriculture and forestry. *Mycorrhizae: Sustainable Agriculture and Forestry*, 1–359. https://doi.org/10.1007/978-1-4020-8770-7

Singh, J., & Thakur, J. K. (2018). Photosynthesis and abiotic stress in plants. *Biotic and Abiotic Stress Tolerance in Plants*, 27–46. https://doi.org/10.1007/978-981-10-9029-5_2/COVER

Smirnoff, N., & Arnaud, D. (2019). Hydrogen peroxide metabolism and functions in plants. *New Phytologist*, *221*(3), 1197–1214. https://doi.org/10.1111/NPH.15488

Smith, S. E., & Read, D. (2008). Mycorrhizal Symbiosis, Third Edition. *Mycorrhizal Symbiosis, Third Edition*, 1–787. https://doi.org/10.1016/B978-0-12-370526-6.X5001-6

Soares, C., Carvalho, M. E. A., Azevedo, R. A., & Fidalgo, F. (2019). Plants facing oxidative challenges—A little help from the antioxidant networks. *Environmental and Experimental Botany*, *161*, 4–25. https://doi.org/10.1016/J.ENVEXPBOT.2018.12.009

Soares, C., Pereira, R., Spormann, S., & Fidalgo, F. (2019b). Is soil contamination by a glyphosate commercial formulation truly harmless to non-target plants? – Evaluation of oxidative damage and antioxidant responses in tomato. *Environmental Pollution*, *247*, 256–265. https://doi.org/10.1016/J.ENVPOL.2019.01.063

Soares, P. M. M., Cardoso, R. M., Ferreira, J. J., & Miranda, P. M. A. (2015). Climate change and the Portuguese precipitation: ENSEMBLES regional climate models results. *Climate Dynamics*, *45*(7–8), 1771–1787. https://doi.org/10.1007/S00382-014-2432-X/FIGURES/10

Song, Y., Chen, Q., Ci, D., Shao, X., & Zhang, D. (2014). Effects of high temperature on photosynthesis and related gene expression in poplar. *BMC Plant Biology*, *14*(1), 1–20. https://doi.org/10.1186/1471-2229-14-111/FIGURES/9

Spormann, S., Nadais, P., Sousa, F., Pinto, M., Martins, M., Sousa, B., Fidalgo, F., & Soares, C. (2023). Accumulation of Proline in Plants under Contaminated Soils—Are We on the Same Page? *Antioxidants*, *12*(3), 666. https://doi.org/10.3390/ANTIOX12030666/S1

Stéfanon, M., Drobinski, P., D'Andrea, F., Lebeaupin-Brossier, C., & Bastin, S. (2014). Soil moisture-temperature feedbacks at meso-scale during summer heat waves over Western Europe. *Climate Dynamics*, *42*(5–6), 1309–1324. https://doi.org/10.1007/S00382-013-1794-9/FIGURES/12

Stirbet, A., Lazár, D., Guo, Y., & Govindjee, G. (2020). Photosynthesis: basics, history and modelling. *Annals of Botany*, *126*(4), 511–537. https://doi.org/10.1093/AOB/MCZ171

Szurman-Zubrzycka, M., Jędrzejek, P., & Szarejko, I. (2023). How Do Plants Cope with DNA Damage? A Concise Review on the DDR Pathway in Plants. *International Journal of Molecular Sciences 2023, Vol. 24, Page 2404*, *24*(3), 2404. https://doi.org/10.3390/IJMS24032404

Taiz, L., Zeiger, E., Møller, I. M., & Murphy, A. (2015). Plant physiology and development. *Plant Physiology and Development. Sinauer Associates Incorporated, Sunderland, MA, USA.*, *Ed. 6*.

Tedersoo, L., & Bahram, M. (2019). Mycorrhizal types differ in ecophysiology and alter plant nutrition and soil processes. *Biological Reviews*, *94*(5), 1857–1880. https://doi.org/10.1111/BRV.12538

Teskey, R., Wertin, T., Bauweraerts, I., Ameye, M., McGuire, M. A., & Steppe, K. (2015). Responses of tree species to heat waves and extreme heat events. *Plant, Cell & Environment*, *38*(9), 1699–1712. https://doi.org/10.1111/PCE.12417

Tommasino, E., López Colomba, E., Carrizo, M., Grunberg, K., Quiroga, M., Carloni, E., Griffa, S., Ribotta, A., & Luna, C. (2018). Individual and combined effects of drought and heat on antioxidant parameters and growth performance in Buffel grass (*Cenchrus ciliaris* L.) genotypes. *South African Journal of Botany*, *119*, 104–111. https://doi.org/10.1016/J.SAJB.2018.08.026

Tripathi, D., Oldenburg, D. J., & Bendich, A. J. (2023). Oxidative and Glycation Damage to Mitochondrial DNA and Plastid DNA during Plant Development. *Antioxidants 2023, Vol. 12, Page 891*, *12*(4), 891. https://doi.org/10.3390/ANTIOX12040891

Tripathy, B. C., & Oelmüller, R. (2012). Reactive oxygen species generation and signaling in plants. *Https://Doi.Org/10.4161/Psb.22455*, *7*(12), 1621–1633. https://doi.org/10.4161/PSB.22455

Uarrota, V. G., Stefen, D. L. V., Leolato, L. S., Gindri, D. M., & Nerling, D. (2018). Revisiting carotenoids and their role in plant stress responses: From biosynthesis to plant signaling mechanisms during stress. *Antioxidants and Antioxidant Enzymes in Higher Plants*, 207–232. https://doi.org/10.1007/978-3-319-75088-0_10/COVER

Urban, J., Ingwers, M., McGuire, M. A., & Teskey, R. O. (2017). Stomatal conductance increases with rising temperature. *Plant Signaling & Behavior*, *12*(8). https://doi.org/10.1080/15592324.2017.1356534

Urban, O., Hlaváčová, M., Klem, K., Novotná, K., Rapantová, B., Smutná, P., Horáková, V., Hlavinka, P., Škarpa, P., & Trnka, M. (2018). Combined effects of drought and high temperature on photosynthetic characteristics in four winter wheat genotypes. *Field Crops Research*, *223*, 137–149. https://doi.org/10.1016/J.FCR.2018.02.029

Usman, M., Ho-Plágaro, T., Frank, H. E. R., Calvo-Polanco, M., Gaillard, I., Garcia, K., & Zimmermann, S. D. (2021). Mycorrhizal Symbiosis for Better Adaptation of Trees to Abiotic Stress Caused by Climate Change in Temperate and Boreal Forests. *Frontiers in Forests and Global Change*, *4*, 742392. https://doi.org/10.3389/FFGC.2021.742392/BIBTEX

van der Heijden, M. G. A., Martin, F. M., Selosse, M. A., & Sanders, I. R. (2015). Mycorrhizal ecology and evolution: The past, the present, and the future. *New Phytologist*, *205*(4), 1406–1423. https://doi.org/10.1111/NPH.13288

Verma, V., Ravindran, P., & Kumar, P. P. (2016). Plant hormone-mediated regulation of stress responses. *BMC Plant Biology*, *16*(1), 1–10. https://doi.org/10.1186/S12870-016-0771-Y/FIGURES/1

Vishwanathan, K., Zienkiewicz, K., Liu, Y., Janz, D., Feussner, I., Polle, A., & Haney, C. H. (2020). Ectomycorrhizal fungi induce systemic resistance against insects on a nonmycorrhizal plant in a CERK1-dependent manner. *New Phytologist*, *228*(2), 728–740. https://doi.org/10.1111/NPH.16715

von Caemmerer, S., & Farquhar, G. D. (1981). Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta*, *153*(4), 376–387. https://doi.org/10.1007/BF00384257/METRICS

Wahid, A., Gelani, S., Ashraf, M., & Foolad, M. R. (2007). Heat tolerance in plants: An overview. *Environmental and Experimental Botany*, *61*(3), 199–223. https://doi.org/10.1016/J.ENVEXPBOT.2007.05.011

Wang, J., Zhang, H., Gao, J., Zhang, Y., Liu, Y., & Tang, M. (2021). Effects of ectomycorrhizal fungi (*Suillus variegatus*) on the growth, hydraulic function, and non-structural carbohydrates of *Pinus tabulaeformis* under drought stress. *BMC Plant Biology*, *21*(1), 1–13. https://doi.org/10.1186/S12870-021-02945-3/FIGURES/8

Wang, Q.-L., Chen, J.-H., He, N.-Y., and Guo, F.-Q. (2018). Metabolic reprogramming in chloroplasts under heat stress in plants. Int. J. Mol. Sci. 19:849. doi: 10.3390/ijms19030849

Wang, Z., Li, G., Sun, H., Ma, L., Guo, Y., Zhao, Z., Gao, H., & Mei, L. (2018). Effects of drought stress on photosynthesis and photosynthetic electron transport chain in young apple tree leaves. *Biology Open*, *7*(11). https://doi.org/10.1242/BIO.035279/259343/AM/EFFECTS-OF-DROUGHT-STRESS-ON-PHOTOSYNTHESIS-AND

Wei, Z., Du, T., Li, X., Fang, L., & Liu, F. (2018). Simulation of stomatal conductance and water use efficiency of tomato leaves exposed to different irrigation regimes and air CO_2 concentrations by a modified “Ball-Berry” model. *Frontiers in Plant Science*, *9*, 341345. https://doi.org/10.3389/FPLS.2018.00445/BIBTEX

Xu, X., Fonseca de Lima, C. F., Vu, L. D., & De Smet, I. (2023). When drought meets heat – a plant omics perspective. *Frontiers in Plant Science*, *14*, 1250878. https://doi.org/10.3389/FPLS.2023.1250878/BIBTEX

Yamori, W. (2016). Photosynthetic response to fluctuating environments and photoprotective strategies under abiotic stress. *Journal of Plant Research*, *129*(3), 379–395. https://doi.org/10.1007/S10265-016-0816-1

Yin, D., Song, R., Qi, J., & Deng, X. (2018). Ectomycorrhizal fungus enhances drought tolerance of *Pinus sylvestris* var. mongolica seedlings and improves soil condition. *Journal of Forestry Research*, *29*(6), 1775–1788. https://doi.org/10.1007/S11676-017-0583-4/FIGURES/7

YUE, X. L., & Gao, Q. X. (2018). Contributions of natural systems and human activity to greenhouse gas emissions. *Advances in Climate Change Research*, *9*(4), 243–252. https://doi.org/10.1016/J.ACCRE.2018.12.003

Zahra, N., Hafeez, M. B., Ghaffar, A., Kausar, A., Zeidi, M. Al, Siddique, K. H. M., & Farooq, M. (2023). Plant photosynthesis under heat stress: Effects and management. *Environmental and Experimental Botany*, *206*, 105178. https://doi.org/10.1016/J.ENVEXPBOT.2022.105178

Zandalinas, S. I., Fritschi, F. B., & Mittler, R. (2020). Signal transduction networks during stress combination. *Journal of Experimental Botany*, *71*(5), 1734–1741. https://doi.org/10.1093/JXB/ERZ486

Zandalinas, S. I., & Mittler, R. (2022). Plant responses to multifactorial stress combination. *New Phytologist*, *234*(4), 1161–1167. https://doi.org/10.1111/NPH.18087

Zandalinas, S. I., Mittler, R., Balfagón, D., Arbona, V., & Gómez-Cadenas, A. (2018). Plant adaptations to the combination of drought and high temperatures. *Physiologia Plantarum*, *162*(1), 2–12. https://doi.org/10.1111/PPL.12540

Zandalinas, S. I., Sengupta, S., Fritschi, F. B., Azad, R. K., Nechushtai, R., & Mittler, R. (2021). The impact of multifactorial stress combination on plant growth and survival. *New Phytologist*, *230*(3), 1034–1048. https://doi.org/10.1111/NPH.17232

Zargar, S. M., Gupta, N., Nazir, M., Mahajan, R., Malik, F. A., Sofi, N. R., Shikari, A. B., & Salgotra, R. K. (2017). Impact of drought on photosynthesis: Molecular perspective. *Plant Gene*, *11*, 154–159. https://doi.org/10.1016/J.PLGENE.2017.04.003

Zhang, H., & Sonnewald, U. (2017). Differences and commonalities of plant responses to single and combined stresses. *The Plant Journal*, *90*(5), 839–855. https://doi.org/10.1111/TPJ.13557

Zhao, X., Chen, T., Feng, B., Zhang, C., Peng, S., Zhang, X., Fu, G., & Tao, L. (2017). Non-photochemical quenching plays a key role in light acclimation of rice plants differing in leaf color. *Frontiers in Plant Science*, *7*, 233665. https://doi.org/10.3389/FPLS.2016.01968/BIBTEX

Zhou, R., Yu, X., Ottosen, C. O., Rosenqvist, E., Zhao, L., Wang, Y., Yu, W., Zhao, T., & Wu, Z. (2017). Drought stress had a predominant effect over heat stress on three tomato cultivars subjected to combined stress. *BMC Plant Biology*, *17*(1), 1–13. https://doi.org/10.1186/S12870-017-0974-X/FIGURES/8

Zittis, G., Bruggeman, A., & Lelieveld, J. (2021). Revisiting future extreme precipitation trends in the Mediterranean. *Weather and Climate Extremes*, *34*, 100380. https://doi.org/10.1016/J.WACE.2021.100380

Zlatev, Z., & Lidon, F. C. (2012). An overview on drought induced changes in plant growth, water relations and photosynthesis. *Emirates Journal of Food and Agriculture*, *24*(1), 57–72. https://doi.org/10.9755/EJFA.V24I1.10599

Annex

Supplementary Material

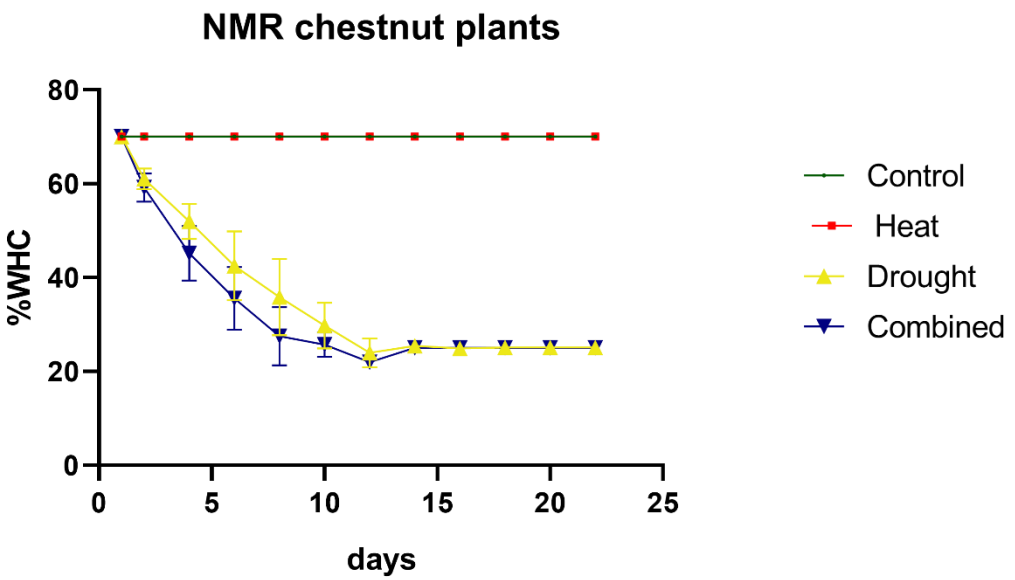
Table S1. Results of the two-way ANOVA for all evaluated parameters in leaves of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d-1); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold.

Parameter	Factors		Interaction
	STRESS	***MYCO***	
RWC	**F (3, 32) = 14,02; $p \leq 0.05$**	**F (1, 32) = 37,81; $p \leq 0.05$**	**F (3, 32) = 3,801; $p \leq 0.05$**
Foliar area	**F (3, 29) = 96,31; $p \leq 0.05$**	**F (1, 29) = 15,28; $p \leq 0.05$**	**F (3, 29) = 18,92; $p \leq 0.05$**
New leaves	**F (3, 52) = 6,832; $p \leq 0.05$**	**F (1, 52) = 37,91; $p \leq 0.05$**	**F (3, 52) = 4,355; $p \leq 0.05$**
E	**F (3, 48) = 19,67; $p \leq 0.05$**	**F (1, 48) = 44,83; $p \leq 0.05$**	**F (3, 48) = 5,459; $p \leq 0.05$**
gs	**F (3, 45) = 37,01; $p \leq 0.05$**	**F (1, 45) = 107,4; $p \leq 0.05$**	**F (3, 45) = 13,00; $p \leq 0.05$**
A	**F (3, 38) = 63,82; $p \leq 0.05$**	**F (1, 38) = 100,6; $p \leq 0.05$**	**F (3, 38) = 10,03; $p \leq 0.05$**
A/gs	**F (3, 35) = 21,34; $p \leq 0.05$**	**F (1, 35) = 418,2; $p \leq 0.05$**	**F (3, 35) = 15,32; $p \leq 0.05$**
F_v/F_m	F (3, 84) = 2,186; $p > 0.05$	F (1, 84) = 1,432; $p > 0.05$	**F (3, 84) = 2,721; $p \leq 0.05$**
NPQ	F (3, 74) = 2,716; $p > 0.05$	F (1, 74) = 3,808; $p > 0.05$	**F (3, 74) = 7,880; $p \leq 0.05$**
ETR	F (3, 75) = 2,076; $p > 0.05$	**F (1, 75) = 19,71; $p \leq 0.05$**	**F (3, 75) = 4,089; $p \leq 0.05$**
ΦPSII	F (3, 79) = 2,120; $p > 0.05$	**F (1, 79) = 17,56; $p \leq 0.05$**	**F (3, 79) = 2,962; $p \leq 0.05$**
Total Chl	F (3, 18) = 2,153; $p > 0.05$	**F (1, 18) = 13,78; $p \leq 0.05$**	F (3, 18) = 1,907; $p > 0.05$
Carotenoids	F (3, 17) = 2,387; $p > 0.05$	**F (1, 17) = 23,31; $p \leq 0.05$**	F (3, 17) = 1,662; $p > 0.05$
LP	**F (3, 33) = 6,444; $p \leq 0.05$**	**F (1, 33) = 5,084; $p \leq 0.05$**	**F (3, 33) = 5,216; $p \leq 0.05$**
H_2O_2	**F (3, 16) = 7,462; $p \leq 0.05$**	**F (1, 16) = 55,41; $p \leq 0.05$**	**F (3, 16) = 7,372; $p \leq 0.05$**
$O_2^{\cdot-}$	**F (3, 30) = 16,39; $p \leq 0.05$**	F (1, 30) = 1,749; $p > 0.05$	**F (3, 30) = 6,033; $p \leq 0.05$**
Pro	**F (1, 17) = 557,8; $p \leq 0.05$**	**F (3, 17) = 77,31; $p \leq 0.05$**	**F (3, 17) = 65,93; $p \leq 0.05$**
GSH	**F (3, 17) = 3,500; $p \leq 0.05$**	F (1, 17) = 3,332; $p > 0.05$	**F (3, 17) = 3,510; $p \leq 0.05$**
AsA	F (3, 16) = 0,9102; $p > 0.05$	**F (1, 16) = 23,72; $p \leq 0.05$**	**F (3, 16) = 14,31; $p \leq 0.05$**

Table S2. Results of the two-way ANOVA for all evaluated parameters in roots of NMR and MR chestnut plants after 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d-1); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants. Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted at bold.

Parameter	Factors		Interaction
	STRESS	***MYCO***	
LP	**F (3, 18) = 15,04; $p \leq 0.05$**	**F (1, 18) = 7,438; $p \leq 0.05$**	**F (3, 18) = 8,495; $p \leq 0.05$**
H_2O_2	**F (3, 18) = 28,98; $p \leq 0.05$**	F (1, 18) = 0,5418; $p > 0.05$	**F (3, 18) = 6,244; $p \leq 0.05$**
$O_2^{\cdot-}$	**F (3, 27) = 42,04; $p \leq 0.05$**	**F (1, 27) = 19,61; $p \leq 0.05$**	**F (3, 27) = 10,56; $p \leq 0.05$**
Pro	**F (3, 18) = 15,04; $p \leq 0.05$**	**F (1, 18) = 7,438; $p \leq 0.05$**	**F (3, 18) = 8,495; $p \leq 0.05$**
GSH	**F (3, 18) = 14,61; $p \leq 0.05$**	**F (1, 18) = 122,6; $p \leq 0.05$**	**F (3, 18) = 9,774; $p \leq 0.05$**
AsA	**F (3, 19) = 21,83; $p \leq 0.05$**	F (1, 19) = 3,233; $p > 0.05$	**F (3, 19) = 10,66; $p \leq 0.05$**

a)



b)

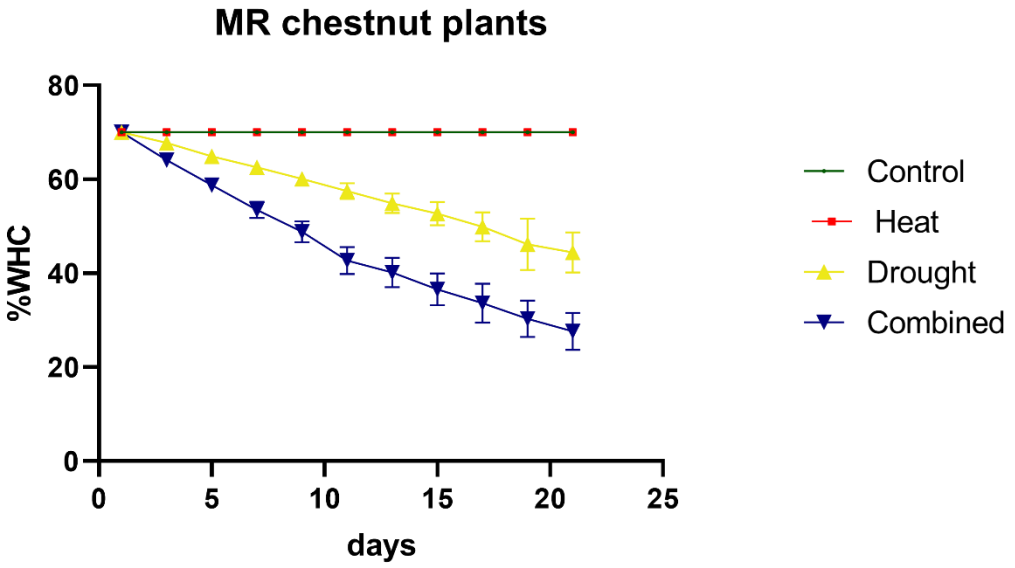


Figure 11 Evolution of the WHC of NMR **a)** and MR **b)** chestnut plants throughout 21 days of stress exposure. CTL – control plants; HEAT – heat-exposed plants (42 ºC; 4h d-1); DROUGHT – drought-exposed plants (no irrigation, until a 25% WHC was reached, for 21 d); COMBINED – heat and drought co-exposed plants.