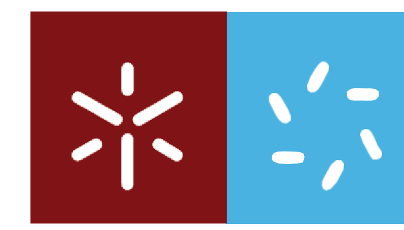

Universidade do Minho

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Francisca Monteiro Rodrigues

Tolerance response of tomato plants (*Solanum lycopersicum* L.) to climate change: biochemical and molecular aspects of salinity- and/or heat-induced stress

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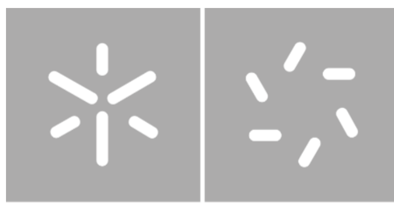

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Tolerance response of tomato plants (*Solanum lycopersicum* L.) to climate change: biochemical and molecular aspects of salinity- and/or heat-induced stress

Dissertação de Mestrado

Mestrado em Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho efetuado sob a orientação da

Professora Doutora Ana Cristina Gomes da Cunha

e da

Professora Doutora Maria Fernanda da Silva Fidalgo Ferro de Beça

Dezembro de 2021

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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RESUMO

Devido à atual instabilidade climática, é esperado que a frequência e intensidade de situações de stresse abiótico, como a salinidade do solo e as temperaturas elevadas, sejam agravadas, colocando em risco a produção agrícola e a segurança alimentar. Apesar dos impactos causados pela exposição individual ao sal e ao calor já terem sido extensivamente estudados, os efeitos da sua potencial interação ainda não são claros. De forma a colmatar esta lacuna de conhecimento, plantas de tomateiro (*Solanum lycopersicum* var. *cerasiforme*) foram expostas a uma situação de salinidade [100 mM cloreto de sódio (NaCl)] e/ou temperatura elevada (42 °C; 4 h d^{-1}) durante 21 dias para a avaliação das respostas fisiológicas e bioquímicas, bem como do desempenho fotossintético. O crescimento das plantas foi negativamente afetado por todos os tratamentos, porém a combinação impôs um efeito mais severo no tamanho e na produção de biomassa de ambos os órgãos, bem como no conteúdo de pigmentos fotossintéticos. Além disso, a co-exposição levou a uma maior desregulação do equilíbrio iónico: o sódio (Na^+) foi muito mais acumulado e o oposto se verificou para o potássio (K^+), magnésio (Mg^{2+}) e cálcio (Ca^{2+}). Apesar disso, não foi observada a sobreacumulação de espécies reativas de oxigénio nem se detetaram sinais de dano oxidativo, devido à potenciação de metabolitos e enzimas antioxidantes. Paralelamente, e no que diz respeito à eficiência fotossintética, o tratamento combinado levou ao aumento do rendimento quântico do fotossistema II (PSII), o que resultou, provavelmente, da diminuição da área foliar específica e de uma convergência ou fortalecimento das vias de defesa. No entanto, a inibição da expressão de genes relacionados com o PSII (*D1* e *CP47*) e o aumento de processos não-fotoquímicos em todas as condições de stresse, levam a crer que o tratamento combinado tenha causado danos no aparelho fotossintético. Por último, um padrão distinto pôde ser observado nos parâmetros relacionados com trocas gasosas, onde apenas a salinidade (individualmente ou em combinação) afetou negativamente a condutância estomática, a taxa de transpiração, e a assimilação de carbono. Relativamente ao perfil de expressão das subunidades da ribulose-1,5-bifosfato carboxilase-oxigenase, todos os tratamentos inibiram os níveis de *RbcS*. Contudo, enquanto o calor diminuiu a expressão de *RbcL*, o sal induziu o efeito contrário, sendo que a sua combinação não afetou a expressão deste gene.

Em suma, a redução drástica no crescimento não parece advir de danos oxidativos nem apenas de danos na maquinaria fotossintética, já que os efeitos negativos observados nas plantas sob stresse combinado não foram mais pronunciados do que nos individuais. Portanto, é plausível que o efeito mais severo no crescimento possa resultar de uma maior realocação de recursos para as vias de defesa ou da interrupção dos mecanismos de crescimento, como a expansão e divisão celular, devido ao aumento da toxicidade de Na^+ e a um desequilíbrio nutricional.

Palavras-chave: fotossíntese; sistema antioxidante; stresse combinado; stresse oxidativo; tomate *cherry.*

ABSTRACT

In the face of climate change, the frequency and intensity of abiotic stresses, such as salinity and high temperatures, are expected to be highly intensified, thus threatening crop production worldwide and putting food security at risk. Even though the impacts of the salt or heat stresses have been widely studied in the past, there is still much to unravel regarding the potential interaction of these stressors, as they are likely to occur simultaneously in natural conditions. Therefore, to address this gap, tomato plants (*Solanum lycopersicum* var. *cerasiforme*) were exposed to salt [100 mM sodium chloride (NaCl)] and/or heat (42 °C; 4 h d^{-1}) for 21 days for the evaluation of physiological and biochemical responses, as well as the photosynthetic performance. Growth was negatively affected by all treatments, but the combination imposed a significant harsher effect on organ elongation and biomass production, as well as in the content of photosynthetic pigments. Furthermore, the combined treatment led to a clear pattern regarding ion balance: sodium (Na^+) was much more accumulated and potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) were more depleted than in any other growth condition. Despite that, no overaccumulation of reactive oxygen species nor signs of oxidative damage were observed, due to an accumulation of antioxidant (AOX) metabolites and the induction of the AOX enzymes. However, this overall maintenance of the redox status was not accompanied by an efficient photosynthetic flow. The chlorophyll fluorescence analysis showed that, while the combined treatment actually led to an increased maximum quantum yield, probably related to the decreased specific leaf area and to a convergence or higher enhancement of defence and physiological pathways, impairments in the photosynthetic apparatus should not be ruled out, as an inhibition of transcript accumulation of two photosystem II-related genes (*D1* and *CP47*) and an increment of non-photochemical quenching in all stress conditions was observed. Lastly, a distinct pattern could be observed in gas-exchange endpoints, where only salinity (single or combined) negatively affected stomatal conductance, transpiration rate and carbon assimilation. Regarding the expression profile of ribulose-1,5-bisphosphate carboxylase-oxygenase subunits, all treatments inhibited *RbcS* accumulation, but only heat stress decreased *RbcL*, with salt-treated plants actually overexpressing this gene only under single expression and the combined treatment remaining unaffected.

In summary, the severe reduction in plant growth does not appear to be the consequence of oxidative damage or be solely explained by photosynthetic disruptions, as the negative effects were not more pronounced than in the individual stressors. Therefore, it is plausible that the harsher effect on growth may result from a higher reallocation of resources to defence pathways or from the disruption of growth mechanisms, like cell expansion and division, due to an increased Na^+ toxicity and nutrient deficiency.

Keywords: antioxidant system; cherry tomato; combined stress; oxidative stress; photosynthesis.

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ABBREVIATIONS, SYMBOLS AND ACRONYMS

ε – molar coefficient factor

$(NH_4)6Mo_7O_{24}$ – ammonium molybdate

$^{•}OH$ – hydroxyl radical

$^1O_2$ – oxygen singlet

$^3Chl^*$ - chlorophyll triplet state

Abs – absorbance

$AlCl_3$ – aluminium chloride

AOX – antioxidant

APX – ascorbate peroxidase

AsA – ascorbate

C – carbon

C_a – atmospheric carbon dioxide

Ca – calcium

CAT – catalase

Chl – chlorophyll

C_i – intracellular carbon dioxide

Cl^- – chlorine

CO_2 – carbon dioxide

CTL – control

Cu – copper

dH_2O – deionised water

DHA – dehydroascorbate

DHAR – dehydroascorbate reductase

DTNB – 5,5-dithio-bis-(2-nitrobenzoic acid)

DTT – dithiothreitol

dw – dry weight

E – transpiration rate

EDTA – ethylenediamine tetraacetic acid

ETC – electron transport chain

F – forward primer

F_0 – minimal fluorescence

Fe – iron

Fe^{2+} – ferrous ion

Fe^{3+} – ferric ion

F_m – maximum fluorescence yield

F_v/F_m – Maximum quantum yield of photosystem II

fw – fresh weight

GPX – glutathione peroxidase

GR – glutathione reductase

g_s – stomatal conductance

GSH – glutathione

GSSG – oxidized glutathione

H_2O_2 – hydrogen peroxide

H_2SO_4 – sulphuric acid

HNO_3 – nitric acid

HSP – heat shock protein

IRGA – infrared gas analyser

K – potassium

KCH_3COO – potassium acetate

LHCII – light-harvesting complex

LP – lipid peroxidation

MDA – malondialdehyde

MDHA - monodehydroascorbate

MDHAR – monodehydroascorbate reductase

Mg – magnesium

N – nitrogen

Na – sodium

Na_2HPO_4 – sodium phosphate

NaCl – sodium chloride

NBT – nitroblue tetrazolium

NPQ – non-photochemical quenching

O_2 – molecular oxygen

$O_2^{\cdot-}$ – superoxide anion

OEC – oxygen evolving complex

PAM – pulse amplitude modulated

PCA – principal component analysis

PMSF – phenylmethylsulfonyl fluoride

P_N – Net carbon dioxide assimilation rate

PO_4^{3-} – phosphate

PPFD – photosynthetic photon flux density

PS – photosystems

PSI – photosystem I

PSII – photosystem II

PVPP – polyvinylpolypyrrolidone

qPCR – quantitative real-time PCR

R – reverse primer

rETR – relative electron transport rate

RLC – rapid light curves

ROS – reactive oxygen species

RT-qPCR – reverse transcription real-time polymerase chain reaction

RuBisCO - ribulose-1,5-bisphosphate carboxylase-oxygenase

SEM – standard error of the mean

-SH – sulfhydryl groups

SLA – specific leaf area

SO_4^{2-} – sulphate

SOD – superoxide dismutase

SOS – salt overly sensitive

SP – saturating light pulse

TAC – total antioxidant capacity

TFC – total flavonoid content

$TiSO_4$ – titanium sulphate

TPC – total phenolic content

WUE_i – intrinsic water use efficiency

ΦPSII – effective quantum yield of PSII

LIST OF SCIENTIFIC PUBLICATIONS

For the elaboration of this master thesis, data present in the following scientific article and communications was used:

Sousa, B.*; **Rodrigues, F.***; Soares, C.; Martins, M.; Azenha, M.; Lino-Neto, T.; Santos, C.; Cunha, A.; Fidalgo, F. – Insights into the combined impacts of heat and salt in tomato plants – a disbalance between nutrient uptake and redox homeostasis [submitted to *Antioxidants;* IF: 6.3].

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Sousa, B.; **Rodrigues, F.**; Soares, C.; Martins, M.; Cunha, A.; Lino-Neto, T.; Santos, C.; Fidalgo, F. (2021). Unravelling the impact of combined salt and heat stresses on tomato plants (*Solanum lycopersicum* L.). Plant Biology Europe. Turin, Italy. (Poster presentation – international congress). *Awarded as one of the best student posters by FESPB*;

Rodrigues, F.; Sousa, B.; Soares, C.; Martins, M.; Lino-Neto, T.; Santos, C.; Cunha, A.; Fidalgo, F. (2021). Tolerance response of tomato plants (*Solanum lycopersicum* L.) to climate change: biochemical aspects of salt- and/or heat-induced stress. II Plant Abiotic Stress Forum: An Integrative Lens Over Plant Adaptation. Porto, Portugal. (Oral presentation – international congress);

Rodrigues, F.; Sousa, B.; Soares, C.; Martins, M.; Cunha, A.; Fidalgo, F. (2021). Tolerance response of tomato plants (*Solanum lycopersicum* L.) to climate change: biochemical aspects of salinity- and/or heat-induced stress. IJUP 21 - Encontro de Jovens Investigadores da Universidade do Porto, Portugal. (Oral presentation – national congress).

CHAPTER I

General Introduction

1.1. The 21st century issues: climate change and food insecurity

Since the beginning of Humanity, when our ancestors lived as hunter-gatherers, humans have been strongly dependent on climate changes, that went from warm periods to ice ages, to survive. However, a life-changing event occurred with the unique climatic stability and warmth of the Holocene: humans became farmers and the development of agriculture allowed civilizations to thrive (Gowdy, 2020; Smith and Archer, 2020). Indeed, for thousands of years, population grew steadily. Nonetheless, over the last century, anthropogenic activity has impacted Earth in an unprecedented way. In the 40 years following the industrial revolution, growth rate tripled, and world population is now expected to reach the 9.8 billion mark until 2050 (Raza et al., 2019; United Nations, Department of Economic and Social Affairs, 2019). Moreover, with the Green Revolution, characterized by the intensification of agriculture through the use of fertilizers and pesticides, high-yield varieties, and the development of mechanized agricultural practices, human population grew exponentially (Smith and Archer, 2020). However, with great development comes a great cost – industrialization led to a severe increase of greenhouse gas emissions, being land clearing, crop production, and fertilization responsible for almost a quarter of it, consequently contributing to global warming and climate change (IPCC, 2021; Tubiello et al., 2021). Increased greenhouse gas emissions, soil depletion, polluted water and loss of biodiversity are just a few of the consequences of a careless industrialization that ultimately drastically influenced and continues to influence climate change at a pace never seen before. In the last years, extreme climatic events such as droughts, floods, cold and heat waves, and storms have been more frequent, thus imposing serious losses in crop yield (up to 70% since 1982) (IPCC, 2021; Raza et al., 2019). Moreover, the overall climatic instability and water use restrictions negatively influence soil systems, leading to salinization, changes in moisture and increased erosion that negatively impact agriculture, either due to nutrient loss, reduced carbon storage or soil degradation (Borrelli et al., 2020; Khan et al., 2021; Kopittke et al., 2019; Lal, 2021). Therefore, abrupt environmental changes, along with an increasing food demand, are already imposing serious challenges to crop production and food security worldwide (Raza et al., 2019).

1.2. Salinity

Soil salinization results from both natural and anthropogenic causes. In fact, the extent and distribution of salt-affected soils has been addressed recently at a universal scale, pointing that more than 424 million Mha of topsoil (0-30 cm) and 833 Mha of subsoil (30-100 cm) are impacted by salinity (FAO, 2021). Moreover, according to Shahid et al. (2018), it is estimated that 76 Mha of the saline soils are affected by secondary salinization – a result of human activities, such as the replacement of perennial vegetation with annual crops, as well as irrigation practices that use salt-rich water. Worryingly, this problem has not only been increasing at a fast pace, but it is also forecasted to be intensified by the present climatic instability (Mukhopadhyay et al., 2021). The consequences are alarming: every year 1.5 Mha of land are becoming unsuitable for crop production due to salinization and half of cultivable lands worldwide are expected to be lost by 2050 (Hossain, 2019; Mukhopadhyay et al., 2021). Indeed, as soil characteristics are strongly dependent on climate – either on wind, precipitation, or temperature – changes in the latter will lead to salt build-up (Mukhopadhyay et al., 2021; Shahid et al., 2018). Additionally, in arid and semi-arid regions, where persistent irrigation with poor-quality groundwater has taken place, the total salinity-affected area grows day by day and, as climate change will impose serious limitations regarding the use of proper water around the globe, this practice is expected to widespread, hence impairing even more soil quality and, consequently, agriculture (Daliakopoulos et al., 2016; FAO and ITPS, 2015; Haddeland et al., 2014; Koutroulis et al., 2013; Lal, 2021). Altogether, the aggravated effects of climate change on soil salinization will affect agroecosystems to an extent where they can no longer fulfill the increased demand for food from the ever-growing world population.

1.3. High temperatures

Since 1970, global surface temperature has been increasing at an unprecedented rate – faster than in any other 50-year period over the last 2,000 years – as a result of the emissions from human activities (IPCC, 2021). The last four decades have been successively warmer than any preceded decade after 1850. In fact, since pre-industrial levels, world temperature rose 1.1 °C, but alarmingly most of it occurred in the last 40 years (Gowdy, 2020; IPCC, 2021). Additionally, global average surface air temperature is expected to rise 0.4 °C until the early 2030 decade and it will continue to increase until at least mid-century, even under the most positive scenario (IPCC, 2021). Indeed, with continued greenhouse gas emissions, projections estimate that atmospheric temperature will rise, at least, 3.3 °C

by the end of the century [Figure 1.1; IPCC (2021)], which, in some regions, may surpass most crop plants' heat tolerance threshold – that rounds 35 °C (Wahid et al., 2007).

Besides, the forecasted global warming will impose more frequent and severe heat waves that will not only directly impact plant productivity, but also provide even more favorable conditions for pests and diseases, while also affecting water availability (Khan et al., 2021; Lal, 2021). As alarming as the increase of Earth's temperature, is the way it affects several aspects of climate. Global warming is already accountable for 50% of sea level rise during 1971-2018 and is expected to contribute to the current climatic instability by reducing snow cover and permafrost, as well as increasing the frequency and intensity of marine heatwaves, heavy precipitation, agricultural and ecological droughts, tropical cyclones, and hot extremes (IPCC, 2021). Such climate-related disasters have already been responsible for a quarter of agricultural losses in developing countries (Lesk et al., 2016), which are especially susceptible to greater losses due to their close location to the equator (Anderson et al., 2020; Cline, 2008), leading to a severe reduction of crop production that will, ultimately, put food security at risk (Raza et al., 2019). Altogether, global warming and its impact on agriculture is a very serious problem that needs to be addressed so that the food supply is assured.

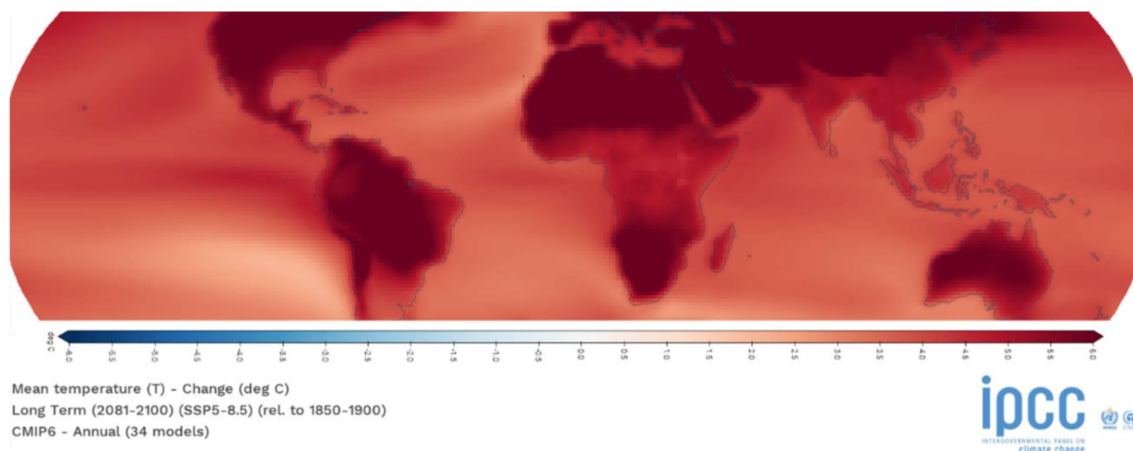

Figure 1.1. The long-term projections for mean surface temperature globally. Retrieved from IPCC (2021).

1.4. Salinity and heat stresses: an overview on physiological disorders

As has been established, the previous environmental conditions represent major threats to crop yield. Both heat and salinity induce changes in several metabolic and physiological routes – such as growth, water relations, nutrient homeostasis, photosynthesis, and oxidative metabolism – which portray the harmful effects of these stressors at the whole-plant level (Hassan et al., 2021; Parihar et al., 2015) , as will be addressed in the sections below.

1.4.1. Growth, water relations and ion imbalance

The devastating implications of heat and salinity exposure limit plant growth and productivity by interfering from an early stage of development. For instance, both stressors negatively affect seed germination, reduce biomass, root elongation, and plant height, which ultimately results in poor yield rates (Fahad et al., 2017; Hassan et al., 2021; Isayenkov, 2012; Parihar et al., 2015; Wahid et al., 2007). Even though similar macroscopic implications on growth may arise from the exposure to salt and high temperatures, the causes underlying such impacts can be quite different. Curiously, salinity stress – defined as the detrimental effects caused by the exposure of plants to excess ions, such as sodium (Na^+) and chlorine (Cl^-) – influences plant growth and development even prior to salt uptake by roots (Parihar et al., 2015). In this way, plant growth is impaired in two distinct phases: firstly, as a consequence of the water deficit effect (osmotic phase), and later due to the salt-specific effects (ionic phase) (Figure 1.2.). Salt build-up in soil decreases plants' water uptake by decreasing soil water potential with increasing salt concentrations (Parihar et al., 2015). Despite this, at low or moderate saline conditions, plants can adjust osmotically and allow the influx of water. However, in soil, salt competes with other essential nutrients, resulting in disrupted ion ratios that may escalate to nutrient disorders [calcium (Ca^{2+}), magnesium (Mg^{2+}), nitrogen (N), phosphate (PO_4^{3-}) and potassium (K^+)] and ion toxicity [Na^+, Cl^- and sulphate (SO_4^{2-})] (Gupta and Huang, 2014). Even though several studies in the past decades addressed the mechanisms by which Na^+ and Cl^- are uptaken, these processes remain unclear (Isayenkov and Maathuis, 2019). Still, it is believed that Na^+ takes advantage of K^+ transporters, for example, the ones from the AKT family, as well as the HAK/KUP/KT transporters, even with low affinity to Na^+ (Isayenkov, 2012). Additionally, in order to maintain the K^+/Na^+ ratio inside the cells, it was thought that plants increased the expression of high affinity K^+ transporters, such as the HKT family carriers, however these transporters have also been reported to uptake Na^+ (Isayenkov, 2012). Besides, it may also be possible that LCT family transporters are involved, as they have been shown to be non-selective cation carriers and that the salt sensitivity of

yeast mutants increased when *LCT1* was overexpressed (Amtmann et al., 2001). Moreover, the NSCC family of non-selective cation channels seem to play a role in Na^+ uptake. Furthermore, both the symplast and the apoplast pathways have been documented to be involved in ion uptake (Isayenkov and Maathuis, 2019). Despite, under physiological conditions, the symplastic pathway being predominant, the apoplastic route gains relevance when transpiration is increased, and could account for 50% of total Na^+ uptake, as it has been reported for rice (Kronzucker and Britto, 2011; Malagoli et al., 2008). Once inside the cells, and due to Na^+ and K^+ similarity in terms of ionic radius and ionic hydration energy, these ions compete for binding sites. As several enzymes are activated by K^+, this competition often results in the disruption of protein synthesis and enzymatic reactions, which are key metabolic processes for plant growth and development (Shabala and Munns, 2017).

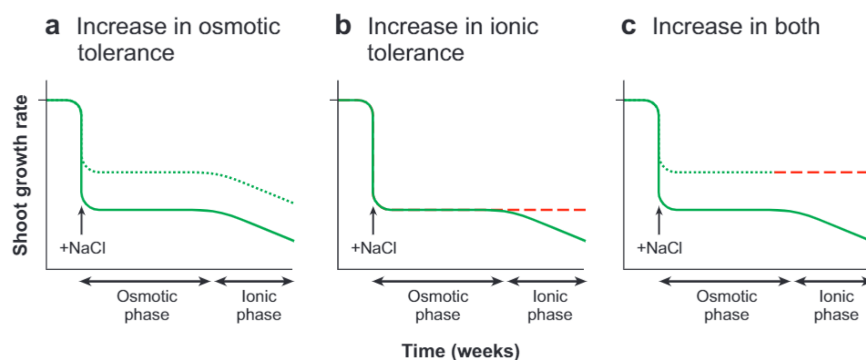

Figure 1.2. The distinct behavior of sensitive and tolerant plants to salinity stress and the impact of the osmotic and ionic phases on growth rate in saline conditions. The full green line represents sensitive species, while the dotted green line indicates the response of osmotic tolerant plants. The red dotted line represents plants with increased ionic tolerance. Retrieved from Munns (2005).

On the other hand, at high temperatures, evapotranspiration rate increases, which, along with the reduction of water uptake, disturbs water balance and, consequently, impacts plant and cell metabolism – photosynthesis, respiration, senescence – resulting in diminished growth (Hassan et al., 2021; Wahid et al., 2007). Despite the knowledge gap regarding the effects of heat stress on roots, it is known that it reduces the activity of nutrient uptake proteins, most likely as a result of the poor translocation of carbohydrates from shoots to roots, as well as impaired root conductance, which not only hampers nutrient uptake itself, but also their ratios (Hasanuzzaman et al., 2013; Hassan et al., 2021). Indeed, a few authors addressing this process reported decreased total nutrient concentrations and attributed such effect to root biomass reduction, as well as diminished root hair surface (Bassirirad, 2000; Klimenko et al., 2011; Rennenberg et al., 2006). Nonetheless, these effects are dependent on nutrient and plant

species and should not be generalized as this topic is still unclear and requires further investigation (Fahad et al., 2017; Hassan et al., 2021).

1.4.2. Oxidative stress and the antioxidant (AOX) system

Among the main plant responses to abiotic stress is the induction of oxidative stress, which gave a bad reputation to reactive oxygen species (ROS), as they are responsible for serious damage to a variety of biomolecules and portray one of the major causes for the impairment of plant growth and development (Hasanuzzaman et al., 2013; Hassan et al., 2021; Parihar et al., 2015). However, even under physiological conditions, these compounds are naturally and continuously produced by the activation or reduction of molecular oxygen (O_2) due to the aerobic and photosynthetic metabolism. Therefore, it is no surprise that the main sources of generation of ROS in plants are the ones where aerobic reactions occurs, namely the chloroplasts, mitochondria, and peroxisomes (Medina et al., 2021; Soares et al., 2019; Xie et al., 2019). In this case, these chemical species, at low concentrations, act as signaling agents; however, under stressful conditions, the disruption of the equilibrium between their production and detoxification leads to an overaccumulation of ROS that culminates in oxidative bursts and triggers damage, such as protein oxidation, peroxidation of membrane lipids and enzyme deactivation, leading, ultimately, to cell unviability (Hasanuzzaman et al., 2013; Medina et al., 2021; Parihar et al., 2015; Soares et al., 2019). Salinity and heat stresses are no exception. Indeed, most studies where the stressors were applied in different plant models (*e.g.* rice, tomato, citrus, pea, and mustard) at distinct intensities and periods of exposure portray an enhancement of ROS production, mostly as a consequence of the disruption of metabolic pathways like photosynthesis and respiration – either due to salt- or heat-induced osmotic stress, Na^+ toxicity or direct high temperature-induced damage (Fahad et al., 2017; Hasanuzzaman et al., 2013; Hassan et al., 2021; Isayenkov, 2012; Medina et al., 2021; Parihar et al., 2015; Shabala and Munns, 2017). Even though there are many processes and sites of production, two chemical phenomena that result in the generation of the singlet oxygen ($^1O_2$) and of the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) stand out: the transference of excessive excitation energy and/or electrons to O_2, respectively (Soares et al., 2019). Among the main ROS, $O_2^{\bullet-}$ and H_2O_2 are considered the first to be generated and, even though their production is associated with electron transport chains (ETC), these ROS are distinct. While $O_2^{\bullet-}$ is moderately reactive due to its low mobility and short half-life, H_2O_2 stability confers a longer half-life and its neutral charge makes it able to cross membranes and, therefore, capable of damaging other molecules far away from its production sites (Soares et al., 2019; Xie et al., 2019). Nonetheless, and more dangerous than $O_2^{\bullet-}$ and H_2O_2, is their

interaction in the presence of redox-active metals, namely copper (Cu) and iron (Fe), which leads to the Haber-Weiss reaction and the consequent production of the most hazardous ROS, the $^{\bullet}OH$, that cannot be scavenged by enzymatic processes. Indeed, along with $O_2^{\bullet-}$, that highly reactive radical triggers a cascade of biochemical events, known as lipid peroxidation (LP), that affect polyunsaturated fatty acids, and not only produce other reactive compounds [*e.g.* malondialdehyde (MDA)], but also compromise membrane integrity, fluidity and selectivity (Soares et al., 2019). In fact, direct damages on membrane structure have been documented under heat stress, but also as a result of Na^+ toxicity, thus leading to the disruption of several metabolic pathways essential for proper growth and development (Ahanger et al., 2019, 2020; Ahmad et al., 2012, 2010; Jin et al., 2016; Liu and Huang, 2000; Raja et al., 2020). Even though LP is mainly caused by $^{\bullet}OH$ and $O_2^{\bullet-}$, $^1O_2$ can also be involved. Additionally, $^1O_2$ damages the photosystems I (PSI) and II (PSII), which are considered the preferential sites of its production. The production of $^1O_2$ is due to the transition of chlorophyll singlet to chlorophyll triplet state (Chl $\rightarrow$ ^{3}Chl*), and the subsequent transference of excitation energy from ^{3}Chl* to $^3O_2$ (^{3}Chl* + $^3O_2$ $\rightarrow$ Chl + $^1O_2$) during photosynthesis as a result of high light or limitation in carbon dioxide (CO_2) assimilation (Soares et al., 2019).

In order to maintain adequate ROS levels and redox homeostasis, plants possess powerful scavenging systems that involve AOX metabolites and enzymes that are often promptly activated under abiotic stress, such as salinity and heat (Fahad et al., 2017; Gupta and Huang, 2014; Hasanuzzaman et al., 2013; Hassan et al., 2021; Isayenkov, 2012; Parihar et al., 2015; Soares et al., 2019; Wahid et al., 2007). The first enzymatic line of defence from ROS is brought up by superoxide dismutase (SOD; EC 1.15.1.1) which catalyses the dismutation of $O_2^{\bullet-}$ into H_2O_2 (Figure 1.3), thus preventing the production of $^{\bullet}OH$ through the Haber-Weiss reaction. The previous reaction, however, may increase the levels of H_2O_2, which are then scavenged enzymatically, for instance, by catalase (CAT; EC 1.11.1.6) and/or ascorbate peroxidase (APX; EC 1.1.11.1) (Figure 1.3). Despite their similar function, these enzymes are quite different. While CAT does not require reducing power, APX uses reduced ascorbate (AsA) to detoxify H_2O_2. Moreover, due to its high catalytic activity when H_2O_2 is severely overproduced, CAT is often correlated with damage prevention, whereas APX activity, as a result of its elevated affinity to H_2O_2, is more related to signaling events (Soares et al., 2019). In order to keep APX activity, AsA must be regenerated enzymatically. When it is being used as substrate for APX, AsA is oxidized into monodehydroascorbate (MDHA), which is either converted back into AsA by monodehydroascorbate reductase (MDHAR; EC 1.6.5.6) or spontaneously forms dehydroascorbate (DHA), that is then reduced to AsA by dehydroascorbate reductase (DHAR; EC

1.8.5.1), using glutathione (GSH) as substrate (Figure 1.3). This thiol is then regenerated by glutathione reductase (GR; EC 1.6.4.2), thus allowing the continuous supply of AsA. This pathway that regenerates two major AOXs is known as the AsA-GSH cycle and often plays a crucial role in ensuring redox homeostasis (Soares et al., 2019). Indeed, the activation of the overall enzymatic component of the AOX system has been reported under different concentrations of sodium chloride (NaCl) (50, 100, 120, 150 and 200 mM) in tomato (*Solanum lycopersicum* L. cvs. Huange 108 and Chibli F1) (Ahanger et al., 2019, 2020; Manai et al., 2014; Mittova et al., 2004), mulberry (*Morus alba* L. cvs. Local and Sujanpuri) (Ahmad et al., 2010), mustard (*Brassica juncea* L. cvs. Varuna, RH-30 and Rohini) (Ahmad et al., 2012), wheat (*Triticum aestivum* L. cvs. KRL-19 and WH-542) (Mandhania et al., 2006), corn (*Zea mays* L.) (Azooz et al., 2009), adzuki bean (*Vigna angularis* Willd.) (Ahanger et al., 2020) and citrus [*Citrus sinensis* (L.) Osbeck] (Gueta-Dahan et al., 1997); and in tomato (Raja et al., 2020; Rivero et al., 2004), moth bean (*Vigna aconitifolia* Jacq.) (Harsh et al., 2016) and mustard (Hayat et al., 2009) exposed to heat stress.

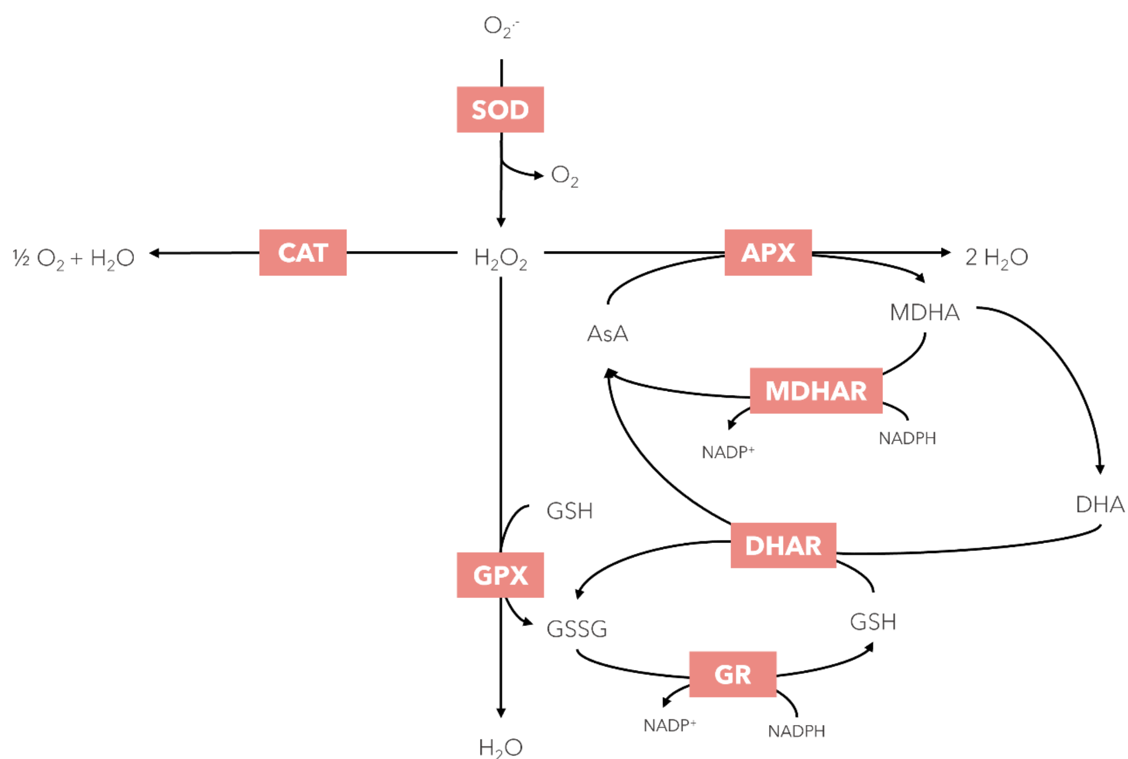

Figure 1.3. Overview of the enzymatic mechanisms for the detoxification of $O_2^{\cdot-}$ and H_2O_2 and the regeneration pathways of the compounds involved. Superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidases (GPX), ascorbate (AsA), monodehydroascorbate (MDHA), dehydroascorbate (DHA), reduced glutathione (GSH), oxidized glutathione (GSSG).

In conjunction, the non-enzymatic components, which comprise low molecular weight cellular compounds, directly detoxify the plant from ROS and/or act indirectly as substrates for the enzymatic system (Soares et al., 2019). Among the many compounds with AOX functions is the powerful osmolyte, proline. This secondary amino acid acts as a ROS scavenger, namely of $^{\bullet}OH$ and $^{1}O_2$, as well as a membrane stabilizer (Soares et al., 2019). Besides, it is believed to stabilize the structure of proteins by acting as a molecular chaperone and it allows the uptake of water by maintaining the hypertonic status of cells (Singh et al., 2015). Due to its properties, and considering the water deficit effect of salinity stress, it is no surprise that this metabolite plays a crucial role in protecting plants against salt-induced damages. Indeed, when different species are exposed to different concentrations of NaCl, there is a high accumulation of this osmoprotectant (Ahmad et al., 2012, 2010; Babu and Devaraj, 2008; Fidalgo et al., 2004). Proline has also proved its importance in plants under heat stress, as moth bean (Harsh et al., 2016), french bean (*Phaseolus vulgaris* L. cv. S-9) (Babu and Devaraj, 2008), mustard (Hayat et al., 2009), wheat cv. WH 711 (Khan et al., 2013) and tomato (Raja et al., 2020) under heat stress, have significantly increased its levels. Another very commonly accumulated AOX metabolite is the above mentioned AsA, better known as vitamin C, which not only serves as substrate for APX, allowing the detoxification of H_2O_2, but also directly interacts with different ROS ($^{1}O_2$, $^{\bullet}OH$ and $O_2^{\bullet-}$) (Soares et al., 2019). Indeed, the exogenous application of AsA mitigates or alleviates the negative effects of both salt and high temperature exposures (Alayafi, 2020; Dolatabadian and Jouneghani, 2009). As forenamed, important for the regeneration of the previous metabolite is GSH. Nonetheless, this non-protein thiol plays other valuable roles. GSH not only directly scavenges H_2O_2, $^{\bullet}OH$ and $O_2^{\bullet-}$, but also, due to its -SH group, maintains the reduced state of numerous compounds by acting as cellular buffer, thus ensuring redox homeostasis (Soares et al., 2019). In fact, crop species, such as adzuki bean (Ahanger et al., 2020), tomato cv. Huange 108 and Tmknvf2 (Ahanger et al., 2019; Ahanger et al., 2020; Rivero et al., 2004) and french bean cv. S-9 (Babu and Devaraj, 2008) under salinity or heat stresses often present elevated levels of this thiol, demonstrating the pivotal role this AOX plays. Besides, specialized compounds, such as phenols, play a variety of roles in plants: from being signaling molecules to protecting against oxidative damage as a consequence of abiotic stress (Soares et al., 2019). Indeed, there is a class of phenols exclusively produced by plants, flavonoids, that apart from interacting with ROS – directly or indirectly – also boost the AOX properties of other metabolites, limit oxidative damage and contribute to membrane lipid homeostasis (Soares et al., 2019).

Although this collection of metabolites represents the major players of the non-enzymatic component of the AOX system, many more compounds are also valuable in response to oxidative stress – among them are sugars, carotenoids (addressed in the next section) and polyamines. These compounds, along with the efficient enzymatic mechanisms, may allow the maintenance of redox homeostasis and the proper functioning of cell metabolism (Medina et al., 2021; Soares et al., 2019). Nonetheless, under severe stressful conditions, it may not be sufficient to fully detoxify ROS, which results in an impairment of physiological, biochemical and molecular networks that reduce productivity and yield (Fahad et al., 2017; Gupta and Huang, 2014; Hasanuzzaman et al., 2013; Hassan et al., 2021; Isayenkov, 2012; Parihar et al., 2015).

1.4.3. Photosynthesis

As photoautotrophic organisms, plants convert solar radiation into chemical energy to produce carbohydrates from carbon dioxide (CO_2) in a process denominated photosynthesis, which occurs in the chloroplast, and comprises two different phases. The light-dependent reactions, or the photochemical phase, that occur in the thylakoid membranes, are involved in the production of reducing power (NADPH) and energy (ATP), while the carbon reduction reactions, or Calvin-Benson cycle, that occur in the stroma, use the products of the former to fix CO_2 into organic molecules (Ashraf and Harris, 2013; Taiz et al., 2015). For this biochemical pathway to start, photosynthetically active radiation must be absorbed by photosynthetic pigments, chlorophylls and carotenoids, which are associated with the two photosystems (PS) located in the thylakoid membranes (Taiz et al., 2015). After photons are absorbed, these photoreceptors change from the ground state to an excited state and then lose their energy by transferring it to other photoreceptors, as heat, or as radiation (fluorescence) (Taiz et al., 2015). The photosynthetic pigments of the antenna transfer the excitation energy to the reaction centers of the PS (Taiz et al., 2015). With this, the obtention of reducing power, through an electron transport chain (ETC) embedded in the thylakoid membrane, begins (Singh and Thakur, 2018). In this chain, two components – the photosystem I (PSI) and photosystem II (PSII) – are responsible for the electron flow. Yet, contrarily to PSI, PSII, which is the first component of the ETC, is able to photooxidize water to receive an electron that will be transported along the ETC to reduce $NADP^+$ to NADPH and produce ATP. In order to do so, this protein complex contains: i) the core complex – where the reaction center P680 is located; ii) the oxygen-evolving complex (OEC) – involved in the water splitting reaction – and iii) the light-harvesting complex (LHCII) – where pigments absorb photons (Derks et al., 2015; Singh and Thakur, 2018; Taiz et al., 2015). Once the ETC fulfills the supply of NADPH and ATP, the carbon reduction reactions begin. The Calvin-Benson

cycle is divided in 3 consecutive steps: carboxylation, reduction and regeneration (Taiz et al., 2015). For carbon (C) fixation to occur, CO_2 must diffuse from the atmosphere to the stroma, entering the leaves by the stomata. Then, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO; EC 4.1.1.39), the most abundant enzyme in plant leaves, is responsible for its fixation (Erb and Zarzycki, 2018; Spreitzer and Salvucci, 2002; Taiz et al., 2015). RuBisCO activity is vital for crop productivity as it catalyses the first step of two competing biochemical pathways: photosynthesis and photorespiration (Erb and Zarzycki, 2018).

Being a crucial biochemical pathway for plants, photosynthesis is greatly affected by salinity – either as a result of the induction of osmotic stress or as a consequence of Na^+ toxicity (Ashraf and Harris, 2013; Parihar et al., 2015; Singh and Thakur, 2018) – but is also highly sensitive to high temperatures (Fahad et al., 2017; Hasanuzzaman et al., 2013; Hassan et al., 2021; Mathur et al., 2014).

In the case of photosynthetic pigments, the impacts of the exposure to salinity and heat are similar, even though the reasons behind it are not. Both stressors are frequently accountable for diminished chlorophyll content by interfering mostly in their biosynthesis, but also in their breakdown (Ashraf and Harris, 2013; Santos, 2004). However, while high temperatures lead to the deactivation of several enzymes involved in both pathways [*e.g.*. 5-aminolevulinic dehydratase (EC 4.2.1.24), porphobilinogen deaminase (EC 2.5.1.61)], Na^+ toxicity reduces the levels of chlorophyll precursors (*e.g.* 5-aminolevulinic acid and glutamate) (Ashraf and Harris, 2013; Santos, 2004). Moreover, salinity may increase chlorophyll degradation by causing nutrient imbalances. Mg^{2+}, whose uptake is often disrupted under salt exposure, is a key component of chlorophylls that is frequently remobilized to young tissues to ensure growth and development in such adverse conditions (Peng et al., 2019).

Given their functions, it is no surprise that both PSI and PSII are essential for the good functioning of the ETC, however the latter is highly sensitive to several environmental conditions, including salinity and, especially, high temperatures (Hasanuzzaman et al., 2013; Parihar et al., 2015). In fact, this component of the ETC is often damaged and/or its repair is inhibited due to the overproduction of the singlet oxygen ($^1O_2$), that occurs as a consequence of low intercellular CO_2 concentration – commonly reported under saline conditions – or due to the formation of chlorophyll triplet state as a result of insufficient energy dissipation in cases of high light intensity (Nishiyama and Murata, 2014; Parihar et al., 2015). Contrastingly, under heat stress, the chloroplast structure suffers major alterations, namely in the organization of thylakoids, grana stacking and swelling, dislodging of LHCII due to changes in the fluidity of the thylakoid membranes (Fahad et al., 2017; Hassan et al., 2021; Mathur et al., 2014), which

consequently affects photosynthesis. In both cases, a general decline in photochemical quenching parameters (maximum quantum yield and effective quantum efficiency of PSII), as well as in the electron transport rate (ETR) takes place, while an increase in non-photochemical quenching (NPQ) parameters is commonly reported (Ashraf and Harris, 2013; Fahad et al., 2017; Hasanuzzaman et al., 2013; Hassan et al., 2021; Parihar et al., 2015; Singh and Thakur, 2018). Such increase can be defined as a tolerance trait as it dissipates excessive light energy in the form of heat so that the overproduction of ROS is avoided. Indeed, besides being important pigments for numerous physiological processes, carotenoids, and especially xantophylls, play a role in the response to abiotic stress. Some xantophylls, namely zeaxanthin, are involved in this process as they are able to quench the excited status of singlet chlorophyll when exposed to excessive radiation (Bassi, 2021; Liu et al., 2015), thus protecting PSII from damage. Additionally, under stress conditions, zeaxanthin is capable of alleviating salt-induced photoinhibition by scavenging $^1O_2$ and/or free radicals in the thylakoid membranes (Liu et al., 2015; Soares et al., 2019). However, carotenoids are frequently reduced upon salt exposure due to the repressed expression of the genes that encode essential enzymes involved in their biosynthetic pathway [*e.g.* phytoene synthase (EC 2.5.1.32), zeta carotene desaturase (EC 1.3.5.6) and lycopene β-cyclase (EC 5.5.1.19)], not being capable of protecting the PSII, and, consequently resulting in limitations to the photosynthetic performance (Ann et al., 2011; Maurya et al., 2015).

Numerous environmental stimuli, namely salt and heat exposure, influence stomatal resistance mostly as a consequence of their interference with water relations (Singh and Thakur, 2018). This triggers stomatal closure, thus preventing excessive water losses through transpiration (Hassan et al., 2021; Mathur et al., 2014; Parihar et al., 2015; Singh and Thakur, 2018). However, a contrasting response has also been documented for heat-stressed plants. When water scarcity is not imposed, higher temperatures may lead to an increase in stomatal conductance and, therefore, in the transpiration rate, allowing cooling down and the alleviation of the stress (Hasanuzzaman et al., 2013). Nonetheless, it is common that heat stressed plants also portray signs of water shortage. In this case, their response is similar to the one perceived upon salinity stress. Under these circumstances, a decrease in stomatal aperture has been frequently reported alongside the impairment of photosynthesis, as this coping mechanism not only limits CO_2 assimilation, thus reducing the synthesis of photoassimilates, but also results in overproducing $^1O_2$, which hampers growth (Hassan et al., 2021; Singh and Thakur, 2018). Besides, both stressors also negatively impact RuBisCO. However, despite the carboxylase activity of this enzyme increasing under heat stress, the relative specificity of this enzyme to CO_2 and solubility of this molecule decrease, when

compared to O_2, favouring the oxygenase activity and, therefore, the photorespiratory pathway (Hassan et al., 2021; Mathur et al., 2014). Although this process is able to drain the products of the photochemical phase, the photosynthetic efficiency is highly diminished and, in severe cases, its machinery may still be saturated by photons, making the reaction centres of the PSI and PSII the main sources of ROS (Hassan et al., 2021).

Overall, even though salinity and heat stresses lead to damages in the photosynthetic apparatus in distinct ways, both stressors greatly hamper this vital physiological process, and directly or indirectly – through the overproduction of ROS – negatively affect growth and development, thus portraying major threats to crop productivity and yield (Fahad et al., 2017; Hasanuzzaman et al., 2013; Hassan et al., 2021; Parihar et al., 2015).

1.4.4. Tolerance mechanisms

Due to their sessile condition, plants are especially vulnerable to biotic or abiotic factors that affect them in several distinct ways (Suzuki et al., 2014). However, during millions of years of evolution, plants developed a set of physiological, biochemical, and molecular mechanisms that allow them to withstand stressful situations, through a multitude of avoidance and tolerance strategies (Fahad et al., 2017; Gupta and Huang, 2014; Hassan et al., 2021; Isayenkov, 2012; Parihar et al., 2015; Wahid et al., 2007).

Under salt exposure, growth and development strongly depend on ion homeostasis, compartmentalization, and transport. As high concentrations of Na^+ in the cytoplasm are harmful to cell metabolism, plants sequester this ion into the vacuole via an Na^+/H^+ antiporter from the NHX family (Isayenkov, 2012). However, the accumulation of cytotoxic levels of Na^+ in the vacuole has its limitations and when maximum capacity is surpassed, this ion is transported to older leaves, which are sacrificed on behalf of the younger tissues (Parihar et al., 2015). Nonetheless, the most relevant strategy for the maintenance of ion homeostasis and salt tolerance is the activation of the Salt Overly Sensitive (SOS) signaling pathway (Figure 1.4), which comprises three key proteins: SOS1, SOS2 and SOS3, that mediate the efflux of Na^+ to the apoplast (Gupta and Huang, 2014; Isayenkov, 2012; Taiz et al., 2015). For it to occur, Ca^{2+} ions must bind to SOS3, so that it activates SOS2, that, consequently, phosphorylates SOS1, which increases its Na^+/H^+ antiporter activity, while diminishing Na^+ toxicity (Gupta and Huang, 2014; Taiz et al., 2015). Besides these tolerance traits, salt-stressed plants often portray an accumulation of compatible solutes, among them proline, glycine betaine and trehalose. The increased biosynthesis of these molecules has the purpose of maintaining osmotic balance, while protecting cell structures (Gupta

and Huang, 2014). Moreover, as described in section 1.3.2., plants under saline conditions often present an increased activity of SOD, CAT, APX, GPX and GR, as well as an accumulation of AOX metabolites, which are correlated with salt tolerance. Therefore, a few of the strategies that allow plants to withstand salinity stress include: the effort to maintain ion homeostasis by compartmentalization and transport, the accumulation of osmoprotectants and compatible solutes and the activation of the AOX system (both enzymatic and non-enzymatic), along with the synthesis of polyamines and hormone modulation (Gupta and Huang, 2014; Parihar et al., 2015).

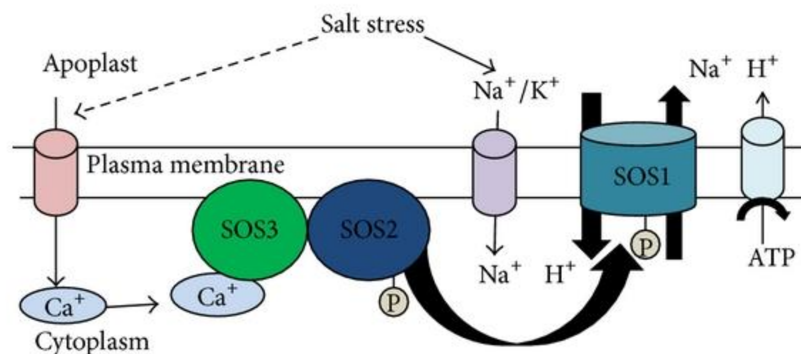

Figure 1.4. The SOS signaling pathway as a tolerance trait for the efflux of Na^+ under saline conditions. Retrieved from Gupta and Huang (2014).

Similarly, in response to high temperatures, plants often increase the activity of AOX enzymes, which seems to max out at 35-40 °C (even though, it may depend on the species), and accumulate AOXs and compatible solutes – that maintain cell turgor and organise both proteins and cellular structures – so as to ensure redox homeostasis (Fahad et al., 2017; Hassan et al., 2021). However, other strategies are also adopted to achieve tolerance, such as changes in the composition and degree of saturation of fatty acids and alterations in membrane permeability (Hassan et al., 2021). Additionally, heat stress triggers the production of heat shock proteins (HSPs), which are divided into five different classes, according to their molecular weight, among them the major ones: HSP90 and HSP70. Even though the mechanisms underlying stress tolerance are yet unclear, it has been reported that tolerant varieties present higher levels of expression of HSPs than the susceptible ones (Al-Whaibi, 2011; Hasan et al., 2021). In fact, it is believed that these proteins act as chaperones, ensuring the functioning and stability of other proteins by preventing their denaturation, while also promoting their renaturation and, in tomato, HSFA1, a transcription factor, seems to be a key regulator of the heat shock response (Al-Whaibi, 2011; Mathur et al., 2014).

1.5. Combined stress: an approach to a more realistic insight into plant physiology

In the face of ongoing and projected climate change, catastrophic events, such as intense heat waves and acute periods of drought, as well as the aggravation of several adverse environmental conditions (*e.g.* soil salinization, strong radiation, elevated atmospheric CO_2 levels) are expected to occur more frequently and often in combination, resulting in major losses for agricultural production, especially in arid and semi-arid regions and in the Mediterranean basin (IPCC, 2021; Rivero et al., 2021; Zandalinas et al., 2020). Prior to the development of efficient strategies to tackle such a disastrous outcome, a more realist approach and understanding of the impacts of climate change on crops adaptation is needed.

Up until now, and as exemplified in the sections above, the effects of individual exposure to environmental stresses have been extensively studied, which allowed an important insight of plants' response to a variety of abiotic factors, such as heat, salinity, cold and drought (Mittler, 2006). Nonetheless, under natural and realistic conditions, plants are exposed to a multitude of abiotic and biotic factors that may interact and trigger synergic, antagonist, and/or combinatory effects of different pathways, networks, and mechanisms that are activated by each of the different stresses, or even by the activation of unique and complex molecular and metabolic responses that are yet unknown and cannot be deduced from the effects of an individual exposure (Mittler, 2006; Rivero et al., 2021; Suzuki et al., 2014; Zandalinas et al., 2020). Recently, several authors tackled this gap of knowledge by evaluating biochemical, physiological, and molecular processes under the combination of a different set of abiotic stresses, namely drought and heat (Correia et al., 2018; Raja et al., 2020; Rizhsky et al., 2004; Zhou et al., 2017), heat and high light (Szymańska et al., 2017), and salinity and heat (Lopez-Delacalle et al., 2021; Rivero et al., 2014). The latter two reported a new and unexpected response to the combination of salinity (75 mM and 120 mM NaCl) and heat (35 °C for 14 days and for 72 h) stresses in tomato plants. Overall, the simultaneous exposure led to a significant improvement of growth and photosynthetic performance, as well as a lesser accumulation of ROS than the individual salt exposure, which was attributed to the prompter activation of important stress-related pathways, such as the proline and AsA metabolisms (Lopez-Delacalle et al., 2021; Rivero et al., 2014). Despite these authors being in accordance, reporting an amelioration of the negative effects of salt exposure, Zandalinas et al. (2020) documented that in *Arabidopsis thaliana* (L.) Heynh. the combination of the same stressors resulted in a harsher effect than that found for the individual treatments. Therefore, the reported effects of the interaction between distinct stressors are not always consistent and may actually be conflicting as the

mechanisms underlying plant response to the combination of stresses depend on the model species and on the extent, sternness and mode of exposure of each stressor (Zandalinas et al., 2020). As the authors that addressed the heat and salinity problematics on tomato (Lopez-Delacalle et al., 2021; Rivero et al., 2014) used hydroponic models and lower temperatures than those predicted in the most recent studies, these findings may be underestimating the effect of the stressors, hence the necessity to study this combination in more realistic and practical scenarios. In conclusion, not only there is an urgent need to investigate and better understand the crosstalk between pathways, networks, and mechanisms affected by each stress but also to address it in the most realistic way, so that new strategies are developed to efficiently tackle the challenges imposed by the present and forecasted climatic instability (Zandalinas et al., 2020).

1.6. Tomato production within the Mediterranean region: Is there a threat?

Due to its unquestionable commercial value, tomato plants (*Solanum lycopersicum* L.) have been widely used as model organisms for fleshy-fruited plants with the objective of increasing fruit quality, productivity, and tolerance to both biotic and abiotic stresses (Kimura and Sinha, 2008). Native of the western edge of the South American continent and later brought to Europe (Peralta and Spooner, 2007), tomato is very much used as a fresh product in several cuisines and is also the main component of processed products like sauces, soups, pastes and juices (Hossain, 2021). Thus, tomato's popularity places it among the most produced crops worldwide just after maize, rice, wheat, potato, soybean, and cassava (FAO, 2020). Indeed, globally, tomato production reached more than 180 million t in 2019 on a cultivated area that surpassed the 5 Mha (FAO, 2020). The temperate climate of the Mediterranean region allowed tomato production to thrive, making countries like Portugal, Spain, and Italy the main producers in Europe, which, together, account for half the production in the continent (FAO, 2020). Nonetheless, in the last few years, production has declined, plausibly due to the unstable climate (Eurostat, 2021). Indeed, the Mediterranean region has not only been considered the most vulnerable one in Europe to soil degradation and desertification (Ferreira et al., 2022) but it is also already affected by soil salinization (Figure 1.5) and this problematic is expected to be further aggravated by climate change, hence imposing serious challenges for crop production (Daliakopoulos et al., 2016; IPCC, 2021).

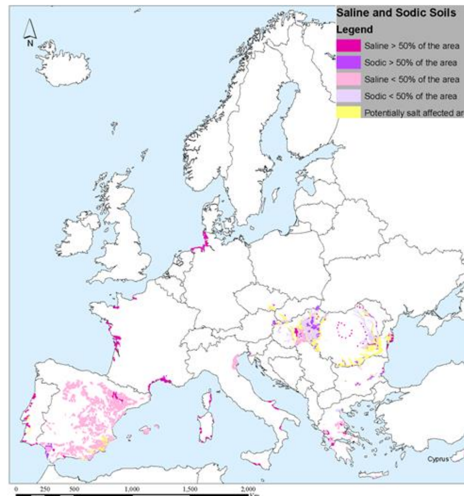

Figure 1.5. Characterization of soils in the European Union based on their salt content. Retrieved from European Soil Data Centre (2008).

Furthermore, this region is expected to face intense and long heat waves, as the average temperature for the Mediterranean region will increase about 5 °C considering the whole year – while between June and August it is forecasted to rise almost 7 °C – (Figure 1.6) (IPCC, 2021). Additionally, maximum daily temperatures above 40 °C are projected for up to 50 days per year (Carvalho et al., 2021). In other words, the Mediterranean region, and especially the Mediterranean basin, is already being affected by both salinity and heat phenomena, that in the future will escalate to an extent that will result in the loss of agricultural productivity, namely in tomato.

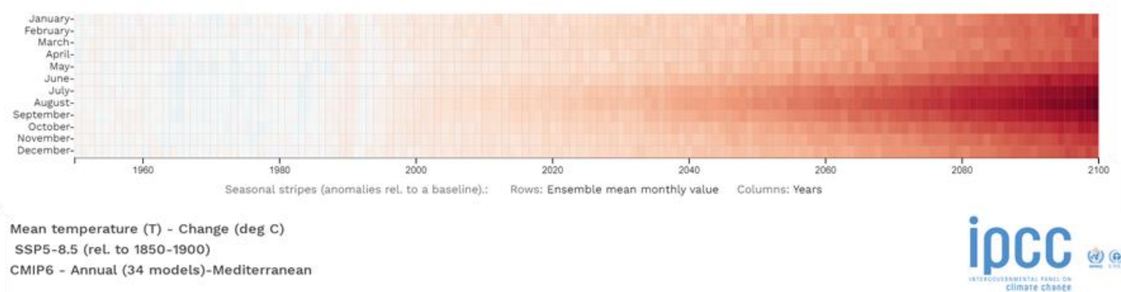

Figure 1.6. The forecasted seasonal surface temperature in the Mediterranean Region. Darker colors represent higher anomalies relative to a baseline. Decreases in temperature are portrayed by white and bluish colors, while reddish colors represent increased temperatures. Retrieved from IPCC (2021).

References

Ahanger, M.A., Aziz, U., Alsahli, A.A., Alyemeni, M.N., Ahmad, P., 2020. Influence of Exogenous Salicylic Acid and Nitric Oxide on Growth, Photosynthesis, and Ascorbate-Glutathione Cycle in Salt Stressed *Vigna angularis*. Biomolecules. https://doi.org/10.3390/biom10010042

Ahanger, M.A., Mir, R.A., Alyemeni, M.N., Ahmad, P., 2020. Combined effects of brassinosteroid and kinetin mitigates salinity stress in tomato through the modulation of antioxidant and osmolyte metabolism. Plant Physiol. Biochem. 147, 31–42. https://doi.org/ 10.1016/j.plaphy.2019.12.007

Ahanger, M.A., Qin, C., Maodong, Q., Dong, X.X., Ahmad, P., Abd-Allah, E.F., Zhang, L., 2019. Spermine application alleviates salinity induced growth and photosynthetic inhibition in *Solanum lycopersicum* by modulating osmolyte and secondary metabolite accumulation and differentially regulating antioxidant metabolism. Plant Physiol. Biochem. 144, 1–13. https://doi.org/10.1016/j.plaphy.2019.09.021

Ahmad, P., Jaleel, C.A., Sharma, S., 2010. Antioxidant defense system, lipid peroxidation, proline-metabolizing enzymes, and biochemical activities in two *Morus alba* genotypes subjected to NaCl stress. Russ. J. Plant Physiol. 57, 509–517. https://doi.org/10.1134/S1021443710040084

Ahmad, P., Kumar, A., Ashraf, M., Akram, N.A., 2012. Salt-induced changes in photosynthetic activity and oxidative defense system of three cultivars of mustard (*Brassica juncea* L.). African J. Biotechnol. 11, 2694–2703. https://doi.org/10.1016/j.sjbs.2013.01.006

Al-Whaibi, M.H., 2011. Plant heat-shock proteins: A mini review. J. King Saud Univ. - Sci. 23, 139–150. https://doi.org/10.1016/j.jksus.2010.06.022

Alayafi, A.A.M., 2020. Exogenous ascorbic acid induces systemic heat stress tolerance in tomato seedlings: transcriptional regulation mechanism. Environ. Sci. Pollut. Res. 27, 19186–19199. https://doi.org/10.1007/s11356-019-06195-7

Amtmann, A., Fischer, M., Marsh, E.L., Stefanovic, A., Sanders, D., Schachtman, D.P., 2001. The Wheat cDNA *LCT1* Generates Hypersensitivity to Sodium in a Salt-Sensitive Yeast Strain. Plant Physiol. 126, 1061–1071. https://doi.org/10.1104/pp.126.3.1061

Anderson, R., Bayer, P.E., Edwards, D., 2020. Climate change and the need for agricultural adaptation. Curr. Opin. Plant Biol. 56, 197–202. https://doi.org/10.1016/j.pbi.2019.12.006

Ann, B.M., Devesh, S., Gothandam, K.M., 2011. Effect of salt stress on expression of carotenoid pathway genes in tomato. J. Stress Physiol. Biochem. 7.

Ashraf, M., Harris, P.J.C., 2013. Photosynthesis under stressful environments: An overview. Photosynthetica 51, 163–190. https://doi.org/10.1007/s11099-013-0021-6

Azooz, M., Ismail, A., Elhamd, M.F.A., 2009. Growth, Lipid Peroxidation and Antioxidant Enzyme Activities as a Selection Criterion for the Salt Tolerance of Maize Cultivars Grown under Salinity Stress. Int. J. Agric. Biol. 11, 21–26.

Babu, R.N., Devaraj, V.R., 2008. High temperature and salt stress response in French bean (*Phaseolus vulgaris*). Aust J Crop Sci 2, 40–48.

Bassi, R., 2021. A new function for the xanthophyll zeaxanthin: glueing chlorophyll biosynthesis to thylakoid protein assembly. Biochem. J. 478, 61–62. https://doi.org/10.1042/BCJ20200803

Bassirirad, H., 2000. Kinetics of nutrient uptake by roots: responses to global change. New Phytol. 147, 155–169. https://doi.org/10.1046/j.1469-8137.2000.00682.x

Borrelli, P., Robinson, D.A., Panagos, P., Lugato, E., Yang, J.E., Alewell, C., Wuepper, D., Montanarella, L., Ballabio,

C., 2020. Land use and climate change impacts on global soil erosion by water (2015-2070). Proc. Natl. Acad. Sci. 117, 21994–22001. https://doi.org/10.1073/pnas.2001403117

Carvalho, D., Cardoso Pereira, S., Rocha, A., 2021. Future surface temperature changes for the Iberian Peninsula according to EURO-CORDEX climate projections. Clim. Dyn. 56, 123–138. https://doi.org/10.1007/s00382-020-05472-3

Cline, W.R., 2008. Global Warming and Agriculture. Finance Dev. 0045, A007. https://doi.org/10.5089/9781451922349.022.A007

Correia, B., Hancock, R.D., Amaral, J., Gomez-Cadenas, A., Valledor, L., Pinto, G., 2018. Combined Drought and Heat Activates Protective Responses in *Eucalyptus globulus* That Are Not Activated When Subjected to Drought or Heat Stress Alone. Front. Plant Sci. 9, 819. https://doi.org/10.3389/fpls.2018.00819

Daliakopoulos, I.N., Tsanis, I.K., Koutroulis, A., Kourgialas, N.N., Varouchakis, A.E., Karatzas, G.P., Ritsema, C.J., 2016. The threat of soil salinity: A European scale review. Sci. Total Environ. 573, 727–739. https://doi.org/10.1016/j.scitotenv.2016.08.177

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. Biochim. Biophys. Acta - Bioenerg. 1847, 468–485. https://doi.org/10.1016/j.bbabio.2015.02.008

Dolatabadian, A., Jouneghani, R.S., 2009. Impact of Exogenous Ascorbic Acid on Antioxidant Activity and Some Physiological Traits of Common Bean Subjected to Salinity Stress. Not. Bot. Horti Agrobot. Cluj-Napoca 37, 165–172. https://doi.org/10.15835/nbha3723406

Erb, T.J., Zarzycki, J., 2018. A short history of RubisCO: the rise and fall (?) of Nature's predominant CO_2 fixing enzyme. Curr. Opin. Biotechnol. 49, 100–107. https://doi.org/10.1016/j.copbio.2017.07.017

European Soil Data Centre, 2008. Saline and Sodic Soils Map for EU-27 [WWW Document]. URL https://esdac.jrc.ec.europa.eu/content/saline-and-sodic-soils-european-union (acessed 25.9.21)

Eurostat, 2021. Crop production in national humidity [WWW Document]. URL https://ec.europa.eu/eurostat/databrowser/view/APRO_CPNH1__custom_1223424/default/table?lang=en (acessed 12.8.21)

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. Front. Plant Sci. 8, 1147. https://doi.org/10.3389/fpls.2017.01147

FAO, 2021. Global map of salt-affected soils. [WWW Document] URL https://www.fao.org/3/cb7247en/cb7247en.pdf (accessed 27.12.21).

FAO, 2020. FAOSTAT Stat. Database. [WWW Document] URL https://www.fao.org/faostat/en/#data/QCL (acessed 16.11.21)

FAO and ITPS, 2015. Status of World's Soil Resources (SWSR) - Main Report. [WWW Document] URL https://www.fao.org/3/i5199e/I5199E.pdf (acessed 16.11.21)

Ferreira, C.S.S., Seifollahi-Aghmiuni, S., Destouni, G., Ghajarnia, N., Kalantari, Z., 2022. Soil degradation in the European Mediterranean region: Processes, status and consequences. Sci. Total Environ. 805, 150106. https://doi.org/10.1016/j.scitotenv.2021.150106

Fidalgo, F., Santos, A., Santos, I., Salema, R., 2004. Effects of long-term salt stress on antioxidant defence systems, leaf water relations and chloroplast ultrastructure of potato plants. Ann. Appl. Biol. 145, 185–192. https://doi.org/10.1111/j.1744-7348.2004.tb00374.x

Gowdy, J., 2020. Our hunter-gatherer future: Climate change, agriculture and uncivilization. Futures 115, 102488. https://doi.org/10.1016/j.futures.2019.102488

Gueta-Dahan, Y., Yaniv, Z., Zilinskas, B.A., Ben-Hayyim, G., 1997. Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in Citrus. Planta 203, 460–469. https://doi.org/10.1007/s004250050215

Gupta, B., Huang, B., 2014. Mechanism of Salinity Tolerance in Plants: Physiological, Biochemical, and Molecular Characterization. Int. J. Genomics 2014, 701596. https://doi.org/10.1155/2014/701596

Haddeland, I., Heinke, J., Biemans, H., Eisner, S., Flörke, M., Hanasaki, N., Konzmann, M., Ludwig, F., Masaki, Y., Schewe, J., Stacke, T., Tessler, Z.D., Wada, Y., Wisser, D., 2014. Global water resources affected by human interventions and climate change. Proc. Natl. Acad. Sci. 111, 3251–3256. https://doi.org/10.1073/pnas.1222475110

Harsh, A., Sharma, Y.K., Joshi, U., Rampuria, S., Singh, G., Kumar, S., Sharma, R., 2016. Effect of short-term heat stress on total sugars, proline and some antioxidant enzymes in moth bean (*Vigna aconitifolia*). Ann. Agric. Sci. 61, 57–64. https://doi.org/10.1016/j.aoas.2016.02.001

Hasanuzzaman, M., Nahar, K., Alam, M.M., Roychowdhury, R., Fujita, M., 2013. Physiological, Biochemical, and Molecular Mechanisms of Heat Stress Tolerance in Plants. Int. J. Mol. Sci. https://doi.org/10.3390/ijms14059643

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., 2021. Heat stress in cultivated plants: nature, impact, mechanisms, and mitigation strategies—a review. Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol. 155, 211–234. https://doi.org/10.1080/11263504.2020.1727987

Hayat, S., Masood, A., Yusuf, M., Fariduddin, Q., Ahmad, A., 2009. Growth of Indian mustard (*Brassica juncea* L.) in response to salicylic acid under high-temperature stress. Brazilian J. Plant Physiol. 21, 187–195. https://doi.org/10.1590/S1677-04202009000300003

Hossain, M.F., 2021. Effect of Planting Time on the Yield and Quality of Cherry Tomato (*Solanum lycopersicum* var. *cerasiforme*). Int. J. Hortic. Sci. Technol. 8, 123–131. https://doi.org/10.22059/ijhst.2020.314445.421

Hossain, M.S., 2019. Present Scenario of Global Salt Affected Soils, its Management and Importance of Salinity Research. Int. Res. J. Biol. Sci. 1, 1–3. https://doi.org/irjbs.2019.1.3

IPCC, 2021: *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* [Masson-Delmotte, V., P. Zhai, A. Pirani, S.L., Connors, C. Péan, S. Berger, N. Caud, Y. Chen, L. Goldfarb, M.I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J.B.R., Matthews, T.K. Maycock, T. Waterfield, O. Yelekçi, R. Yu, and B. Zhou (eds.)]. Cambridge University Press. In Press.

Isayenkov, S. V, 2012. Physiological and molecular aspects of salt stress in plants. Cytol. Genet. 46, 302–318. https://doi.org/10.3103/S0095452712050040

Isayenkov, S. V, Maathuis, F.J.M., 2019. Plant Salinity Stress: Many Unanswered Questions Remain. Front. Plant Sci. 10, 80. https://doi.org/10.3389/fpls.2019.00080

Jin, R., Wang, Y., Liu, R., Gou, J., Chan, Z., 2016. Physiological and Metabolic Changes of Purslane (*Portulaca oleracea* L.) in Response to Drought, Heat, and Combined Stresses. Front. Plant Sci. 6, 1123. https://doi.org/10.3389/fpls.2015.01123

Khan, M.I.R., Iqbal, N., Masood, A., Per, T.S., Khan, N.A., 2013. Salicylic acid alleviates adverse effects of heat stress on photosynthesis through changes in proline production and ethylene formation. Plant Signal. Behav.

8, e26374. https://doi.org/10.4161/psb.26374

Khan, N., Ray, R.L., Sargani, G.R., Ihtisham, M., Khayyam, M., Ismail, S., 2021. Current Progress and Future Prospects of Agriculture Technology: Gateway to Sustainable Agriculture. Sustain. . https://doi.org/10.3390/su13094883

Kimura, S., Sinha, N., 2008. Tomato (*Solanum lycopersicum*): A Model Fruit-Bearing Crop. CSH Protoc. 2008, pdb.emo105. https://doi.org/10.1101/pdb.emo105

Klimenko, N.V., Peshkova, S.B., Dorofeev, A.A., 2011. Nitrate reductase activity during heat shock in winter wheat. J. Stress Physiol. Biochem. 2, 50–55.

Kopittke, P.M., Menzies, N.W., Wang, P., McKenna, B.A., Lombi, E., 2019. Soil and the intensification of agriculture for global food security. Environ. Int. 132, 105078. https://doi.org/10.1016/j.envint.2019.105078

Koutroulis, A.G., Tsanis, I.K., Daliakopoulos, I.N., Jacob, D., 2013. Impact of climate change on water resources status: A case study for Crete Island, Greece. J. Hydrol. 479, 146–158. https://doi.org/10.1016/j.jhydrol.2012.11.055

Kronzucker, H.J., Britto, D.T., 2011. Sodium transport in plants: a critical review. New Phytol. 189, 54–81. https://doi.org/10.1111/j.1469-8137.2010.03540.x

Lal, R., 2021. Chapter 31 - Climate change and agriculture, Letcher, T.M., (Eds.). Climate Change: Observed Impacts on Planet Earth Elsevier, pp. 661–686. https://doi.org/10.1016/B978-0-12-821575-3.00031-1

Lesk, C., Rowhani, P., Ramankutty, N., 2016. Influence of extreme weather disasters on global crop production. Nature 529, 84–87. https://doi.org/10.1038/nature16467

Liu, L., Shao, Z., Zhang, M., Wang, Q., 2015. Regulation of Carotenoid Metabolism in Tomato. Mol. Plant 8, 28–39. https://doi.org/10.1016/j.molp.2014.11.006

Liu, X., Huang, B., 2000. Heat Stress Injury in Relation to Membrane Lipid Peroxidation in Creeping Bentgrass. Crop Sci. 40, 503–510. https://doi.org/10.2135/cropsci2000.402503x

Lopez-Delacalle, M., Silva, C.J., Mestre, T.C., Martinez, V., Blanco-Ulate, B., Rivero, R.M., 2021. Synchronization of proline, ascorbate and oxidative stress pathways under the combination of salinity and heat in tomato plants. Environ. Exp. Bot. 183, 104351. https://doi.org/10.1016/j.envexpbot.2020.104351

Malagoli, P., Britto, D.T., Schulze, L.M., Kronzucker, H.J., 2008. Futile Na^+ cycling at the root plasma membrane in rice (*Oryza sativa* L.): kinetics, energetics, and relationship to salinity tolerance. J. Exp. Bot. 59, 4109–4117. https://doi.org/10.1093/jxb/ern249

Manai, J., Kalai, T., Gouia, H., Corpas, F.J., 2014. Exogenous nitric oxide (NO) ameliorates salinity-induced oxidative stress in tomato (*Solanum lycopersicum*) plants. J. soil Sci. plant Nutr. 14, 433–446. http://dx.doi.org/10.4067/S0718-95162014005000034

Mandhania, S., Madan, S., Sawhney, V., 2006. Antioxidant defense mechanism under salt stress in wheat seedlings. Biol. Plant. 50, 227–231. https://doi.org/10.1007/s10535-006-0011-7

Mathur, S., Agrawal, D., Jajoo, A., 2014. Photosynthesis: Response to high temperature stress. J. Photochem. Photobiol. B Biol. 137, 116–126. https://doi.org/10.1016/j.jphotobiol.2014.01.010

Maurya, V.K., Srinvasan, R., Ramesh, N., Anbalagan, M., Gothandam, K.M., 2015. Expression of Carotenoid Pathway Genes in Three Capsicum Varieties under Salt Stress. Asian J. Crop Sci. 7, 286–294. https://doi.org/10.3923/ajcs.2015.286.294

Medina, E., Kim, S.-H., Yun, M., Choi, W.G., 2021. Recapitulation of the Function and Role of ROS Generated in Response to Heat Stress in Plants. Plants. https://doi.org/10.3390/plants10020371

Mittler, R., 2006. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 11, 15–19. https://doi.org/10.1016/j.tplants.2005.11.002

Mittova, V., Guy, M., Tal, M., Volokita, M., 2004. Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. J. Exp. Bot. 55, 1105–1113. https://doi.org/10.1093/jxb/erh113

Mukhopadhyay, R., Sarkar, B., Jat, H.S., Sharma, P.C., Bolan, N.S., 2021. Soil salinity under climate change: Challenges for sustainable agriculture and food security. J. Environ. Manage. 280, 111736. https://doi.org/10.1016/j.jenvman.2020.111736

Munns, R., 2005. Genes and salt tolerance: bringing them together. New Phytol. 167, 645–663. https://doi.org/10.1111/j.1469-8137.2005.01487.x

Nishiyama, Y., Murata, N., 2014. Revised scheme for the mechanism of photoinhibition and its application to enhance the abiotic stress tolerance of the photosynthetic machinery. Appl. Microbiol. Biotechnol. 98, 8777–8796. https://doi.org/10.1007/s00253-014-6020-0

Parihar, P., Singh, S., Singh, R., Singh, V.P., Prasad, S.M., 2015. Effect of salinity stress on plants and its tolerance strategies: a review. Environ. Sci. Pollut. Res. 22, 4056–4075. https://doi.org/10.1007/s11356-014-3739-1

Peng, Y.Y., Liao, L.L., Liu, S., Nie, M.M., Li, J., Zhang, L.D., Ma, J.F., Chen, Z.C., 2019. Magnesium Deficiency Triggers SGR–Mediated Chlorophyll Degradation for Magnesium Remobilization. Plant Physiol. 181, 262–275. https://doi.org/10.1104/pp.19.00610

Peralta, I.E., Spooner, D.M., 2007. History, origin and early cultivation of tomato (Solanaceae). Razdan, M.K., Mattoo, A.K. (Eds.), Genetic Improvement of Solanaceous Crops. Science Publishers. 2, 1–24.

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 10, 208. https://doi.org/10.1007/s13205-020-02206-4

Raza, A., Razzaq, A., Mehmood, S.S., Zou, X., Zhang, X., Lv, Y., Xu, J., 2019. Impact of Climate Change on Crops Adaptation and Strategies to Tackle Its Outcome: A Review. Plants . https://doi.org/10.3390/plants8020034

Rennenberg, H., Loreto, F., Polle, A., Brilli, F., Fares, S., Beniwal, R.S., Gessler, A., 2006. Physiological Responses of Forest Trees to Heat and Drought. Plant Biol. 8, 556–571. https://doi.org/10.1055/s-2006-924084

Rivero, R.M., Mestre, T.C., Mittler, R., Rubio, F., Garcia-Sanchez, F., Martinez, V., 2014. The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. Plant. Cell Environ. 37, 1059–1073. https://doi.org/10.1111/pce.12199

Rivero, R.M., Mittler, R., Blumwald, E., Zandalinas, S.I., 2021. Developing climate-resilient crops: improving plant tolerance to stress combination. Plant J. https://doi.org/10.1111/tpj.15483

Rivero, R.M., Ruiz, J.M., Romero, L., 2004. Oxidative metabolism in tomato plants subjected to heat stress. J. Hortic. Sci. Biotechnol. 79, 560–564. https://doi.org/10.1080/14620316.2004.11511805

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., Mittler, R., 2004. When Defense Pathways Collide. The Response of Arabidopsis to a Combination of Drought and Heat Stress. Plant Physiol. 134, 1683–1696. https://doi.org/10.1104/pp.103.033431

Santos, C., 2004. Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. Sci. Hortic. (Amsterdam). 103, 93–99. https://doi.org/10.1016/j.scienta.2004.04.009

Shabala, S., Munns, R., 2017. Salinity stress: physiological constraints and adaptive mechanisms. Shabala, S.

(Eds.), Plant Stress Physiology. Cabi. https://doi.org/http://dx.doi.org/10.1079/9781845939953.0059

Shahid, S.A., Zaman, M., Heng, L., 2018. Soil Salinity: Historical Perspectives and a World Overview of the Problem - Guideline for Salinity Assessment, Mitigation and Adaptation Using Nuclear and Related Techniques, in: Zaman, M., Shahid, S.A., Heng, L. (Ed.). Springer International Publishing, Cham, pp. 43–53. https://doi.org/10.1007/978-3-319-96190-3_2

Singh, J., Thakur, J.K., 2018. Photosynthesis and Abiotic Stress in Plants Biotic and Abiotic Stress Tolerance in Plants, Vats, S. (Ed.). Springer Singapore, Singapore, pp. 27–46. https://doi.org/10.1007/978-981-10-9029-5_2

Singh, M., Kumar, J., Singh, S., Singh, V.P., Prasad, S.M., 2015. Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. Rev. Environ. Sci. Bio/Technology 14, 407–426. https://doi.org/10.1007/s11157-015-9372-8

Smith, G.R., Archer, R., 2020. Climate, population, food security: adapting and evolving in times of global change. Int. J. Sustain. Dev. World Ecol. 27, 419–423. https://doi.org/10.1080/13504509.2020.1712558

Soares, C., Carvalho, M.E.A., Azevedo, R.A., Fidalgo, F., 2019. Plants facing oxidative challenges—A little help from the antioxidant networks. Environ. Exp. Bot. 161, 4–25. https://doi.org/10.1016/j.envexpbot.2018.12.009

Spreitzer, R.J., Salvucci, M.E., 2002. RuBisCO: Structure, Regulatory Interactions, and Possibilities for a Better Enzyme. Annu. Rev. Plant Biol. 53, 449–475. https://doi.org/10.1146/annurev.arplant.53.100301.135233

Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and biotic stress combinations. New Phytol. 203, 32–43. https://doi.org/10.1111/nph.12797

Szymańska, R., Ślesak, I., Orzechowska, A., Kruk, J., 2017. Physiological and biochemical responses to high light and temperature stress in plants. Environ. Exp. Bot. 139, 165–177. https://doi.org/10.1016/j.envexpbot.2017.05.002

Taiz, L., Zeiger, E., Møller, I.M., Murphy, A., 2015. Plant physiology and development. 6th ed. Sinauer Associates Incorporated, Sunderland, MA, USA.

Tubiello, F.N., Rosenzweig, C., Conchedda, G., Karl, K., Gütschow, J., Xueyao, P., Obli-Laryea, G., Wanner, N., Qiu, S.Y., Barros, J. De, Flammini, A., Mencos-Contreras, E., Souza, L., Quadrelli, R., Heiðarsdóttir, H.H., Benoit, P., Hayek, M., Sandalow, D., 2021. Greenhouse gas emissions from food systems: building the evidence base. Environ. Res. Lett. 16, 65007. https://doi.org/10.1088/1748-9326/ac018e

United Nations, Department of Economic and Social Affairs, 2019. World Population Prospects 2019: Highlights. [WWW Document] URL https://population.un.org/wpp/Publications/Files/WPP2019_Highlights.pdf (acessed 23.9.21)

Wahid, A., Gelani, S., Ashraf, M., Foolad, M.R., 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 61, 199–223. https://doi.org/10.1016/j.envexpbot.2007.05.011

Xie, X., He, Z., Chen, N., Tang, Z., Wang, Q., Cai, Y., 2019. The Roles of Environmental Factors in Regulation of Oxidative Stress in Plant. Biomed Res. Int. 2019, 9732325. https://doi.org/10.1155/2019/9732325

Zandalinas, S.I., Fritschi, F.B., Mittler, R., 2020. Signal transduction networks during stress combination. J. Exp. Bot. 71, 1734–1741. https://doi.org/10.1093/jxb/erz486

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 Biol. 17, 24. https://doi.org/10.1186/s12870-017-0974-x

CHAPTER II

Biological Questions and Main Goals

Given the lack of knowledge in climate-related damages in important crops and, most importantly, the necessity of filling those gaps in the state of the art so that new strategies can be developed to ensure that food security is maintained, this MSc dissertation aims at unravelling the mechanisms underlying tomato plants' response to combined salt and heat stress, under pot conditions. Among the many varieties and cultivars of this species, cherry tomato (*Solanum lycopersicum* L. var. *cerasiforme*) plants were chosen as model, since interest in its cultivation is growing stronger due to their ability to produce tasty and nutritious fruits. To tackle this main goal, several questions need to be answered:

a) Is the combination of heat and salinity merely the sum of its parts or if there are new and complex mechanisms triggered by this situation that need to be considered?

b) How does the combination of heat and salinity influence the growth and development of tomato plants?

c) How is nutrient uptake influenced by the combined stress?

d) How does it affect the redox homeostasis of plant cells?

e) How does the antioxidant system respond to the combination of these stressors?

f) How does the simultaneous exposure impact the photosynthetic performance?

CHAPTER III

Insights into the combined impacts of heat and salt in tomato plants – a disbalance between nutrient uptake and redox homeostasis

Abstract

Currently, salinity and heat are two critical threats to crop production and food security that are being aggravated by the global climatic instability. In this scenario, prior to the development of stress-tolerant crops, it is imperative to understand plant responses to the simultaneous exposure to different stressors and the crosstalk between underlying functional mechanisms. Thus, in this study, the physiological and biochemical response of potted tomato plants (*Solanum lycopersicum* L.) to the combination of salinity [100 mM sodium chloride (NaCl)] and heat (42 °C; 4 h d^{-1}) stress was evaluated. After 21 days of co-exposure, the concentration of sodium (Na^{+}) was severely increased, while the levels of calcium (Ca^{2+}), potassium (K^{+}) and magnesium (Mg^{2+}) were depleted. In fact, the accumulation of Na^{+} in plant tissues was superior when salt-treated plants were also exposed to high temperatures than in the individual saline treatment, leading to a harsher negative effect of both factors on growth (length and dry weight). Despite that, neither oxidative damage nor a major accumulation of reactive oxygen species (ROS) was registered in the stressed plants, mostly due to the overall accumulation of antioxidant (AOX) metabolites (proline, thiols, glutathione) alongside the activation of several AOX enzymes (catalase, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase). Nonetheless, the accumulation of toxic ions, coupled with the high energy costs associated with the stimulation of osmolytes and the maintenance of the redox homeostasis, heavily impaired the ability of tomato plants to grow properly when the combination of salinity and high temperatures was imposed. Thus, it is clear that, under a climate change scenario, the simultaneous exposure to different abiotic stressors can severely threaten the growth and productivity of crop plants since, at least in these specific – but highly common – stressors, the co-exposure appears to exacerbate the negative effects of the individual factors.

Keywords: antioxidant system; climate change; high temperatures; oxidative stress; salinity; *Solanum lycopersicum*.

1. Introduction

Human societies, since millennia ago, have been built around stable and efficient agricultural practices meeting a wide range of human needs, most notably food, fibres, fuels, and raw materials. Up until recently, agriculture has been evolving and serving its purpose, but the increasing world population associated with a frightening scenario of climatic instability is taking a heavy toll on the ability of this sector to efficiently respond to the needs of our modern society (FAO, 2009; Prosekov and Ivanova, 2018). In fact, total arable area has been rapidly declining worldwide due to soil degradation (*e.g.* heavy salinization, nutrient deficiency, contamination) and the higher occurrence of drastic climatic events, such as extreme temperatures, drought or floods (Qafoku, 2015; St.Clair and Lynch, 2010).

For example, it is estimated that around 4 Mha of European soils are moderate to highly degraded by secondary salinization, mostly due to irrigation with saline water and poor drainage conditions, which is one of the main factors driving the desertification of the Mediterranean coast (Daliakopoulos et al., 2016). Although this factor itself is already worrying in what concerns agriculture demand, this trend will escalate even more due to the impacts of other climate change-related variables. For instance, the projected continuous increases in global temperatures will affect the hydrological cycle and reduce the extent of watercourses, while intensifying water demand for crop irrigation, leading to an increased use of poor-quality water and to higher salt build-ups after evaporation (Daliakopoulos et al., 2016; Haddeland et al., 2014; Koutroulis et al., 2013). This is particularly problematic in regions with low rainfall and high evapotranspiration, such as the Mediterranean basin, where projections indicate that, throughout this century, this region could face up to 50 days per year with maximum daily temperatures above 40 °C (an increase of 10–25 days per year considering the present scenario) (Carvalho et al., 2021). In fact, even when not taking into account the cumulative or synergistic effects of other stress variables, most crops are not adapted to such drastic increases in temperature, with the heat-stress threshold of most crops being around 25-35 °C (Wahid et al., 2007).

Up to now, there is extensive literature regarding the effects of salinity or high temperatures on the growth and development of several plants, as both conditions can vastly affect the germination and developmental processes, impair photosynthetic performance, and compromise water relations and the nutrient balance, ultimately leading to reduced yield and loss of viability [as reviewed by Parihar et al. (2015) and Wahid et al. (2007)]. Indeed, a proper nutrient supply is of extreme importance for an optimal development and growth. However, salinity deeply affects nutrient balance, by lowering the assimilation of potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}), which are of high importance in numerous

pathways and networks (Isayenkov and Maathuis, 2019; Parihar et al., 2015), while simultaneously increasing the uptake of sodium (Na^+) and chloride (Cl^-), which can be highly toxic and interfere with several essential cellular processes (Isayenkov and Maathuis, 2019). Nonetheless, and while both stressors may lead to similar end results in plant growth through different affected pathways, one feature that is commonly and similarly affected by the exposure to salt or heat is the cellular redox status, whose disruption prompts oxidative bursts that can severely damage cell integrity. In fact, and while in non-stressful conditions there is a tight regulation between the generation of reactive oxygen species (ROS) and their detoxification, in situations of stress this balance can be threatened through an over-production of ROS and/or the inhibition of the antioxidant (AOX) machinery, ultimately leading to the loss of cell viability and death (Soares et al., 2019a).

However, and despite the knowledge regarding the effects of different abiotic stressors, it is important to have in mind that in a real environmental context crops are exposed to a multitude of factors whose impacts on the plants' physiological performance are not always easily extrapolated from what occurs in the presence of an individual stressor (Jin et al., 2016; Rizhsky et al., 2004; Sun et al., 2015; Suzuki et al., 2014; Zhou et al., 2017). Nonetheless, very few authors have tackled the impacts of a consistently warmer and more saline environment (either through soil salinization or poor water quality) on plants (Li et al., 2011; Lopez-Delacalle et al., 2021; Rivero et al., 2014; Suzuki et al., 2016; Zhao et al., 2010).

Thus, and considering all that has been mentioned, more studies must focus on important crops that are seriously threatened by the changing climate. For instance, countries in the Mediterranean region are highly associated with tomato (*Solanum lycopersicum* L.) production, where it has been cultivated for centuries, with Spain and Portugal consistently being in the top five of tomato producers in Europe. However, this crop faces serious threats, being reported that the forecasted climate change will severely affect tomato yield, with high temperatures and soil salinity being the major stress factors acting in this region (Carvalho et al., 2021; Daliakopoulos et al., 2016). Nevertheless, in this species, only two studies have been conducted so far. Rivero et al. (2014) and Lopez-Delacalle et al. (2021) showed that, in comparison to the individual treatments, the combination of salt and heat can differentially affect several pathways and improve water efficiency. However, the use of hydroponic growing systems and persistent but lower temperatures (35 °C) than those considered in current projections, might not accurately reflect the response of a usually potted and Summer-grown plant, especially when these stressors are only applied for a short duration.

In this sense, the main goal of this work is to understand how periodic exposure to high temperatures (42 °C) and irrigation with saline water [100 mM sodium chloride (NaCl)] affects the performance of tomato plants, under pot conditions. To address these objectives, several biological questions need to be answered throughout this research – a) How does the combination of heat and salinity affect the growth and development of tomato plants?; b) Does this combination of stressors disrupt the redox and nutrient balance of these plants?; c) How does the AOX system respond to these stress-induced redox fluctuations?; and d) Is the combination of heat and salinity merely the sum of its parts or are there new and complex mechanisms triggered by this situation that need to be carefully considered?

2. Materials and Methods

2.1. Plant material and growth conditions

Seeds of *Solanum lycopersicum* L. var. *cerasiforme* (cherry tomato) were surface disinfected by immersion in 70% (v/v) ethanol for 5 min, followed by a 5 min incubation in 20% (v/v) commercial bleach (5% active chloride), containing 0.02% (w/v) tween®-20. Both procedures were performed under constant agitation, followed by successive clean-ups with deionized water (dH_2O). Then, seeds were placed in Petri dishes (10 cm diameter) containing solidified [0.675% (w/v) agar] 0.5x MS medium, including Gamborg B5 vitamins (pH 5.5-6.0) (Murashige and Skoog, 1962), and left to germinate for 7 days in a growth chamber, under controlled conditions (16 h light/8 h dark, 25 °C, 150 μmol m^{-2} s^{-1}). After this period, plantlets with similar size and development were transferred to plastic pots filled with 600 mL Siro Royal universal substrate (SIRO©, Portugal; physicochemical characteristics in Supplementary Table S3.1) and grown under the same controlled conditions as above. To ensure replicability and avoid competition, three plants were sown per pot. During the first week, plantlets were acclimated to the new conditions, being irrigated only with dH_2O. A total of 28 pots were prepared.

2.2. Experimental design

After the 7-day acclimation period, pots were randomly divided into four trays (one per experimental condition), each containing at least four pots, and plants were grown for the next 21 days under the following treatments:

CTL (Control) **–** Plants were irrigated every other day with dH_2O;

SALT – Plants were irrigated every other day with a 100 mM NaCl (11 dS m^{-1}) solution (60 mL per pot);

HEAT – Plants were irrigated every other day with dH_2O and transferred to a twin growth chamber at 42 °C, for 4 h, every day;

COMBINED – Plants were irrigated every other day with 100 mM NaCl (60 mL per pot) and transferred to a twin growth chamber at 42 °C, for 4 h, every day.

The selection of NaCl concentration was based on previous bibliographic records (Debouba et al., 2006; Khavari-Nejad and Mostofi, 1998; Tanveer et al., 2020) and on preliminary assays performed in our laboratory (Supplementary Figure S3.1). Moreover, according to Ayers and Westcot (1985), the level of salinity applied here (equivalent to 11 dS m^{-1}, measured with CDM210 MeterLab electrical conductivity meter) in the irrigation water is just slightly above the tolerance threshold for moderately sensitive species (5 to 10 dS m^{-1} irrigation water electric conductivity), such as tomato. Thus, 100 mM NaCl is an adequate concentration to impose salt stress, while maintaining an environmentally relevant experimental design. Regarding the heat stress, this was induced by a daily 4 h exposure to 42 °C, based on the projections already mentioned for the Mediterranean region and was imposed between the 5th and 9th h of light, mimicking the hottest hours in a field-situation.

2.3. Plant harvest and biometric analysis

After 21 days of growth, plants were collected, thoroughly washed, divided into roots and shoots and the length and fresh weight (fw) of both parts were determined for all plants. Then, part of the plant material from each replicate of all experimental conditions was: i) left to dry in an oven at 60 °C, until reaching stable weight, to determine the dry weight (dw) and the water content; ii) immediately used for the estimation of superoxide anion ($O_2^{\bullet-}$) content; or iii) frozen and macerated in liquid nitrogen and stored at -80 °C until further use.

Since plant water content was affected by the applied stressors, biochemical parameters were expressed on a dw basis – estimated from the tissues' water content.

2.4. Element quantification – Na^+, K^+, Ca^{2+} and Mg^{2+}

For the quantification of inorganic elements (Na^+, K^+, Ca^{2+} and Mg^{2+}), four dried samples of roots and shoots of tomato plants (each sample comprising three plants) were crushed with an ultracentrifuge mill at 8,000 rpm (ZM 200, Retsch) and, then, three sub-samples (0.3-0.5 g) were digested in a microwave oven with 4 mL of concentrated nitric acid (HNO_3) and 2 mL 30% (w/v) hydrogen peroxide (H_2O_2). The digestion proceeded at 800 W during 10 min, followed by 5 min at 1,000 W and a cooling period of 15 min. Each clear solution obtained was quantitatively transferred to 50 mL volumetric flasks. The analysis

was performed by flame furnace atomic absorption spectroscopy (FAAS), operated at the optical and flame parameters recommended for the instrument used (Thermo Scientific, ICE 3300). Calibration was performed with external standards [in 0.5% (w/v) HNO_3] in the following ranges: Na^+ (0.1-0.8 mg L^{-1}), K^+ (0.2-1.6 mg L^{-1}), Ca^{2+} (0.3-2.5 mg L^{-1}) and Mg^{2+} (0.075-0.5 mg L^{-1}). Results were expressed as mg g^{-1} dw.

2.5. Determination of ROS content – superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2)

The estimation of $O_2^{\bullet-}$ content was performed in fresh samples of roots and shoots by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{\bullet-}$, in accordance to the protocol described by Sharma et al. (2017). In order to estimate $O_2^{\bullet-}$ levels, a standard curve was prepared using sodium nitrite and the absorbance (Abs) was read at 530 nm. Results were expressed as μmol g^{-1} dw.

The levels of H_2O_2 were determined by the titanium sulphate ($TiSO_4$) colorimetric method in accordance to de Sousa et al. (2013). The Abs of the yellowish complex, formed when an acidic solution of titanyl ions is mixed with H_2O_2, was read at 410 nm and results were expressed as μmol g^{-1} dw, using 0.28 μM^{-1} cm^{-1} as extinction coefficient (ε).

2.6. Estimation of the lipid peroxidation (LP) degree

LP was evaluated in accordance with Heath and Packer (1968), based on the determination of malondialdehyde (MDA) content, an end-product of this process (Soares et al., 2019a). Abs was read at 532 and 600 nm, with the latter being subtracted to the first to avoid the effects of non-specific turbidity. MDA content was expressed as nmol g^{-1} dw, using ε =155 mM^{-1} cm^{-1}.

2.7. Quantification of proline, ascorbate (AsA) and reduced glutathione (GSH)

Proline levels were determined via a ninhydrin-based colorimetric assay, first described by Bates et al. (1973). Abs were read at 520 nm, and proline content was estimated using a standard curve, prepared with known proline concentrations. The results were then expressed as mg g^{-1} dw.

Reduced ascorbate (AsA) was quantified through the methodology described by Gillespie and Ainsworth (2007), based on the AsA-mediated reduction of the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), which then forms a complex with 2-2'-bipyridyl, measurable at 525 nm. The same method was applied to determine the total AsA content, after samples were treated with dithiothreitol (DTT) to reduce the oxidized portion of this AOX [dehydroascorbate (DHA)]. Results were expressed as μmol g^{-1} dw, after preparing a standard curve with known AsA concentrations. DHA content was calculated by subtracting the reduced AsA to the total AsA pool.

The quantification of GSH (free and reduced glutathione) was performed in accordance with the protocol optimized by Soares et al. (2019b), which is based on the Glutathione Assay Kit (CS0260; Sigma-Aldrich®). Here, the complex formed between GSH and 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) was measured at 412 nm and GSH levels were estimated from a calibration curve prepared with known GSH concentrations. Results were expressed as nmol g^{-1} dw.

2.8. Quantification of total thiols and non-protein/protein-bound thiols ratio

Total thiol quantification was accomplished as described by Zhang et al. (2009), using DTNB to determine the concentration of sulfhydryl groups (-SH). Non-protein thiol quantification was performed according to the same method, but with the addition of 10% (w/v) sulfosalicylic acid to allow for protein precipitation. Both quantifications were done by measuring $Abs_{412\ nm}$ (using ε of 13,600 M^{-1} cm^{-1}) and the results were expressed as µmol g^{-1} dw. Protein-bound thiols were subsequently calculated by subtracting non-protein thiols to the total thiol content.

2.9. Determination of total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC)

The quantification of TPC, TFC and TAC was performed as described by Zafar et al. (2016). First, frozen shoot and root samples were homogenised with 80% (v/v) methanol, centrifuged for 10 min (2,500 *g*) and the supernatant collected and stored at -20 °C. Then, TPC was assessed through the Folin-Ciocalteu reaction, with Abs being read at 725 nm. Regarding TFC, the methanolic extracts were mixed with 10% (w/v) aluminium chloride ($AlCl_3$), 1 M potassium acetate (KCH_3COO), and dH_2O, and after a 30 min incubation in the dark, Abs were read at 415 nm. Lastly, TAC was estimated by mixing the supernatant with a reaction solution composed of 0.6 M sulphuric acid (H_2SO_4), 4 mM ammonium molybdate [$(NH_4)_6Mo_7O_{24}$] and 28 mM sodium phosphate (Na_2HPO_4). After a 90 min incubation, at 95 °C, Abs were read at 695 nm. The final values for all three parameters were estimated through standard curves that were prepared using gallic acid, quercetin and ascorbic acid for TPC, TFC and TAC, respectively, and expressed on a dw basis.

2.10. Enzymatic activity - superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.1.11.1), glutathione reductase (GR; EC 1.6.4.2) and dehydroascorbate reductase (DHAR; EC 1.8.5.1)

The extraction of the main AOX enzymes was performed, under cold conditions, by an adaptation of the method described by Fidalgo et al. (2011). Here, ≈ 200 mg of frozen shoot and root samples were mixed with 1.5 mL of an extraction buffer composed of 100 mM potassium phosphate buffer (pH 7.3) and supplemented with 1 mM ethylenediamine tetraacetic acid (EDTA), 8% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM AsA and 2% (w/v) polyvinylpolypyrrolidone (PVPP). After centrifugation (16,000 *g* for 25 min at 4 °C), the supernatant was collected and used for protein quantification and determination of enzymatic activity. Soluble proteins were estimated using the method described by Bradford (1976), using bovine serum albumin as standard.

The activity of SOD was determined via a spectrophotometric assay based on the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) (Donahue et al., 1997). Here, Abs were recorded at 560 nm and the results were expressed as units of SOD mg^{-1} protein, with one unit of SOD being defined as the amount of enzyme necessary to cause a 50% inhibition of NBT photoreduction.

CAT and APX activities were estimated spectrophotometrically by monitoring the over-time H_2O_2 ($\varepsilon_{240\ nm}$ = 39.4 mM^{-1} cm^{-1}) degradation and AsA ($\varepsilon_{290\ nm}$ = 0.49 M^{-1} cm^{-1}) oxidation, respectively. In both cases, H_2O_2 was added to start the reaction and results were expressed as μmol H_2O_2 min^{-1} mg^{-1} protein or nmol AsA min^{-1} mg^{-1} protein. These determinations were performed according with the Aebi (1984) and Nakano and Asada (1981) methods for CAT and APX activity assessment, respectively, being downscaled for microplates, as optimized by Murshed et al. (2008).

In a similar way, GR and DHAR activity were also determined through spectrophotometric enzyme kinetics, downscaling the Foyer and Halliwell (1976) and Ma and Cheng (2004) methods for UV microplates, respectively, as described by Murshed et al. (2008). For GR, NADPH oxidation was monitored over-time at 340 nm after adding oxidized glutathione (GSSG) to the mixture, and results were expressed as nmol NADPH min^{-1} mg^{-1} protein, using 6.22 mM^{-1} cm^{-1} as ε. DHAR activity levels were determined by adding DHA to the mixture and following its reduction to AsA at 265 nm. Results were expressed as nmol AsA min^{-1} mg^{-1} protein, considering $\varepsilon_{265\ nm}$ = 14 mM^{-1} cm^{-1}.

2.11. Statistical analyses

Every parameter was assessed using at least three biological replicates ($n \geq 3$) – here defined as a mixture of the 3 plants of each pot – with at least three technical repetitions per assay. Results were expressed as mean ± standard error of the mean (SEM). Differences among treatments were assessed by two-way ANOVA [SALT – 0 mM and 100 mM NaCl; HEAT – 25 °C and 42 °C (4 h d^{-1})], after checking the normality and homogeneity assumptions. When $p \leq 0.05$, differences between groups were assessed by Tukey's post-hoc test. When significance was found for the interaction, a correction for the simple main effects was performed. These analyses were carried out using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and the results of the ANOVAs are detailed in Supplementary Material (Tables S3.2 and S3.3).

A principal component analysis (PCA) was performed to assess the similarities between conditions and the major associations between variables that are responsible for the observed similarities/differences. For this, the average values for each evaluated parameter were plotted and the first two components were used to make biplots. This analysis was carried out using XLSTAT 2021.2.2 (http://www.xlstat.com, Addinsoft USA, New York, NY).

3. Results

3.1. Biometric analysis – organ length, dry biomass and water content

The individual stress treatments induced similar growth inhibitions, as seen by the significant decrease in organ elongation (17% and 26% in SALT; 24% and 27% in HEAT for roots and shoots, respectively), in relation to the CTL (Figure 3.1a,d). The exposure to salt or heat stress also led to identical decreases in dry weight when compared to CTL plants (Figure 3.1b,e), with inhibition values of around 40% and 30% in roots and shoots, respectively. The combination of stressors imposed a more severe negative effect on both length and dry weight of tomato plant primary organs (decreases of 46% and 77%; 58% and 71% in roots and shoots, respectively), in comparison with the CTL, although no significant differences could be found for the interaction between both factors (Tables S3.2 and S3.3). In what concerns water content (Figure 3.1c,f), no effects were observed in roots, while the treatment with salt, alone or in combination, led to a significant reduction of water content in the aerial parts of the plants.

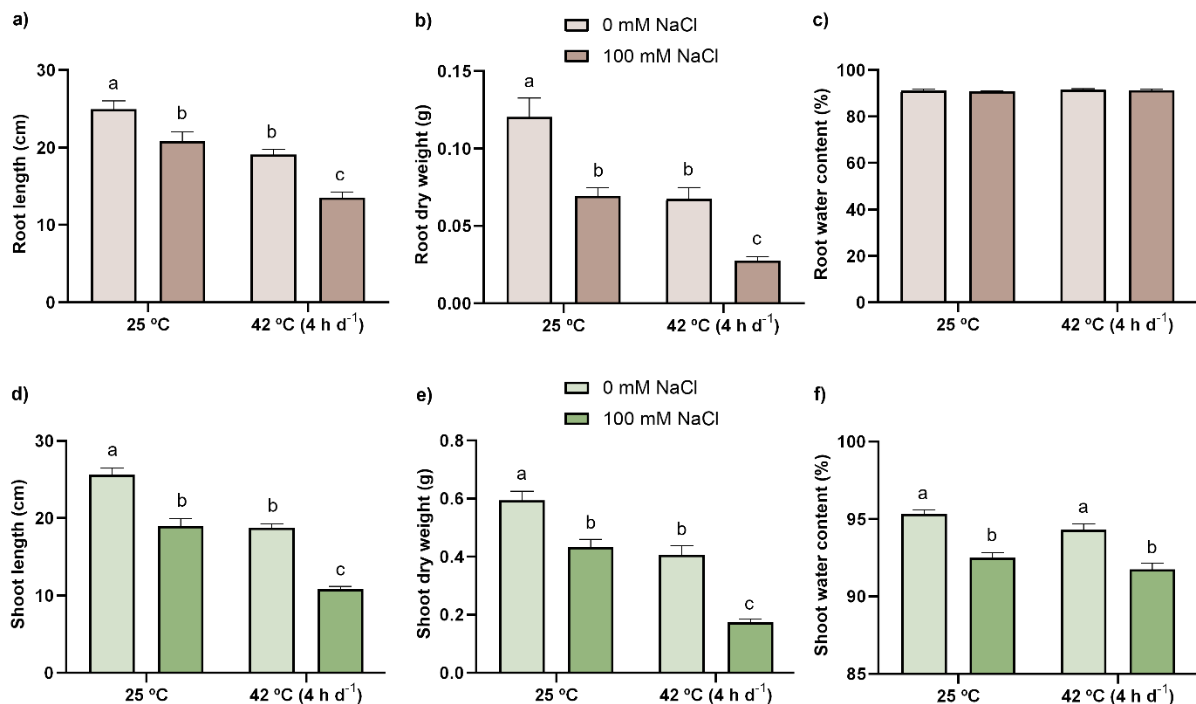

Figure 3.1. Length (a,d), dry weight (b,e) and water content (c,f) in roots (brown bars) and in shoots (green bars) of tomato plants after a 21-day exposure to 42 °C (4 h d^{-1}) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

3.2. Element quantification – Na^+, K^+, Ca^{2+} and Mg^{2+}

Plants from both salt treatments (single or combined) presented a severe increase of the levels of Na^+ in roots (almost 9-fold and 10-fold for SALT and COMBINED, respectively, and in relation to CTL), as well as in shoots, where plants under combined exposure were, once again, more affected (accumulation of around 8-fold) than those under individual salinity stress (increment of 5-fold), with statistical significance being attributed to the interaction between HEAT and SALT (Tables S3.2 and S3.3). On the other hand, even though the heat treatment also resulted in an altered accumulation of Na^+ (11% increase in roots and a 19% decrease in shoots, in comparison with CTL), its levels were much lower than those found in SALT and COMBINED. Curiously, the concentration of K^+ in plants exposed to salt, single or in combination with heat, decreased 32-39% in roots and 31-35% in shoots, while heat imposed a 14% increment of this element in roots but a decrease in shoots (14%). A different pattern was observed for Ca^{2+}, which was accumulated when plants were exposed to heat (14% and 38% in roots and shoots, respectively) and in the shoots of the individual salt treatment (17%), even though it was decreased in the roots (34%). However, upon combination, the stressors led to diminished levels of Ca^{2+} in both organs when compared to CTL (63% in roots and 12% in shoots). Lastly, levels of Mg^{2+} in heat-stressed plant tissues were either

unaltered (roots) or reduced (by 6% in shoots), while salt stress increased the concentration of this element by 25% and 11% in roots and shoots. The interaction between stressors was significant in both organs (Tables S3.2 and S3.3), with plants treated simultaneously with salt and heat presenting 6-10% less Mg^{2+} than control plants.

Table 3.1. Effect of 21 days of salt (irrigation with 100 mM NaCl), heat (exposure to 42 °C for 4 h d^{-1}) and combined stresses on the content of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} in roots and shoots of tomato plants. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

Parameter	CTL	SALT	HEAT	COMBINED
Root Na^{+} (mg g^{-1} dw)	1.793 ± 0.003 d	15.920 ± 0.021 b	1.990 ± 0.026 c	17.693 ± 0.015 a
Shoot Na^{+} (mg g^{-1} dw)	5.163 ± 0.5003 c	25.380 ± 0.044 b	4.180 ± 0.012 d	40.050 ± 0.015 a
Root K^{+} (mg g^{-1} dw)	3.833 ± 0.019 b	2.607 ± 0.015 c	4.800 ± 0.012 a	2.332 ± 0.002 d
Shoot K^{+} (mg g^{-1} dw)	11.583 ± 0.019 a	8.050 ± 0.015 c	9.917 ± 0.018 b	7.570 ± 0.025 d
Root Ca^{2+} (mg g^{-1} dw)	0.487 ± 0.004 b	0.323 ± 0.001 c	0.557 ± 0.009 a	0.181 ± 0.001 d
Shoot Ca^{2+} (mg g^{-1}dw)	2.000 ± 0.015 c	2.343 ± 0.018 b	2.757 ± 0.007 a	1.767 ± 0.012 d
Root Mg^{2+} (mg g^{-1} dw)	2.547 ± 0.009 b	3.193 ± 0.037 a	2.527 ± 0.007 b	2.397 ± 0.012 c
Shoot Mg^{2+} (mg g^{-1} dw)	6.097 ± 0.054 b	6.737 ± 0.038 a	5.730 ± 0.052 c	5.473 ± 0.026 d

3.3. ROS content

Regarding $O_2^{\bullet -}$ (Figure 3.2a,d), all plants exhibited similar levels of this ROS in roots, independently of the applied treatment. In shoots, its levels were decreased by 20% when plants were exposed to salt, whilst the combination of both conditions led to a further reduction (52% in comparison with CTL). Concerning H_2O_2 (Figure 3.2b,e), heat stress, either single or combined with salt, resulted in an equal increment of this ROS in roots (63% in relation to CTL). In shoots, however, H_2O_2 levels decreased similarly with all treatments (33% in SALT and HEAT, and 36% in COMBINED), over the CTL.

3.4. LP

LP degree, which was estimated by the MDA content, is shown in Figure 3.2c,f. When plants were exposed to the stresses, LP equally diminished in shoots, in relation to the CTL (56%, 52% and 67% in SALT, HEAT and COMBINED, respectively). In roots, the simultaneous exposure to the stressors led to significantly lower values (29%), in comparison with the CTL.

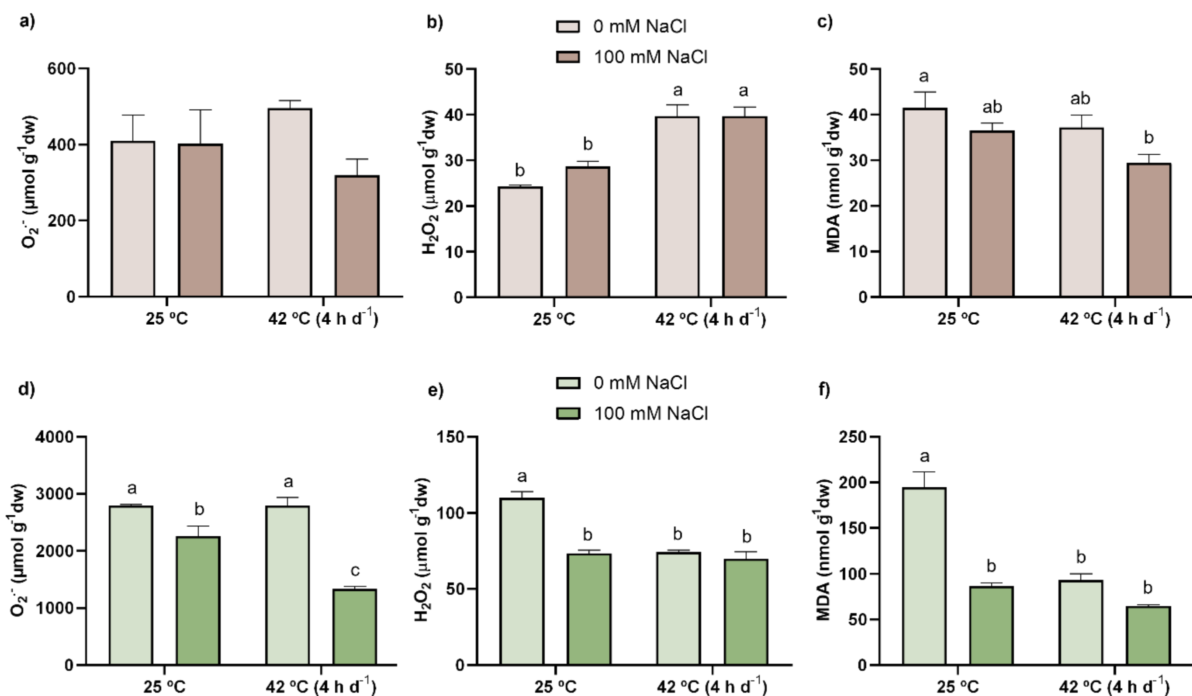

Figure 3.2. Levels of oxidative stress markers of tomato plants: $O_2^{\cdot-}$ (a,d), H_2O_2 (b,e) and MDA (c,f) content in roots (brown bars) and in shoots (green bars) of tomato plants after a 21-day exposure to 42 °C (4 h d^{-1}) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

3.5. Proline, AsA and GSH

Proline levels were severely affected by salt in shoots (27-fold) and, especially, in roots (59-fold) in relation to the CTL (Tables 3.2 and 3.3). Under the co-exposure scenario, the accumulation of proline was not as pronounced as the single treatment with salt (44 and 17-fold changes being noted in shoots and roots, correspondingly), with the ANOVA results showing significant interaction between SALT and HEAT (Tables S3.2 and S3.3). Regarding heat treatment alone, no significant differences were found in relation to the CTL, either in roots or shoots.

Total AsA (Tables 3.2 and 3.3) was only negatively affected in shoots of tomato plants by salt, single or combined, where significant decreases of around 30%, in comparison with CTL, were recorded (Table 3.3). Moreover, a 36% and 31% decrease could be found in DHA content in shoots of these two treatments (SALT and COMBINED; Table 3.3). Lastly, and although shoots of every treatment tended to present lower reduced AsA content than CTL (Table 3.3), no statistical significance was achieved.

Concerning GSH, the ANOVA results (Tables S3.2 and S3.3) showed a positive interaction between both treatments. Indeed, its content in roots was only altered upon the simultaneous exposure to salt and heat, being 48% higher than in the CTL (Table 3.2). On the contrary, this thiol was decreased in shoots

of plants exposed to all treatments (Table 3.3). However, as can be seen, salinity led to a greater reduction (29%) than that found in heat-related treatments (13% and 16%).

3.6. Thiols

Total thiols content is presented in Tables 3.2 and 3.3. In roots, heat stress led to an increase of around 35% in total thiols regardless of the salt co-exposure. In shoots, total thiols were only negatively affected by heat alone (32% in comparison with CTL), although a significant interaction was perceived between both SALT and HEAT (Table S3.3), related to the relatively higher values found the COMBINED treatment. The ratio between non-protein and protein-bound thiols remained unaffected, the exception being the shoots of heat-treated plants (increase of 76% in relation to the untreated plants).

3.7. TPC, TFC and TAC

TPC was not affected by any treatment in roots (Table 3.1). However, in shoots of plants under salt stress, single or combined with heat, TPC decreased 29% and 22%, respectively (Table 3.2). Concerning TFC, it was influenced by both stressors (Tables 3.1 and 3.2). In roots (Table 3.1), salt caused a 34% reduction of these antioxidants, while heat led to a decrease of 28% in comparison to CTL plants. Similarly, in shoots, salinity stress, single or combined with heat, resulted in a reduction in TFC (47% in SALT and 43% in COMBINED), as can be observed in Table 3.2. TAC values were only negatively affected (43%) by heat stress alone in shoots (Table 3.2). On the other hand, in roots, an increment of 52% was reported in plants co-treated with salt and heat (Table 3.1), in comparison to CTL plants. Lastly, for the abovementioned parameters, the interaction between stressors was significant in both organs (Tables S3.2 and S3.3).

Table 3.2. Effect of 21 days of salt (irrigation with 100 mM NaCl), heat (exposure to 42 °C for 4 h d^{-1}) and combined stresses on the content of proline, AsA (total, AsA, DHA and AsA/DHA), GSH, thiols (total and protein/non-protein), TPC, TFC and TAC in roots of tomato plants. Values represent mean ± SEM (n ≥ 3). Significant differences (*p* ≤ 0.05) between treatments are indicated by different letters.

Parameter (roots)	CTL	SALT	HEAT	COMBINED
Proline (mg g^{-1} dw)	0.099 ± 0.02 c	5.820 ± 0.114 a	0.088 ± 0.039 c	4.320 ± 0.357 b
Total AsA (μg g^{-1} dw)	7.273 ± 0.500	7.803 ± 0.444	8.607 ± 0.406	8.240 ± 0.633
AsA (μg g^{-1} dw)	1.980 ± 0.665	1.867 ± 0.044	1.933 ± 0.079	1.960 ± 0.269
DHA (μg g^{-1} dw)	5.917 ± 0.173	6.023 ± 0.3868	6.723 ± 0.3480	6.280 ± 0.6201
AsA/DHA	0.338 ± 0.047	0.297 ± 0.012	0.280 ± 0.012	0.263 ± 0.019
GSH (nmol g^{-1} dw)	252.5 ± 13.5 b	233.2 ± 1.95 b	295.0 ± 18.2 ab	374.5 ± 36.4 a
Total thiols (μmol g^{-1} dw)	1.306 ± 0.023 b	1.116 ± 0.038 b	1.785 ± 0.111 a	1.739 ± 0.032 a
Non protein/Protein thiols	0.232 ± 0.045	0.300 ± 0.050	0.179 ± 0.018	0.146 ± 0.021
TPC (μg gallic acid equivalents g^{-1} dw)	824.4 ± 34.40 ab	791.9 ± 41.69 ab	708.8 ± 7.687 b	902.4 ± 53.22 a
TFC (μg quercetin equivalents g^{-1} dw)	458.2 ± 27.81 a	302.5 ± 40.94 b	328.2 ± 15.32 b	392.5 ± 18.14 ab
TAC (μg AsA equivalents g^{-1} dw)	1399 ± 67.48 b	1623 ± 77.64 b	1261 ± 17.58 b	2123 ± 163.1 a

Table 3.3. Effect of 21 days of salt (irrigation with 100 mM NaCl), heat (exposure to 42 °C for 4 h d^{-1}) and combined stresses on the content of proline, AsA (total, AsA, DHA and AsA/DHA), GSH, thiols (total and protein/non-protein), TPC, TFC and TAC in shoots of tomato plants. Values represent mean ± SEM (n ≥ 3). Significant differences (*p* ≤ 0.05) between treatments are indicated by different letters.

Parameter (shoots)	CTL	SALT	HEAT	COMBINED
Proline (mg g^{-1} dw)	1.112 ± 0.154 c	29.930 ± 2.265 a	0.593 ± 0.023 c	18.540 ± 1.117 b
Total AsA (μg g^{-1} dw)	18.84 ± 0.93 a	13.20 ± 1.65 b	14.19 ± 1.03 ab	12.86 ± 0.47 b
AsA (μg g^{-1} dw)	6.497 ± 0.224	5.340 ± 0.932	4.173 ± 0.376	4.397 ± 0.095
DHA (μg g^{-1} dw)	12.34 ± 0.80 a	7.87 ± 0.73 b	10.01 ± 0.67 ab	8.46 ± 0.38 b
AsA/DHA	0.53 ± 0.03 ab	0.67 ± 0.06 a	0.42 ± 0.02 b	0.52 ± 0.01 ab
GSH (nmol g^{-1} dw)	1039.0 ± 9.8 a	733.1 ± 18.6 c	904.6 ± 41.6 b	870.0 ± 28.2 b
Total thiols (μmol g^{-1} dw)	7.862 ± 0.720 a	7.292 ± 0.350 ab	5.324 ± 0.438 b	8.809 ± 0.198 a
Non protein/Protein thiols	0.110 ± 0.004 b	0.094 ± 0.009 b	0.194 ± 0.007 a	0.113 ± 0.006 b
TPC (μg gallic acid equivalents g^{-1} dw)	2508 ± 101.1 a	1786 ± 117.2 b	2030 ± 34.16 ab	1947 ± 143.5 b
TFC (μg quercetin equivalents g^{-1} dw)	2313 ± 41.04 a	1225 ± 95.39 b	2319 ± 109.6 a	1321 ± 105.3 b
TAC (μg AsA equivalents g^{-1} dw)	3471 ± 232.0 a	2771 ± 327.6 ab	1966 ± 99.06 b	2922 ± 240.1 ab

3.8. Enzymatic activity (SOD, CAT, APX, DHAR and GR)

Results regarding the activity of the AOX enzymes are presented in Figures 3.3 and 3.4. Although no effect was found for SOD in roots (Figure 3.3a), significant changes were observed in shoots (Figure 3.3c). In fact, when compared with CTL plants, SOD activity was inhibited by 45% upon heat single exposure, but a higher activity (28%) of this enzyme was recorded in response to the co-treatment, over the CTL, with the ANOVA showing a significant interaction between both stress factors (Table S3.3). CAT activity was similarly enhanced in roots of all stressed plants (Figure 3.3b) up to almost 100%, with a positive interaction being detected for this organ (Table S3.2). Contrarily, in shoots, CAT was inhibited by 26% and 60% in response to heat single and co-exposure, respectively (Figure 3.3d), although no interaction was recorded (Table S3.3).

APX activity (Figure 3.4a,d) was greatly enhanced in response to the combined action of the two stress factors in shoots (62%), but mainly in roots, where an increment of 129% in relation to the CTL was observed. The elevated activity of APX was also reported in roots upon the individual exposure to heat (90%). Regarding DHAR activity (Figure 3.4b,e), compared to CTL, it was noticeably enhanced only by the simultaneous exposure to the stressors (100% and 112% in roots and shoots, respectively). Indeed, the statistical analysis (Tables S3.2 and S3.3) shows that in both organs there was a significant interaction between SALT and HEAT. Lastly, the individual salt stress inhibited GR by 25% in roots, however, when combined with heat, an increase of 31% was observed in relation to the CTL (Figure 3.4c) being the interaction of both conditions (salt and heat) significant (Table S3.2). In shoots (Figure 3.4f), the activity of this enzyme was elevated by 51% and 38% in plants under salt treatment and simultaneous exposure to both stress factors, correspondingly.

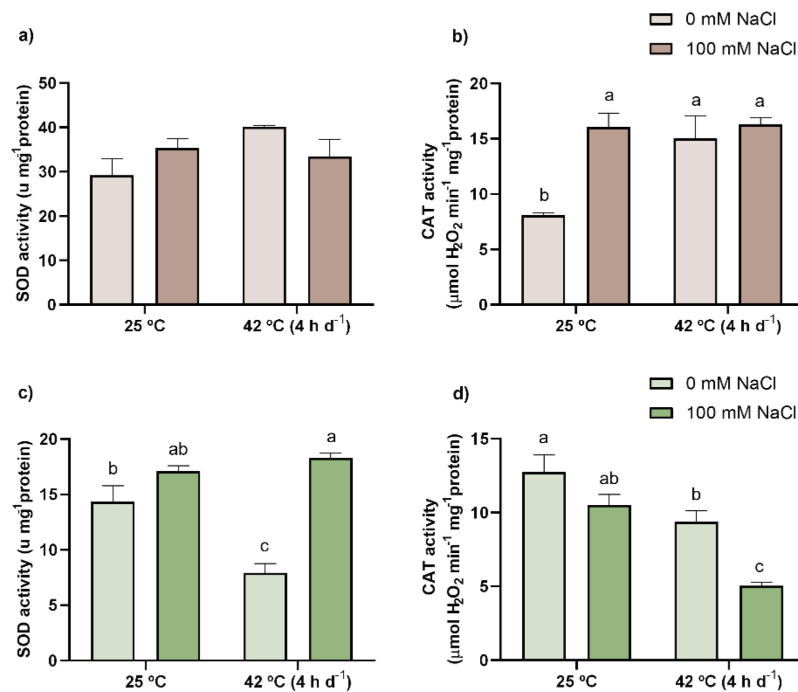

Figure 3.3. Activity levels of SOD (a,c) and CAT (b,d) in roots (brown bars) and in shoots (green bars) of tomato plants after a 21-day exposure to 42 °C (4 h d-1) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

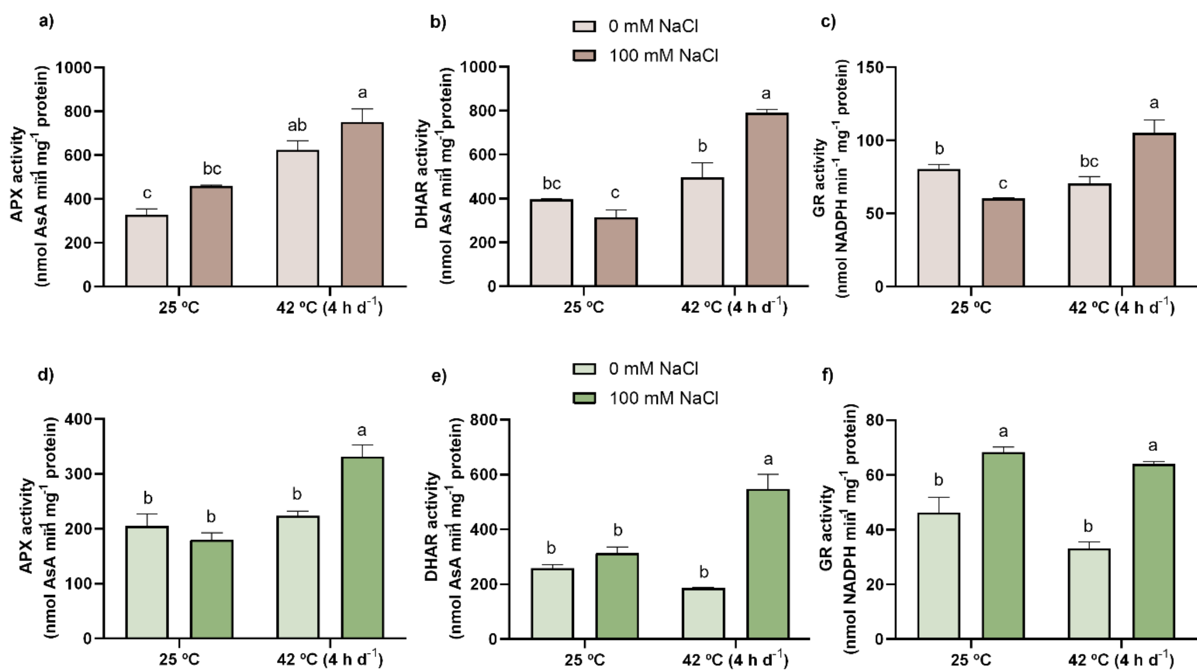

Figure 3.4. Activity levels of APX (a,d), DHAR (b,e) and GR (c,f) in roots (brown bars) and in shoots (green bars) of tomato plants after a 21-day exposure to 42 °C (4 h d-1) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

3.9. Principal component analysis (PCA)

To understand how different conditions vary between them, and also to infer the correlation between all tested parameters, a PCA was carried out (Figure 3.5). The data obtained showed that the first component explained 49.74% and 60.06% of variance in roots and shoots, respectively, while the second accounted for 29.74% and 23.34%. Furthermore, it was observed that, for roots (Figure 3.5a), SALT and CTL plants were grouped in the same quadrant (fourth), while HEAT and COMBINED plants were grouped separately at the first and third quadrants, respectively. In shoots (Figure 3.5b), although some proximity can be observed between SALT and COMBINED, the four treatments were distributed by the four quadrants (CTL in the first, COMBINED in the second, SALT in the third and HEAT in the fourth) revealing that the dependent variables were affected differently by each experimental condition, but also when comparing plant organs. It is also worth noticing that more variables are related to CTL in shoots (namely, water content, CAT, K^+, AsA, GSH, flavonoids, phenols and H_2O_2) than in roots, and that this group of plants is characterized by higher values of length, dry weight, and MDA in both organs. Interestingly, Na^+ is associated with salinity treatments, especially COMBINED, in both organs and presents opposite relations to K^+ and Ca^{2+} in roots. Lastly, in *S. lycopersicum* plants the differences between the COMBINED and the remaining treatments in what concerns the redox status and lack of oxidative damage can be explained through the perceived negative correlation between MDA content and the general activation of the AOX system in roots, while in shoots this was mostly observed for the enzymatic component of this system, along with proline and thiols.

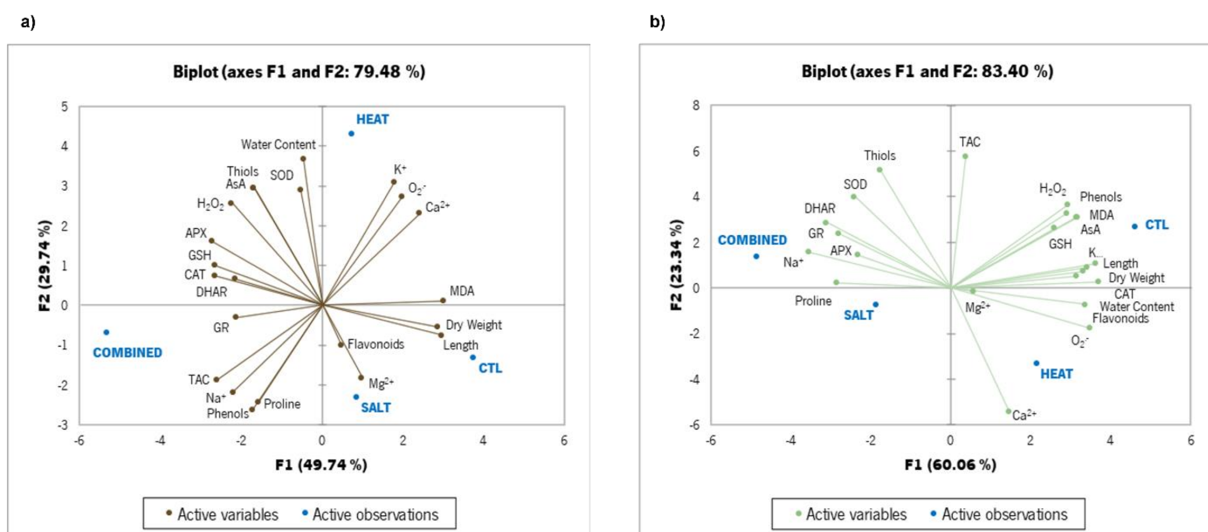

Figure 3.5. Biplot-based PCA with first two principal components showing the differential response of roots (a) and shoots (b) of tomato plants to salt (irrigation with 100 mM NaCl), heat (exposure to 42 °C for 4 h d^{-1}) and combined stresses for 21 days.

4. Discussion

Climate change is an unavoidable calamity imposing new and aggravated challenges to crop production and food security. In this scenario, high temperatures and salinization of soils and water are amongst the major environmental factors causing agricultural losses around the globe (Hassan et al., 2021; Hernández, 2019). Despite the individual heat and salinity stresses have been extensively explored, little is known regarding the effects of the interaction of these two stressors, which frequently occur simultaneously. Therefore, in this study, the response of tomato plants (*S. lycopersicum* var. *cerasiforme*) to the combination of heat and salinity was assessed, in terms of growth and physiological performance, to understand how plants cope and adjust their metabolism towards the co-occurrence of both stressors.

4.1. The combination of heat and salt led to a harsher effect on growth-related parameters

Here, plant growth, in what concerns root and stem elongation and biomass (Figure 3.1a,b,d,e), was impaired upon exposure to both salt and heat, but especially by the co-exposure treatment. Equivalent salt-induced declines in growth-related parameters have been reported in several crop plants, for instance in *Glycine max* (L.) Merr. (soybean) (Dolatabadian et al., 2011), *Hordeum vulgare* L. (barley) (Tavakkoli et al., 2011), *Oryza sativa* L. (rice) (Hasanuzzaman et al., 2009), *Brassica juncea* L. (mustard) (Ahmad et al., 2012), and even tomato (Mittova et al., 2004). Often, such growth inhibitions are primarily correlated with a reduced water uptake, along with a negative interference in nutrient and ion ratios caused by the build-up of salts in the soil. Indeed, Na^+ competes with K^+ for transporters (AKT and HKT) due to their similarity in terms of ionic radius and hydration energy (Gupta and Huang, 2014), resulting in depleted levels of the latter and increased levels of the former, as herein reported upon exposure to salt, a result aligned with those observed in other tomato cultivars such as Bush Beefsteak (Chaichi et al., 2017) and Target F1 (Tuna et al., 2007). Once inside the plant, excessive salt becomes toxic as a result of the growing inability of cells to avoid the accumulation of Na^+ and Cl^- ions in the cytoplasm and transpiration stream (Parihar et al., 2015). This portrays a major threat for key metabolic processes involved in plant growth and development, as K^+ is a cofactor of several enzymes and its replacement with Na^+ leads to the disruption of protein synthesis and enzymatic reactions (Shabala and Munns, 2017). Additionally, in accordance with our findings, it is known that Ca^{2+} deficiency is often salt-induced, which may limit the efflux of Na^+ to the apoplast via the Ca^{2+}-dependent Salt Overly Sensitive (SOS) signaling pathway (Gupta and Huang, 2014; Parihar et al., 2015; Taiz et al., 2015). In fact, plants under salt exposure but supplemented with exogenous Ca^{2+} have been documented to improve growth and development, giving relevance to the important role of this macronutrient in salinity tolerance (Cachorro

et al., 1994; Tuna et al., 2007). Moreover, as the salt accumulation in soil hampers water uptake, by decreasing soil water potential, it is not surprising that a reduced water content (Figure 3.1f) was perceived in the aerial part of tomato plants exposed to salt. In fact, such effect has already been documented by Ahmad et al. (2012), Amirjani (2011) and Hasanuzzaman et al. (2009) in various plant models. Lastly, and even though there is still a lot to unravel regarding the plants' uptake of Mg (Mao et al., 2014), as well as the impacts of salinity on this process, it is generally expected to have a negative effect [as reviewed by Parihar et al. (2015)]. Curiously, the results herein presented show an opposite pattern, but a higher uptake of this nutrient might be related to its important role in plant growth, enzymatic activity, and photosynthesis – both as a key component of chlorophylls and as a vital player in CO_2 fixation (Sigel and Sigel, 1990) – which are usually affected by Na^+ toxicity (Parihar et al., 2015).

Similar to the previous stressor, high temperatures significantly impaired tomato plants' growth performance in both shoots and roots. Based on previous records, these heat-induced impacts are mostly due to disrupted water relations, damaged photosynthetic machinery, changes in membrane permeability, oxidative stress and nutrient imbalances (Ashraf and Hafeez, 2004; Hassan et al., 2021; Hayat et al., 2009; Nagesh Babu and Devaraj, 2008; Wahid et al., 2007). Nonetheless, and even though in the present study, all ions analysed (Na^+, K^+, Ca^{2+} and Mg^{2+}) were altered upon heat exposure depending on the tissue, there appears to be no significant effect in nutrient uptake when looking at the whole plant – in accordance with the lack of macroscopic signs of nutrient deficiency. Indeed, the mechanisms by which high temperatures disturb nutrient uptake are yet unclear and seem to be inconsistent, as documented by Giri et al. (2017) and Matías et al. (2021), and reviewed by Hassan et al. (2021). Additionally, water content (Figure 3.1c,f) was unaffected by heat, which is in accordance with other research on different tomato cultivars, namely that by Zhou et al. (2017) and Rivero et al. (2014), and is possibly related to the non-limiting irrigation. Moreover, as this stress was applied periodically, simulating field conditions, unlike the persistent exposure described in most research up to date, it is possible that this has contributed to a better acclimation ability and/or recovery leading to the maintenance of proper water relations and, as further discussed below, redox status, although at the cost of reduced biomass. In fact, Parrotta et al. (2020) reported that when tomato plants cv. Micro-Tom were subjected to periodic high temperatures – 8 h d^{-1} at 40 °C, for 6 days – the highest accumulation of heat shock protein 70 (HSP70), a chaperone that possesses cytoprotective functions under harmful conditions (Usman et al., 2017), occurred during the recovery periods, suggesting a role in restoring cell stability and adaptation to subsequent stress episodes. Nonetheless, it is important to take in account that, even with a possible

enhancement in defence pathways and a generally unaffected nutrient uptake, heat stressed plants still presented growth reductions, which might be related to heat-induced damage in the photosynthetic apparatus, leading to impaired carbon metabolism and reduced photoassimilate production (Hassan et al., 2021; Wahid et al., 2007). This hypothesis will be further discussed in this dissertation (Chapter IV).

Interestingly, when both stress factors were applied simultaneously, a stronger negative effect could be perceived on mineral absorption patterns (Table 3.1) and, consequently, on plant growth (Figure 3.1a,b,d,e). Indeed, we observed that Ca^{2+} and K^+ uptake was decreased in a harsher way than that found in SALT, and, curiously, these plants, exposed to combined stressors, also presented higher concentrations of Na^+ than the single treatment. This may be a result of the ability of heat to reduce the activity of nutrient uptake proteins, most likely due to a lower root conductance or damage in enzymes, allowing a greater influx of Na^+ and a diminished uptake of Ca^{2+} and K^+ (Hasanuzzaman et al., 2013; Hassan et al., 2021). This would limit the SOS pathway, while also increasing the levels of Na^+ in the cytosol, which portrays a higher risk of toxicity and an increased competition between Na^+ and K^+ for the binding sites of several key enzymes, culminating in a severe reduction of plant growth. However, the knowledge regarding the effects of heat stress on roots is limited, as well as on its impact on membrane transporters. Moreover, by impairing water uptake and leading to increased stomata resistance, salinity could also have negatively influenced transpiration rate – an important cooling and nutrient distribution mechanism (Parihar et al., 2015; Sterling, 2005), increasing their susceptibility to heat stress. Although there are only few records exploring the dynamics, in terms of physiological and biometrical impacts, of heat and salt co-exposure in *S. lycopersicum,* our data contrasts with the reports of Rivero et al. (2014) and Lopez-Delacalle et al. (2021). These authors, when exposing tomato plants cv. Optima for 72 h at 35 °C and 120 mM NaCl and cv. Boludo for 14 days at 35 °C and 75 mM NaCl, respectively, showed that the combination of both stressors prompted a better growth, photosynthetic efficiency, and water and nutrient relations than those grown only under saline conditions. Nonetheless, it is also important to consider that such contrasting results may arise from distinct tolerance threshold between cultivars or varieties, as well as the employment of different experimental conditions that affect plant response and acclimation differently, namely growing plants on a soil-based system instead of hydroponics, as well as using a persistent or periodic exposure to high temperatures. Here, as the increased toxicity of Na^+ may be affecting different processes – among them, water relations and those related with the photosynthetic machinery (as supported by the lower concentrations of Mg^{2+} found in these plants) – growth might have been compromised due to the disruption of vital mechanisms, through a lack of resources or due to their

allocation into defence pathways [*e.g.* accumulation of AOXs, osmolytes, and HSPs, explaining the lack of macroscopic toxicity symptoms and oxidative damage (section 4.2)], so that plant survival was ensured under these adverse conditions. In fact, and as reviewed by Margalha et al. (2019), in conditions of disrupted nutrient uptake or ratios, the crosstalk between the two central nutrient-sensing kinases in plants leads to the induction of the one that ensures optimal nutrient allocation strategies and the inhibition of the one regulating nutrient use to promote cell growth and proliferation.

4.2. The co-exposure of tomato plants to heat and salinity, individually or in combination, did not result in a severe oxidative stress condition

Even though the primary effects of salinity and heat are not related to oxidative stress, an excessive accumulation of ROS is fairly connected to a decline in growth and productivity in salt- (Parihar et al., 2015) and heat- (Hassan et al., 2021) exposed plants. However, in the present study, no major signs of ROS overaccumulation and membrane damage (measured as LP) were detected in plants subjected to either individual stressor, except in roots of heat-stressed plants, where H_2O_2 levels were enhanced (Figure 3.2b). Nonetheless, the higher content of this ROS appears to be in equilibrium with the AOX capacity of tomato plants, as no oxidative damage, translated into LP, could be detected in this situation (Figure 3.2a).

Concerning the combined exposure, as in heat-exposed plants, a higher accumulation of H_2O_2 was found in roots, though $O_2^{\bullet-}$ content remained unaltered; also, in shoots, plants simultaneously subjected to salinity stress and high temperatures experienced a very noticeable decrease of this ROS, in relation to all other experimental conditions (Figure 3.2a,b,d,e). In fact, the reduced $O_2^{\bullet-}$ content is in accordance with an increased SOD activity – responsible for the dismutation of this ROS into H_2O_2 (Soares et al., 2019a). Thus, and while this would imply an increase in H_2O_2 content, the levels of this ROS were also reduced, possibly due to an efficient AOX response (as discussed in sections 4.3 and 4.4). Although in some cases, a reduced content in $O_2^{\bullet-}$ and/or H_2O_2 might be related to the production of other ROS, such as the hydroxyl radical ($^{\bullet}OH$), which is the main factor causing LP (Soares et al., 2019a), no signs of oxidative damage could be found, namely at the MDA production (Figure 3.2c,f), suggesting that tomato plants are much likely investing on potent defence mechanisms to prevent salt- and/or heat-induced stresses.

4.3. The simultaneous effect of heat and salinity on tomato plants results in differential activation patterns of AOX metabolites

Under water stress, which is often a consequence of salinity, heat, and drought stress, plants tend to accumulate compatible organic solutes, such as proline (Claussen, 2005). In fact, proline is not only a powerful osmoprotectant but also a ROS scavenger – namely of •OH and singlet oxygen ($^1O_2$) – and a membrane stabilizer (Soares et al., 2019a). Thus, the exacerbated increase in the levels of this metabolite in plants exposed to salt, individually and in combination with heat (Tables 3.2 and 3.3), is not surprising and may suggest a major role of proline in tomato's tolerance response to this stressor. Such dramatic accumulation has already been documented in response to different concentrations of salt for several plant models (Ahmad et al., 2012, 2010; Ashraf et al., 2012; Fidalgo et al., 2004; Nxele et al., 2017; Yazici et al., 2007), among them distinct tomato varieties (Al Hassan et al., 2015; Gharsallah et al., 2016). Indeed, proline acts on several fronts, including LP prevention, which probably explains the absence of membrane damage. Although similar results could be expected after heat treatment (Harsh et al., 2016; Khan et al., 2013; Raja et al., 2020; Rajametov et al., 2021), since water content remained similar to that of the control and the overaccumulation of ROS was not sufficient to induce LP, it seems that proline was not a key player in tomato plants exposed to heat stress. Curiously, when both stressors were applied simultaneously, the levels of this osmoprotectant were noticeably enhanced in relation to CTL and HEAT treatment but such increase was not as pronounced as in SALT situation. Thus, and as already reported by Rivero et al. (2014) and Lopez-Delacalle et al. (2021), it is possible that other defence pathways are acting *in tandem* with proline in this response, as these authors report that decreased proline content (in comparison with the individual salt treatment) was accompanied by the increase in other osmolytes, such as glycine betaine. Also, and considering not only the importance of proline accumulation but also the role of its catabolism in providing energy to the cell during stress conditions, particularly under situations of nutrient depletion [30 ATP equivalents are generated from the oxidation of one proline molecule (Liang et al., 2013)], it seems that proline metabolism is playing an important role in the response of *S. lycopersicum* to the combined action of salt and heat stress. Nonetheless, the lower accumulation of this osmolyte – either resulting from its catabolism or from reduced synthesis, due to the negative effects of heat on the photosynthetic apparatus and the toxic levels of Na^+ in these plants – can also be detrimental to these plants, as proline balances turgor pressure (affected by excess salt) and acts as a chaperone, preventing protein aggregation and denaturation, and enzyme inhibition (Liang et al., 2013). Furthermore, and as reviewed by Singh et al. (2015), the exogenous application of this

amino acid is associated with decreased uptake of Na^+, which is also possibly related to the higher content of this ion in the situation where proline levels were inferior.

Equally relevant in the response against abiotic stress is the most abundant AOX metabolite, AsA, which effectively scavenges the accumulated ROS via direct or indirect pathways (Noctor and Foyer, 1998). In the present study, it is possible to observe that neither stress (individual or combined) had any effect on the synthesis and regeneration of this AOX (Tables 3.2 and 3.3). In fact, and although the opposite is normally found (Khan and Panda, 2007; Mishra et al., 2013; Zhao et al., 2010), these results fall in accordance with the similar pattern of activity between the enzymes mediating AsA oxidation (APX) and its reduction (DHAR). Curiously, in heat-treated plants, especially in combination with salt, a slight tendency for a higher oxidation of AsA was observed, this being in line with a higher APX activity than in the SALT situation and in the CTL. However, a different pattern was observed in shoots of tomato plants. In this organ, both salt-related treatments negatively influenced AsA accumulation, as has already been observed in *Brassica napus* L. (Hasanuzzaman et al., 2011) and *Vigna angularis* (Willd.) Ohwi & H. Ohashi (Ahanger et al., 2020a) exposed to 100 mM NaCl. Nonetheless, as no signs of oxidative stress could be perceived and no major changes were found regarding the redox status of this AOX, it can be hypothesised that AsA biosynthesis might be only slightly downregulated, allocating energy and resources to other pathways. Indeed, even in the combined treatment, where APX activity was greatly enhanced, the lower AsA pool was still sufficient to ensure redox homeostasis of the cell, being accompanied by a great regeneration effort by DHAR.

Furthermore, knowing that no relevant symptoms of redox disorders were found upon the exposure to both stresses, either single or combined, the hypothesis of plant cells being able to ensure a proper redox state of proteins and other metabolites was raised. The maintenance of reduced conditions within cells is of major importance in stressful conditions, being thiols (-SH) excellent stress biomarkers (Soares et al., 2019a). The major non-protein thiol is GSH, also regarded as one of the main water soluble AOXs (Soares et al., 2019a). With respect to salt stress, this metabolite appears to be more relevant in the aerial part of the plants, as no differences could be found in roots neither in its content nor its regeneration (Table 3.2). Nonetheless, in shoots, GSH levels severely decreased (Table 3.3) alongside a small decrease in total thiols and the increase in GR activity (Figure 3.4f) attempting to maintain the GSH pool. In fact, similar results were found in different salt-stressed tomato cvs. (Gran brix and Marmande RAF) and var. (*Super 2270*), as de la Torre-González et al. (2017) and Ghorbani et al. (2018) also reported lower GSH content, with the latter, along with Yazici et al. (2007) and Ahmad et al. (2010), noting an enhanced GR

performance. This points towards a high oxidation rate, rather than degradation of this thiol, indicating that, since APX and DHAR activity remained unaltered (Figure 3.4a,b,d,e), GSH can be acting directly as a ROS scavenger or as a substrate for the ROS scavenging function of glutathione-peroxidase (GPX; EC 1.11.1.9), as H_2O_2 content was lower in this situation (SALT).

When under heat stress, either individual or in combination with salt, the levels of total thiols increased in roots of tomato plants, due to a high accumulation of protein-bound thiols (Table 3.2). Indeed, the main protein-thiols are glutaredoxins and thioredoxins (Zagorchev et al., 2013), with the latter having already been described as being important players in thermotolerance reactions (Ferreira et al., 2006; Lee et al., 2007), and thus contributing for the maintenance of the redox homeostasis. Aside from that, it is also important to note that in the combined treatment, GSH levels arose in roots (Table 3.2) alongside an overall upregulation of the AsA-GSH cycle (Table 3.2 and Figure 3.4a,b,c), highlighting its important role in ROS-scavenging reactions and in the maintenance of the redox homeostasis. In shoots (Table 3.3), both temperature-related conditions presented a similar decrease in GSH content, although the rationale behind that reduction can be different for both situations. Indeed, while the small decrease in GSH content in the heat treatment can be ascribed to a general irrelevance of the AsA-GSH cycle in this situation, coupled with a slight, but not significant reduction in the activity of substrate-regenerating enzymes, the same did not occur in the combined treatment. Here, there was a clear induction of all enzymes pertaining to the AsA-GSH cycle (Figure 3.4), indicating a major role of this thiol in the proper functioning of this cycle, while also possibly acting by itself as a ROS scavenger or as a substrate for GPX. Actually, similar results have been reported when plants were exposed to high temperatures (Zou et al., 2016) even though a possible effect of heat stress on the biosynthesis of this thiol was suggested. However, in studies performed in tomato exposed to heat as an individual stressor (Rivero et al., 2004) or in combination with salt (Lopez-Delacalle et al., 2021) showed an accumulation of this thiol, with or without the activation of the enzymatic cycle, granting more strength to the oxidation hypothesis than to that related to GSH degradation. Lastly, the protein class of thiols was severely affected (non-protein/protein thiols ratio almost doubled) only in the individual heat treatment in shoots (Table 3.3). In fact, several studies on heat-stressed plants have documented the potential of high temperatures to induce protein denaturation (Hassan et al., 2021), which contrasts with our data in roots, but can be related to the greater exposure of the aerial parts to this stressor.

Aside from the above-mentioned compounds, specialized metabolites, such as phenols, play a variety of roles in plants: from being signaling molecules to attracting pollinators and protecting against oxidative

damage as a consequence of abiotic stress (Waśkiewicz et al., 2013). In shoots (Table 3.2), these metabolites (measured as TPC and TFC) were only negatively affected in salt-treated tomato plants, with or without the exposure to heat. Being among the most common polyphenolic compounds, it is no surprise that this pattern was also observed for the flavonoid content. However, and although inconsistent results are found in the literature (Ahanger et al., 2020b; Bistgani et al., 2019; Frary et al., 2010), a review by Waśkiewicz et al. (2013) describes these parameters (TPC and TFC) as being extremely sensitive to differences in experimental conditions, especially in high salt concentrations. Nonetheless, as different phenols possess different characteristics and different AOX potential, it is possible that the resource allocation led to a shift in the phenolic pattern towards a higher relevance of those more important to the situation at hand and to the detriment of other types of phenolic compounds. In roots, however, both individual stressors affected the content in phenols and flavonoids but the same did not occur in the combined treatment. As salt and heat stressors are known to regularly induce the production and accumulation of different types of phenolic compounds, including flavonoids (Sharma et al., 2019; Waśkiewicz et al., 2013) it is possible that, although under these experimental situations the individual stressors led to their degradation, the convergence of defence pathways in the combined treatment efficiently restored TPC and TFC to control levels.

Overall, the results described above are mostly in agreement with the estimation of TAC, which assesses the global contribution of compounds such as phenols, flavonoids, some thiols and α-tocopherol (Young, 2001). In fact, in roots of *S. lycopersicum* plants only the combined treatment led to enhanced AOX capacity, with this possibly being ascribed to the increased accumulation of protein-thiols, proline and relatively higher phenolic compounds. In shoots, no effects were detected aside from a severe reduction in the heat treatment, which can likewise be related with a slightly reduced TPC and a much lower accumulation of protein-thiols.

4.4. Combined exposure to the stressors resulted in a prompter activation of the enzymatic AOX response, especially the AsA-GSH cycle enzymes

Classified as the first line of defence, SOD catalyses the detoxification of $O_2^{\bullet-}$ into H_2O_2 (Soares et al., 2019a). Upon salt exposure, this enzyme was not activated in tomato plants, which is in accordance with the maintenance of the content of $O_2^{\bullet-}$ and H_2O_2 in roots. However, a slight tendency for salt-treated plants to present a higher SOD catalytic activity in shoots is also correlated with the small decrease in $O_2^{\bullet-}$ content that is herein reported. Considering the H_2O_2-scavenging enzymes, and even though several authors report the enhancement of various AOX enzymes in response to salt (Ahanger et al., 2019; Manai

et al., 2014; Shalata and Tal, 1998), in the present work only CAT was activated, and just in roots, explaining the maintenance of H_2O_2 in an organ that, since it is responsible for salt uptake, is commonly associated with salinity-induced oxidative stress.

After exposure to high temperatures, $O_2^{\bullet-}$ levels remained unchanged (although a tendency to increase can be perceived), but a rise in the content of H_2O_2 was noticed in roots. This might be related to a SOD-mediated transformation of the former to the latter, and while no changes in its activity were reported, high basal SOD levels could be sufficient to deal with moderate stress conditions. Nonetheless, a tendency for SOD to possess higher activity in this treatment and organ can be noticed, as supported by Zhao et al. (2010) and Liu and Huang (2000). Unsurprisingly, due to ROS accumulation, CAT and APX activities were enhanced in an effort to detoxify H_2O_2 and prevent oxidative damage. In fact, heat stress has already been shown to result in increased APX in roots (Goyal and Asthir, 2010), although for CAT the opposite pattern is more common (Goyal and Asthir, 2010; Liu and Huang, 2000; Yuan et al., 2016). Moreover, APX activation did not result in an insufficient AsA pool, as both DHAR and GR remained unaffected, with efficient AsA regeneration possibly being ascribed to monodehydroascorbate (MDHAR; EC 1.6.5.4) action (Soares et al., 2019a). Contrarily to what was observed in roots, in shoots, no ROS were overaccumulated, and APX was not activated, while SOD and CAT were actually inhibited, effects also documented by Liu and Huang (2000) and Djanaguiraman et al. (2010) using 35 °C/ 25 °C and 40 °C/ 30 °C (day/night).

When plants were exposed to the combination of stressors, $O_2^{\bullet-}$ levels in roots were unaffected, which seems to be in agreement with the maintenance of SOD activity also reported in this organ, an effect that opposes the activation of this AOX enzyme documented by Zhao et al. (2010) in rice roots. Moreover, and though there was an activation of both H_2O_2-scavenging enzymes, APX and CAT, the AOX system did not fully detoxify this ROS, since H_2O_2 content was still higher than in CTL plants, but not at high enough levels to induce noticeable oxidative damage. In fact, is it possible that H_2O_2, under these still higher concentrations, might serve as a signalling agent to prepare the plant for subsequent ROS bursts (Foyer and Noctor, 2005). In shoots, and similarly to what Lopez-Delacalle et al. (2021) reported, SOD was as activated as upon the single salinity treatment, thus, explaining the highly diminished levels of $O_2^{\bullet-}$. Nonetheless, it would be expected an accumulation of H_2O_2, which did not occur, possibly due to the enhanced activity of APX, a result also documented by Lopez-Delacalle et al. (2021). Oppositely, CAT was inhibited in shoots, however this might have little impact on the global AOX response due to its lower affinity to H_2O_2 (Soares et al., 2019a), and the ability of these plants to maintain the redox homeostasis through other mechanisms. Overall, in plants exposed to the combination of salt and heat, the AOX

enzymes, especially the ones involved in the AsA-GSH cycle, seem to be determinant to maintain redox homeostasis in shoots, while in roots the enzymatic and the non-enzymatic components play together an important role in the response of tomato plants to the combined challenge of heat and salt stress.

5. Conclusion

Considering the data here presented, it is possible to assume that the AOX system, especially the AsA-GSH cycle, was of major importance in the response of *S. lycopersicum* L. plants to the co-exposure to heat and salt. The combination of these stressors not only resulted in higher impacts at both growth and biochemical levels, but also led to a higher accumulation of Na^+ than the individual stresses (Figure 3.6). In fact, when observing the PCA (Figure 3.5), the combined treatment was plotted apart from all other treatments, with the main differences being associated with the higher accumulation of Na^+ in both organs, which is paralleled by a decrease in the uptake of Ca^{2+}, K^+ and Mg^{2+}, as well as a drastic reduction in plant growth. Nonetheless, the co-exposure also resulted in an efficient activation of the AOX enzymes in the aerial part of plants and the collective stimulation of both enzymatic and non-enzymatic components of the AOX system in the roots. Thus, these results might suggest that, despite the accumulation of toxic ions, the already limited plant resources are being allocated towards defensive pathways to ensure survival under these adverse conditions. Nonetheless, and very alarmingly, growth was more negatively affected by the combination than by the sum of both individual stressors and imposes a worrying trend in a world facing an increasing climatic instability. In this sense, it is imperative that further studies are undertaken in order to complement what has been here reported (*e.g.* analysis of photosynthetic machinery and possible tolerance traits to these stressors), as well as to develop new and efficient ways to successfully alleviate the negative effects of these abiotic stresses, thus minimizing losses in crop productivity. Since tomato plants seemed to heavily invest on AOX mechanisms to counteract heat- and salt co-exposure, the evaluation of AOX-promoting agents, such as biostimulants, phytohormones or beneficial elements, could also represent a feasible tool to increase tomato tolerance to these two stressors.

Figure 3.6. Overview of the main results of the present chapter.

References

Aebi, H., 1984. Catalase *in vitro*. Methods Enzymol. 105, 121–6. https://doi.org/10.1016/S0076-6879(84)05016-3

Ahanger, M.A., Aziz, U., Alsahli, A.A., Alyemeni, M.N., Ahmad, P., 2020a. Influence of Exogenous Salicylic Acid and Nitric Oxide on Growth, Photosynthesis, and Ascorbate-Glutathione Cycle in Salt Stressed *Vigna angularis*. Biomolecules. https://doi.org/10.3390/biom10010042

Ahanger, M.A., Mir, R.A., Alyemeni, M.N., Ahmad, P., 2020b. Combined effects of brassinosteroid and kinetin mitigates salinity stress in tomato through the modulation of antioxidant and osmolyte metabolism. Plant Physiol. Biochem. 147, 31–42. https://doi.org/10.1016/j.plaphy.2019.12.007

Ahanger, M.A., Qin, C., Maodong, Q., Dong, X.X., Ahmad, P., Abd-Allah, E.F., Zhang, L., 2019. Spermine application alleviates salinity induced growth and photosynthetic inhibition in *Solanum lycopersicum* by modulating osmolyte and secondary metabolite accumulation and differentially regulating antioxidant metabolism. Plant Physiol. Biochem. 144, 1–13. https://doi.org/10.1016/j.plaphy.2019.09.021

Ahmad, P., Jaleel, C.A., Sharma, S., 2010. Antioxidant defense system, lipid peroxidation, proline-metabolizing enzymes, and biochemical activities in two *Morus alba* genotypes subjected to NaCl stress. Russ. J. Plant Physiol. 57, 509–517. https://doi.org/10.1134/S1021443710040084

Ahmad, P., Kumar, A., Ashraf, M., Akram, N.A., 2012. Salt-induced changes in photosynthetic activity and oxidative defense system of three cultivars of mustard (*Brassica juncea* L.). African J. Biotechnol. 11, 2694–2703. https://doi.org/10.5897/AJB11.3203

Al Hassan, M., Martínez Fuertes, M., Ramos Sánchez, F.J., Vicente, O., Boscaiu, M., 2015. Effects of Salt and Water Stress on Plant Growth and on Accumulation of Osmolytes and Antioxidant Compounds in Cherry Tomato. Not. Bot. Horti Agrobot. Cluj-Napoca. 43. https://doi.org/10.15835/nbha.43.1.9793

Amirjani, M.R., 2011. Effect of salinity stress on growth, sugar content, pigments and enzyme activity of rice. Int. J. Bot. 7, 73–81. https://doi.org/10.3923/ijb.2011.73.81

Ashraf, M., Hafeez, M., 2004. Thermotolerance of pearl millet and maize at early growth stages: growth and nutrient relations. Biol. Plant. 48, 81–86. https://doi.org/10.1023/B:BIOP.0000024279.44013.61

Ashraf, M.A., Ashraf, M., Shahbaz, M., 2012. Growth stage-based modulation in antioxidant defense system and proline accumulation in two hexaploid wheat (*Triticum aestivum* L.) cultivars differing in salinity tolerance. Flora - Morphol. Distrib. Funct. Ecol. Plants 207, 388–397. https://doi.org/https://doi.org/10.1016/j.flora.2012.03.004

Ayers, R.S., Westcot, D.W., 1985. Water quality for agriculture. FAO. Rome.

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

Bistgani, Z.E., Hashemi, M., DaCosta, M., Craker, L., Maggi, F., Morshedloo, M.R., 2019. Effect of salinity stress on the physiological characteristics, phenolic compounds and antioxidant activity of *Thymus vulgaris* L. and *Thymus daenensis* Celak. Ind. Crops Prod. 135, 311–320. https://doi.org/10.1016/j.indcrop.2019.04.055

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Cachorro, P., Ortiz, A., Cerdá, A., 1994. Implications of calcium nutrition on the response of *Phaseolus vulgaris* L. to salinity. Plant Soil 159, 205–212. https://doi.org/10.1007/BF00009282

Carvalho, D., Cardoso Pereira, S., Rocha, A., 2021. Future surface temperature changes for the Iberian Peninsula according to EURO-CORDEX climate projections. Clim. Dyn. 56, 123–138. https://doi.org/10.1007/s00382-020-05472-3

Chaichi, M.R., Keshavarz-Afshar, R., Lu, B., Rostamza, M., 2017. Growth and nutrient uptake of tomato in response to application of saline water, biological fertilizer, and surfactant. J. Plant Nutr. 40, 457–466. https://doi.org/10.1080/01904167.2016.1246567

Claussen, W., 2005. Proline as a measure of stress in tomato plants. Plant Sci. 168, 241–248. https://doi.org/10.1016/j.plantsci.2004.07.039

Daliakopoulos, I.N., Tsanis, I.K., Koutroulis, A., Kourgialas, N.N., Varouchakis, A.E., Karatzas, G.P., Ritsema, C.J., 2016. The threat of soil salinity: A European scale review. Sci. Total Environ. 573, 727–739. https://doi.org/10.1016/j.scitotenv.2016.08.177

de la Torre-González, A., Navarro-León, E., Albacete, A., Blasco, B., Ruiz, J.M., 2017. Study of phytohormone profile and oxidative metabolism as key process to identification of salinity response in tomato commercial genotypes. J. Plant Physiol. 216, 164–173. https://doi.org/10.1016/j.jplph.2017.05.016

de Sousa, A., Teixeira, J., Regueiras, M.T., Azenha, M., Silva, F., Fidalgo, F., 2013. Metalaxyl-induced changes in the antioxidant metabolism of *Solanum nigrum* L. suspension cells. Pestic. Biochem. Physiol. 107, 235–243. https://doi.org/10.1016/J.PESTBP.2013.07.006

Debouba, M., Gouia, H., Suzuki, A., Ghorbel, M.H., 2006. NaCl stress effects on enzymes involved in nitrogen assimilation pathway in tomato "*Lycopersicon esculentum*" seedlings. J. Plant Physiol. 163, 1247–1258. https://doi.org/10.1016/j.jplph.2005.09.012

Djanaguiraman, M., Prasad, P.V.V., Seppanen, M., 2010. Selenium protects sorghum leaves from oxidative damage under high temperature stress by enhancing antioxidant defense system. Plant Physiol. Biochem. 48, 999–1007. https://doi.org/10.1016/j.plaphy.2010.09.009

Dolatabadian, A., Modarres Sanavy, S.A.M., Ghanati, F., 2011. Effect of Salinity on Growth, Xylem Structure and Anatomical Characteristics of Soybean. Not. Sci. Biol. 3. https://doi.org/10.15835/nsb315627

Donahue, J.L., Okpodu, C.M., Cramer, C.L., Grabau, E.A., Alscher, R.G., 1997. Responses of Antioxidants to Paraquat in Pea Leaves (Relationships to Resistance). Plant Physiol. 113, 249–257.

https://doi.org/10.1104/PP.113.1.249

FAO, 2009. How to Feed the World in 2050 [WWW Document]. URL http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf (accessed 6.29.21).

Ferreira, S., Hjernø, K., Larsen, M., Wingsle, G., Larsen, P., Fey, S., Roepstorff, P., Salomé Pais, M., 2006. Proteome Profiling of *Populus euphratica* Oliv. Upon Heat Stress. Ann. Bot. 98, 361–377. https://doi.org/10.1093/aob/mcl106

Fidalgo, F., Freitas, R., Ferreira, R., Pessoa, A.M., Teixeira, J., 2011. *Solanum nigrum* L. antioxidant defence system isozymes are regulated transcriptionally and posttranslationally in Cd-induced stress. Environ. Exp. Bot. 72, 312–319. https://doi.org/10.1016/j.envexpbot.2011.04.007

Fidalgo, F., Santos, A., Santos, I., Salema, R., 2004. Effects of long-term salt stress on antioxidant defence systems, leaf water relations and chloroplast ultrastructure of potato plants. Ann. Appl. Biol. 145, 185–192. https://doi.org/10.1111/j.1744-7348.2004.tb00374.x

Foyer, C.H., Halliwell, B., 1976. The presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. Planta 133, 21–25. https://doi.org/10.1007/BF00386001

Foyer, C.H., Noctor, G., 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant. Cell Environ. 28, 1056–1071. https://doi.org/10.1111/j.1365-3040.2005.01327.x

Frary, A., Göl, D., Keleş, D., Ökmen, B., Pınar, H., Şığva, H.Ö., Yemenicioğlu, A., Doğanlar, S., 2010. Salt tolerance in *Solanum pennellii*: antioxidant response and related QTL. BMC Plant Biol. 10, 58. https://doi.org/10.1186/1471-2229-10-58

Gharsallah, C., Fakhfakh, H., Grubb, D., Gorsane, F., 2016. Effect of salt stress on ion concentration, proline content, antioxidant enzyme activities and gene expression in tomato cultivars. AoB Plants 8. https://doi.org/10.1093/aobpla/plw055

Ghorbani, A., Razavi, S.M., Omran, V.O.G., Pirdashti, H., 2018. *Piriformospora indica* Alleviates Salinity by Boosting Redox Poise and Antioxidative Potential of Tomato. Russ. J. Plant Physiol. 65, 898–907. https://doi.org/10.1134/S1021443718060079

Gillespie, K.M., Ainsworth, E.A., 2007. Measurement of reduced, oxidized and total ascorbate content in plants. Nat. Protoc. 2, 871–874. https://doi.org/10.1038/nprot.2007.101

Giri, A., Heckathorn, S., Mishra, S., Krause, C., 2017. Heat Stress Decreases Levels of Nutrient-Uptake and -Assimilation Proteins in Tomato Roots. Plants. https://doi.org/10.3390/plants6010006

Goyal, M., Asthir, B., 2010. Polyamine catabolism influences antioxidative defense mechanism in shoots and roots of five wheat genotypes under high temperature stress. Plant Growth Regul. 60, 13–25. https://doi.org/10.1007/s10725-009-9414-8

Gupta, B., Huang, B., 2014. Mechanism of Salinity Tolerance in Plants: Physiological, Biochemical, and Molecular Characterization. Int. J. Genomics 2014, 701596. https://doi.org/10.1155/2014/701596

Haddeland, I., Heinke, J., Biemans, H., Eisner, S., Flörke, M., Hanasaki, N., Konzmann, M., Ludwig, F., Masaki, Y., Schewe, J., 2014. Global water resources affected by human interventions and climate change. Proc. Natl. Acad. Sci. 111, 3251–3256. https://doi.org/10.1073/pnas.1222475110

Harsh, A., Sharma, Y.K., Joshi, U., Rampuria, S., Singh, G., Kumar, S., Sharma, R., 2016. Effect of short-term heat stress on total sugars, proline and some antioxidant enzymes in moth bean (*Vigna aconitifolia*). Ann. Agric. Sci. 61, 57–64. https://doi.org/10.1016/j.aoas.2016.02.001

Hasanuzzaman, M., Fujita, M., Islam, M.N., Ahamed, K.U., Nahar, K., 2009. Performance of four irrigated rice varieties under different levels of salinity stress. Int. J. Integr. Biol. 6, 85–90.

Hasanuzzaman, M., Hossain, M.A., Fujita, M., 2011. Selenium-Induced Up-Regulation of the Antioxidant Defense and Methylglyoxal Detoxification System Reduces Salinity-Induced Damage in Rapeseed Seedlings. Biol. Trace Elem. Res. 143, 1704–1721. https://doi.org/10.1007/s12011-011-8958-4

Hasanuzzaman, M., Nahar, K., Alam, M.M., Roychowdhury, R., Fujita, M., 2013. Physiological, Biochemical, and Molecular Mechanisms of Heat Stress Tolerance in Plants. Int. J. Mol. Sci. 14. https://doi.org/10.3390/ijms14059643

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., 2021. Heat stress in cultivated plants: nature, impact, mechanisms, and mitigation strategies—a review. Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol. 155, 211–234. https://doi.org/10.1080/11263504.2020.1727987

Hayat, S., Masood, A., Yusuf, M., Fariduddin, Q., Ahmad, A., 2009. Growth of Indian mustard (*Brassica juncea* L.) in response to salicylic acid under high-temperature stress. Brazilian J. Plant Physiol. 21, 187–195. https://doi.org/10.1590/S1677-04202009000300003

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198. https://doi.org/10.1016/0003-9861(68)90654-1

Hernández, J.A., 2019. Salinity Tolerance in Plants: Trends and Perspectives. Int. J. Mol. Sci. 20, 10. https://doi.org/10.3390/ijms20102408

Isayenkov, S. V, Maathuis, F.J.M., 2019. Plant Salinity Stress: Many Unanswered Questions Remain. Front. Plant Sci. 10, 80. https://doi.org/10.3389/fpls.2019.00080

Jin, R., Wang, Y., Liu, R., Gou, J., Chan, Z., 2016. Physiological and Metabolic Changes of Purslane (*Portulaca oleracea* L.) in Response to Drought, Heat, and Combined Stresses. Front. Plant Sci. 6, 1123. https://doi.org/10.3389/fpls.2015.01123

Khan, M.H., Panda, S.K., 2007. Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl-salinity stress. Acta Physiol. Plant. 30, 81. https://doi.org/10.1007/s11738-007-0093-7

Khan, M.I.R., Iqbal, N., Masood, A., Per, T.S., Khan, N.A., 2013. Salicylic acid alleviates adverse effects of heat stress on photosynthesis through changes in proline production and ethylene formation. Plant Signal. Behav. 8, e26374. https://doi.org/10.4161/psb.26374

Khavari-Nejad, R.A., Mostofi, Y., 1998. Effects of NaCl on photosynthetic pigments, saccharides, and chloroplast ultrastructure in leaves of tomato cultivars. Photosynthetica 35, 151–154. https://doi.org/10.1023/A:1006846504261

Koutroulis, A.G., Tsanis, I.K., Daliakopoulos, I.N., Jacob, D., 2013. Impact of climate change on water resources status: A case study for Crete Island, Greece. J. Hydrol. 479, 146–158. https://doi.org/10.1016/j.jhydrol.2012.11.055

Lee, D.G., Ahsan, N., Lee, S.H., Kang, K.Y., Bahk, J.D., Lee, I.J., Lee, B.H., 2007. A proteomic approach in analyzing heat-responsive proteins in rice leaves. Proteomics 7, 3369–3383. https://doi.org/10.1002/pmic.200700266

Li, W., Zhang, C., Lu, Q., Wen, X., Lu, C., 2011. The combined effect of salt stress and heat shock on proteome profiling in *Suaeda salsa*. J. Plant Physiol. 168, 1743–1752. https://doi.org/10.1016/j.jplph.2011.03.018

Liang, X., Zhang, L., Natarajan, S.K., Becker, D.F., 2013. Proline mechanisms of stress survival. Antioxid. Redox Signal. 19, 998–1011. https://doi.org/10.1089/ars.2012.5074

Liu, X., Huang, B., 2000. Heat Stress Injury in Relation to Membrane Lipid Peroxidation in Creeping Bentgrass. Crop Sci. 40, 503–510. https://doi.org/10.2135/cropsci2000.402503x

Lopez-Delacalle, M., Silva, C.J., Mestre, T.C., Martinez, V., Blanco-Ulate, B., Rivero, R.M., 2021. Synchronization of proline, ascorbate and oxidative stress pathways under the combination of salinity and heat in tomato plants. Environ. Exp. Bot. 183, 104351. https://doi.org/10.1016/j.envexpbot.2020.104351

Ma, F., Cheng, L., 2004. Exposure of the shaded side of apple fruit to full sun leads to up-regulation of both the xanthophyll cycle and the ascorbate–glutathione cycle. Plant Sci. 166, 1479–1486. https://doi.org/10.1016/j.plantsci.2004.01.024

Manai, J., Kalai, T., Gouia, H., Corpas, F.J., 2014. Exogenous nitric oxide (NO) ameliorates salinity-induced oxidative stress in tomato (*Solanum lycopersicum*) plants. J. Soil Sci. Plant Nutr. 14, 433–446. http://dx.doi.org/10.4067/S0718-95162014005000034

Mao, D., Chen, J., Tian, L., Liu, Z., Yang, L., Tang, R., Li, J., Lu, C., Yang, Y., Shi, J., Chen, L., Li, D., Luan, S., 2014. *Arabidopsis* Transporter MGT6 Mediates Magnesium Uptake and Is Required for Growth under Magnesium Limitation. Plant Cell 26, 2234–2248. https://doi.org/10.1105/tpc.114.124628

Margalha, L., Confraria, A., Baena-González, E., 2019. SnRK1 and TOR: modulating growth–defense trade-offs in plant stress responses. J. Exp. Bot. 70, 2261–2274. https://doi.org/10.1093/jxb/erz066

Matías, J., Rodríguez, M.J., Cruz, V., Calvo, P., Reguera, M., 2021. Heat stress lowers yields, alters nutrient uptake and changes seed quality in quinoa grown under Mediterranean field conditions. J. Agron. Crop Sci. 207, 481–491. https://doi.org/10.1111/jac.12495

Mishra, P., Bhoomika, K., Dubey, R.S., 2013. Differential responses of antioxidative defense system to prolonged salinity stress in salt-tolerant and salt-sensitive Indica rice (*Oryza sativa* L.) seedlings. Protoplasma 250, 3–19. https://doi.org/10.1007/s00709-011-0365-3

Mittova, V., Guy, M., Tal, M., Volokita, M., 2004. Salinity up-regulates the antioxidative system in root mitochondria and peroxisomes of the wild salt-tolerant tomato species *Lycopersicon pennellii*. J. Exp. Bot. 55, 1105–1113. https://doi.org/10.1093/jxb/erh113

Murashige, T., Skoog, F., 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol. Plant. 15, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Murshed, R., Lopez-Lauri, F., Sallanon, H., 2008. Microplate quantification of enzymes of the plant ascorbate–glutathione cycle. Anal. Biochem. 383, 320–322. https://doi.org/10.1016/j.ab.2008.07.020

Nagesh Babu, R., Devaraj, V.R., 2008. High temperature and salt stress response in French bean (*Phaseolus vulgaris*). Aust J Crop Sci 2, 40–48.

Nakano, Y., Asada, K., 1981. Hydrogen Peroxide is Scavenged by Ascorbate-specific Peroxidase in Spinach Chloroplasts. Plant Cell Physiol. 22, 867–880. https://doi.org/10.1093/oxfordjournals.pcp.a076232

Noctor, G., Foyer, C.H., 1998. Ascorbate and Glutathione: Keeping Active Oxygen Under Control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249–279. https://doi.org/10.1146/annurev.arplant.49.1.249

Nxele, X., Klein, A., Ndimba, B.K., 2017. Drought and salinity stress alters ROS accumulation, water retention, and osmolyte content in sorghum plants. South African J. Bot. 108, 261–266. https://doi.org/10.1016/j.sajb.2016.11.003

Parihar, P., Singh, S., Singh, R., Singh, V.P., Prasad, S.M., 2015. Effect of salinity stress on plants and its tolerance strategies: a review. Environ. Sci. Pollut. Res. 22, 4056–4075. https://doi.org/10.1007/s11356-014-3739-1

Parrotta, L., Aloisi, I., Faleri, C., Romi, M., Del Duca, S., Cai, G., 2020. Chronic heat stress affects the

photosynthetic apparatus of *Solanum lycopersicum* L. cv Micro-Tom. Plant Physiol. Biochem. 154, 463–475. https://doi.org/10.1016/j.plaphy.2020.06.047

Prosekov, A.Y., Ivanova, S.A., 2018. Food security: The challenge of the present. Geoforum 91, 73–77. https://doi.org/10.1016/j.geoforum.2018.02.030

Qafoku, N.P., 2015. Chapter Two - Climate-Change Effects on Soils: Accelerated Weathering, Soil Carbon, and Elemental Cycling, in: Sparks, D.L. (Ed.), Advances in Agronomy. Academic Press, pp. 111–172. https://doi.org/10.1016/bs.agron.2014.12.002

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 10, 208. https://doi.org/10.1007/s13205-020-02206-4

Rajametov, S.N., Yang, E.Y., Jeong, H.B., Cho, M.C., Chae, S.Y., Paudel, N., 2021. Heat Treatment in Two Tomato Cultivars: A Study of the Effect on Physiological and Growth Recovery. Hortic.. https://doi.org/10.3390/horticulturae7050119

Rivero, R.M., Mestre, T.C., Mittler, R., Rubio, F., Garcia-Sanchez, F., Martinez, V., 2014. The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. Plant. Cell Environ. 37, 1059–1073. https://doi.org/10.1111/pce.12199

Rivero, R.M., Ruiz, J.M., Romero, L., 2004. Oxidative metabolism in tomato plants subjected to heat stress. J. Hortic. Sci. Biotechnol. 79, 560–564. https://doi.org/10.1080/14620316.2004.11511805

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., Mittler, R., 2004. When Defense Pathways Collide. The Response of Arabidopsis to a Combination of Drought and Heat Stress. Plant Physiol. 134, 1683–1696. https://doi.org/10.1104/pp.103.033431

Shabala, S., Munns, R., 2017. Salinity stress: physiological constraints and adaptive mechanisms. Shabala, S. (Eds.), Plant Stress Physiology. Cabi. http://dx.doi.org/10.1079/9781845939953.0059

Shalata, A., Tal, M., 1998. The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. Physiol. Plant. 104, 169–174. https://doi.org/10.1034/j.1399-3054.1998.1040204.x

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

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 Biol. 17, 56. https://doi.org/10.1186/s12870-017-1003-9

Sigel, H., Sigel, A., 1990. Metal ions in biological systems: Volume 26: Compendium on Magnesium and Its Role in Biology: Nutrition and Physiology. CRC Press.

Singh, M., Kumar, J., Singh, S., Singh, V.P., Prasad, S.M., 2015. Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. Rev. Environ. Sci. Bio/Technology 14, 407–426. https://doi.org/10.1007/s11157-015-9372-8

Soares, C., Carvalho, M.E.A., Azevedo, R.A., Fidalgo, F., 2019a. Plants facing oxidative challenges — A little help from the antioxidant networks. Environ. Exp. Bot. 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. Environ. Pollut. 247, 256–265. https://doi.org/10.1016/j.envpol.2019.01.063

St.Clair, S.B., Lynch, J.P., 2010. The opening of Pandora's Box: climate change impacts on soil fertility and crop nutrition in developing countries. Plant Soil 335, 101–115. https://doi.org/10.1007/s11104-010-0328-z

Sterling, T.M., 2005. Transpiration: Water Movement through Plants. J. Nat. Resour. Life Sci. Educ. 34, 123. https://doi.org/10.2134/jnrlse.2005.0123

Sun, C., Gao, X., Fu, J., Zhou, J., Wu, X., 2015. Metabolic response of maize (*Zea mays* L.) plants to combined drought and salt stress. Plant Soil 388, 99–117. https://doi.org/10.1007/s11104-014-2309-0

Suzuki, N., Bassil, E., Hamilton, J.S., Inupakutika, M.A., Zandalinas, S.I., Tripathy, D., Luo, Y., Dion, E., Fukui, G., Kumazaki, A., Nakano, R., Rivero, R.M., Verbeck, G.F., Azad, R.K., Blumwald, E., Mittler, R., 2016. ABA Is Required for Plant Acclimation to a Combination of Salt and Heat Stress. PLoS One 11, e0147625. https://doi.org/10.1371/journal.pone.0147625

Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and biotic stress combinations. New Phytol. 203, 32–43. https://doi.org/10.1111/nph.12797

Taiz, L., Zeiger, E., Møller, I.M., Murphy, A., 2015. Plant physiology and development., 6th ed. Sinauer Associates Incorporated, Sunderland, MA, USA.

Tanveer, K., Gilani, S., Hussain, Z., Ishaq, R., Adeel, M., Ilyas, N., 2020. Effect of salt stress on tomato plant and the role of calcium. J. Plant Nutr. 43, 28–35. https://doi.org/10.1080/01904167.2019.1659324

Tavakkoli, E., Fatehi, F., Coventry, S., Rengasamy, P., McDonald, G.K., 2011. Additive effects of Na^+ and Cl^- ions on barley growth under salinity stress. J. Exp. Bot. 62, 2189–2203. https://doi.org/10.1093/jxb/erq422

Tuna, A.L., Kaya, C., Ashraf, M., Altunlu, H., Yokas, I., Yagmur, B., 2007. The effects of calcium sulphate on growth, membrane stability and nutrient uptake of tomato plants grown under salt stress. Environ. Exp. Bot. 59, 173–178. https://doi.org/10.1016/j.envexpbot.2005.12.007

Usman, M.G., Rafii, M.Y., Martini, M.Y., Yusuff, O.A., Ismail, M.R., Miah, G., 2017. Molecular analysis of Hsp70 mechanisms in plants and their function in response to stress. Biotechnol. Genet. Eng. Rev. 33, 26–39. https://doi.org/10.1080/02648725.2017.1340546

Wahid, A., Gelani, S., Ashraf, M., Foolad, M.R., 2007. Heat tolerance in plants: an overview. Environ. Exp. Bot. 61, 199–223. https://doi.org/10.1016/j.envexpbot.2007.05.011

Waśkiewicz, A., Muzolf-Panek, M., Goliński, P., 2013. Phenolic Content Changes in Plants Under Salt Stress, in: Ahmad, P., Azooz, M.M., Prasad, M.N.V. (Eds.), Ecophysiology and Responses of Plants under Salt Stress. Springer New York, New York, NY, pp. 283–314. https://doi.org/10.1007/978-1-4614-4747-4_11

Yazici, I., Türkan, I., Sekmen, A.H., Demiral, T., 2007. Salinity tolerance of purslane (*Portulaca oleracea* L.) is achieved by enhanced antioxidative system, lower level of lipid peroxidation and proline accumulation. Environ. Exp. Bot. 61, 49–57. https://doi.org/10.1016/j.envexpbot.2007.02.010

Young, I.S., 2001. Measurement of total antioxidant capacity. J. Clin. Pathol. 5, 339. https://doi.org/10.1136/jcp.54.5.339

Yuan, L., Liu, S., Zhu, S., Chen, G., Liu, F., Zou, M., Wang, C., 2016. Comparative response of two wucai (*Brassica campestris* L.) genotypes to heat stress on antioxidative system and cell ultrastructure in root. Acta Physiol. Plant. 38, 223. https://doi.org/10.1007/s11738-016-2246-z

Zafar, H., Ali, A., Ali, J.S., Haq, I.U., Zia, M., 2016. Effect of ZnO Nanoparticles on *Brassica nigra* Seedlings and Stem Explants: Growth Dynamics and Antioxidative Response. Front. Plant Sci. 7, 535. https://doi.org/10.3389/fpls.2016.00535

Zagorchev, L., Seal, C.E., Kranner, I., Odjakova, M., 2013. A Central Role for Thiols in Plant Tolerance to Abiotic Stress. Int. J. Mol. Sci. 14, 7405-7432. https://doi.org/10.3390/ijms14047405

Zhang, H., Lian, C., Shen, Z., 2009. Proteomic identification of small, copper-responsive proteins in germinating embryos of *Oryza sativa*. Ann. Bot. 103, 923–930. https://doi.org/10.1093/aob/mcp012

Zhao, F.Y., Liu, T., Xu, Z.J., 2010. Modified responses of root growth and reactive oxygen species-scavenging system to combined salt and heat stress in transgenic rice. Russ. J. Plant Physiol. 57, 518–525. https://doi.org/10.1134/S1021443710040096

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 Biol. 17, 24. https://doi.org/10.1186/s12870-017-0974-x

Zou, M., Yuan, L., Zhu, S., Liu, S., Ge, J., Wang, C., 2016. Response of osmotic adjustment and ascorbate-glutathione cycle to heat stress in a heat-sensitive and a heat-tolerant genotype of wucai (*Brassica campestris* L.). Sci. Hortic. (Amsterdam). 211, 87–94. https://doi.org/10.1016/j.scienta.2016.08.011

Supplementary Material

Table S3.4. Physicochemical characteristics of the Siro Royal universal substrate (SIRO®, Portugal) used for the plant assay.

PHYSICOCHEMICAL CHARACTERISTICS	
pH ($CaCl_2$)	5.5 – 6.5
Electric conductivity	50 – 100 µs cm^{-1}
Granulometry	0 – 15 mm
Organic matter	> 70%
NPK	19 – 7 – 10

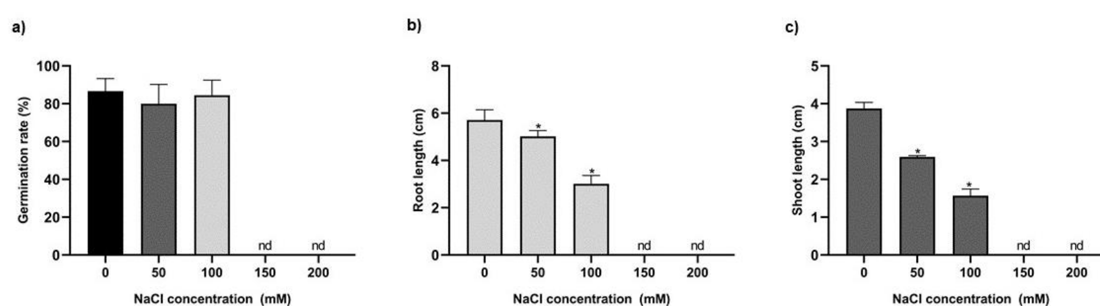

Figure S3.1. Percentage of germination (a), root length (b) and shoot length (c) of tomato seedlings grown for 7 days in solid MS nutritive medium, supplemented with different concentrations of NaCl (0, 50, 100, 150 and 200 mM). Data presented are mean ± SEM (n ≥ 3). Asterisks above the error bars indicate significant statistical differences between treatments and the control at $p \leq 0.05$, assessed through Tukey post-hoc test, following a one-way ANOVA.

Table S3.2. Results of the two-way ANOVA for all evaluated parameters in roots of *Solanum lycopersicum* L. var. *cerasiforme* after 21 days of exposure to 42 °C (4 hours per day) and irrigation with (100 mM) or without NaCl. Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted in bold.

PARAMETER (roots)	Factors		Interaction
	SALT	***HEAT***	
Length	**F (1, 43) = 28.26; *p* < 0.0001**	**F (1, 43) = 52.66; *p* < 0.0001**	F (1, 43) = 0.6012; *p* = 0.4424
Dry weight	**F (1, 43) = 36.84; *p* < 0.0001**	**F (1, 43) = 40.38; *p* < 0.0001**	F (1, 43) = 0.5333; *p* = 0.4692
Water content	F (1, 19) = 0.4653; *p* = 0.4653	F (1, 19) = 0.9359; *p* = 0.3455	F (1, 19) = 0.007945; *p* = 0.9299
Na^+	**F (1, 8) = 656421; *p* < 0.0001**	**F (1, 8) = 2863; *p* < 0.0001**	**F (1, 8) = 1834; *p* < 0.0001**
K^+	**F (1, 8) = 19837; *p* < 0.0001**	**F (1, 8) = 691.1; *p* < 0.0001**	**F (1, 8) = 2244; *p* < 0.0001**
Ca^{2+}	**F (1, 8) = 3321; *p* < 0.0001**	**F (1, 8) = 55.13; *p* < 0.0001**	**F (1, 8) = 496.1; *p* < 0.0001**
Mg^{2+}	**F (1, 8) = 165.7; *p* < 0.0001**	**F (1, 8) = 414.0; *p* < 0.0001**	**F (1, 8) = 374.4; *p* < 0.0001**
$O_2^{\cdot-}$	F (1, 8) = 2.350; *p* = 0.1638	F (1, 8) = 0.0004534; *p* = 0.9835	F (1, 8) = 1.930; *p* = 0.2022
H_2O_2	F (1, 8) = 1.694; *p* = 0.2293	**F (1, 8) = 61.16; *p* < 0.0001**	F (1,8) = 1.731; *p* = 0.2248
MDA	**F (1, 11) = 5.810; *p* = 0.0346**	F (1, 11) = 4.635; *p* = 0.0544	F (1, 11) = 0.2805; *p* = 0.6069
Proline	**F (1, 9) = 830.0; *p* < 0.0001**	**F (1, 9) = 19.13; *p* = 0.018**	**F (1, 9) = 18.55; *p* = 0.0020**
Total AsA	F (1, 10) = 0.02314; *p* = 0.8821	F (1, 10) = 2.693; *p* = 1318	F (1, 10) = 0.6917; *p* = 0.4250
AsA	F (1, 10) = 0.06414; *p* = 0.8052	F (1, 10) = 0.01829; *p* = 0.8951	F (1, 10) = 0.1727; *p* = 0.6865
DHA	F (1, 9) = 0.1271; *p* = 0.7296	F (1, 9) = 1.268; *p* = 0.2892	F (1, 9) = 0.3393; *p* = 0.5745
AsA/DHA	F (1, 9) = 0.7880; *p* = 0.3978	F (1, 9) = 1.967; *p* = 0.1944	F (1, 9) = 0.1392; *p* = 0.7177
GSH	F (1, 9) = 2.005; *p* = 0.1905	**F (1, 9) = 18.68; *p* = 0.0019**	**F (1, 9) = 5.400; *p* = 0.0452**
Total thiols	F (1, 8) = 3.618; *p* = 0.0937	**F (1, 8) = 79.12; *p* < 0.0001**	F (1, 8) = 1.373; *p* = 0.2749
Non-protein/ Protein thiols	F (1, 10) = 0.1765; *p* = 0.6833	**F (1, 10) = 6.380; *p* = 0.0301**	F (1, 10) = 1.509; *p* = 0.2474
Phenols	F (1, 11) = 4.437; *p* = 0.0590	F (1, 11) = 0.004443; *p* = 0.9481	**F (1, 11) = 8.747; *p* = 0.0130**
Flavonoids	F (1, 12) = 2.770; *p* = 0.1219	F (1, 12) = 0.5301; *p* = 0.4805	**F (1, 12) = 16.07; *p* = 0.0017**
TAC	**F (1, 11) = 26.70; *p* = 0.0003**	F (1, 11) = 2.968; *p* = 0.1129	**F (1, 11) = 9.226; *p* = 0.0113**
SOD	F (1, 11) = 0.006482; *p* = 0.9373	F (1, 11) = 2.057; *p* = 0.1793	F (1, 11) = 4.219; *p* = 0.0645
CAT	**F (1, 10) = 15.01; *p* = 0.0031**	**F (1, 10) = 9.137; *p* = 0.0128**	**F (1, 10) = 7.859; *p* = 0.0187**
APX	**F (1, 9) = 8.434; *p* = 0.0175**	**F (1, 9) = 43.25; *p* = 0.0001**	F (1, 9) = 0.001389; *p* = 0.9711
DHAR	**F (1, 9) = 7.860; *p* = 0.0206**	**F (1, 9) = 58.39; *p* <0.0001**	**F (1, 9) = 24.81; *p* = 0.0008**
GR	F (1, 9) = 2.326; *p* = 0.1616	**F (1, 9) = 13.90; *p* = 0.0047**	**F (1, 9) = 34.27; *p* = 0.0002**

Table S3.3. Results of the two-way ANOVA for all evaluated parameters in shoots of *Solanum lycopersicum* L. var. *cerasiforme* after 21 days of exposure to 42 °C (4 hours per day) and irrigation with (100 mM) or without NaCl. Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted in bold.

PARAMETER (shoots)	Factors		Interaction
	SALT	*HEAT*	
Length	**$F_{(1, 36)} = 107.0$; $p < 0.0001$**	**$F_{(1, 36)} = 112.6$; $p < 0.0001$**	$F_{(1, 36)} = 0.8989$; $p = 0.3494$
Dry weight	**$F_{(1, 42)} = 59.36$; $p < 0.0001$**	**$F_{(1, 42)} = 76.05$; $p < 0.0001$**	$F_{(1, 42)} = 1.909$; $p = 0.1743$
Water content	**$F_{(1, 19)} = 56.06$; $p < 0.0001$**	**$F_{(1, 19)} = 6.082$; $p = 0.0233$**	$F_{(1, 19)} = 0.1518$; $p = 0.7011$
Na^{+}	**$F_{(1, 8)} = 1381045$; $p < 0.0001$**	**$F_{(1, 8)} = 82240$; $p < 0.0001$**	**$F_{(1, 8)} = 107573$; $p < 0.0001$**
K^{+}	**$F_{(1, 8)} = 22713$; $p < 0.0001$**	**$F_{(1, 8)} = 3027$; $p < 0.0001$**	**$F_{(1, 8)} = 925.1$; $p < 0.0001$**
Ca^{2+}	**$F_{(1, 8)} = 570.2$; $p < 0.0001$**	**$F_{(1, 8)} = 44.18$; $p = 0.0002$**	**$F_{(1, 8)} = 2424$; $p < 0.0001$**
Mg^{2+}	**$F_{(1, 8)} = 19.00$; $p < 0.0024$**	**$F_{(1, 8)} = 343.6$; $p < 0.0001$**	**$F_{(1, 8)} = 104.0$; $p < 0.0001$**
$O_2^{\cdot-}$	**$F_{(1, 8)} = 78.00$; $p < 0.0001$**	**$F_{(1, 8)} = 16.65$; $p = 0.0035$**	**$F_{(1, 8)} = 16.59$; $p = 0.0036$**
H_2O_2	**$F_{(1, 9)} = 42.32$; $p = 0.0001$**	**$F_{(1, 9)} = 38.81$; $p = 0.0002$**	**$F_{(1, 9)} = 26.58$; $p = 0.0006$**
MDA	**$F_{(1, 9)} = 57.70$; $p < 0.0001$**	**$F_{(1, 9)} = 47.30$; $p < 0.0001$**	**$F_{(1, 9)} = 19.71$; $p = 0.0016$**
Proline	**$F_{(1, 9)} = 408.4$; $p < 0.0001$**	**$F_{(1, 9)} = 26.47$; $p = 0.0006$**	**$F_{(1, 9)} = 22.06$; $p = 0.0011$**
Total AsA	**$F_{(1, 9)} = 7.639$; $p = 0.0220$**	$F_{(1, 9)} = 3.930$; $p = 0.0788$	$F_{(1, 9)} = 2.925$; $p = 0.1214$
AsA	$F_{(1, 9)} = 0.5398$; $p = 0.4812$	**$F_{(1, 9)} = 6.613$; $p = 0.0301$**	$F_{(1, 9)} = 1.180$; $p = 0.3056$
DHA	**$F_{(1, 9)} = 19.01$; $p = 0.0018$**	$F_{(1, 9)} = 1.573$; $p = 0.2414$	$F_{(1, 9)} = 4.470$; $p = 0.0636$
AsA/DHA	**$F_{(1, 10)} = 5.243$; $p = 0.0450$**	**$F_{(1, 10)} = 11.76$; $p = 0.0064$**	$F_{(1, 10)} = 0.004423$; $p = 0.9483$
GSH	**$F_{(1, 10)} = 32.78$; $p = 0.0002$**	$F_{(1, 10)} = 0.002158$; $p = 0.9639$	**$F_{(1, 10)} = 20.78$; $p = 0.0010$**
Total thiols	**$F_{(1, 11)} = 8.443$; $p = 0.0143$**	$F_{(1, 11)} = 1.035$; $p = 0.3309$	**$F_{(1, 11)} = 16.34$; $p = 0.0019$**
Non-protein/ Protein thiols	**$F_{(1, 10)} = 52.82$; $p < 0.0001$**	**$F_{(1, 10)} = 58.90$; $p < 0.0001$**	**$F_{(1, 10)} = 34.17$; $p = 0.0002$**
Phenols	**$F_{(1, 11)} = 12.14$; $p = 0.0051$**	$F_{(1, 11)} = 1.886$; $p = 0.1970$	**$F_{(1, 11)} = 7.649$; $p = 0.0184$**
Flavonoids	**$F_{(1, 11)} = 111.3$; $p < 0.0001$**	$F_{(1, 11)} = 0.2694$; $p = 0.6140$	**$F_{(1, 11)} = 0.1965$; $p = 0.6662$**
TAC	$F_{(1, 11)} = 0.2497$; $p = 0.6271$	**$F_{(1, 11)} = 6.931$ $p = 0.0233$**	**$F_{(1, 11)} = 10.37$; $p = 0.0082$**
SOD	**$F_{(1, 10)} = 61.00$; $p < 0.0001$**	**$F_{(1, 10)} = 9.535$; $p = 0.0115$**	**$F_{(1, 10)} = 20.44$; $p = 0.0011$**
CAT	**$F_{(1, 11)} = 16.66$; $p = 0.0018$**	**$F_{(1, 11)} = 30.00$; $p = 0.0002$**	$F_{(1, 11)} = 1.695$; $p = 0.2196$
APX	**$F_{(1, 8)} = 6.117$; $p = 0.0385$**	**$F_{(1, 8)} = 25.41$; $p = 0.0010$**	**$F_{(1, 8)} = 15.42$; $p = 0.0044$**
DHAR	**$F_{(1, 10)} = 40.70$; $p < 0.0001$**	**$F_{(1, 10)} = 6.185$; $p = 0.0322$**	**$F_{(1, 10)} = 21.95$; $p = 0.0009$**
GR	**$F_{(1, 11)} = 58.88$; $p < 0.0001$**	**$F_{(1, 11)} = 6.330$; $p = 0.0287$**	$F_{(1, 11)} = 1.646$; $p = 0.2259$

CHAPTER IV

Unravelling the effects of combined salinity and heat stresses on the photosynthetic performance of cherry tomato

Abstract

The combination of abiotic stresses, such as heat and salinity, portrays a serious threat to crop productivity worldwide, with emphasis in the Mediterranean region. However, although the effects of individual stressors are well known, there is still much to unravel regarding their potential interaction. To address this, the photosynthetic performance of tomato plants (*Solanum lycopersicum* var. *cerasiforme*) exposed to salt [100 mM sodium chloride (NaCl)] and heat (42 °C; 4 h d^{-1}), individually or in combination, for 21 days was evaluated. A similar pattern was found for growth and for photosynthetic pigments: both endpoints were diminished in all stressed plants, but the co-exposure led to a harsher effect. Nonetheless, specific leaf area equally decreased in all treatments. Regarding photosystem II (PSII), while transcript accumulation of *CP47* and *D1* was repressed in all stressful conditions, the effective and maximum quantum yield of PSII and relative electron transport rate were only not negatively affected under co-exposure. Despite that, non-photochemical quenching increased in all treatments. *In vivo* gas-exchange parameters were negatively affected by salt, both at single and combined exposures, being characterized by a decreased stomatal conductance and a reduced transpiration and net carbon dioxide assimilation rate. Concerning ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO; EC 4.1.1.39), all treatments equally repressed the expression of *RbcS*, while *RbcL* transcripts diminished under heat stress but increased upon salt exposure. Overall, as the impacts on the photosynthetic apparatus of tomato plants exposed to simultaneous heat and salt were milder than those caused by the individual stressors *per se*, it appears that other mechanisms, such as cell expansion and division, or an efficient resource allocation to ensure survival might be the cause behind the severe growth impairment herein observed.

Keywords: abiotic stress; chlorophyll fluorometry; gas-exchange; photochemistry; photosystem II; RuBisCO; *Solanum lycopersicum*.

1. Introduction

The world we live in is in constant change and so adaptation to the surrounding environmental conditions is key to ensure the survival of any living organism. For instance, plants reach their optimum growth under certain levels of abiotic environmental factors (*e.g.* temperature or nutrient and water availability) and, as sessile beings, rely on a high plasticity to be able to grow and reproduce when this balance is disrupted (Zhang et al., 2021). This ability of plants to adapt, in conjunction with societal and scientific efforts, has allowed agriculture to efficiently respond to human needs throughout the ages. However, natural and anthropogenic-forced climate change has been continuously – now at an alarmingly faster rate – pushing agriculture beyond its limits.

For instance, the Iberian Peninsula is renowned for tomato (*Solanum lycopersicum* L.) production and consumption. In fact, both Portugal and Spain are consistently in the yearly top five of highest producers of this crop in Europe. Yet, tomato production in these countries has been declining in the recent years (Eurostat, 2021), mostly due to unfavourable climate conditions. This is especially worrying since the latest IPCC report (IPCC, 2021) predicts that, until the end of the 21st century, the average temperature for the Mediterranean region may increase up to 5 °C, with an estimated average of 32 days per year with maximum temperatures above 40 °C, which is well above the overall heat-stress threshold for most crops (25-35 °C) (Wahid et al., 2007). This rise in temperature, increasing evapotranspiration rate, will also increase the need for irrigation water, leading to a greater use of poor quality water and to salt build-up after evaporation, contributing to the already worrying scenario prospect of desertification in the region (Daliakopoulos et al., 2016; Haddeland et al., 2014; Koutroulis et al., 2013). In this sense, it is imperative that more studies focus on the effects of hotter and more saline environments on crop yield and physiology, as well as on the identification of possible tolerance traits that help improving plant selection and agricultural practices. As photosynthesis is the basis of plant productivity, studies underpinning the modulation of the photosynthetic machinery in response to climate change, including the combination of abiotic stresses, are needed. Actually, photosynthesis is very sensitive to alterations in climate factors, such as increases in temperature and soil salinization (Hassan et al., 2021; Mathur et al., 2014; Parihar et al., 2015; Singh and Thakur, 2018). Plants exposed to heat- or salt-induced stress are known to experience degradation and/or impaired biosynthesis of photosynthetic pigments, as well as the disruption of chloroplast ultrastructure (Hassan et al., 2021; Parihar et al., 2015), along with consequences in photochemistry, carbon (C) metabolism, stomatal conductance, water status, and protein biosynthesis – impacting, among others, ribulose-1,5-bisphosphate carboxylase-oxygenase

(RuBisCO; EC 4.1.1.39), the enzyme responsible for carbon dioxide (CO_2) fixation (Mathur et al., 2014; Parihar et al., 2015; Singh and Thakur, 2018).

In the context of climate change, we are facing not only an increased severity of stress events, pushing away plants from homeostasis-permissive conditions, but also the co-existence of several disruptive ones – such as the abovementioned temperature increase and soil salinization of the Mediterranean region – whose effects can surpass plants' tolerance limits to either stressor (Suzuki et al., 2014). Curiously, Lopez-Delacalle et al. (2021) and Rivero et al. (2014) have shown that *S. lycopersicum* exposed to salt [hydroponics - 75 and 120 mM sodium chloride (NaCl), respectively] and heat (35 °C) for 14 days or 72 h responded similarly to those only exposed to heat, being much less affected than those treated with salt, concerning growth and photosynthetic ability. However, results obtained by our research group (see Chapter III) have shown that when potted tomato plants were irrigated with saline water (100 mM NaCl) for 3 weeks and exposed to 42 °C during 4 h d^{-1}, the effects of the stress combination on plant growth were actually more severe than the sum of the individual stressors. In this sense, it becomes clear that there is still a lot to unravel regarding the interaction between these stress factors.

For this reason, this study aims at providing a thorough evaluation of the impacts of combined salt- and heat- stress on the photosynthetic performance of tomato plants through a) the quantification of photosynthetic pigments; b) gene expression analyses of *D1*, *CP47* and both RuBisCO subunits; c) chlorophyll fluorescence analysis and d) gas-exchange measurements.

2. Material and Methods

2.1. Plant material and growth conditions

Solanum lycopersicum L. var *cerasiforme* (cherry tomato) seeds were surface-disinfected in 70% (v/v) ethanol for 5 min, then 20% (v/v) commercial bleach (5% active chloride) containing 0.02% (w/v) tween®-20, also for 5 min. Both steps were carried out under constant agitation, followed by successive washings with deionized water (dH_2O). After disinfection, seeds were distributed in Petri dishes (10 cm diameter) containing solidified [0.675% (w/v) agar] half-strength MS medium containing Gamborg B5 vitamins (pH 5.5-6.0) (Murashige and Skoog, 1962) and placed in a growth chamber [16 h light/8 h dark; 25 °C, photosynthetic photon flux density (PPFD): 150 µmol m^{-2} s^{-1}] for one week. Then, seedlings with similar size were randomly distributed in plastic pots (3 plants per pot), containing 600 mL Siro Royal universal substrate (SIRO©, Portugal; physicochemical characteristics can be found in Table S3.1 of the Chapter III), and maintained under the conditions above referred.

2.2. Experimental setup

For the first 7 days, seedlings were left to acclimate to pot conditions and irrigated only with dH_2O. After the first week, pots were randomly divided into different trays, and plants were grown for the next 21 days under the following treatments:

CTL (Control) **–** Plants were irrigated every other day with dH_2O;

SALT – Plants were irrigated every other day with a 100 mM NaCl (11 dS m^{-1}) solution (60 mL per pot);

HEAT – Plants were irrigated every other day with dH_2O and transferred to a twin growth chamber at 42 °C, for 4 h, every day;

COMBINED – Plants were irrigated every other day with 100 mM NaCl (60 mL per pot) and transferred to a twin growth chamber at 42 °C, for 4 h, every day.

The treatment conditions were previously optimized by the research group (see Chapter III) and were based on the existing literature (Ayers and Westcot, 1985), the current projections for the Mediterranean region (Carvalho et al., 2021), and taking into account that high temperatures – above 35 °C – do not occur during the entirety of the day, to ensure an environmentally relevant experimental design.

After the growth period (21 days), cell death assessment, chlorophyll fluorescence analyses, and gas-exchange measurements were performed *in vivo* in fully expanded leaves (2nd and 3rd from the apex) of all plants. Then, plants were harvested, carefully washed and then shoots of half of the plants were grounded in liquid nitrogen and stored at -80 °C, while the remaining plants were dried in a forced-air oven at 60 °C until constant weight for the determination of tissue water content.

2.3. Histochemical detection of cell death

To evaluate if salt- and/or heat-stress could affect cell viability, 6 fully expanded leaves were detached from randomly chosen plants of each experimental situation and treated as described in Soares et al. (2016). Briefly, leaves were placed in falcon tubes filled with 0.25% (w/v) Evans Blue and left in the dark for 4 h. Then, leaves were cleared with boiling 96% (v/v) ethanol to ensure removal of pigments, washed with dH_2O, and photographed with a digital camera. Since Evans Blue is unable to penetrate intact viable cells, the presence of blue spots in the plant material is an indicator of cell death.

2.4. Quantification of photosynthetic pigments – chlorophylls and carotenoids

Chlorophylls (*a* and *b*) and carotenoids were extracted in 80% (v/v) acetone from frozen shoot samples (ca. 200 mg) and quantified in accordance with Lichtenthaler (1987). Absorbance (Abs) of each sample was measured at 664, 647 and 470 nm, with the formulas of Lichtenthaler (1987) being applied to

determine the content of chlorophyll *a* and *b* and carotenoids. Results were expressed in mg g^{-1} dry weight (dw). The conversion from fresh weight to dw was performed after determination of plant water content for each experimental condition (data not shown).

2.5. Chlorophyll fluorescence analyses

2.5.1. Maximum quantum yield of photosystem II (F_v/F_m), effective quantum yield of photosystem II (ΦPSII), relative electron transport rate (rETR) and non-photochemical quenching (NPQ)

Pulse amplitude modulated (PAM) fluorometry was utilized to assess chlorophyll fluorescence-related parameters in fully expanded (2nd and 3rd) leaves of tomato plants grown under the different experimental conditions, as described in Soares et al. (2020). These analyses were carried out using a PAM-210 fluorometer (Heinz Walz GmbH, 1997), which was controlled through the PAMWin software. PAM-210 was equipped with a red measuring LED with short-pass filter (< 690 nm), peaking at around 650 nm, an actinic red LED (unfiltered, peaking at around 665 nm), a far-red LED with a long-pass filter (> 710 nm, peaking at around 730 nm) and a PIN photodiode and dichroic filter, reflecting fluorescence at 90° towards the detector.

In order to open all the photosystem II (PSII) reaction centres and relax possible non-photochemical quenching mechanisms, plants were dark-adapted for at least 30 min before starting the measurements. The minimal fluorescence (F_0) was recorded and, after the application of a saturating light pulse (SP; 3500 μmol m^{-2} s^{-1}, 800 ms) to register maximum fluorescence yield (F_m), the maximum quantum yield of PSII (F_v/F_m) was determined – $F_v/F_m = (F_m\text{-}F_0)/F_m$ (Kitajima and Butler, 1975). Upon this, leaves were adapted to actinic light (PPFD: 128 μmol m^{-2} s^{-1}) for 10 min followed by a SP to register F'_m and F_t and to calculate the effective quantum yield of PSII [$\Phi PSII = (F'_m\text{-}F_t)/F'_m$; (Genty et al., 1989)] and relative electron transport rate [rETR = ΦPSII X PPFD; (Genty et al., 1989)]. Non-photochemical quenching (NPQ) was calculated as $(F_m\text{-}F'_m)/F'_m$ (Müller et al., 2001).

2.5.2. Rapid light curves (RLC)

In order to understand how plants exposed to different conditions would respond to increasing light intensities, RLC were studied. After the abovementioned determinations (2.5.1), leaves were exposed to sequential incremental steps of actinic light (PPFD: 18; 68; 98; 128; 158; 218; 318; 448; 608; 858 and 1258 μmol m^{-2} s^{-1}) for 20 s, followed by a SP at the end of each step to determine the respective ΦPSII, rETR and NPQ.

2.6. Gas-exchange measurements

The determination of the impacts of salt- and/or heat-induced stress on tomato plants' gas-exchange was carried out with an infrared gas analyser (IRGA; LC pro-T, ADC, Hoddersdon, UK) in fully expanded (2nd and 3rd) leaves of three plants per biological replicate, under atmospheric CO_2 concentration and a saturating PPFD of 255 µmol m^{-2} s^{-1}. Transpiration rate (*E*, mmol g^{-2} s^{-1}), stomatal conductance (g_s, mmol g^{-2} s^{-1}), net CO_2 assimilation rate (P_N, µmol g^{-2} s^{-1}) and the ratio between intracellular and atmospheric CO_2 (C_i/C_a) were estimated using the equations of von Caemmerer and Farquhar (1981). Intrinsic water use efficiency ($WUE_i = P_N/g_s$.) and specific leaf area [SLA = leaf area (cm^2)/dw (g)] were also determined.

2.7. Gene expression analysis – reverse transcription real-time polymerase chain reaction (RT-qPCR)

2.7.1. Extraction and purification of RNA and synthesis of cDNA

RNA from shoots of tomato plants (ca. 50-100 mg) was extracted using the NZYol (NZytech®, Portugal) method, in accordance with the manufacturer's instructions, and purified using the GRS Total RNA kit – Plant from GRiSP® (GRiSP Research Solutions, Portugal), according to the supplied protocol.

RNA was quantified spectrophotometrically ($Abs_{260\ nm}$ of 1.0 = 40 ng µL^{-1}) using a DS-11 Microvolume Abs Spectrophotometer (DeNovix Inc., USA) and its purity was determined calculating $Abs_{260/280\ nm}$ and $Abs_{260/230\ nm}$, as described by Martins et al. (2020). RNA integrity was assessed by a 0.8% (w/v) agarose gel electrophoresis.

Then, the reverse transcription reaction to obtain cDNA was performed with the Xpert cDNA Synthesis Kit (GRiSP), in accordance with the supplied instructions, using 1 µg of RNA in a final volume of 20 µL. Lastly, cDNA samples were stored at -20 °C until used for real-time PCR (qPCR) expression analysis.

2.7.2. Analysis of gene expression by qPCR

Transcript accumulation of several photosynthesis-related genes was monitored through qPCR, carried out in a CFX96 Real-Time Detection System (Bio-Rad®, Portugal). *D1* and *CP47* encode proteins associated to the PSII reaction centre, while *RbcS* and *RbcL* encode the small and large, subunits of RuBisCO, respectively.

All reactions were performed in triplicate and each well contained 1 µL of diluted (1:10) cDNA in a reaction mixture of 1x PowerUp™ SYBR® Green Master Mix and 0.4 µM primers (Table 4.1) to a final volume of 20 µL. The qPCR conditions were: 2 min at 50 °C, 2 min at 95 °C, followed by 35 cycles of 3 s at 95 °C and 30 s at 60 °C. Melting curves analysis was carried out with a gradual (0.5 °C s^{-1}) 60-95

°C increment to verify primer specificity, revealing a single peak for each gene. Transcript levels were then quantified through the $2^{(-\Delta\Delta Ct)}$ formula of Livak and Schmittgen (2001), using *ACTIN* and *UBIQUITIN* as reference genes (Løvdal and Lillo, 2009), as they were previously validated and tested.

Table 4.1. Gene-specific primers for photosynthesis-related genes used in qPCR analysis.

Name	Sequence	Amplicon Size (bp)	Melting T (° C)	Reference
D1	**F:** TGG ATG GTT TGG TGT TTT GAT G	191	54.03	Mariz-Ponte et al. (2021)
	R: CCG TAA AGT AGA GAC CCT GAA AC		54.83	
CP47	**F:** CCT ATT CCA TCT TAG CGT CCG	142	54.90	
	R: TTG CCG AAC CAT ACC ACA TAG		54.87	
RbcS	**F:** TGA GAC TGA GCA CGG ATT TG	148	54.90	
	R: TTT AGC CTC TTG AAC CTC AGC		54.79	
RbcL	**F:** ATC TTG CTC GGG AAG GTA ATG	81	54.68	
	R: TCT TTC CAT ACC TCA CAA GCA G		54.64	

F: forward primer; R: reverse primer; bp: base pairs; T: temperature.

2.8. Statistical analyses

All parameters were estimated using at least three biological replicates (n ≥ 3), with at least three technical replicates per assay. Results were expressed as mean ± standard error of the mean (SEM). Treatment effects – SALT (0 mM and 100 mM NaCl) and HEAT (25 °C and 42 °C (4 h d^{-1}) – were tested by two-way ANOVA, after confirmation of homoscedasticity by the Brown-Forsythe test, followed by Tukey's post-hoc test to assess differences between groups (significant for $p \leq 0.05$). If significance was found for the interaction, a correction for the simple main effects was performed. These analyses were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and the results of the ANOVAs are detailed in Supplementary Material (Table S4.1). A principal component analysis (PCA) was carried out to assess similarities between groups and the variables that are most responsible for the clustering pattern. For this, the average values for each determined parameter were plotted and the first two components were used to make biplots. This analysis was done using XLSTAT 2021.2.2 (http://www.xlstat.com, Addinsoft USA, New York, NY).

3. Results

3.1. Visual assessment of plant growth, SLA and cell viability

The inhibitory effects of the tested stressors on plant growth and leaf structure were clear (Figure 4.1). Individually, heat and salt exposure visibly impaired plant growth, with the co-exposure leading to a more severe outcome. Additionally, plants under salt stress (sole and combined with heat) seemed to have narrower stems than those found in HEAT and CTL. Also, in relation to CTL, a similar reduction of leaf area was observed in all stressed plants, although no other severe macroscopic toxicity symptom could be detected. Also, SLA (Figure 4.1b) indicates that leaves from stressed plants, regardless of the treatment, exhibited a thicker and/or denser mesophyll with a significantly smaller (30-42%) area per gram of leaf. Regarding cell viability, results showed that no cell death was induced by heat and/or salinity stress, as no bluish areas were observed in the leaves of any treatment (Figure 4.1c).

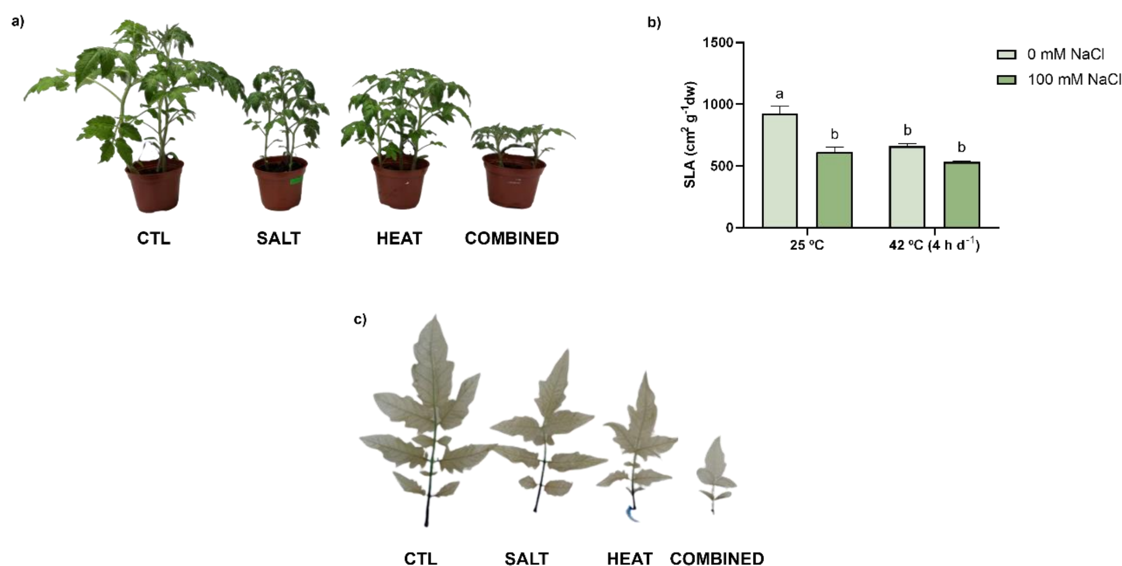

Figure 4.1. Plant growth (a), specific leaf area (b) and cell death (c) in tomato plants after a 21-day exposure to 42 °C (4 h d^{-1}) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

3.2. Photosynthetic pigments – chlorophylls and carotenoids

By analysing the content of photosynthetic pigments (Table 4.2), it can be noticed that heat stress did not lead to a significant impact in chlorophylls and carotenoids content, although a clear tendency for lower levels could be perceived. Nonetheless, plants treated with 100 mM NaCl presented a significant decrease in chlorophylls (*a*, *b* and total) and carotenoid levels, of 31% and 38%, respectively, while a much more severe effect was detected in combination with heat (around 71% for both pigment groups).

Lastly, no differences were perceived regarding the chlorophyll *a/b*, indicating that both chlorophyll *a* and *b* were affected equally.

Table 4.2. Effect of 21 days of salt (irrigation with 100 mM NaCl), heat (exposure to 42 °C for 4 h d^{-1}) and combined stresses on total chlorophyll, chlorophyll *a* and *b*, chlorophyll *a/b* and carotenoids of tomato plants. Values represent mean ± SEM (n ≥ 3). For each variable, significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

Parameter	CTL	SALT	HEAT	COMBINED
Total chlorophyll (mg g^{-1} dw)	27.883 ± 2.783 a	19.293 ± 1.743 b	21.411 ± 0.469 ab	7.875 ± 0.411 c
Chlorophyll *a* (mg g^{-1}d dw)	20.517 ± 2.080 a	14.174 ± 1.274 b	15.858 ± 0.399 ab	5.808 ± 0.314 c
Chlorophyll *b* (mg g^{-1}d dw)	7.366 ± 0.707 a	5.119 ± 0.475 b	5.553 ± 0.072 ab	2.068 ± 0.097 c
Chlorophyll *a/b*	2.781 ± 0.035 a	2.774 ± 0.046 a	2.855 ± 0.037 a	2.808 ± 0.030 a
Carotenoids (mg g^{-1} dw)	4.765 ± 0.891 a	2.931 ± 0.394 b	3.607 ± 0.209 ab	1.387 ± 0.090 c

3.3. Chlorophyll fluorescence analysis

3.3.1. Photochemical and non-photochemical efficiencies and rETR at plant growth light conditions

Data reveals that even though the individual stressors negatively affected ΦPSII and rETR (ca. 8% for SALT and 22% for HEAT), the combination of salt and heat led to similar results to those found in CTL leaves (Figure 4.2a,b), with a significant interaction between both stress factors (Table S4.1). Furthermore, there was a small (3%) but significant increase in F_v/F_m in the combined treatment (Figure 4.2c). Nonetheless, all stressed plants present increased energy dissipation by non-photochemical mechanisms, as rises of 193%, 103% and 119% for SALT, HEAT and COMBINED, respectively, were recorded for NPQ, in relation to the CTL (Figure 4.2d).

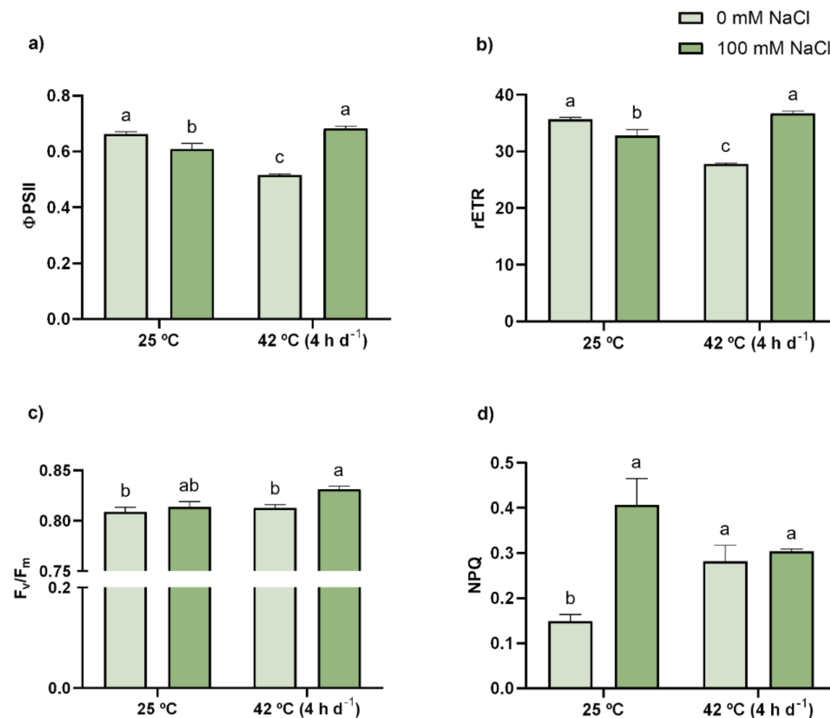

Figure 4.2. ΦPSII (a), rETR (b) F_v/F_m (c) and NPQ (d) of tomato plants after a 21-day exposure to 42 °C (4 h d^{-1}) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

3.3.2. RLC

Figure 4.3 shows data regarding overall photosynthetic and non-photochemical performance in response to increasing light intensities. Both CTL and SALT plants appear to be saturated, in terms of rETR, after the nineth or tenth actinic light step (PPFD around 600 µmol photons m^{-2} s^{-1}), while HEAT and COMBINED only began to saturate at the highest PPFD analysed, with ETR at 1258 µmol m^{-2} s^{-1} being 24% and 54% higher, respectively. Furthermore, as can also be observed, this apparent saturation in terms of electron flow was accompanied by constant reductions of ΦPSII for all treatments, although not as much in plants exposed to both stressors. In fact, ΦPSII (1258 µmol m^{-2} s^{-1}) was up to 57% higher in these plants than in CTL and SALT and 27% in relation to CTL . At low PPFD (up to 158 µmol m^{-2} s^{-1}), all stressed plants were dissipating more radiation than CTL (Figure 4.3c), however, at higher light intensities, only SALT plants consistently presented higher NPQ values than any other treatment (31 to 34% increase).

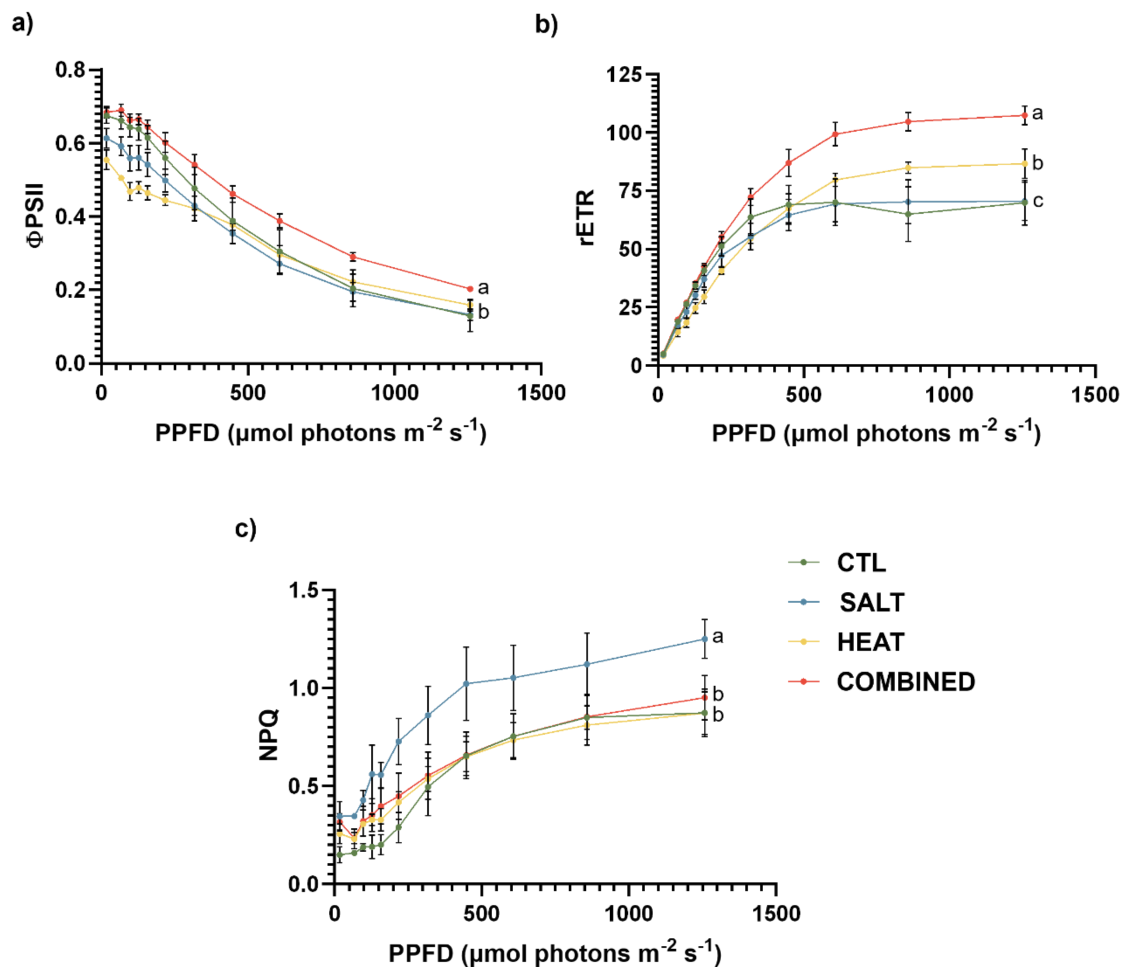

Figure 4.3. ΦPSII (a), rETR (b) and NPQ (c) of tomato plants subjected to increasing PPFD after a 21-day exposure to 42 °C (4 h per d^{-1}) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

3.4. Gas-exchange measurements

Concerning gas-exchange measurements (Figure 4.4), the similarity between CTL and HEAT, as well as SALT and COMBINED was noteworthy, highlighting the strong effect of salt stress on these parameters. Salt-stressed plants, regardless of the heat treatment, exhibited decreases of 73-82% in E (Figure 4.4a) and g_s (Figure 4.4b), of 53-59% in P_N (Figure 4.4c), of 14% in C_i/C_a (Figure 4.4d), and increases of ca. 80% on WUE_i (Figure 4.4e) in relation to CTL plants.

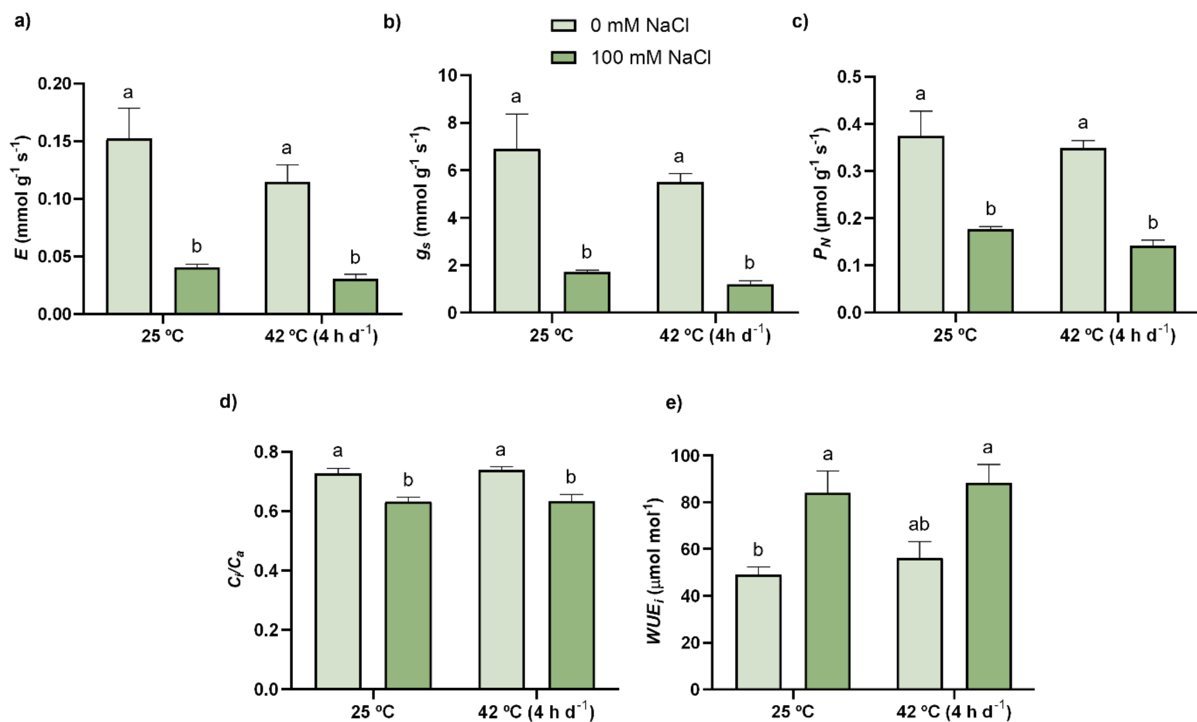

Figure 4.4. *E* (a), g_s (b), P_N (c), C_i/C_a (d) and WUE_i (e) of tomato plants after a 21-day exposure to 42 °C (4 h d^{-1}) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

3.5. Expression pattern of photosynthesis-related genes

In Figure 4.5, it is possible to observe a strong impact of both tested factors on the expresssion levels of the analysed genes. PSII-related genes, *D1* and *CP47* (Figure 4.5a,b), were strongly repressed by single salt (inhibitions of 85-90%) and heat (72%), although the combination of both factors showed less impact, with reductions of 73% and 55% for *D1* and *CP47*, respectively, with a significant interaction between factors (Table S4.1). In what concerns RuBisCO subunits (Figure 4.5c,d), *RbcS* expression was similarly affected by both stressors (compromised by 40 to 55% in relation to CTL) but a different pattern could be percieved for *RbcL*: heat strongly repressed gene expression (76%) as seen for PSII-related genes, but salinity led to a higher accumulation of *RbcL* transcripts (74% more than in CTL).

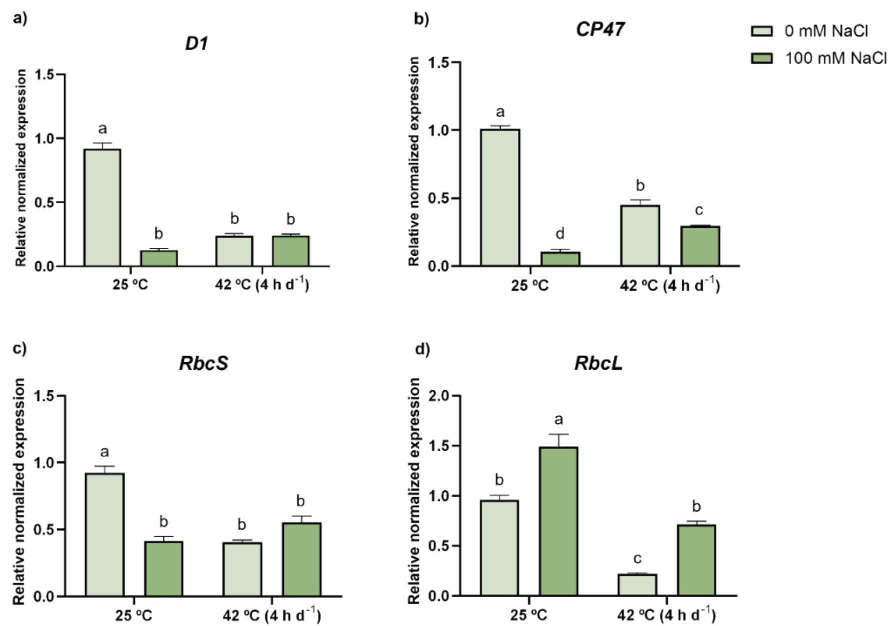

Figure 4.5. *D1* (a), *CP47* (b), *RbcS* (c) and *RbcL* (d) transcript accumulation in tomato plants after a 21-day exposure to 42 °C (4 h d^{-1}) and irrigation with (darker bars) or without (lighter bars) 100 mM NaCl. Values represent mean ± SEM (n ≥ 3). Significant differences ($p \leq 0.05$) between treatments are indicated by different letters.

3.6. Principal component analysis (PCA)

To understand the correlation between different conditions and all tested dependent variables, a PCA was carried out (Figure 4.6). The analysis showed that the first component explained 61.51% of variance and the second accounted for 20.06%. Furthermore, the dependent variables were affected differently by each condition, as it was observed that the four treatments were distributed by the four quadrants (Figure 4.6). Nonetheless, the small distance that can be observed between SALT and COMBINED is mostly a result of an enhanced expression of *RbcL* and higher NPQ values at both 128 and 1258 μmol photons m^{-2} s^{-1}. Contrarily to these conditions, CTL plants appeared to portray higher SLA and increased expression levels of *CP47*, *D1* and *RbcS*. Overall, in *S. lycopersicum* plants, the combined and the remaining treatments were plotted in different quadrants, being associated with increased rETR and ΦPSII at growth PPFD and at high light intensity, as well as high values of F_v/F_m and WUE_i and the harsh decrease of chlorophylls and carotenoids.

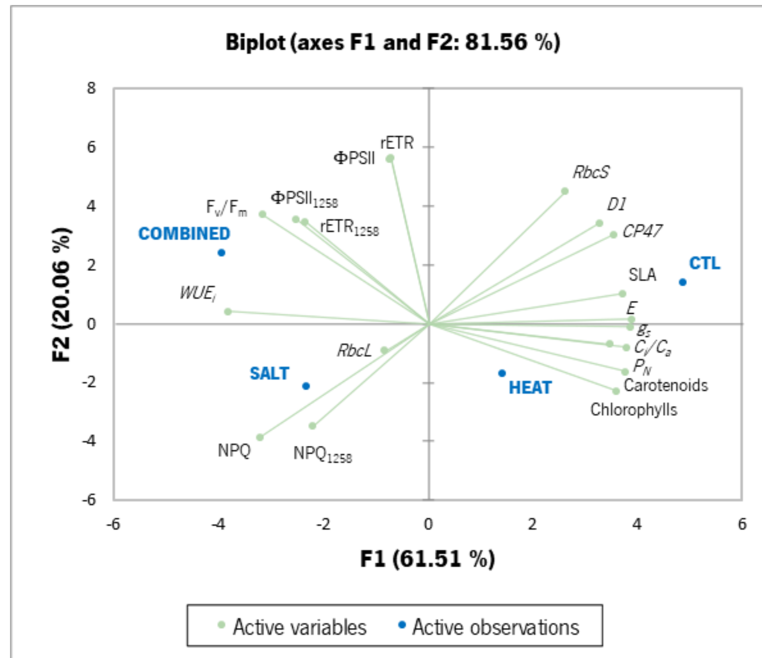

Figure 4.6. Biplot-based PCA with first two principal components showing the differential response in the photosynthetic performance of tomato plants under salt (irrigation with 100 mM NaCl), heat (exposure to 42 ˚C for 4 h d^{-1}) and combined stresses for 21 days.

4. Discussion

Plant growth and development are strongly affected by environmental variations, especially if intense and unpredictable, and therefore, the present climatic scenario will aggravate agricultural losses around the globe. Among the most devastating abiotic factors conditioning crop production are high temperatures and soil salinization, which have been widely studied individually but the effects of their combination are still unclear. Recently, our research group has provided key findings concerning the main cellular and biochemical pathways involved in heat and salt co-exposure in tomato plants (Chapter III). Based on the data collected, a marked decline in growth and development was observed, this being concomitant with a generalized investment of defence mechanisms to prevent oxidative damage. Thus, this work emerges as a follow-up study aiming at unravelling the modulation of photosynthetic mechanisms in response to these stressors, pinpointing the effects on both photochemical and chemical phases of photosynthesis.

4.1. Despite all treatments equally diminishing SLA, growth was more negatively affected upon combination

By visually assessing the set of plants in this study, it is clear that the individual exposure to salt and heat negatively affected plant height, which was aggravated by the combination of the stressors. Our group already reported such effect on plant growth-related parameters, which, in response to salinity, was

associated with the disruption of water status (measured by water content), but mostly with the imbalance of nutrient and ion uptake, alongside salt-induced toxicity (see Chapter III). Likewise, and as addressed in Chapter III, heat exposure impaired root and stem elongation. Even though the causes are not as clear and apparently oxidative stress was not induced, it was hypothesised that it could result from the disruption of ion homeostasis and damages in the photosynthetic apparatus (discussed in the following sections), despite water content being maintained. Nonetheless, plant growth was not only visibly reduced in terms of height but also in terms of leaf area and thickness, as revealed by the determination of the SLA. Indeed, it has been reported that salinity increases the leaf lamina thickness, either because of an increased size of mesophyll cells or the existence of multiple cell layers [references in Bayuelo-Jiménez et al. (2012)]. When Bayuelo-Jiménez et al. (2012) exposed *Phaseolus* species to increasing concentrations of NaCl, a decreased SLA was also observed. Curiously, these authors, along with Longstreth and Nobel (1979), have suggested that such effect on SLA could portray a tolerance trait, since salt-tolerant genotypes present lower values than salt-sensitive ones. It was hypothesized that, as very often carbon assimilation is reduced due to salt-induced stomata closure, plants may present thicker mesophylls in order to reduce its resistance to CO_2 by increasing the internal surface area for higher diffusion rates. Contrarily, it is common that plants exposed to high temperatures often benefit from increased SLA to enhance their evaporative and light capture potential. Indeed, such effect has already been documented for 16 different species at 23 °C and 28 °C (Loveys et al., 2002), but here it did not occur. Nonetheless, SLA was already negatively correlated with relative growth rate, which is strongly dependent on photosynthesis (Bayuelo-Jiménez et al., 2012). Indeed, individual salt- and heat-treated tomato plants showed reduced photosynthetic performance as will be discussed later in sections 4.2. and 4.3. Despite that, according to Tardieu et al. (1999), a diminished SLA is frequently a result of a higher impact of abiotic stresses on cell expansion rate than on photosynthesis. Therefore, since no signs of oxidative damage were perceived (see Chapter III), the reduction of growth may be related to the interference of the stressors on other vital growth mechanisms, such as cell division.

When both stressors were applied simultaneously, a more severe effect was observed in terms of growth, as these plants were visibly shorter than those exposed only to heat or salt, however, this phenotype could not be attributed to a reduction in stomatal conductance solely. Indeed, the hypothesis previously raised regarding salinity negatively influencing transpiration rate (see Chapter III) gains strength with the present data. Tomato plants under combined exposure did, in fact, reduce stomatal conductance due to salt treatment, which consequently diminished transpiration and C assimilation rates. Nonetheless,

no significant aggravation of these parameters occurred when tomato plants were simultaneously exposed to salt and heat stress (COMBINED). Although light reactions of photosynthesis may have suffered from the combined exposure, further discussed in section 4.2., the combined effect of the stressors does not seem harsher than the individual ones. Therefore, the observed diminished plant growth may be ascribed to a combination of factors, like the reduction in the content of photosynthetic pigments affecting overall C budget, reduction in cell expansion and division (Tardieu et al., 1999), but also, the mobilization of resources to defence pathways, as hypothesised previously (Chapter III).

Furthermore, despite salinity commonly leading to cell death, as well as moderate heat stress, Evans Blue vital dye indicated the lack of substantial membrane damage, and consequently the absence of cell death. This information agrees with previous assays (see Chapter III), which showed no signs of lipid peroxidation in salt- and heat-stressed tomato plants, coupled with an enhanced antioxidant system, including increased proline content, responsible for membrane stabilization and protection against salt stress-induced cell damage (Banu et al., 2009).

4.2. The co-exposure reduced the expression of *D1* and *CP47* and pigment content, but did not inhibit the photochemical reactions of photosynthesis

Plants rely on photosynthesis to produce carbohydrates, so that they can grow and develop at optimal rates. This vital process includes two sequential phases: photochemical phase – involved in obtaining reducing power (NADPH) and energy (ATP) – and the C reduction phase or Calvin-Benson cycle – responsible for sugar synthesis using CO_2 and the products of light reactions (Ashraf and Harris, 2013). As a non-spontaneous redox process, photosynthesis requires external energy to start, thus the first step is sunlight absorption by the photosynthetic pigments. However, chlorophylls and carotenoids are highly sensitive and are often negatively affected by stressful conditions (Singh and Thakur, 2018). Heat and salt affect these molecules in a similar extent, although usually for different reasons. In the present study, both stresses either led to a tendency for diminished (HEAT) or a significant decrease (SALT) of chlorophyll and carotenoid content, plausibly due to their interference in the biosynthesis and/or breakdown of these pigments, their effect being more relevant on the former than the latter. According to Ashraf and Harris (2013) and Santos (2004), while salinity leads to Na^+ toxicity, which mostly reduces the levels of chlorophyll precursors (*e.g.* 5-aminolevulinic acid and glutamate), high temperatures not only affect them in a similar way but also damage and degrade several enzymes [*e.g.* 5-aminolevulinate dehydratase (EC 4.2.1.24), porphobilinogen deaminase (EC 2.5.1.61)] involved in the biosynthetic pathway of chlorophylls. Additionally, as the presence of excessive salt in the soil results in an hampered water and

nutrient uptake, which often correlates with diminished Mg^{2+} levels in plants, this nutrient, a key component of chlorophylls, is often remobilized to younger tissues, culminating in pigment degradation (Peng et al., 2019). Curiously, under salinity stress, no deficiency in Mg^{2+} was observed, however tomato plants simultaneous exposed to the stressors portrayed diminished levels of this nutrient (see Chapter III). In this sense, it is reasonable that the severe reduction of chlorophyll content arises from the combination of the inhibition of its biosynthesis – due to high temperature and saline conditions – and a higher degradation rate associated with Mg^{2+} mobilization. Additionally, it is also worth noticing that no treatment led to the disruption of chlorophyll *a*/*b* ratio, indicating no changes in the pigment composition of the photosynthesis apparatus, in accordance with what was previously documented for both individual stressors (Camejo et al., 2005; Del Zoppo et al., 1999). Although most studies focus on chlorophylls, carotenoids are also important pigments involved in several physiological processes, such as plant growth, photosynthesis and response to abiotic factors (Liu et al., 2015). In photosynthesis these pigments allow greater light harvesting, but more importantly, xantophylls are able to protect PSII from damage by quenching the excited status of singlet chlorophyll when exposed to excessive radiation (Liu et al., 2015). Nonetheless, salt treatment decreases the expression levels of carotenoid pathways genes, thus leading to its decline (Ann et al., 2011; Vaibhav et al., 2015), which is in accordance with our data. Curiously, under combined stress, the negative effect on carotenoid content was harsher than the one observed in SALT, however, when exposed to heat alone and alike other tomato cvs (Arvento and LA2093) (Zhou et al., 2017), these pigments remained unaffected, suggesting a heat-induced enhancement of the salt-related effects on carotenoid metabolism.

When the chlorophylls of the antenna absorb energy from sunlight, the excited molecules can dissipate their energy by heat or light (fluorescence) or by transferring the energy to other chlorophyll molecules by inductive resonance. Once the excited electron reaches the reaction centre of the photosystems, the chlorophylls may lose the it to an acceptor, initiating the electron transport chain (ETC), that ultimately reduces $NADP^+$ to NADPH, using H_2O as electron donor (Taiz et al., 2015). Photosystem II (PSII) contains the core complex – where D1 and CP47 proteins are located –, the oxygen-evolving complex (OEC) and the light-harvesting complex (LHCII) bound to the core complex by CP47 proteins (Derks et al., 2015). Indeed, D1, as an electron carrier between the OEC and the reaction centre P680, and CP47, as a part of the PSII core antenna protein complexes, play important roles during the photochemical reactions of photosynthesis and changes in its transcripts may compromise the ETC functioning (Pospíšil and Prasad, 2014). Moreover, it has been reported that despite direct damages to

PSII only occur as a result of light stress, its repair is negatively influenced by other abiotic stresses (Nishiyama and Murata, 2014). In fact, adverse conditions (*e.g.* salinity, high and low temperatures, CO_2 limitation) are related to the inhibition of the *de novo* synthesis of proteins, particularly of D1, by the suppression of gene expression, as proved in the present study for *D1* and *CP47* in all treatments. Additionally, it has been reported that such effect is commonly related to the overproduction of singlet oxygen ($^1O_2$) due to excess light energy under CO_2 limitation, as reviewed by Nishiyama and Murata (2014). Indeed, here, as intercellular CO_2 was decreased due to a low carbon diffusion in both treatments exposed to salt, it can be suggested that $^1O_2$ was accumulated and negatively regulated the expression of both *CP47* and *D1.* Moreover, the overproduction of this reactive oxygen species (ROS) may have also been enhanced as a consequence of the diminished levels of crucial inhibitors of its generation – carotenoids – in SALT and COMBINED. Interestingly, in the analysis of rETR at increasing light intensities, SALT and CTL plants appear to be saturated at PPFD around 600 µmol photons m^{-2} s^{-1}, indicating a slower adaptation to high light in relation to HEAT and COMBINED, since only at the highest PPFD saturation appears to be starting. Nonetheless, this saturation of electron flow was not accompanied by a constant increase of ΦPSII, suggesting that NPQ gains relevance at increasing light conditions. When analysing these parameters at the growth PPFD, and considering that SLA decreased in among treatments, leaves of stressed plants were thicker than those found in CTL, which could explain the increment of F_v/F_m in SALT (tendency) and COMBINED (significant), as the increased number of cells, and thus higher amount of this protein complex, in the area subjected to PAM analysis might overestimate F_v/F_m in these situations. From this perspective, the positive effect on F_v/F_m perceived in the combined treatment does not, in all certainty, indicate a lack of damages in the PSII, as shown by the inhibition of the *CP47* and *D1* transcription, that might negatively affect ΦPSII and rETR. Indeed, several plants species have been reported to portray injuries in the photosynthetic apparatus as a result of heat (Fahad et al., 2017; Hassan et al., 2021) or salt exposure (Parihar et al., 2015). When Rivero et al. (2014) induced heat and salt combined stress on tomato cv. Optima, decreased levels of F_v/F_m, were observed. Additionally, according to Rivero et al. (2014), the combination of stressors led to a similar reduction of F_v/F_m and ΦPSII to that found in the single heat treatment in tomato cv. Optima plants. However, these authors also reported the accumulation of ROS and the induction of lipid peroxidation, which might have caused direct damages to the photosynthetic machinery and affected chloroplast structure, leading to the dislodging of the PSII. Thus, given the high susceptibility of PSII to oxidative damage, the lack of lipid peroxidation and accumulation of superoxide anion and hydrogen peroxide perceived, in the present study – due to the

efficient activation of the antioxidant system (Chapter III) –, may be accountable for milder effects on the PSII. Nonetheless, and due to the abovementioned effects on the expression of *CP47* and *D1*, the hypothesis raised regarding the overestimation of PSII-related parameters when considering the different thickness of tomato leaves, should not be discarded. Moreover, such plausible explanation seems to be in accordance with the evident increase of dissipated energy, NPQ, observed in all stressed plants and also documented by Jahan et al. (2021) for heat stress and by Zribi et al. (2009) for salinity stress. In fact, such increase can be considered a tolerance trait as it dissipates excessive light energy in the form of heat so that the overproduction of ROS is avoided, as well as the photodamage of PSII.

4.3. Gas-exchange parameters were equally affected by salt, solely or in combination with heat

The photosynthetic reduction of CO_2 to carbohydrates in the stroma is divided in 3 main phases: carboxylation, reduction and regeneration (Taiz et al., 2015). Firstly, CO_2 must diffuse from the atmosphere to the chloroplasts of the mesophyll cells via stomata; however, several abiotic conditions, including salinity and heat, influence stomatal resistance to gas diffusion (Mathur et al., 2014). Heat-stressed plants often suffer from water stress due to an increased evaporation of soil water that, paired with a reduced root conductance, leads to a diminished uptake, resulting in stomata closure (Hassan et al., 2021). Although a slight tendency to decrease g_s and E could be perceived, HEAT plants did not suffer any significant changes regarding g_s, E, P_N and C_i/C_a nor WUE_i , probably because water supply was not limiting. In fact, in plants under single heat exposure, water content was maintained in shoots (Chapter III). When water deficit was not an issue, tomato plants cv. Boludo tended to increase stomatal conductance, cooling down through transpiration, elevating their evaporative potential, as shown by Lopez-Delacalle et al. (2021), when plants were consistently exposed to 35 °C. Yet, contrarily to the continuous heat treatment applied by these authors, in our study, plants were only temporarily exposed to 42 °C, so it might have allowed their recovery at some extent. Indeed, the maintenance of CO_2 assimilation has already been documented by Kreslavski et al. (2008) when studying the post-heat stress recovery of photosynthesis on wheat seedlings. Moreover, our data clearly showed an analogous pattern between CTL and HEAT with respect to gas-exchange parameters, which were significantly different from the outcomes observed for SALT and COMBINED. Indeed, salinity stress (solely or combined with heat) led to the disruption of water relations (measured by water content) in shoots (see Chapter III), decreasing g_s thus diminishing E, P_N and C_i/C_a, and increasing WUE_i, as also resported by Lopez-Delacalle et al. (2021) upon exposure to salt. Nonetheless, plants need to synthesise carbohydrates, which requires CO_2

fixation by RuBisCO (Taiz et al., 2015). This enzyme, making up to 50% of total soluble protein in plant leaves (Erb and Zarzycki, 2018), consists of eight small subunits (RbcS) encoded by nuclear genome and eight large subunits (RbcL) encoded by plastidial genes (Spreitzer and Salvucci, 2002). Desimone et al. (1996) reported that ROS overaccumulation could be accountable for the degradation of RbcL, which comprises the carboxy-terminus – a key component for stability and maximal activity of RuBisCO. Heat is often associated with the inhibithion of photosynthesis as a result of damages in the photosynthetic apparatus and the inactivation of RuBisCO (Mathur et al., 2014). In the present study, the expression of both subunits was decreased upon exposure to high temperatures, an effect also reported by Jahan et al. (2021) in tomato seedlings under 42 °C for 24 h. In fact, changes in the mesophyll capacity for photosynthesis – dependent on RuBisCO activity – have been reported to be responsible for alterations in P_N (von Caemmerer and Farquhar, 1981). Curiously, here, although tomato plants presented significantly lower levels of expression of *RbcS* and *RbcL*, in response to heat, the assimilation of CO_2 was not affected. Interestingly, Danilova et al. (2018) reported that some genes encoded in the chloroplast, like *RbcL*, presented lower transcript levels after 3 h of treatment, even though they can recover to steady-state levels, which could possibly explain the maintenance of P_N. On another hand, while *RbcS* was inhibited in all stressed plants, the expression of the large subunit was enhanced or maintained upon salt and combined exposure, respectively. Contrastingly, in *Sorghum bicolor* (L.) Moench (sweet sorghum) (ElSayed et al., 2019) and *Phaseolus vulgaris* L. (green bean) (ElSayed et al., 2021), salinity (100 mM or 200 mM given through irrigation with Hoagland nutrient solution) repressed the expression of both *RbcS* and *RbcL* and the activity of this enzyme, thus hampering photosynthetic performance. ElSayed et al. (2021) hypothesised that the diminished intercelular concentration of CO_2 was caused by a low carboxylation efficency and reduced activity of RuBisCO due to the decreased expression of *RbcL.* Nevertheless, according to von Caemmerer and Farquhar (1981), changes in P_N may also arise from alterations in g_s. Indeed, in the present work, intracellular CO_2 concentration was decreased regardless of the accumulation of *RbcL* transcripts of tomato plants exposed to salt individually or combined with heat, which is most likely a consequence of limited CO_2 assimilation due to the reduced g_s than a result of changes in the expression of *RbcL*.

5. Conclusion

The results obtained in the present study helped disclosing the consequences of the combination of heat and salinity stresses in *S. lycopersicum* plants in what concerns photosynthetic performance. Overall, PSII seemed to be more affected by high temperatures than any other treatment, while gas-exchange parameters were predominantly affected by salt. Nonetheless, the response of tomato plants to the co-exposure was unique, as highlighted in the PCA (Figure 4.6). Here, the combined treatment was plotted apart from all other treatments, with the main differences being associated with the higher values of F_v/F_m, ϕPSII and rETR than what can be found for the individual stressors. Altogether, the effects of the combination of high temperatures and soil salinization on the photosynthetic performance of tomato plants are singular (Figure 4.7), but do not appear harsher than those of the individual exposure. Therefore, the severe growth reduction under co-exposure may ascribe from the reallocation of resources from growth to defence pathways or from the disruption of other vital growth-related mechanisms (*e.g.* cell expansion and division, synthesis of cell wall). In this sense, so that a full understanding of the impacts of the combination of these stresses and the development of strategies to alleviate its negative effects are achieved, it is imperative that further studies are undertaken to complement what has been here reported.

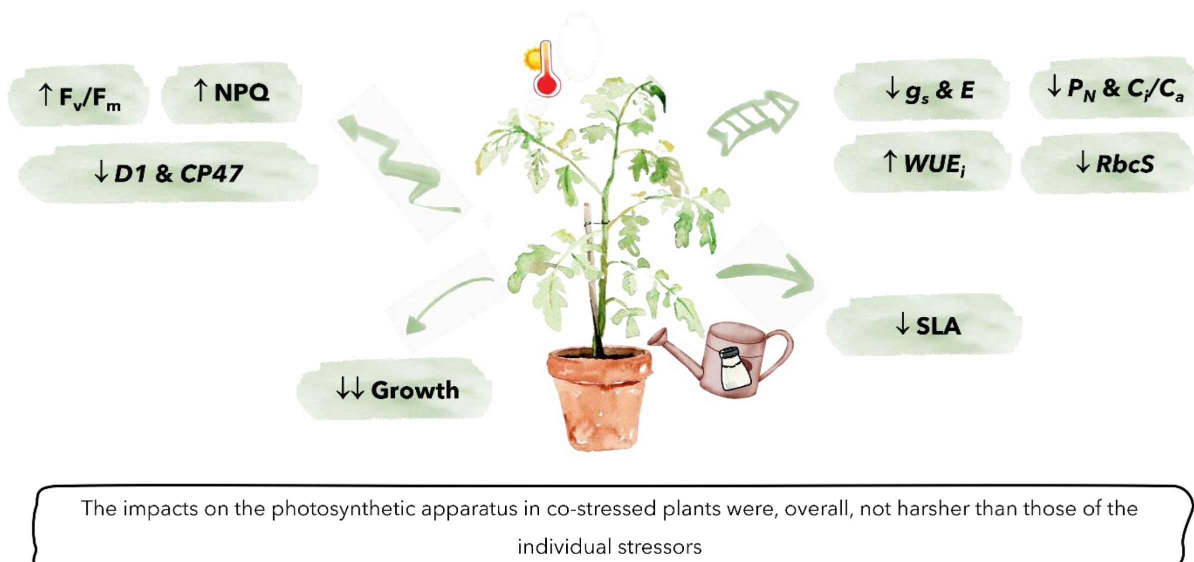

Figure 4.7. Overview of the main results of the present chapter.

References

Ann, B.M., Devesh, S., Gothandam, K.M., 2011. Effect of salt stress on expression of carotenoid pathway genes in tomato. J. Stress Physiol. Biochem. 7, 87-94.

Ashraf, M., Harris, P.J.C., 2013. Photosynthesis under stressful environments: An overview. Photosynthetica 51, 163–190. https://doi.org/10.1007/s11099-013-0021-6

Ayers, R.S., Westcot, D.W., 1985. Water quality for agriculture. FAO. Rome.

Banu, M.N.A., Hoque, M.A., Watanabe-Sugimoto, M., Matsuoka, K., Nakamura, Y., Shimoishi, Y., Murata, Y., 2009. Proline and glycinebetaine induce antioxidant defense gene expression and suppress cell death in cultured tobacco cells under salt stress. J. Plant Physiol. 166, 146–156. https://doi.org/10.1016/j.jplph.2008.03.002

Bayuelo-Jiménez, J.S., Jasso-Plata, N., Ochoa, I., 2012. Growth and Physiological Responses of *Phaseolus* Species to Salinity Stress. Int. J. Agron. 2012, 527673. https://doi.org/10.1155/2012/527673

Camejo, D., Rodríguez, P., Angeles Morales, M., Miguel Dell'Amico, J., Torrecillas, A., Alarcón, J.J., 2005. High temperature effects on photosynthetic activity of two tomato cultivars with different heat susceptibility. J. Plant Physiol. 162, 281–289. https://doi.org/10.1016/j.jplph.2004.07.014

Carvalho, D., Cardoso Pereira, S., Rocha, A., 2021. Future surface temperature changes for the Iberian Peninsula according to EURO-CORDEX climate projections. Clim. Dyn. 56, 123–138. https://doi.org/10.1007/s00382-020-05472-3

Daliakopoulos, I.N., Tsanis, I.K., Koutroulis, A., Kourgialas, N.N., Varouchakis, A.E., Karatzas, G.P., Ritsema, C.J., 2016. The threat of soil salinity: A European scale review. Sci. Total Environ. 573, 727–739. https://doi.org/10.1016/j.scitotenv.2016.08.177

Danilova, M.N., Kudryakova, N. V, Andreeva, A.A., Doroshenko, A.S., Pojidaeva, E.S., Kusnetsov, V. V, 2018. Differential impact of heat stress on the expression of chloroplast-encoded genes. Plant Physiol. Biochem. 129, 90–100. https://doi.org/10.1016/j.plaphy.2018.05.023

Del Zoppo, M., Galleschi, L., Onnis, A., Pardossi, A., Saviozzi, F., 1999. Effect of Salinity on Water Relations, Sodium Accumulation, Chlorophyll Content and Proteolytic Enzymes in a Wild Wheat. Biol. Plant. 42, 97. https://doi.org/10.1023/A:1002179728270

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. Biochim. Biophys. Acta - Bioenerg. 1847, 468–485. https://doi.org/10.1016/j.bbabio.2015.02.008

Desimone, M., Henke, A., Wagner, E., 1996. Oxidative Stress Induces Partial Degradation of the Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase in Isolated Chloroplasts of Barley. Plant Physiol. 111, 789–796. https://doi.org/10.1104/pp.111.3.789

ElSayed, A.I., El-hamahmy, M.A.M., Rafudeen, M.S., Ebrahim, M.K.H., 2019. Exogenous spermidine enhances expression of Calvin cycle genes and photosynthetic efficiency in sweet sorghum seedlings under salt stress. Biol. Plant. 63, 511–518. https://doi.org/10.32615/bp.2019.046

ElSayed, A.I., Rafudeen, M.S., Gomaa, A.M., Hasanuzzaman, M., 2021. Exogenous melatonin enhances the reactive oxygen species metabolism, antioxidant defense-related gene expression, and photosynthetic capacity of *Phaseolus vulgaris* L. to confer salt stress tolerance. Physiol. Plant. 173, 1369–1381. https://doi.org/10.1111/ppl.13372

Erb, T.J., Zarzycki, J., 2018. A short history of RubisCO: the rise and fall (?) of Nature's predominant CO_2 fixing

enzyme. Curr. Opin. Biotechnol. 49, 100–107. https://doi.org/10.1016/j.copbio.2017.07.017

Eurostat, 2021. Crop production in national humidity [WWW Document]. URL https://ec.europa.eu/eurostat/databrowser/view/APRO_CPNH1__custom_1223424/default/table?lang=en (acessed 12.8.21).

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. Front. Plant Sci. 8, 1147. https://doi.org/10.3389/fpls.2017.01147

Genty, B., Briantais, J.M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta - Gen. Subj. 990, 87–92. https://doi.org/10.1016/S0304-4165(89)80016-9

Haddeland, I., Heinke, J., Biemans, H., Eisner, S., Flörke, M., Hanasaki, N., Konzmann, M., Ludwig, F., Masaki, Y., Schewe, J., Stacke, T., Tessler, Z.D., Wada, Y., Wisser, D., 2014. Global water resources affected by human interventions and climate change. Proc. Natl. Acad. Sci. 111, 3251 LP – 3256. https://doi.org/10.1073/pnas.1222475110

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., 2021. Heat stress in cultivated plants: nature, impact, mechanisms, and mitigation strategies—a review. Plant Biosyst. - An Int. J. Deal. with all Asp. Plant Biol. 155, 211–234. https://doi.org/10.1080/11263504.2020.1727987

IPCC, 2021: *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* [Masson-Delmotte, V., P. Zhai, A. Pirani, S.L., Connors, C. Péan, S. Berger, N. Caud, Y. Chen, L. Goldfarb, M.I. Gomis, M. Huang, K. Leitzell, E. Lonnoy, J.B.R., Matthews, T.K. Maycock, T. Waterfield, O. Yelekçi, R. Yu, and B. Zhou (eds.)]. Cambridge University Press. In Press.

Jahan, M.S., Guo, S., Sun, J., Shu, S., Wang, Y., El-Yazied, A.A., Alabdallah, N.M., Hikal, M., Mohamed, M.H.M., Ibrahim, M.F.M., Hasan, M.M., 2021. Melatonin-mediated photosynthetic performance of tomato seedlings under high-temperature stress. Plant Physiol. Biochem. 167, 309–320. https://doi.org/10.1016/j.plaphy.2021.08.002

Kitajima, M., Butler, W.L., 1975. Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. Biochim. Biophys. Acta - Bioenerg. 376, 105–115. https://doi.org/10.1016/0005-2728(75)90209-1

Koutroulis, A.G., Tsanis, I.K., Daliakopoulos, I.N., Jacob, D., 2013. Impact of climate change on water resources status: A case study for Crete Island, Greece. J. Hydrol. 479, 146–158. https://doi.org/10.1016/j.jhydrol.2012.11.055

Kreslavski, V., Tatarinzev, N., Shabnova, N., Semenova, G., Kosobryukhov, A., 2008. Characterization of the nature of photosynthetic recovery of wheat seedlings from short-term dark heat exposures and analysis of the mode of acclimation to different light intensities. J. Plant Physiol. 165, 1592–1600. https://doi.org/10.1016/j.jplph.2007.12.011

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

Liu, L., Shao, Z., Zhang, M., Wang, Q., 2015. Regulation of Carotenoid Metabolism in Tomato. Mol. Plant 8, 28–39. https://doi.org/10.1016/j.molp.2014.11.006

Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR

and the $2^{-\Delta\Delta C_T}$ Method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Longstreth, D.J., Nobel, P.S., 1979. Salinity Effects on Leaf Anatomy: Consequences for Photosynthesis. Plant Physiol. 63, 700–703. https://doi.org/10.1104/pp.63.4.700

Lopez-Delacalle, M., Silva, C.J., Mestre, T.C., Martinez, V., Blanco-Ulate, B., Rivero, R.M., 2021. Synchronization of proline, ascorbate and oxidative stress pathways under the combination of salinity and heat in tomato plants. Environ. Exp. Bot. 183, 104351. https://doi.org/10.1016/j.envexpbot.2020.104351

Løvdal, T., Lillo, C., 2009. Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. Anal. Biochem. 387, 238–242. https://doi.org/10.1016/j.ab.2009.01.024

Loveys, B.R., Scheurwater, I., Pons, T.L., Fitter, A.H., Atkin, O.K., 2002. Growth temperature influences the underlying components of relative growth rate: an investigation using inherently fast- and slow-growing plant species. Plant. Cell Environ. 25, 975–988. https://doi.org/10.1046/j.1365-3040.2002.00879.x

Mariz-Ponte, N., Mendes, R.J., Sario, S., Correia, C.V., Correia, C.M., Moutinho-Pereira, J., Melo, P., Dias, M.C., Santos, C., 2021. Physiological, Biochemical and Molecular Assessment of UV-A and UV-B Supplementation in *Solanum lycopersicum*. Plants 10, 918. https://doi.org/10.3390/plants10050918

Martins, M., Sousa, B., Lopes, J., Soares, C., Machado, J., Carvalho, S., Fidalgo, F., Teixeira, J., 2020. Diclofenac shifts the role of root glutamine synthetase and glutamate dehydrogenase for maintaining nitrogen assimilation and proline production at the expense of shoot carbon reserves in *Solanum lycopersicum* L. Environ. Sci. Pollut. Res. 27, 29130–29142. https://doi.org/10.1007/s11356-020-09136-x

Mathur, S., Agrawal, D., Jajoo, A., 2014. Photosynthesis: Response to high temperature stress. J. Photochem. Photobiol. B Biol. 137, 116–126. https://doi.org/10.1016/j.jphotobiol.2014.01.010

Müller, P., Li, X.P., Niyogi, K.K., 2001. Non-Photochemical Quenching. A Response to Excess Light Energy. Plant Physiol. 125, 1558–1566. https://doi.org/10.1104/pp.125.4.1558

Murashige, T., Skoog, F., 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol. Plant. 15, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nishiyama, Y., Murata, N., 2014. Revised scheme for the mechanism of photoinhibition and its application to enhance the abiotic stress tolerance of the photosynthetic machinery. Appl. Microbiol. Biotechnol. 98, 8777–8796. https://doi.org/10.1007/s00253-014-6020-0

Parihar, P., Singh, S., Singh, R., Singh, V.P., Prasad, S.M., 2015. Effect of salinity stress on plants and its tolerance strategies: a review. Environ. Sci. Pollut. Res. 22, 4056–4075. https://doi.org/10.1007/s11356-014-3739-1

Peng, Y.Y., Liao, L.L., Liu, S., Nie, M.M., Li, J., Zhang, L.D., Ma, J.F., Chen, Z.C., 2019. Magnesium Deficiency Triggers SGR–Mediated Chlorophyll Degradation for Magnesium Remobilization. Plant Physiol. 181, 262–275. https://doi.org/10.1104/pp.19.00610

Pospíšil, P., Prasad, A., 2014. Formation of singlet oxygen and protection against its oxidative damage in Photosystem II under abiotic stress. J. Photochem. Photobiol. B Biol. 137, 39–48. https://doi.org/10.1016/j.jphotobiol.2014.04.025

Rivero, R.M., Mestre, T.C., Mittler, R., Rubio, F., Garcia-Sanchez, F., Martinez, V., 2014. The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. Plant. Cell Environ. 37, 1059–1073. https://doi.org/10.1111/pce.12199

Santos, C., 2004. Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. Sci. Hortic. (Amsterdam). 103, 93–99. https://doi.org/10.1016/j.scienta.2004.04.009

Singh, J., Thakur, J.K., 2018. Photosynthesis and Abiotic Stress in Plants, Vats, S. (Ed.), Biotic and Abiotic Stress Tolerance in Plants. Springer Singapore, Singapore, pp. 27–46. https://doi.org/10.1007/978-981-10-9029-5_2

Soares, C., Branco-Neves, S., de Sousa, A., Pereira, R., Fidalgo, F., 2016. Ecotoxicological relevance of nano-NiO and acetaminophen to *Hordeum vulgare* L.: Combining standardized procedures and physiological endpoints. Chemosphere 165, 442–452. https://doi.org/10.1016/j.chemosphere.2016.09.053

Soares, C., Pereira, R., Martins, M., Tamagnini, P., Serôdio, J., Moutinho-Pereira, J., Cunha, A., Fidalgo, F., 2020. Glyphosate-dependent effects on photosynthesis of *Solanum lycopersicum* L. — An ecophysiological, ultrastructural and molecular approach. J. Hazard. Mater. 398, 122871. https://doi.org/10.1016/j.jhazmat.2020.122871

Spreitzer, R.J., Salvucci, M.E., 2002. RUBISCO: Structure, Regulatory Interactions, and Possibilities for a Better Enzyme. Annu. Rev. Plant Biol. 53, 449–475. https://doi.org/10.1146/annurev.arplant.53.100301.135233

Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and biotic stress combinations. New Phytol. 203, 32–43. https://doi.org/10.1111/nph.12797

Taiz, L., Zeiger, E., Møller, I.M., Murphy, A., 2015. Plant physiology and development. 6th ed. Sinauer Associates Incorporated, Sunderland, MA, USA.

Tardieu, F., Grainer, C., Muller, B., 1999. Modelling leaf expansion in a fluctuating environment: are changes in specific leaf area a consequence of changes in expansion rate? New Phytol. 143, 33–43. https://doi.org/10.1046/j.1469-8137.1999.00433.x

Vaibhav Kumar Maurya, R. Srinvasan, N. Ramesh, M.A. and Gothandam, K.M., 2015. Expression of Carotenoid Pathway Genes in Three Capsicum Varieties under Salt Stress. Asian J. Crop Sci. 7, 286–294. https://doi.org/10.3923/ajcs.2015.286.294

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

Wahid, A., Gelani, S., Ashraf, M., Foolad, M.R., 2007. Heat tolerance in plants: An overview. Environ. Exp. Bot. 61, 199–223. https://doi.org/10.1016/j.envexpbot.2007.05.011

Wang, D., Heckathorn, S.A., Mainali, K., Tripathee, R., 2016. Timing Effects of Heat-Stress on Plant Ecophysiological Characteristics and Growth. Front. Plant Sci. 7, 1629. https://doi.org/10.3389/fpls.2016.01629

Zhang, H., Zhu, J., Gong, Z., Zhu, J.K., 2021. Abiotic stress responses in plants. Nat. Rev. Genet. https://doi.org/10.1038/s41576-021-00413-0

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 Biol. 17, 24. https://doi.org/10.1186/s12870-017-0974-x

Zribi, L., Fatma, G., Fatma, R., Salwa, R., Hassan, N., Néjib, R.M., 2009. Application of chlorophyll fluorescence for the diagnosis of salt stress in tomato "*Solanum lycopersicum* (variety Rio Grande)." Sci. Hortic. (Amsterdam). 120, 367–372. https://doi.org/10.1016/j.scienta.2008.11.025

Supplementary Material

Table S4.1. Results of the two-way ANOVA for all evaluated parameters in *Solanum lycopersicum* L. var. *cerasiforme* plants after 21 days of exposure to 42 °C (4 h d^{-1}) and irrigation with (100 mM) or without NaCl. Parameters where significant differences ($p \leq 0.05$) were recorded are highlighted in bold.

Parameter	Factors		Interaction
	SALT	*HEAT*	
Chl *a*	**F (1, 10) = 31.6; *p* < 0.0002**	**F (1, 10) = 19.9; *p* = 0.0012**	F (1, 10) = 1.61; *p* = 0.2326
Chl *b*	**F (1, 10) = 32.0; *p* = 0.0002**	**F (1, 10) = 23.1; *p* = 0.0007**	F (1, 10) = 1.50; *p* = 0.2493
Total Chl	**F (1, 10) = 31.9; *p* = 0.0002**	**F (1, 10) = 20.8; *p* = 0.0010**	F (1, 10) = 1.59; *p* = 0.2357
Carotenoids	**F (1, 10) = 47.7; *p* < 0.0001**	**F (1, 10) = 21.2; *p* = 0.0010**	F (1, 10) = 0.423; *p* = 0.5256
F_v/F_m	**F (1, 15) = 7.09; *p* = 0.0178**	**F (1, 15) = 5.95; *p* = 0.0276**	F (1, 15) = 2.34; *p* = 0.1469
rETR	**F (1, 17) = 20.0; *p* = 0.0003**	**F (1, 17) = 8.35; *p* = 0.0102**	**F (1, 17) = 75.2; *p* < 0.0001**
ΦPSII	**F (1, 17) = 20.3; *p* = 0.0003**	**F (1, 17) = 8.47; *p* = 0.0097**	**F (1, 17) = 75.8; *p* < 0.0001**
NPQ	**F (1, 15) = 13.6; *p* = 0.0022**	F (1, 15) = 0.751; *p* = 0.3997	**F (1, 15) = 9.16; *p* = 0.0085**
$\Phi PSII_{1258}$	**F (1, 19) = 5.37; *p* = 0.0318**	**F (1, 19) = 23.0; *p* = 0.0001**	F (1, 19) = 3.68; *p* = 0.0703
$rETR_{1258}$	**F (1, 16) = 10.5; *p* = 0.0050**	**F (1, 16) = 67.2; *p* < 0.0001**	**F (1, 16) = 9.36; *p* = 0.0075**
NPQ_{1258}	**F (1, 15) = 19.0; *p* = 0.0006**	**F (1, 15) = 8.22; *p* = 0.0118**	**F (1, 15) = 8,09; *p* = 0.0123**
E	**F (1, 12) = 41.4; *p* < 0.0001**	F (1, 12) = 2.45; *p* = 0.1438	F (1, 12) = 0.820; *p* = 0.3830
g_s	**F (1, 11) = 33.8; *p* = 0.0001**	F (1, 11) = 1.36; *p* = 0.2683	F (1, 11) = 0.300; *p* = 0.5946
P_N	**F (1, 10) = 38.5; *p* < 0.0001**	F (1, 10) = 0.822; *p* = 0.3858	F (1, 10) = 0.0208; *p* = 0.8883
WUE_i	**F (1, 8) = 22.0; *p* = 0.0016**	F (1, 8) = 0.664; *p* = 0.4386	F (1, 8) = 0.0386; *p* = 0.8492
C_i/C_a	**F (1, 10) = 28.5; *p* = 0.0003**	F (1, 10) = 0.145; *p* = 0.7115	F (1, 10) = 0.0525; *p* = 0.8233
SLA	**F (1, 11) = 34.2; *p* = 0.0001**	**F (1, 11) = 21.2; *p* = 0.0008**	**F (1, 11) = 5.97; *p* < 0.0001**
D1	**F (1, 8) = 229; *p* < 0.0001**	**F (1, 8) = 118; *p* < 0.0001**	**F (1, 8) = 233; *p* < 0.0001**
CP47	**F (1, 8) = 509; *p* < 0.0001**	**F (1, 8) = 62.5; *p* < 0.0001**	**F (1, 8) = 253; *p* < 0.0001**
RbcS	**F (1, 8) = 21.2; *p* = 0.0018**	**F (1, 8) = 23.4; *p* = 0.0013**	**F (1, 8) = 70.2; *p* < 0.0001**
RbcL	**F (1, 8) = 56.3; *p* < 0.0001**	**F (1, 8) = 124; *p* < 0.0001**	F (1, 8) = 0.114; *p* = 0.7444

CHAPTER V

Concluding Remarks and Future Perspectives

1. Concluding Remarks

- **Growth and nutrient balance**
  - The combined treatment exacerbated the negative effects of the individual exposure to heat and salt in plant growth in both shoots and roots;
  - Exposure of tomato plants to combined salinity and heat stresses led to a higher accumulation of sodium (Na^+) and lower concentrations of potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) than those found with the individual stress conditions.
- **Oxidative stress and antioxidant (AOX) defences**
  - No higher accumulation of reactive oxygen species was perceived, nor lipid peroxidation was induced in all stressed plants;
  - The co-exposure to salt and heat differentially affected the AOX system in roots and shoots and its response was, overall, more intense than that perceived in the single treatments;
  - In roots, both the accumulation of AOX metabolites and activation of enzymes were induced by the combination of stressors;
  - Upon combined stress, in shoots, a general activation of the enzymatic component of the AOX system was observed;
  - The ascorbate-glutathione (AsA-GSH) cycle played a key role in ensuring redox homeostasis in tomato plants under simultaneous exposure to salt and heat.
- **Photosynthetic performance**
  - Individually, heat induced the most severe adverse effects on the photochemical efficiency of photosystem II (PSII);
  - Gas-exchange parameters were predominantly and very negatively affected by salt exposure;
  - PSII-related genes and *RbcS* were repressed under all treatments, while *RbcL* transcripts were accumulated under salinity stress and reduced upon heat exposure;
  - The implications of the co-exposure to the stressors were not harsher than those of the individual exposure.

Overall, the combination of salinity and heat stress induced a unique response that could not have been extrapolated from the individual exposure to the stressors. Additionally, even though an activation of AOX defences ensured redox homeostasis and photosynthetic performance was not majorly compromised – at least not more than what was observed for the individual stressors – tomato plants' growth was hampered to an extent that surpassed the single treatments, and the nutrient balance was harshly disrupted. Thus, it is possible that other cellular processes (*e.g.* cell expansion and division) are being negatively affected by the stressors or the resource pool is being mobilized from growth to ensure survival through the induction of the AOX system.

2. Future Perspectives

Taking everything into consideration, with this work, a clear and elucidative perspective of the implications of the forecasted salinity and high temperatures on cherry tomato plants was achieved. Nonetheless, there are still questions that remain unanswered that, hopefully, plant physiologists will tackle in the near future. With the intent to fully understand the mechanisms underlying tomato plants' response to combined salt and heat stresses, so that the development of tolerant crops and strategies to improve productivity is possible, it would be interesting to pursue the following key ideas:

- Complement the study of the impact of the stressors on the photosynthetic apparatus by assessing, through Western Blotting, the protein content of D1, CP47 and RbcL;
- Evaluate the expression profile of genes encoding proteins involved in the uptake and transport of Na^+, as well as heat shock proteins and their transcription factors;
- Unravel the implications of heat and salinity on cellular division and expansion, on the synthesis of cell wall, and on the phytohormone metabolism;
- Further investigate the effects of heat and salinity on plant water relations and cell wall characteristics, by measuring water potential and performing pressure/volume curves;
- Confirm the lab-scaled results in open-field trials to fully validate the obtained data;
- Evaluate the potential of exogenous application of growth promoters to mitigate the negative effects of the combination of these stress factors;
- Screen other tomato cultivars and varieties or wild species for tolerance traits to the stressors.

It is worth mentioning that the first two points are currently being analysed within the research group to complement the results obtained in this dissertation and for the preparation of a research paper encompassing that data and the one presented in Chapter IV.