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Metabolic changes induced by the combination of abiotic stresses and identification of tolerance mechanisms in tomato plants.

Cambios metabólicos inducidos por la combinación de estreses abióticos e identificación de mecanismos de tolerancia en plantas de tomate.

María López de la Calle

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“Todas las cosas son imposibles,

mientras lo parecen”

Concepción Arenal (1820 – 1893)

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Trabajo realizado para obtener el Título de Doctor
por la Universidad de Murcia

María López de la Calle

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GENERAL INDEX

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RESUMEN

RESUMEN

El cambio climático y los fenómenos meteorológicos extremos afectan a los ecosistemas comprometiendo la seguridad alimentaria y la energética, especialmente en las regiones pobres y con menos recursos. En el año 2020, uno de los tres más cálidos de los que se tiene registro, la temperatura media mundial aumentó 1,2 °C sobre los niveles preindustriales de referencia, según la Declaración de la Organización Meteorológica Mundial (WMO) sobre el estado del clima mundial en 2020 (WMO 2021). En ella se destaca el deterioro acelerado de los indicadores del cambio climático y los fenómenos meteorológicos extremos, así como la consecuente repercusión socioeconómica. La previsión de un aumento de la temperatura global, acompañada de las olas de calor previstas, hace prever que la demanda de agua para los cultivos aumentará, lo que conllevará un aumento de la competencia entre la agricultura y otros sectores. Todo esto provoca, además, una importante reducción de la calidad de las aguas de riego, que, junto con las estimaciones en el incremento de la población mundial, hacen necesaria la búsqueda de alternativas en la generación de cultivos con una mayor tolerancia a los distintos estreses abióticos.

Numerosas investigaciones y avances científicos han señalado cómo los distintos estreses abióticos y bióticos aplicados de forma individual afectan a los cultivos a diversos niveles, con la consiguiente generación de gran conocimiento sobre los sistemas implicados en estas respuestas. Sin embargo, en los últimos años, se ha descubierto que la respuesta de las plantas a la combinación de dos o más estreses es altamente específica y no puede ser deducida de la respuesta que tiene cada organismo al someterlo a los mismos estreses de manera individual. Concretamente, las zonas áridas y semiáridas del planeta, como son las pertenecientes a la Cuenca del Mediterráneo, se encuentran especialmente afectadas por condiciones ambientales como la salinidad y las altas temperaturas, condiciones que suelen darse sinérgicamente. Es por ello que el estudio de esta combinación de estreses concreta, presenta un gran valor científico y agronómico para esta Región.

La salinidad y las altas temperaturas reducen el crecimiento de las plantas, así como la actividad fotosintética, induciendo la acumulación de especies reactivas de oxígeno (ROS), inhibiendo la actividad de importantes enzimas metabólicas, alterando la eficiencia de las reacciones enzimáticas dentro de la célula, cambiando la composición de los metabolitos sintetizados y acumulados y afectando a la estabilidad de diversas proteínas, lo que provoca un estado de desequilibrio metabólico general. La respuesta de las plantas al ambiente que las rodea afecta a todos estos mecanismos, los cuales deben actuar de manera sincronizada a nivel fisiológico, bioquímico y molecular. Por eso, el metabolismo de las plantas debe estar estrictamente regulado e interconectado para permitir una aclimatación a estos entornos cambiantes.

Por otro lado, según la FAO, el tomate es una de las frutas más consumidas en el mundo, lo que le aporta valor agrícola y económico. Además, a nivel científico constituye una potente herramienta molecular ya que su genoma es conocido y público, lo que la convierte en una planta modelo para su estudio.

Teniendo en consideración estas premisas, esta Tesis Doctoral tiene como objetivo principal el ahondar en la respuesta fisiológica, bioquímica y molecular de las plantas de tomate a la combinación de salinidad y alta temperatura mediante el uso de plantas de tomate de una variedad de interés comercial (*Solanum lycopersicum* L. cv Boludo, Monsanto) y el de líneas isogénicas recombinantes (RILs) caracterizadas por su diferente sensibilidad a estos estreses combinados. Se pretende integrar los resultados obtenidos a nivel fisiológico, bioquímico y molecular en diferentes rutas metabólicas conocidas para obtener una información completa y detallada de estos mecanismos específicos involucrados en el desarrollo de la tolerancia de las plantas a la combinación de salinidad y alta temperatura, dos de los estreses abióticos más devastadores en la producción agrícola en el sureste de España.

Este objetivo principal se ha desarrollado a través de los siguientes objetivos específicos:

I. Estudiar la vía de asimilación de N para comprender mejor cómo esta ruta metabólica esencial puede verse afectada por la combinación específica de salinidad y alta temperatura en plantas con distinto grado de tolerancia a dichas condiciones.
II. Dilucidar cómo la combinación de la salinidad y las altas temperaturas afectan al metabolismo y la acumulación de azúcares en los frutos del tomate, así como su relevancia en la tolerancia de la planta y en los rasgos de calidad final del fruto.
III. Establecer las posibles relaciones entre mecanismos de señalización implicados en la tolerancia a la combinación de salinidad y alta temperatura en una variedad comercial de tomate.

El nitrógeno (N) es uno de los elementos más limitantes para el desarrollo de las plantas, por ello, en un primer experimento, se realizó un estudio en una selección de plantas RILs obtenidas mediante el cruzamiento de una línea silvestre (*S. pimpinellifollium* L.), tolerante a condiciones abióticas extremas, y una línea comercial (*S. lycopersicum* L.). Esta población de líneas RILs se convierte así en una herramienta muy útil para el análisis de rasgos cualitativos de una especie, ya que poseen diferente sensibilidad a la combinación de estreses abióticos. Mediante el uso de aproximaciones fisiológicas y bioquímicas en experimentos sucesivos, se seleccionaron dos líneas, una tolerante (RIL-76) y otra sensible (RIL-66) a la combinación de salinidad y alta temperatura, las cuales fueron usadas para determinar cómo la absorción y asimilación de nitrógeno (N) se veía afectada por esta combinación de estreses. La combinación de salinidad y alta temperatura afectó de diferente manera a la línea sensible y a la tolerante, mostrando en la tolerante un mayor crecimiento y rendimiento fotosintético. De igual manera, la línea tolerante también mostró una mayor actividad enzimática de las enzimas nitrato reductasa (NR), glutamato sintasa (GOGAT) y glutamato deshidrogenasa (GDH) que podría guardar relación con los altos niveles encontrados de glutamina (Gln), ácido glutámico (Glu), asparagina (Asn), ácido γ-aminobutírico (GABA), valina (Val), prolina (Pro) e isoleucina (Isoleu). Por otro lado, la línea sensible manifestó un déficit de los nutrientes K^+, Mg^{2+} y contenido en N orgánico, parámetros esenciales en el crecimiento y desarrollo de cualquier cultivo.

Debido a la implicación y a la importancia de los azúcares como parámetros de calidad, además de su papel como osmoprotectores y mensajeros secundarios, se consideró relevante el estudio del metabolismo de los azúcares bajo condiciones combinadas de estrés por salinidad y alta temperatura. Por ello, seguidamente al estudio del metabolismo del N, se realizó un experimento agronómico en condiciones reales de campo para determinar cómo afectaban los estreses de salinidad y alta temperatura combinados al metabolismo de los azúcares en los frutos de dos líneas RILs seleccionadas nuevamente como tolerante (RIL-76) y como sensible (RIL-18) y en dos estadios de maduración (fruto rojo y verde). La línea tolerante mostró un aumento significativo en la actividad de las enzimas fructosa-1,6-bisfosfatasa (FBPasa), sacarosa fosfato sintasa (SPS), sacarosa sintasa 3 (SUS3), glucosa-6-fosfato deshidrogenasa (G6PDH) e invertasa ácida (AI) en comparación con la línea sensible, lo que resultó en una mayor acumulación de fructosa, glucosa y UDP-glucosa.

Analizando todos los parámetros en un sentido amplio y de conjunto, la línea seleccionada como tolerante (RIL-76), mostró en ambos experimentos una mayor adaptación a las condiciones de estrés que la línea sensible. Estos resultados pueden ser debidos a la presencia en su genoma de rasgos del parental silvestre, más resistente a las condiciones de estrés, pero con carencias en cuanto a valor comercial.

Dada la relevancia de los hallazgos realizados previamente en la población de RILs, en esta Tesis se planteó el objetivo de integrar todos los datos y obtener una caracterización general de cómo afecta la combinación de salinidad y alta temperatura a plantas de una variedad comercial de tomate. En este caso, en lugar de centrar el ensayo en una única ruta metabólica, como se hizo previamente en los ensayos con las líneas RILs, se realizó un análisis de todo el perfil transcripcional y metabolómico, cumplimentado con ensayos bioquímicos y fisiológicos, que validó y amplió los resultados previos. Los resultados mostraron que la combinación de salinidad y alta temperaturas daba lugar a una reprogramación única de todas vías metabólicas con cambios en la expresión de 1388 genes y la acumulación de 568 compuestos moleculares. La integración de los datos mostró una sincronización entre las vías de la prolina, el ascorbato y el metabolismo oxidativo para mantener la homeostasis celular. En los últimos años, diversas investigaciones han demostrado la implicación de los factores de transcripción (TF) en la respuesta al estrés. En este ensayo se ha demostrado que las familias de TFs MYB, bZYP y bHLH se expresaron diferencialmente con respecto a las plantas sometidas a condiciones control. Además, únicamente para las condiciones experimentales de combinación de estrés, se expresaron diferencialmente varios genes de las familias TF C2H2 o Trihelix. Muchas de estas familias de TF se han relacionado con la división celular o con diversos procesos fisiológicos, pero también en respuesta a diferentes estreses abióticos y podrían ser reguladores de los genes diferencialmente expresados bajo la combinación de salinidad y alta temperatura.

Los resultados obtenidos en esta Tesis Doctoral y recogidos en los Capítulos III, IV y V concluyeron que:

I. La línea de tomate tolerante, RIL-76, bajo condiciones de estrés combinado de salinidad y alta temperatura, mostró mayor crecimiento en comparación con las líneas sensibles RIL-66 y RIL-18 sometidas a las mismas condiciones.

II. Bajo la combinación de estrés por salinidad y alta temperatura, se indujo una disminución en el contenido de K^+, Mg^{2+}, N total y N orgánico en la línea sensible RIL-66 con respecto a su control. Sin embargo, esta disminución no se produjo en la línea tolerante RIL-76 que mantuvo los niveles del control.

III. La línea tolerante RIL-76, bajo la combinación de salinidad y alta temperatura, incrementó la eficiencia el metabolismo del nitrógeno a niveles transcripcionales y postranscripcional. Específicamente, la línea tolerante RIL-76 mostró un aumento en la actividad enzimática NR, GOGAT y GDH que podría estar relacionada con los niveles de Gln, Glu, Asn, GABA, Val, Pro e Isoleu encontrados bajo la combinación de estos estreses.

IV. La línea tolerante, RIL-76 presentó una mayor actividad enzimática de FBPasa, SPS, SUS3, G6PDH y AI en comparación con la línea sensible, RIL-18 en condiciones de campo.

V. La línea tolerante, RIL-76 presentó una mayor concentración de los azúcares fructosa, glucosa y UDP-glucosa en frutos de tomate bajo la combinación de salinidad y alta temperatura en condiciones de campo en comparación con la línea sensible RIL-18.

VI. La línea tolerante RIL-76 presentó una sobreexpresión de los genes que codifican para los transportadores de azúcar *SlGLCT*, *SlINT4*, *SlSUT4*, *SlTMT1* y *SlVGT1* en condiciones de estrés en campo, lo que no se observó en la línea sensible RIL-18.

VII. La variedad comercial Boludo mostró un aumento de los parámetros fotosintéticos bajo la combinación de salinidad y alta temperatura en comparación con aquellas plantas sujetas únicamente a la condición de salinidad.

VIII. La salinidad y la alta temperatura aplicadas en combinación mostraron un perfil transcriptómico único y específico con un total de 1388 genes expresados diferencialmente, los cuales no presentaron alteraciones bajo las condiciones de salinidad o alta temperatura aplicadas individualmente.

IX. La combinación de salinidad y alta temperatura indujo un perfil metabólico específico con una concentración diferencial y significativa de 568 compuestos moleculares específicamente acumulados que no presentaron alteraciones bajo las condiciones de estrés aplicadas individualmente.

X. La integración de datos metabolómicos y transcriptómicos en vías biosintéticas conocidas bajo la combinación de salinidad y alta temperatura reveló una sincronización entre las vías de ascorbato y prolina para mantener la homeostasis celular de las ROS.

XI. La respuesta a la combinación de la salinidad y la alta temperatura en plantas de tomate comercial se asoció con la regulación positiva de factores de transcripción específicos incluidos en las familias bZIP, C2H2 y Trihelix.

Los parámetros más importantes a considerar para la selección de variedades de interés agronómico en el pasado han sido exclusivamente el rendimiento y la calidad de la cosecha. Los nuevos escenarios que plantea el cambio climático, junto con las herramientas moleculares que existen actualmente, nos permiten seleccionar variedades considerando también la tolerancia a nuevas condiciones ambientales y el uso de marcadores genéticos y metabólicos para este fin.

El conjunto de estos tres ensayos demuestra la relevancia del estudio los estreses abióticos en combinación y sirve como base para la identificación de posibles mecanismos de señalización de tolerancia al estrés.

SUMMARY

SUMMARY

Climate change and extreme weather events affect ecosystems, compromising food and energy security, especially in poor regions with fewer resources. In 2020, one of the three warmest on record, the global mean temperature increased 1.2°C above pre-industrial reference levels, according to the World Meteorological Organization (WMO) on the report State of the Global Climate 2020 (WMO 2021). It highlights the accelerated deterioration of the indicators of climate change and extreme meteorological phenomena, as well as the subsequent socio-economic repercussions. The forecast of an increase in global temperature, accompanied by the expected heat waves, suggests that the demand for water for crops will increase, which will lead to increased competition between agriculture and other sectors. All of this will lead to a significant reduction in the quality of irrigation water, which, together with the estimated increase in the world population, make it necessary to search for alternatives in the generation of crops with greater tolerance to the different abiotic stresses.

Numerous investigations and scientific advances have indicated how the different abiotic and biotic stresses applied individually affect crops at different levels, with the subsequent generation of great knowledge about the systems involved in these responses. However, in recent years, it has been discovered that the response of plants to the combination of two or more stresses is highly specific and cannot be deduced from an organism’s response when subjected to the same stresses individually. Specifically, the arid and semi-arid regions of the planet, such as those belonging to the Mediterranean Basin, are especially affected by environmental conditions such as salinity and high temperatures, conditions that tend to occur simultaneously. That is why the study of this specific combination of stresses has great scientific and agronomic values for these regions.

Salinity and high temperatures reduce plant growth and photosynthetic activity, thereby inducing the accumulation of reactive oxygen species (ROS), inhibiting the activity of important metabolic enzymes, altering the efficiency of

enzymatic reactions within the cell, changing the composition of the synthesized and accumulated metabolites, and affecting the stability of various proteins, which causes a state of general metabolic imbalance. The response of plants to the environment that surrounds them affects all of these mechanisms, which must act in synergistically at the physiological, biochemical and molecular levels. Therefore, the metabolism of plants must be strictly regulated and interconnected to allow them to acclimatize to these changing environments.

According to the FAO, the tomato is one of the most consumed fruits in the world, which grants it agricultural and economic value. In addition, on a scientific level, it constitutes a powerful molecular analysis tool, as its genome has been sequenced and made public, defining it as a model plant for various types of research studies.

Taking these premises into consideration, the main objective of this Doctoral Thesis is to delve into the physiological, biochemical and molecular response of tomato plants to the combination of salinity and heat through the use of a commercial variety of tomato (*Solanum lycopersicum* L. cv Boludo, Monsanto) and its recombinant inbred lines (RILs), characterized by their different sensitivity to these combined stresses. The aim is to integrate the results obtained at the physiological, biochemical and molecular levels in different known metabolic pathways to obtain complete and detailed information on the specific mechanisms involved in the development of tolerance of plants to the combination of salinity and high temperature, two of the most devastating abiotic stresses in agricultural production in Southeastern Spain.

This main objective was developed through the following secondary objectives:

I. To study the N assimilation pathway to better understand how this essential metabolic pathway can be affected by the specific combination of salinity and high temperature in plants with different degrees of tolerance to these conditions.

II. To elucidate how the combination of salinity and high temperatures affect the metabolism and the accumulation of sugars in tomato fruits, as well as their relevance in the tolerance of the plant and in the final quality traits of the fruit.

III. To establish the possible relationships between the signaling mechanisms involved in tolerance to the combination of salinity and high temperature in a commercial tomato variety.

Nitrogen (N) is one of the most limiting elements for the development of plants. Therefore, in the first experiment, a study was carried out on a selection of RILs plants obtained by crossing a wild line (*S. pimpinellifollium* L.), tolerant to extreme abiotic conditions, and a commercial line (*S. lycopersicum* L.). This population of RILs lines became a very useful tool for the analysis of qualitative traits of a species, as they had different sensitivities to the combination of abiotic stresses. Through the use of physiological and biochemical approaches in successive experiments, two lines were selected, one tolerant (RIL-76) and another sensitive (RIL-66) to the combination of salinity and heat, which were used to determine how N absorption and assimilation were affected by this combination of stresses. The combination of salinity and heat affected the sensitive and tolerant lines differently, with the tolerant line showing greater growth and photosynthetic performance. Similarly, the tolerant line also showed higher enzymatic activities of the enzymes nitrate reductase (NR), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH), which could be related to the high levels of glutamine (Gln), glutamic acid (Glu), asparagine (Asn), γ-aminobutyric acid (GABA), valine (Val), proline (Pro) and isoleucine (Isoleu) found in the plants. On the other hand, the sensitive line showed a deficit of the nutrients K^+, Mg^{2+} and organic N content, essential parameters in the growth and development of any crop.

Due to the involvement and importance of sugars as quality parameters, in addition to their role as osmoprotectors and secondary messengers, the study of sugar metabolism under combined conditions of salinity stress and heat was considered relevant. Therefore, following the study of N metabolism, an agronomic experiment was carried out under real field conditions to determine how the combined stresses of salinity and high temperature affected the metabolism of sugars in the fruits of two RILs lines, once again RIL-76 as the tolerant line, and RIL-18 as the sensitive one, and at two stages of maturation (red and green fruit). The tolerant line showed a significant increase in the activity of the enzymes fructose-1,6-bisphosphatase (FBPase), sucrose phosphate synthase (SPS), sucrose synthase 3 (SUS3), glucose-6-phosphate dehydrogenase (G6PDH), and acid invertase (AI) as compared to the sensitive line, which showed a higher accumulation of fructose, glucose and UDP-glucose.

After the analysis of all the parameters in a broad and overall sense, the tolerant line (RIL-76), showed a greater adaptation to stress conditions than the sensitive line in both experiments. These results may be due to the presence of traits from the wild parent in its genome, as it is more resistant to stress conditions, but lacking in commercial value.

Given the relevance of the findings in the population of RILs, the objective of the present Thesis was to integrate all the data and obtain a general characterization of how the combination of salinity and high temperature affects plants of a commercial tomato variety. In this case, instead of focusing the trial on a single metabolic pathway, as previously done in the trials with the RILs lines, an analysis of the entire transcriptional and metabolomic profile was performed, and completed with biochemical and physiological assays, which validated and expanded on the previous results. The results showed that the combination of salinity and heat led to a unique reprogramming of all the metabolic pathways, with changes in the expression of 1,388 genes and the accumulation of 568 molecular compounds. The integration of the data showed a synchronization between the proline (Pro), ascorbate (ASC) and oxidative metabolism pathways to maintain cellular homeostasis. In recent years, various studies have demonstrated the involvement of transcription factors (TF) in the stress response. The results showed that the TF families MYB, bZYP and bHLH were differentially expressed with respect to the plants subjected to control conditions. Furthermore, several genes from the TF families C2H2 or Trihelix were differentially expressed only for the stress combination experimental conditions. Many of these TF families have been associated to cell division or various physiological processes, but also in response to different abiotic stresses, and could therefore be regulators of genes that are differentially expressed under the combination of salinity and high temperature.

The present study is of special scientific and agronomic interest, as it was carried out on a commercial variety of tomato. This allows the results to be easily transferred to real field situations, as they provide solutions to current problems in agriculture.

The set of these three trials demonstrates the importance of the study of abiotic stresses in combination and serves as a basis for the identification of stress tolerance signaling mechanisms.

The results obtained in this Doctoral Thesis and collected in Chapters III, IV and V concluded that:

I. The tolerant tomato line, RIL-76, under the combination of salinity and heat stress, showed greater growth compared to the sensitive lines, RIL-66 and RIL-18, subjected to the same conditions.

II. Under the combination of salinity and heat stress, a decrease in the content of K^+, Mg^{2+}, total N and organic N was induced in the sensitive line, RIL-66, compared to its control. However, this decrease did not occur in the tolerant line RIL-76, which maintained control levels.

III. The tolerant line, RIL-76, under the combination of salinity and heat, increased the efficiency of nitrogen metabolism at transcriptional and post-transcriptional levels. Specifically, the tolerant line RIL-76 showed an increase in the enzymatic activity of NR, GOGAT and GDH that could be associated to the levels of Gln, Glu, Asn, GABA, Val, Pro and Isoleu found under the combination of these stresses.

IV. The tolerant line, RIL-76, presented a higher enzymatic activity of FBPase, SPS, SUS3, G6PDH and AI compared to the sensitive line, RIL-18 under field conditions.

V. The tolerant line, RIL-76 presented a higher concentration of the sugars fructose, glucose and UDP-glucose in tomato fruits under the combination of salinity and heat in field conditions as compared to the sensitive line RIL-18.

VI. The tolerant line RIL-76 presented an overexpression of the genes that code for the sugar transporters *SlGLCT*, *SlINT4*, *SlSUT4*, *SlTMT1* and *SlVGT1* under stress conditions in the field, which was not observed in the sensitive line RIL-18.

VII. The commercial variety Boludo showed an increase in photosynthetic parameters under the combination of salinity and heat compared to those plants that were subjected only to the salinity condition.

VIII. Salinity and heat applied in combination showed a unique and specific transcriptomic profile with a total of 1388 differentially expressed genes, which did not show alterations under the salinity or heat conditions applied individually.

IX. The combination of salinity and heat induced a specific metabolic profile with a differential and significant concentration of 568 specifically accumulated molecular compounds that did not show alterations under these stress conditions applied individually.

X. The integration of metabolomic and transcriptomic data into known biosynthetic pathways under the combination of salinity and heat revealed a synchronization between the ascorbate and proline pathways to maintain ROS cell homeostasis.

XI. The response to the combination of salinity and heat of commercial tomato plants was associated with the positive regulation of specific transcription factors included in the bZIP, C2H2 and Trihelix families.

The most important parameters to consider for the selection of varieties of agronomic interest in the past have been exclusively the yield and the quality of the harvest. The new scenarios posed by climate change, together with the molecular tools that currently exist, allow us to select varieties by also considering tolerance to new environmental conditions and the use of genetic and metabolic markers for this purpose.

The set of these three experiments demonstrates the importance of the study of abiotic stresses in combination, and serves as a basis for the identification of possible stress tolerance signaling mechanisms

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LIST OF ABBREVIATIONS

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AI:	Acid invertase
APX:	Ascorbate peroxidase
ASC:	Ascorbate
Asn:	Asparagine
ATP:	Adenosine triphosphate
ATPase:	Adenosine triphosphatase
AVRDC:	The World Vegetable Center
bHLH:	Basic helix–loop–helix
bZIP:	Basic Leucine Zipper Domain
C2H2:	Cys2His2
CAT:	Catalase
DHA:	Dehydroascorbate
DHAP:	Dihydroxyacetone phosphate
DHAR:	Dehydroascorbate reductase
DNA:	Deoxyribonucleic acid
DW:	Dry weight
ETR:	Electron transport rate
EUFIC:	European Food Information Council
FAO:	Food and Agriculture Organization
FBPase:	Fructose 1,6-bisphosphatase
Fd:	Ferredoxin
FK2:	Fructokinase 2
Fv’/Fm’:	Maximum quantum efficiency rate
FW:	Fresh weight
GA3P:	Glyceraldehyde 3-phosphate
GABA:	γ-aminobutyric acid
GDH:	Glutamate dehydrogenase
pGlcT:	Plastidic glucose translocators proteins
GlcT:	Glucose transposter

Gln:	Glutamine
Glu:	Glutamic acid
GOGAT:	Glutamate synthase
GR:	Glutathione reductase
GS:	Glutamine synthetase
GSA:	Glutamyl semialdehyde
GSA-DH:	Glutamyl semialdehyde dehydrogenase
GSH:	Glutathione
GSSG:	Oxidized glutathione
Ile:	Isoleucine
INT4:	Inositol transporter 4
IPCC:	Intergovernmental Panel on Climate Change
Kha:	Thousands of hectares
MAPKs:	Mitogen activate protein kinases
MDA:	Malondialdehyde
MDHAR:	Monodehydroascorbate reductase
Mt:	Millions of tons
mRNA:	Messenger ribonucleic acid
NAD(P)H:	Nicotinamide adenine dinucleotide phosphate in its reduced form
NAD$^+$:	Nicotinamide adenide dinucleotide (oxidized form)
NADH:	Nicotinamide adenide dinucleotide (reduced form)
NI:	Neutral invertase
NiR:	Nitrito reductase
NR:	Nitrato reductase
NUE:	Nitrogen Use Efficiency
OAT:	Arnithine aminotransferase
P5C:	Δ1-pyrroline-5-carboxylate
P5CDH:	Δ1-pyrroline-5-carboxylate dehydrogenase
P5CR:	Δ1-pyrroline-5-carboxylate reductase
P5CS:	Δ1-pyrroline-5-carboxylate synthetase
PDX:	Peroxidases

PO:	Proline oxidase
Pro:	Proline
PRODH:	Proline dehydrogenase
RBOHD:	Respiratory burst homolog D
RIL:	Recombinant inbred line
RNA:	Ribonucleic acid
ROS:	Reactive Oxygen Species
SOD:	Superoxide dismutase
SPS:	Sucrose phosphate synthase
SUS3:	Sucrose synthase 3
SUT4:	Sucrose transporter 4
TBARS:	Thiobarbituric acid
TF:	Transcription Factors
TMT:	Tonoplast localized monosaccharide transporters
Trp:	Tryptophan
Tyr:	Tyrosine
UDP-Glucose:	Uracil-diphosphate glucose
USDA:	United States Department of Agriculture
Val:	Valine
VGT1:	Vacuolar glucose transporter 1
ϒ-GK:	γ-glutamyl kinase
ΦPSII:	Photosystem II efficiency

CHAPTER I:

INTRODUCTION

1. Chapter I. Introduction

1.1. Botanical description of tomato

Tomato (*Solanum lycopersicum L.*) is a dicotyledonous plant belonging to the Solanaceae family, and is usually cultivated as an annual plant, although under optimal conditions, its cultivation can last longer (JV Maroto 1995).

The generally accepted taxonomy is:

i. Class: *Dicotyledoneas.*
ii. Order: *Solanales (Personatae).*
iii. Family: *Solanaceae.*
iv. Subfamily: *Solanoideae.*
v. Genus: *Solanum.*
vi. Species: *Lycopersicum.*

The tomato is a shrubby perennial plant, and its growth can be determined or indeterminate, depending on the species. The most common tomato types chosen by farmers worldwide are those with an indeterminate growth pattern, where the stems show uniform patterns formed by three leaves or buds and a racemose-type inflorescence, ending with the vegetative apex, where the plant will continue its vegetative growth (Lapuerta 1995). These indeterminate growth tomatoes are usually cultivated in greenhouses, because, as they produce inflorescences continuously, they are perfect for fresh tomato consumption (Kinet and Peet 1997). The determined plant has fewer leaves per inflorescence and a limited growth. In the first stages of the plant, the stem has an herbaceous consistency, but in the adult stage, it is semi-woody and covered with glandular and non-glandular hairs. The root system is pivotal, and is composed of the main root from which secondary roots and adventitious roots emerge. The leaves are compound and odd-pinnate, presenting a terminal leaflet and 7 to 9 lateral leaflets (Lapuerta 1995).

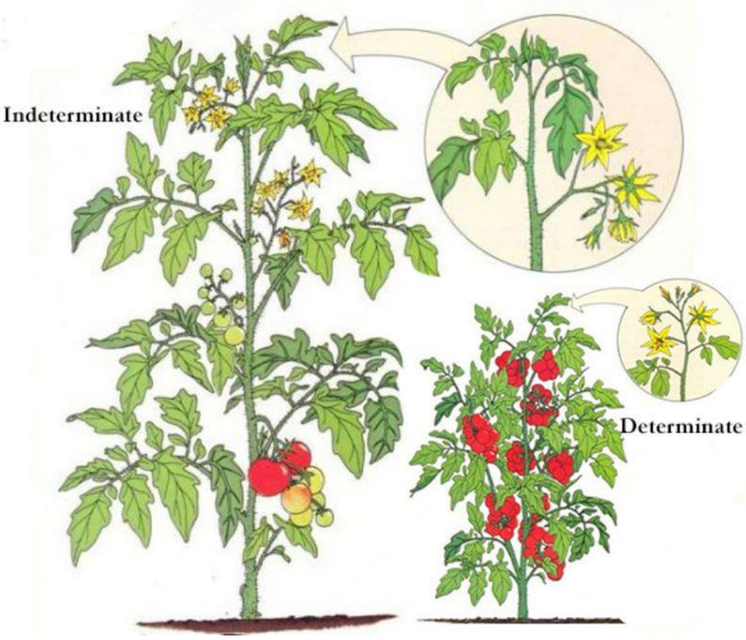

Image 1. Indeterminate versus determinate tomato growth type (source: www.ucanr.edu)

The inflorescence has 4 to 12 radial flowers with five stamens each, grouped in racemes or bouquets (Image 2a). It consists of 5 or more sepals arranged in a helical way and an equal number of alternate welded stamens between the petals; all this forms a staminal cone that surrounds the gynoecium (Image 2b) (Jaramillo et al. 2007). The ovary is super bicarpenal and contains numerous seminal primordia-producing polysperm berries. The flower is attached to the floral axis by a pedicel with a jointed thickening that has an abscission layer. It has a compressed lenticular seed and a rolled embryo (Lapuerta 1995).

The tomato plant is self-pollinating, i.e. pollination occurs with pollen from the same flower. The fruit is a globular berry and presents a diversity of sizes, shapes and colors depending on the tomato variety. In the same bunch, small fruits and open flowers can grow at the same time. The tomato fruit is formed by the exocarp, parenchymal mesocarp and endocarp, which surround the locules and seeds (Lapuerta 1995).

Image 2. Tomato inflorescence

1.2. Tomato cultivation

Tomato is one of the most-consumed fruit in the world and represents one of the most important horticultural crops (Calvo-Polanco et al. 2016). Its demand, production and commercialization are constantly increasing, which makes it the crop with one of the highest economic value (Foolad 2004). The annual increase in its production in recent years is mainly due to the increase in yield and, to a lesser extent, to the increase in the cultivated area.

According to data from the Food and Agriculture Organization of the United Nations (FAOSTAT, 2021), the total cultivated area in hectares in 2017 was 4.8 million, and the world production of tomatoes for fresh consumption amounted to 198 MMt. China is the main tomato producer worldwide, with a production of 59.62 MMt, followed by India, with a total production of 20.70 MMt, and Turkey, with 12.75 MMt. Spain ranks eighth, with 5.16 MMt, which represents a 24% increase in production as compared to the data recorded in 2017. Spain is also the second-most important producer in the European Union, producing more tomatoes after Italy. Figure 1 shows the percentage of production of the main tomato-producing countries, and in Table 1, the production data of these countries in millions of tons (Mt) and the area dedicated to tomato cultivation in thousands of hectares (Kha).

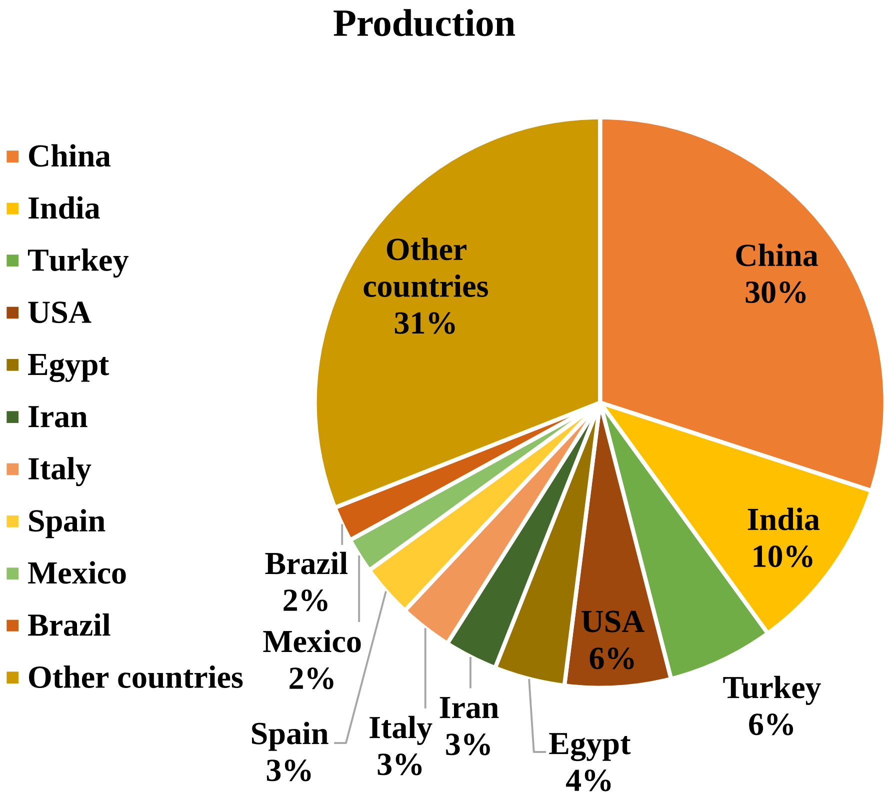

Figure 1. Distribution of tomato production among the main producing countries (FAOSTAT, 2021).

Table 1. Main tomato-producing countries in the world (FAOSTAT, 2021). The total production is estimated in millions of tons and the cultivated area in thousands of hectares.

Rank	*Country*	*Production (Mt)*	*Cultivated area (Kha)*
1	China	59.63	1033.28
2	India	20.71	797
3	Turkey	12.75	187.07
4	USA	10.91	126.07
5	Egypt	7.30	182.44
6	Iran	6.18	153.73
7	Italy	6.01	99.75
8	Spain	5.16	60.85
9	Mexico	4.24	93.00

10	Brazil	4.23	61.53
	Other countries	61.20	2053.66

According to statistical data collected by the Ministry of Agriculture, Fisheries and Food of the Government of Spain in 2020, the regions that produce tomatoes for fresh consumption are Almeria, the Region of Murcia, Alicante and the Canary Islands. Also, these regions export their products to other countries, which has led to the modernization of many companies in the agricultural sector dedicated to it. In other areas such as Navarra, Zaragoza and Extremadura, tomato cultivation is mainly focused on the processed tomato industry, where it is made into juices, pasta or sauces. Depending on the final destination of the tomato fruits, farmers adapt their cultivation techniques to the specific needs of the sector and market demand. The Region of Murcia, with around 325,000 tons produced in 2019, is the third most important tomato-producing region in Spain. Its production is destined for the fresh market, with a large portion of it exported to other countries.

1.3. Commercial and nutritional tomato value

In recent years, the market and consumers have increasingly demanded fresh, healthier and highly nutritious products, and many studies have been conducted for improving the quality of horticultural fruits, such as tomatoes (Gruda 2005). The current tomato cultivars grown for fresh consumption show large phenotypic differences in their fruits' characteristics, such as their color, size or shape. However, it is the organoleptic properties that determine the high consumption of some products with respect to others.

Tomatoes are classified as functional foods at a nutritional and pharmacological level, as it produces beneficial effects to the human diet and it is related to the reduction of chronic diseases thanks to its content of antioxidant compounds (Bramley 2000; Canene-Adams et al. 2005). When referring to the nutritional and functional quality of a product, we are talking about the usefulness of these foods to in the proper functioning of the human or animal body system. Although nutritional quality is imperceptible to consumers, it is considered of great importance for human health**.**

Tomato is rich in lycopene, β-carotene, vitamin C and phenolic compounds (Dumas et al. 2003; Bhowmik et al. 2012), and its consumption represents 85% of the total lycopene in the human diet. Lycopene is a tomato-specific carotenoid with great antioxidant capacity (Bramley 2000; Canene-Adams et al. 2005), and it has been shown to induce cell-to-cell communications, regulate hormonal functions, and is involved in the immune system and other important metabolic pathways (Rao and Agarwal 1999). β-carotene is involved in the formation of vitamin A, which prevents vision problems, enhances the immune system and protects against certain types of skin and mucous membrane-related cancers (Sies 1991; Martí et al. 2016). Vitamin C is involved in anti-inflammatory processes and it reduces cell oxidative damage. Additionally, vitamin C participates in the biosynthesis of hormones, collagen and other biological functions (Aguirre and May 2008). Phenolic compounds are very present in vegetables, and therefore, in the human diet, and they also play an important role in the prevention of certain diseases due to their antioxidant function (Fernandez-Panchon et al. 2008). Tomato is also a rich source of folates, potassium and vitamin E, although its availability may vary depending on the processes and treatments to which the products derived from its commercial transformation have been subjected (Willcox et al. 2003). For all these reasons, its consumption is an indicator of a good diet and a good lifestyle (George et al. 2004).

According to the United States Department of Agriculture (USDA, 2018), tomatoes have a low caloric intake (18 kcal per 100g) and a high content of water (95%), sugars, minerals, vitamins and lipids (Table 2), which makes it a food with a great nutritional value. Glucose and fructose are the major sugars in tomato fruit, representing almost 50% of its dry matter. On the other hand, malic acid and citric acid are the main organic acids, representing 10% of the dry matter (Lapuerta 1995).

Sugars and acids do not add to the great nutritional value in tomato, but they do play a large role in their taste. The mean content of proteins, amino acids and lipids of tomato is very poor, around 0.6% of the fresh weight (Davies and Hobson 1981). Minerals also represent a small fraction of the fresh weight, approximately 0.4% (Davies and Hobson 1981), but they play an important role in the nutritional composition of the fruit. The increase in the content of dry matter, proteins, amino acids, lipids and minerals of the tomato is largely conditioned by the fertilization practices used during its cultivation and by the influence of the abiotic factors to which the plant was subjected during its life cycle.

Table 2. USDA tomato nutritional values, 2018.

Nutritional composition	Value per 100g
Water	94.52 g
Energy	18 kcal
Protein	0.88 g
Total lipids	0.20 g
Carbohydrates	3.89 g
Total fiber	1.2 g
Sugars	2.63 g
Vitamins	96.79 mg
Calcium, Ca	10 mg
Iron, Fe	0.27 mg
Magnesium, Mg	11 mg
Phosphorus, P	24 mg
Potassium, K	237 mg
Sodium, Na	5 mg
Zinc, Zn	0.17 mg

1.4. Tomato domestication

The tomato is native to South America, between the regions of Chile, Ecuador and Colombia, but its domestication began in southern Mexico and northern Guatemala. The wild forms of "cherry tomato", *Lycopersicum esculentum* var. *cerasiforme*, native to Peru, migrated through Ecuador, Colombia, Panama and Central America until reaching Mexico, where it is believed it was first domesticated by man. This cherry tomato (*S. lycopersicum* var. *cerasiforme*) is considered the probable ancestor of the tomato that we know today, which in turn, was domesticated from the wild species of red fruit *Solanum pimpinellifolium* (Figure 2) (Ranc et al. 2008). The tomato reached an advanced stage of domestication in Mexico before being brought to Europe and Asia (Parks et al. 1975).

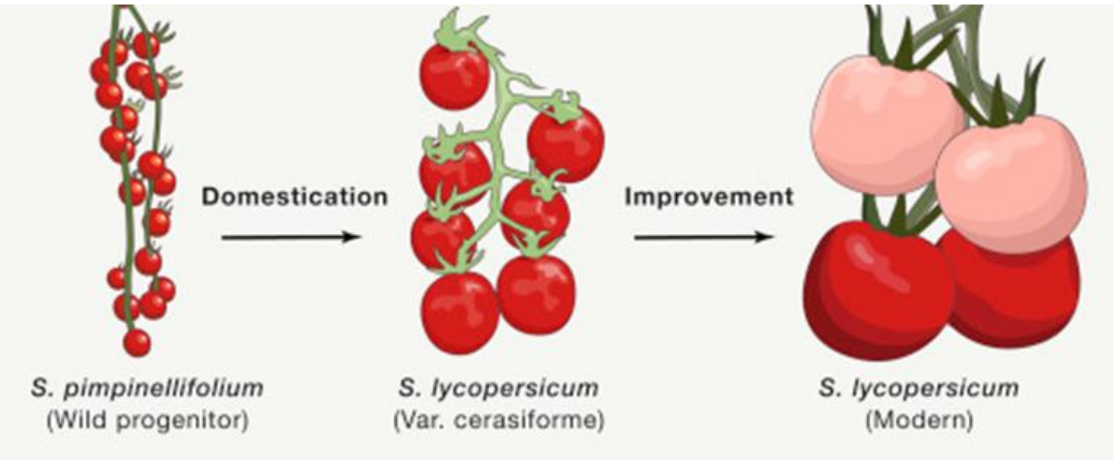

Figure 2. Domestication of the tomato from the wild ancestor (*S. pimpinellifolium* L.) to the current tomato (Giovannoni 2018).

The domestication of crops was carried out by man to obtain food, feed or fibers by selecting desirable traits in plants to improve some characteristics, such as tolerance to environmental stresses, tolerance to pathogens or to improve fruit yield and quality (Bai and Lindhout 2007).

The information presented in the first European herbaria, from the second half of the 16th century, revealed that the first types of cultivated tomatoes in Europe had soft skins, with a wide variety of shapes and colors. The introduction of the tomato to the European continent probably occurred through Spain (Blanca et al. 2012), between 1523, the year of the conquest of Mexico by Spain, and 1524, when the first descriptions were published by the Italian Pier Andrea Mattioli (EUFIC 2001).

The consumption of tomato as a food source occurred approximately in 1850 in the United States, and from this date on, we count with scientific and agronomic data. The different agronomic and climatological conditions in which tomato was cultivated, have caused the fruit to evolve into a great diversity of phenotypes. The genetic diversity of tomato plants allows their crossings to minimize genetic characteristics that are not desirable and to enhance the traits that are commercially interesting (Giovannoni 2018).

Tomato plants are an excellent example of domestication, since their wild ancestor, *Solanum pimpinellifolium L.* has few similarities with the plant genus that is currently used for tomato fruit production: *Solanum lycopersicum L.* (Zuriaga et al. 2009).

The tomato fruits that we consume today are about 100 times larger than the original tomato fruit (Lin et al. 2014). Gao et al (2019a) demonstrated that the loss (or negative selection) of genes that occurred during the tomato domestication process favored the development of more important traits, such as resistance to diseases and/or abiotic stresses. To increase crop yields and improve their characteristics, the crossing of wild plants with commercial plants has been studied; however, the mechanism of how the tomato genome has changed remains largely unknown.

1.4.1. Specie *Solanum pimpinellifolium* L.

Solanum pimpinellifolium L. belongs to the genus Solanum (Miller 1754; Luckwill 1943), within the Solenaceae family (Bohs and Olmstead 1997; Spooner and Peralta 2000). It is a small shrub typical of the coastal areas of Peru and Ecuador (Luckwill 1943). There are several differences between the Ecuadorian and

Peruvian regions: the greatest diversities are found in northern Peru, where it is considered to be the origin of the tomato plant. In the Ecuadorian region there is a greater genetic diversity in the species, which is related to the different climatic diversity found in the coastal areas of Ecuador (Zuriaga et al. 2009; Lin et al. 2014).

The species *S. pimpinellifolium L.* is not cultivated for human consumption, as the organoleptic characteristics of its fruits are not as marketable as the commercial tomato, but both species are closely related. The main differences are in the size of its fruits, leaves and flowers (Luckwill 1943). *Solanum pimpinellifolium* is currently considered to be the ancestor of the species that we cultivate today (Bretó et al. 1993), since the current tomato has characteristics inherited from the species, such as resistance to some strains of pathogens (Langford 1937; Bohn and Tucker 1940), genes for fruit color (Tanksley et al. 1982), and traits that facilitate tomato harvesting (Luckwill 1943). To date, *S pimpinerifollium L.* is often used in the field of research, since it hybridizes very well with *S lycopersicum L.* (Zuriaga et al. 2009) and also, because it has many economically important traits for the commercial tomato varieties (Mieslerova et al. 2000).

1.4.2. Recombinant inbred lines (RILs)

In recent years, recombinant inbred lines (RILs) have been developed as a very useful tool for the study and selection of certain genes and/or characters (Broman 2005). These lines are obtained by a first cross of a wild line with a commercial line (to be improved), and, later, by a series of self-fertilizations or backcrosses of the F2 progeny are carried out until the lines reach genetic stability. Parentals are selected based on phenotype, marker availability, and compatibility, and may be genetically engineered to eliminate unwanted variations or to introduce reporter genes (Pollard 2012).

The use of RILs has certain advantages over other populations used for genetic maps, since they are genetically homozygous lines that can be propagated without the need to use segregation techniques, by the propagation of selectively viable seeds (Colomé-Tatché and Johannes 2016). In this way, these lines can be distributed and replicated in different experiments, in different laboratories and in different environments. This characteristic is especially useful for the analysis of qualitative traits of a species, since it allows the reduction of the environmental component to the total phenotypic variability.

In some studies performed with RILs from tomato plants, it was shown that resequencing the next generation of parental lines allowed the majority of polymorphisms to be characterized and used to move from qualitative trait loci (QTL) programs to the causative gene (Kevei et al. 2015).

Thus, RILs can be very useful in the study of the phenotypic, physiological, biochemical and molecular traits under certain conditions of environmental stresses, as the provide a wide range of plants that are tolerant and sensitive to some of these selected conditions.

1.5. Stress in higher plants: generalities

Environmental fluctuations usually occur in the surrounding habitat in which plants develop. This causes unfavorable situations for their development and optimal functioning (Manzoor Alam 1999). These unfavorable conditions are known in Plant Physiology as `stress´ and it causes important and constant physiological, biochemical and metabolic reprograming and adjustments. Biotic stresses are those that are induced by pathogenic organisms, and they can be accentuated due to the climatic conditions in which the infection occurs. On the other hand, plants can face also abiotic stresses, such as extreme temperatures (above or below the optimum for a specific plant species growth), water scarcity (total or partial), or the salinity of soils and/or irrigation waters, among others. Stresses can act individually or, more frequently, in combination, i.e. when two or more stresses act simultaneously on plants (Mittler 2006; Miller et al. 2010; Choudhury et al. 2017; Lopez-Delacalle et al. 2020, 2021b).

The different plant species differ in their optimal environmental conditions, showing different degrees of susceptibility and adaptability to any kind of stress.

Commonly, a plant is considered to be stressed when its gene expression, cellular metabolism, growth rates, and crop yields changes with respect to that observed under optimal conditions.

1.5.1. Salinity stress

Salinity is one of the main factors affecting plant growth and production. The regions most affected by salinity in the world are located in arid and semi-arid regions. The main salts that negatively affect plant production correspond to sodium chlorides ($NaCl$) and sulfates (Na_2SO_4), calcium (Ca^{2+}), magnesium (Mg^{2+}) and potassium (K^+) salts.

Additionally, the Mediterranean area is highly vulnerable to human activity (agricultural and fishing exploitation, tourism, etc.), which causes potential environmental problems, such as salinity of its soils. This Mediterranean area is one of the most important areas worldwide for tomato cultivation. Currently, the problems derived from salinity, either in the soil or in the irrigation water, are being decisive for the crops' quality and yield (Cuartero and Fernández-Muñoz 1998). The causes linked to these salinization processes are usually an excessive use of fertilizers, use of poor quality water due to excess salts, poor soil drainage and the felling of tree vegetation, which allows the leaching of salts into groundwater. According to Cuartero and Fernández-Muñoz (1998), tomato can be used in areas where the quality of the water and soil is not suitable for other types of cultivation, since these plants are moderately tolerant to salinity, with optimal conductivity values of the irrigation solution ranging between 3 and 5 dSm^{-1}.

Salinity affects crops through different mechanisms: either by inducing water stress due to an osmotic effect and/or because of the absorption and accumulation of sodium ions by plants, resulting in toxicity due to the imbalance of ions inside the plant cells (Acosta-Motos et al. 2017) (Figure 3). As a consequence of the osmotic stress induced by salinity, the plant increases the production of certain organic molecules named osmoprotectants, which do not interfere with the cellular metabolism, and whose function is to decrease its cellular osmotic potential (Saxena et al. 2013; Rivero et al. 2014; Singh et al. 2015a). Some of the ions accumulated under conditions of osmotic stress are essential for the optimal plant growth and development, such as K^+, although most of the accumulated compatible compounds are organic solutes, such as soluble sugars (mainly glucose and fructose), alcohols derived from sugars, and complex sugars (i.e mannitol), as well as different amino acids, such as proline, polyamines and quaternary ammonium compounds, such as glycine betaine (Rivero et al. 2014). The accumulation of these compounds allows the cells to decrease their osmotic potential, which will facilitate the water movement into the root cells, a fundamental physiological process for plant growth and survival, and which is strongly inhibited under salinity conditions.

As previously mentioned, salinity also induces ionic stress in plants, normally due to the massive absorption and accumulation of ions such as Cl^- and Na^+, in toxic concentrations for plant cells. This causes a series of secondary effects, such as specific toxicity problems and nutritional imbalances, related to a lower absorption of ions essential for plant growth and development (Yokoi et al. 2002; Alemán et al. 2009). Thus, high concentrations of Na^+ in the external solution, cause a decrease in the concentrations of K^+ and Ca^{2+} in plant tissues (Cramer 2006). These reductions may be due to the antagonism described between Na^+ and K^+ at the absorption sites in the roots, to the effect of Na^+ on the transport of K^+ in the xylem (Lynch and Läuchli 1984), or the inhibition of the absorption processes (Suhayda et al. 1990; Alemán et al. 2009). Ca^{2+} availability would be seriously reduced by the shift from Na^+ to Ca^{2+} at extracellular binding sites, especially with a low Ca^{2+}:Na^+ ratio (Cramer 2006). Furthermore, the reduction in Ca^{2+} absorption under saline conditions could be due to its precipitation and to the increase in ionic strength that reduces its activity (Hu and Schmidhalter 2005). It is known that salinity stress induces other ions deficiencies or produces a nutritional imbalance due to competition between nutrients such as K^+, Ca^{2+} and NO_3^- with the toxic ions Na^+ and Cl^- being accumulated. As salinity causes water deficit in plants, K^+ is very important in maintaining the turgor pressure of the plant, so a high K^+:Na^+ ratio would also increase the plant's resistance to salinity. Compared with N, P, K^+ and Ca^{2+}, micronutrients could be less important in plant tolerance to salinity. However, some studies have revealed that the addition of some macronutrients in the growth media under saline conditions can alleviate the adverse effects of this stress on plant growth (Hu and Schmidhalter 2005; García-Martí et al. 2019). In this sense, García-Martí et al. (2019) showed that an increase in K^+ and Ca^{2+} concentration in the nutrient solution may have a positive effect on the amelioration of oxidative stress, cell homeostasis and in the final adaptation of tomato plants to salinity stress.

Other researchers have shown that a high concentration of Na^+ not only inhibits nutrient uptake directly by interfering with root plasma membrane transporters, such as selective K^+ channels, but also by inhibiting root growth due to the osmotic effect of Na^+ and the adverse effects this cation on the soil structure (Tester and Davenport 2003; Alemán et al. 2009).

When plants are exposed to salinity during their reproductive stage, the number, size, and weight of their fruits decrease dramatically (Zhang et al., 2016) as a result of a reduction in the length of time between anthesis and maturation. The quality parameters of these fruits are also highly affected, by an increase in the concentration of total soluble solids and reducing sugars (Islam et al. 2018), and an increase in acidity and chloride ions concentration (Magán et al. 2008). On the other hand, it has been also demonstrated that when tomato plants grow with small amounts of salt in the medium, the flavor of their fruits improves, even though their size is smaller (Mizrahi 1982), mainly due to the increase in the accumulation of soluble sugars (Ghoname Elsayed et al. 2019; Meza et al. 2020).

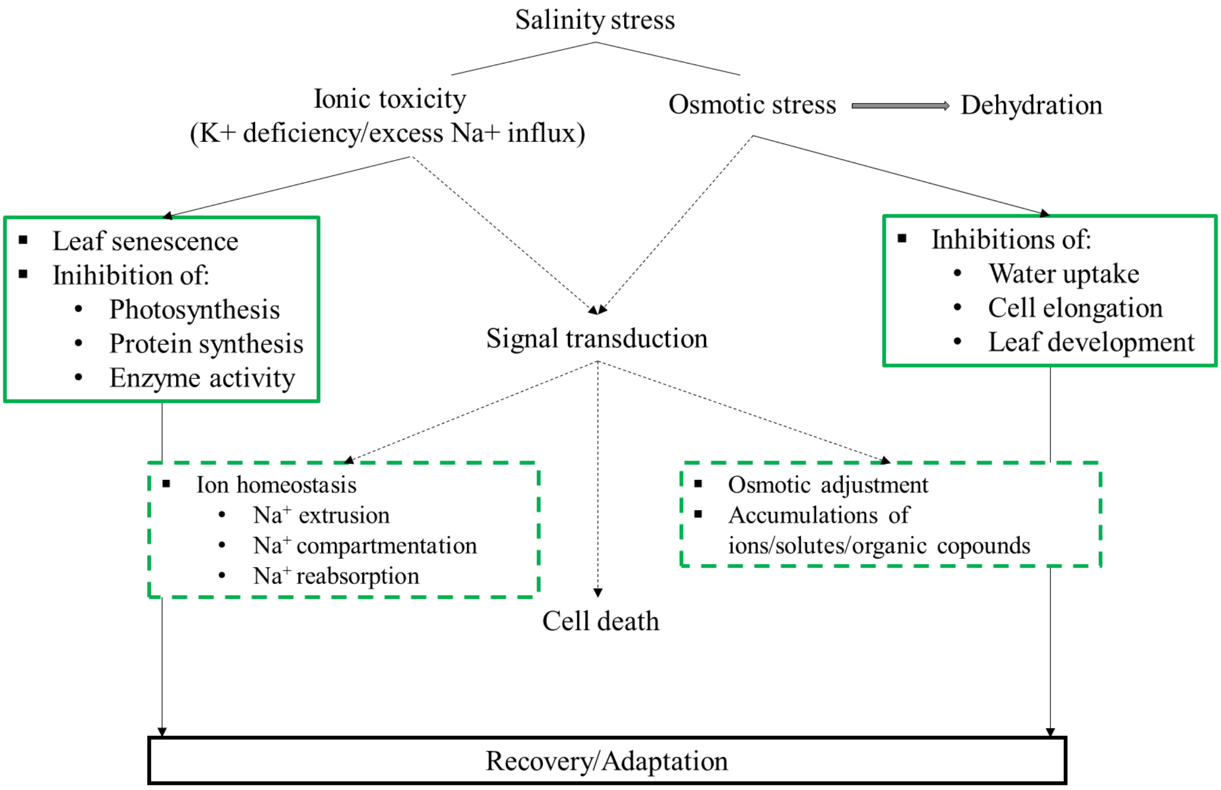

Figure 3. Effects and responses produced by salinity in plants. Modified scheme from Munns and Tester (2008).

1.5.2. Heat stress

In the coming years, the increase in temperature due to climate change is expected to affect all the cultivated areas worldwide (Balfagón et al. 2019), which could have devastating consequences for food production and quality, conservation of agriculture and crop irrigation (IPCC 2019). The increase in seasonal temperatures will also have an impact on economy and food security, since it could become a prolonged situation that will derive into the exhaustion of the plant adaptation processes to this temperature increase. The future of agriculture will be bleak in hot regions, unless significant investments are made in developing crop varieties that are tolerant to heat and the consequent water-related stress (Battisti and Naylor 2009).

Tomato is a crop whose optimum growth temperature lies between 23-26ºC (Camejo et al. 2005). However, the main areas where it is grown are arid and semi-arid areas, where the increase in temperature above 40ºC during some periods is a difficult factor to control.

Heat stress causes a large number of changes at physiological, biochemical and molecular levels, and at cellular and subcellular levels (Tiroli-Cepeda and Ramos 2010) (Figure 4), which are mainly negative for a crops' growth and production (Penfield 2008). Under heat stress situations, all the plant parts are affected, as observed in the viability of the seeds and pollen, the fertility of flowers, and the fruit yield and quality (Kumar Singh et al. 2017). Heat stress effects will vary depending on the genotype of the plant, the plant growth stage and the affected organ, with the root of a young plant and the flowering stage being the most sensitive ones (Zhou et al. 2017).

Temperature stress also varies depending on its duration and intensity, inducing photosynthesis inhibition due to stomatal and non-stomatal limitations, decreasing fruit set and fruit size, shortening the ripening periods, and decreasing fruit yield (Adams et al. 2001; Camejo et al. 2005).

Photosynthesis inhibition induced by heat stress is usually associated with stomatal closure as a consequence of water deficit (Wahid et al., 2007), which will reduce the amount of CO_2 available for assimilation by the plant, and the concomitant decrease of the CO_2/O_2 ratio in the chloroplasts (Foyer and Noctor 2005). These changes can greatly affect photosynthesis efficiency, with this process being perhaps the most sensitive to heat (Wise et al. 2004). Seed germination and seedling emergence are also negatively affected by temperature stress, with a considerable reduction in the germination rate (Wahid et al. 2007). Also, an increase in the environmental temperature might improve the fruit growth rate and reduce the time needed for ripening; as both processes occur at different speeds, the result will be smaller fruits with a lower weight (Adams et al. 2001; Bhattarai et al. 2021).

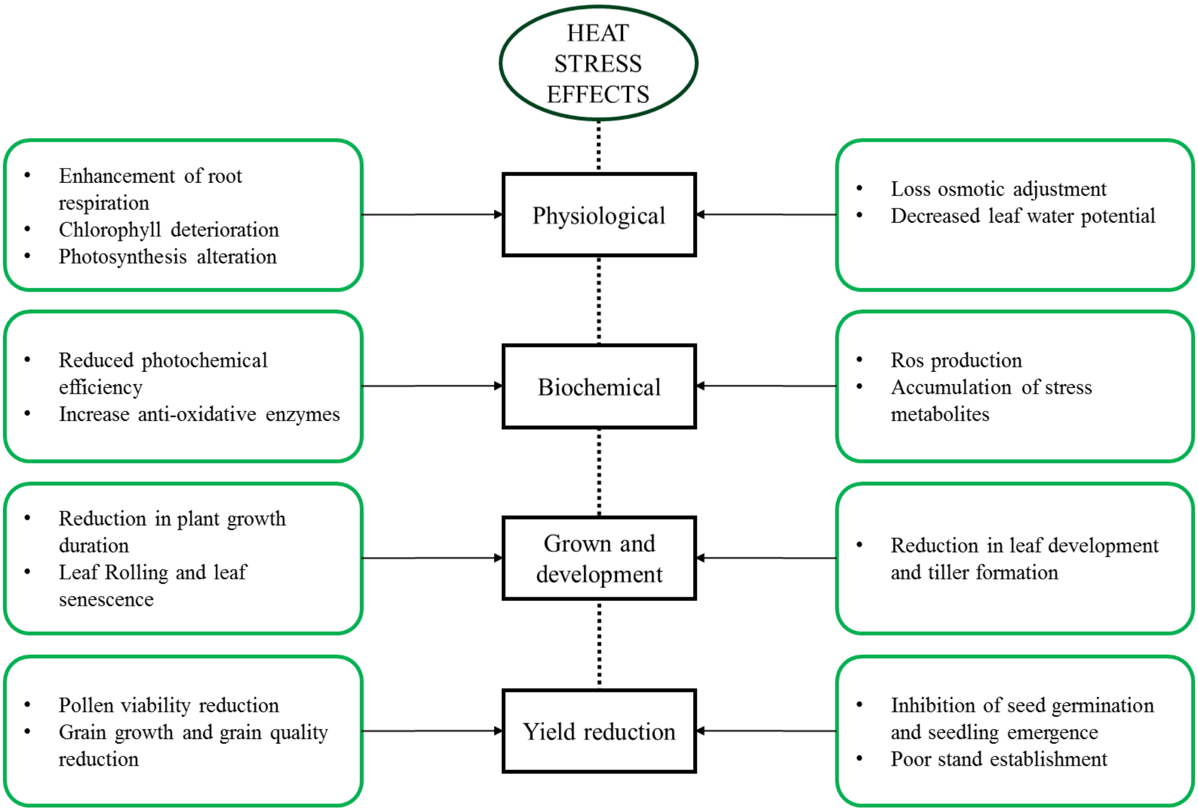

Figure 4. Major effects of heat stress on plants growth and development.

1.5.3. Abiotic stress combination and the field environment

As commented previously, the most devastating environmental stresses for crops are those associated with a water deficit, such as water shortage, extreme temperatures or salinity. All of these cause a depletion of the cell water content and an increase in the cell osmotic potential, thus causing generalized osmotic stress and, ultimately, significant losses in production (Kumar et al. 2019; Singh et al. 2020). In recent decades, a large number of studies have focused on the response of plants to different environmental stresses, increasing our knowledge on this subject. However, most studies have focused on the study of the response mechanisms (both at a physiological, biochemical or molecular level) of plants to single-stress (Singh et al. 2020; Ayenan et al. 2021). These studies have been and are very relevant for the understanding of the plant tolerance mechanisms to these specific abiotic stresses, but they are far from what truly occurs in the open field, where abiotic stresses usually act in combination. Therefore, given the current climate change emergency, we must assume that the combination of stresses will become more common under natural conditions. Thus, there is an urgent need to carry out studies based on the effects of stresses applied in combination, which is also supported by the scientific evidence that the plant's response to various simultaneous stresses is specific and therefore cannot be explained or deduced simply by combining the plant responses to the stresses applied individually (Rivero et al. 2014; García-Martí et al. 2019; Lopez-Delacalle et al. 2020, 2021b).

In arid and semi-arid areas of the planet, such as the Mediterranean area, environmental conditions such as salinity (caused by both low-quality water for irrigation and/or salinized soils) and periods of high environmental temperatures above 40ºC are frequent. These stresses reduce crop development and negatively affect agricultural production, considerably reducing the economic benefits of agricultural products. These adverse environmental conditions often act together in the open field, and they can lead to devastating economic losses in global agriculture (Rivero et al. 2014). These losses, added to the climatic predictions provided by the IPCC (AR5 Climate Change 2014: Impacts, Adaptation, and Vulnerability — IPCC) for the next 50-80 years, which indicate a worsening of current stress conditions and a significant increase in world population, make it necessary to obtain crops with greater tolerance to abiotic stress conditions. In this context, plants have developed multiple physiological, metabolic and molecular response mechanisms to counteract salinity and temperature stresses, which are integrated and act in a synchronized manner to produce a specific, final response that makes the plant acclimatize to the surrounding environment. As indicated above, to respond to the present problems in agriculture, stress combination research should focus on the most relevant stress combinations for each particular region (Rivero et al. 2014).

Suzuki et al. (2014) reviewed numerous studies on the combination of different biotic and abiotic stresses and concluded that salinity had a potentially positive effect in combination with boron, high CO2, and heat stresses. However, the study also reported that it had a potentially negative effect in combination with pathogens, ozone, heat and nutrient stresses (Suzuki et al. 2014). On the other hand, in the same review, Suzuki et al. (2014), indicated that heat has a potentially negative effect when interacting with the stresses of high light, UV, pathogen and ozone.

1.6. Changes induced by abiotic stress

The response of plants to the surrounding environment involves several mechanisms that act synchronously at physiological, biochemical and molecular levels. For this, plant metabolism must be tightly regulated and interconnected to help plants acclimatize to these changing environments.

The response to each combination of stresses is highly specific. Both heat stress and salinity induce alterations in ion transport and ion compartmentalization (Munns and Tester 2008). Salinity causes toxicity in plant cells due to a massive accumulation of Na^+, which ultimately interrupts the absorption of K^+ in the roots due to the antagonistic relationship between these two elements (Yokoi et al. 2002; Alemán et al. 2009; Belouchrani et al. 2020).

Also, both salinity and heat stress reduce plant growth and photosynthetic activity, inducing the accumulation of reactive oxygen species (ROS) (Miller et al. 2010; Suzuki et al. 2012; Lopez-Delacalle et al. 2021b), inhibit the activity of important metabolic enzymes, and alter the efficiency of enzymatic reactions within the cell (Laluk et al. 2011; García-Martí et al. 2019; Lopez-Delacalle et al. 2020), change the composition of synthesized and accumulated metabolites (Shulaev et al. 2008; Lopez-Delacalle et al. 2020, 2021b), and affect the stability of various proteins (Lugan et al. 2010; Peleg et al. 2011; Gautam et al. 2020), causing a state of general metabolic imbalance.

Faced with these imbalances, plants have developed complex and diverse systems in response to abiotic stress. Among some of the most common and important responses of plants to both salinity and heat stress are the accumulation of osmoprotectants, antioxidant responses through the activation of the oxidative metabolism for the detoxification of reactive oxygen species (ROS), accumulation of phenolic acids with antioxidant capacity, activation of heat shock proteins and mitogen activate protein kinases (MAPKs), the synthesis of several second messengers, activation of transcription factors (TFs), and transcriptional and translational reprogramming, among others (Demirel et al. 2020; Gupta et al. 2020; Sewelam et al. 2020).

All of these mechanisms must occur in a cascade and must be specifically regulated to translate the stress signal into a response mechanism (Demirel et al. 2020; Zandalinas et al. 2020b). The present work, due to the importance of studying abiotic stress in combination, is focused on the response of tomato plants to the combination of salinity and heat stress. It places a special emphasis on the main mechanisms involved in some important aspects of plant metabolism and homeostasis, such as nitrogen, proline, oxidative and carbohydrate metabolism, considered of great importance for plant survival. Due to their importance, these metabolisms will be explained in more detail in this Introduction section.

1.6.1. Nitrogen metabolism

Nitrogen (N) is the fourth element in quantitative importance within the components of living matter, preceded only by C, H and O. Only plants and certain organisms can synthesize natural amino acids from inorganic nitrogen sources. Given that N is one of the most limiting factors for plant growth, plants have developed specific mechanisms to maximize its absorption and assimilation (Miflin and Lea 1976; Mokhele et al. 2012). It is also known that environmental conditions can limit the optimal N supply, uptake and assimilation by plants, so knowledge on how these main processes are affected is of vital importance (Giri et al. 2017).

N is the main component of important biomolecules, such as proteins, amino acids, nucleic acids, coenzymes and pigments. The inorganic forms of N, such as nitrate (NO_3^-) and ammonium (NH_4^+) present in the growth media are the basic N forms absorbed by plants (Crawford and Glass 1998; Glass 2003). NO_3^- is absorbed by the plant roots through an active symport mechanism mediated by an Adenosine Triphosphatase (ATPase) H^+-P, with the consumption of H^+ and Adenosine Triphosphate (ATP) (Crawford and Glass 1998).

The excess NO_3^- is transported via the xylem vessels to the mesophilic cells, where the NO_3^- is temporarily stored in the vacuole. NO_3^- is then reduced to NO_2^- in the cytosol and then to NH_4^+ in the chloroplast, to be subsequently incorporated into amino acids. The reduction of NO_3^- to NO_2^- is catalyzed by nitrate reductase (NR, EC 1.7.1.1), with the transferring of electrons from NADH to NO_3^- (Srivastava 1980). Later, the enzyme nitrite reductase (NiR, EC 1.7. 1.15) catalyzes the reduction of NO_2^- to NH_4^+. It is well known that chloroplastic NiR transfers electrons from ferredoxin (Fd) to NH_4^+ in this reduction process. NO_3^-, light, and glucose induce the synthesis of NR, which, in turn, is repressed by the presence of glutamine, NH_4^+, and other amino acids (Aslam et al. 1976). The assimilation of NH_4^+ constitutes the final stage of the nitrogen assimilation process and consists of the incorporation of NH_4^+ into carbon skeletons which are carried out, fundamentally, by the consecutive action of the enzymes glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 2.6.1.53). This ammonium assimilation route is known as the GS-GOGAT cycle, and has been widely studied (Miflin and Lea 1976; Liu et al. 2017; Lopez-Delacalle et al. 2020).

The net product derived from the activity of the GS-GOGAT cycle is one glutamate molecule per cycle. GS catalyzes the NH_4^+ incorporation into a glutamate molecule through an ATP-dependent hydrolization and amidation process, with glutamine being the end product. In plants, this enzyme is encoded by a small family of 13 genes responsible for the existence of different types of cytosolic (GS1) or plastidic (GS2) isoforms in the different plant organs. In higher plants, there are two forms of GOGAT, one dependent on ferredoxin (Fd-GOGAT, EC 1.4.7.1) and the other dependent on $NADH^+$ as electron donor (known as NADH-GOGAT, EC 1.4.1.14) (Temple et al. 1998). On its part, glutamine is used by GOGAT together with a 2-oxoglutarate molecule and two reduction equivalents provided either by reduced ferredoxin (Fd-GOGAT) or $NADH^+$ (NADH-GOGAT), generating two glutamate molecules, one of which will be used by GS, and the other will be the net result of the cycle (Temple et al. 1998). Glutamine and glutamate are thus the two primary organonitrogenated products of nitrogen assimilation, and will be the N-based molecule for the successive synthesis of N-containing metabolites and amino acids (Miflin and Lea 1976).

Glutamate dehydrogenase (GDH, EC 1.4.1.2.) also plays a fundamental role in glutamate homeostasis in plants, as it catalyzes the reversible amination/deamination reaction of glutamate, so this enzyme could participate both in the synthesis or the catabolism of this amino acid (Robinson et al. 1991).

As other physiological and biochemical processes, N uptake and assimilation (Figure 5) is a process that is strongly sensitive to environmental stresses, as a variation in the optimum conditions for each plant species will adversely affect it. Thus, an increase in environmental temperature will affect the absorption of this macronutrient from the soil, since the enzymes involved in its assimilation will strongly reduce their activity due to heat (Gong et al. 2020).

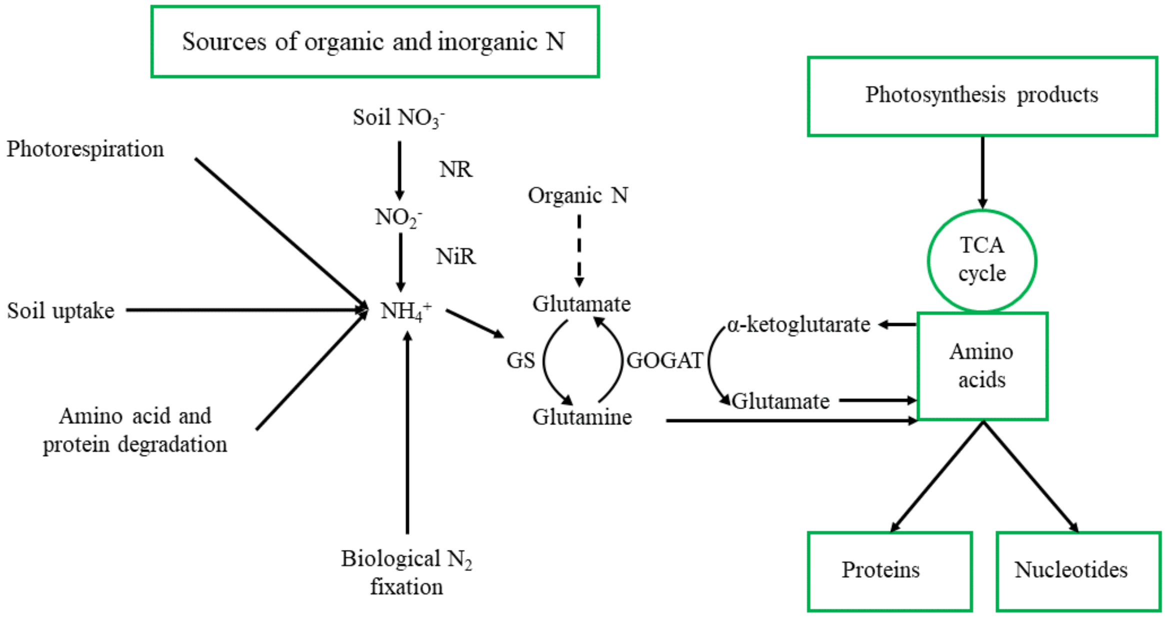

Figure 5. Main reactions involved in nitrogen assimilation in higher plants. NO_3^-, nitrate; NO_2^-, nitrite; NH_4^+, ammonium, N_2, atmospheric dinitrogen. NR, nitrate reductase; NiR , nitrite reductase; Nase , nitrogenase; GS , glutamine synthetase; GOGAT , glutamate synthase. The ultimate source of inorganic N available to the plant is ammonium, which is incorporated into organic molecules in the form of Glutamine and Glutamate through the

combined action of the two enzymes, GS and GOGAT. Carbon originating from photosynthesis through the tricarboxylic acid cycle (TCA cycle) provides the α–ketoglutarate needed for the reaction catalyzed by the enzyme GOGAT. Modified from Hirel et al. (2011).

The different temperatures that plants have to face during their development also affect NO_3^- absorption. Thus, Polissety (1985) observed in maize plants that a variation in environmental temperature led to a decrease in the absorption of NO_3^-, which led to an inhibition of the root and aerial part development.

Similarly, high temperatures also affect the distribution of N through the different plant organs. It has been shown, for example, that an increase in temperature from 30 to 41ºC caused a decrease in the N content in the aerial part of *Vigna radiata* (Hafeez et al. 1991).

Lopez-Delacalle et al. (2020) have also shown that all the activities of the enzymes involved in the N assimilation are seriously affected by high temperatures, salinity and their combination. For example, NR decreases its activity significantly when tomato plants were subjected to salinity stress, which translated into a decrease in the concentration of glutamine, asparagine and arginine (Lopez-Delacalle et al. 2020).

In different plant species, it has been shown that N assimilation, and, in particular, GS, play a determining role concerning abiotic stress tolerance (Yousfi et al. 2012). In this sense, a clear role for cytosolic GS in proline synthesis has been described (Brugière et al. 1999). Likewise, it has been shown that the overexpression of chloroplastic GS in tobacco and rice resulted in an increase in plant tolerance to photooxidation (Kozaki and Takeba 1996) and salinity stress (Hoshida et al. 2000). The work of Kozaki and Takeba (1996) is particularly interesting since it emphasizes the possible participation of photorespiration and GS2 in protection against abiotic stress, as a mechanism to prevent stromal over-reduction, and therefore photoinhibition. This mechanism was also previously suggested by other authors under different stress situations and control conditions (Douce and Neuburger 1999; Wingler et al. 2000; Maurino and Peterhansel 2010). Also, it has been shown that photorespiration can be important for avoiding the suppression of the repair of photosystem II that has been photo-damaged under abiotic stress (Takahashi et al. 2007).

Also noteworthy is the relationship between NH_4^+ accumulation and ROS production in the peroxisome. Yang et al. (2020) have shown in rice plants that a high concentration of NH_4^+ is usually accompanied by an overproduction of ROS and the consequent stress signaling cascade.

1.6.2. Proline metabolism

One of the most common responses at the metabolic level to any abiotic stress condition, is the accumulation of compatible solutes, with proline being of special importance (Delauney and Verma 1993). Proline is an amino acid that is widely described as an osmoprotectant, and it is highly accumulated under any stress conditions, not only by plants, but also by eubacteria, marine invertebrates, protozoa and algae, when these are exposed to drought, salinity, high temperatures, freezing, ultraviolet radiation and heavy metals (Delauney and Verma 1993; Saradhi et al. 1995; Kuznetsov and Shevyakova 1997; Yancey 2005; Rivero et al. 2014).

Proline can be synthesized from glutamate or ornithine (Delauney and Verma 1993). Under normal conditions, the synthesis of proline in higher plants is carried out from ornithine, while under stress, glutamate is mainly used as a precursor for the synthesis of this amino acid (Delauney and Verma 1993). Glutamate is phosphorylated by the kinase activity of a bifunctional enzyme named Δ1-pyrroline-5-carboxylate synthetase (P5CS, EC 2.7.2.11), which has a double enzymatic activity: γ-glutamyl kinase (γ-GK) and glutamyl semialdehyde dehydrogenase (GSA-DH), produce L-glutamyl phosphate. The L-glutamyl phosphate is then transformed into glutamyl semialdehyde (GSA) by the reductase activity of P5CS (GSA-DH). Subsequently, GSA is transformed into Δ1-pyrroline-5-carboxylate (P5C) by a spontaneous reaction. Finally, P5C is reduced by the enzyme Δ1-pyrroline-5-carboxylate reductase (P5CR) to produce proline (Delauney and Verma 1993; Zhang et al. 1995). The cycle of proline synthesis from ornithine begins through the transformation of the amino acid ornithine into GSA, by the enzyme ornithine aminotransferase (OAT, EC 2.6.1.13) (Delauney and Verma 1993; Hare et al. 1998) and, later, GSA is incorporated into the cycle of proline synthesis from glutamate as described above. The catabolism of proline occurs in the mitochondria thanks to the activity of the enzymes proline dehydrogenase (PDH, EC 1.5.5.2) or proline oxidase (PO) to produce P5C from proline, and through the activity of Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH, EC 1.2.1.88) to transform P5C into glutamate (Figure 6) (Sharma et al. 2011).

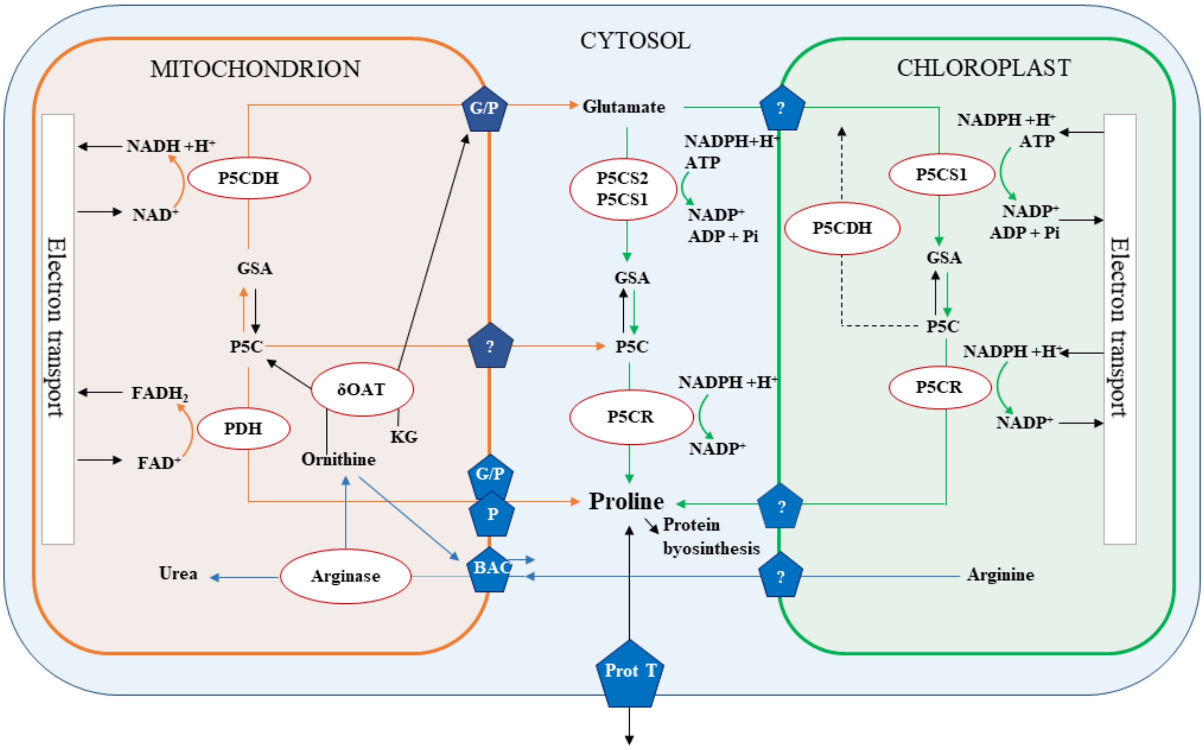

Figure 6. Proline metabolism in higher plants. Biosynthetic pathway is marked with green lines, the catabolic pathway with red lines and the ornithine pathway with blue lines. BAC, basic amino acid transporter involved in arginine and ornithine exchange; Glu, glutamate; G/P, mitochondrial glutamate/proline antiporter; KG, alpha-ketoglutarate; P, mitochondrial proline transporter; Pi, inorganic phosphate; ProT, plasma membrane proline transporter; ?, predicted transporters (Szabados and Savouré 2010).

Proline (Pro) accumulation has been linked to stress tolerance in different ways. Findings that relate Pro synthesis and accumulation to stress tolerance in plants have been demonstrated by genetic manipulation of its synthesis pathway and through the exogenous supply to the plant to this amino acid (Kim et al. 2017; Zouari et al. 2019; Mansour and Salama 2020; Cacefo et al. 2021). Pro is rich in N, which allows its use in protein synthesis. In addition, it acts as a molecular chaperone, protecting the integrity of proteins and improving the activities of different enzymes under certain unfavorable circumstances for the cells (Verbruggen and Hermans 2008; Mansour and Salama 2020). Pro also mediates osmotic adjustment (Mansour and Salama 2020). Some studies have attributed some antioxidant characteristics to proline, suggesting a ROS detoxifying activity and its action as a singlet oxygen deactivator (Smirnoff and Cumbes 1989; Matysik et al. 2002). It has also been proposed that Pro can stabilize ROS detoxifying enzymes and activate alternative detoxification routes (Hoque et al. 2008; Islam et al. 2009), thus contributing to the maintenance of the cellular redox balance. Apart from its protective or detoxifying characteristics, Pro contributes to the regulation of cell development and is an essential component in the metabolic signaling of mitochondrial pathways (Verbruggen and Hermans 2008). Pro involvement as a metal chelator has also been shown (Hayat et al. 2012). On the other hand, the catabolism of Pro also seems to be directly involved in the provision of energy when plants are subjected to stress, by the release of NADPH and ATP through the action of the enzyme PRODH (Jaspers et al. 2010). However, it is still necessary to delve deeper into the study of the mechanisms that regulate its involvement in stress, especially under those situations where stresses act in combination, which still generates discrepancies.

1.6.3. Oxidative metabolism

The generation of ROS is known as a key process that is shared between biotic and abiotic stress responses. Oxidative stress is defined as the toxic effect caused by highly reactive chemical species produced during the reduction of molecular oxygen (O_2) in aerobic organisms (Halliwell 1987). Reactive oxygen species (ROS) derived from partially reduced or activated oxygen are highly reactive and toxic to the cellular environment and can lead to oxidative destruction of the cell (Figure 7) (Garg and Manchanda 2009).

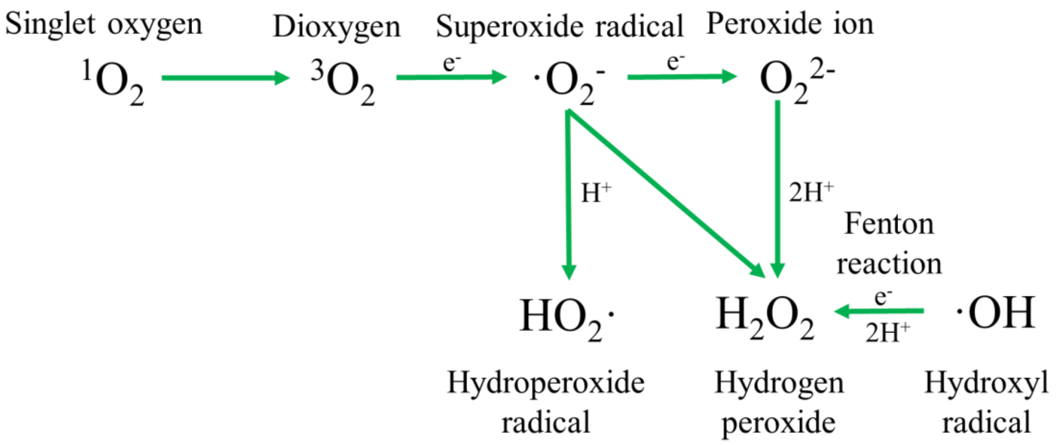

Figure 7. Reactive oxygen species pathway.

Exposure of plants to stress conditions increases ROS production. Two of the main ROS molecules produced in aerobic organisms are the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2). O_2^- radicals are very harmful to cells, and their toxic effects reside in their ability to initiate cascade reactions that result in the production of other destructive species, such as a singlet oxygen ($^1O_2$), a hydroxyl radical (HO•), or H_2O_2, which are highly toxic and reactive molecules that cause cellular damage through the oxidation of lipids, proteins and DNA (Pitzschke et al. 2006).

When ROS are accumulated in cells, aside from their toxic effects described above, they can also function as signaling molecules for numerous important biological processes when these concentrations are maintained at homeostatic levels by antioxidants enzymes and redox compounds (Mittler 2017). Thus, they intervene in the signaling processes involved in cell growth, hormonal homeostasis and the response to several abiotic and biotic stresses (Mittler 2017).

Among all the ROS produced in plant cells, the superoxide ion ($O\bullet^-$) and hydrogen peroxide (H_2O_2) are the most important due to their high toxicity, their permeability through the membranes and their accumulation levels in the cell. H_2O_2 is moderately reactive and its excess in plant cells produces a significant degree of oxidative stress (Gill and Tuteja 2010).

One of the processes considered most damaging to a living organism is the peroxidation of the membrane lipids. This is mainly caused by their reaction with H_2O_2 and hydroxyl radicals ($OH^·$). The generated lipid hydroperoxides are easily decomposed into several reactive species such as lipid alkoxyl radicals, malondialdehyde, alkanes, epoxides and lipid alcohols (Davis and Swanson 2001; Fam and Morrow 2005). The overall effects of lipid peroxidation are a reduction in membrane fluidity (which makes it easier for phospholipids to exchange between the two parts of the lipid bilayer), an increase in the passage of substances through the membrane (that would not cross it under normal conditions) and damage to membrane proteins, which would lead to the inactivation of receptors, enzymes and ion channels (Gill and Tuteja 2010), which finally results in membrane disruption and loss of integrity.

Lipid peroxidation involves the formation and accumulation of peroxylipid radicals, double bonds in unsaturated lipids, and the eventual destruction of phospholipids membranes, producing a variety of degradation products, including alcohols, ketones, aldehydes, and ethers (Buege and Aust 1978). One of these degradation products, which is produced in large amounts due to the peroxidation of fatty acids, is malondialdehyde (MDA), which is capable of reacting with thiobarbituric acid (TBARS) to produce colored compounds. Thus, the analysis of the total concentration of MDA can be used to measure damage to cell membranes caused by oxidative stress (Davis and Swanson 2001).

Faced with the damage caused by oxidative stress, and with the intention of maintaining ROS at a low level in the cells, plants have developed a powerful antioxidant defense system. In this system, both enzymatic or non-enzymatic antioxidants are involved, which work in coordination with uncontrolled oxidation cascades, thus protecting plant cells from oxidative damage by the effective cellular detoxification of ROS. Among the antioxidant enzymes, we can highlight the superoxide dismutases (SODs, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidases (PDXs, EC 1.11.1.1) and the enzymes belonging to the ascorbate-glutathione cycle, such as ascorbate peroxidase (APX, EC 1.11.1.11), dehydro and monodehydro ascorbate reductase (DHAR, EC 1.8.5.1; MDHAR, EC 1.6.5.4), and glutathione reductase (GR, EC 1.8.1.7) (Figure 8).

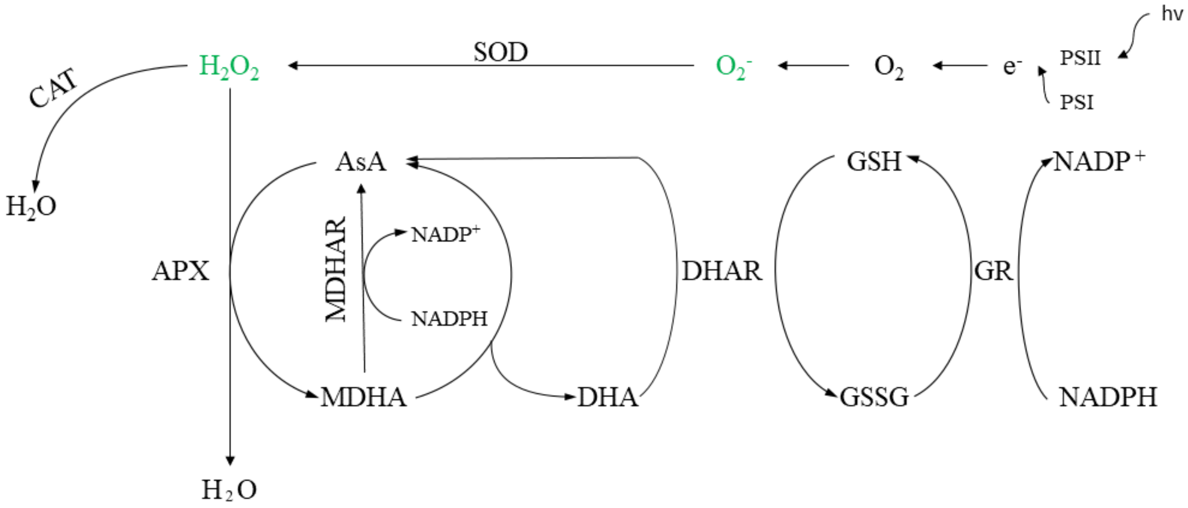

Figure 8. Enzymes involved in the oxidative metabolism in plants. CAT, catalase; SOD, superoxide dismutase, APX, ascorbate peroxidase; AsA, ascorbate, MDHAR, monodehydro ascorbate reductase; MDHA, monodehydro ascorbate; DHA, dehydro ascorbate; DHAR, dehydro ascorbate reductase; GSH, glutathione; GSSG, oxidized glutathione; GR, glutathione reductase; NADPH, Nicotinamide adenine dinucleotide phosphate.

Among the non-enzymatic antioxidant compounds, the main ones are ascorbate (AsA), glutathione (GSH), phenolic compounds, non-protein amino acids and α-tocopherol (Mehla et al. 2017). AsA and GSH are actively used by the ascorbate-glutahione cycle (figure 8) in a series of redox reactions that effectively detoxify the excess of H_2O_2 produced during stress The existence of alterations in the regeneration process of the antioxidant compounds induced by abiotic stress during the ascorbate-glutathione cycle can lead to a redox alteration, decompensating the optimal ratio of AsA/dehydroascorbate (DHA) or oxidized GSH / glutathione (GSSG) of the cells (Rivero et al. 2007; Tausz et al. 2009). Several studies carried out in different species of plants grown under abiotic stress conditions have elucidated that a high ratio of GSH/GSSG and/or AsA/DHA is due to an increase in GSH and AsA content or the decrease in the enzymatic activities of the proteins involved of redox state to regenerate the oxidized forms, GSSG and DHA, and may be the key factor in efficient protection against stress-induced accumulation of ROS (Szalai et al. 2009) (Figure 8).

Heat and salinity induce the accumulation of H_2O_2, which leads to lipid peroxidation and causes membrane instability and cell death, as well as a significant reduction in plant biomass (Nxele et al. 2017). Heat stress also causes damage to plant cell membranes due to the action of lipid peroxidation (Xu et al. 2006).

Recently, it has been shown that the changes in plant metabolism caused by abiotic stress occur in a very fast and dynamic way, within a few minutes or even seconds. For the survival and adaptation of plants to these conditions, these fast stress signaling mechanisms and changes are essential. Stress can take place in one organ of the plant or at the entire plant level, which is more common. It has recently been shown that the affected tissue generates a systemic signal through oxidative metabolism, which travels to other plant areas, activating defense mechanisms, even when the other plant parts have not yet come in contact with the stress (Mittler and Blumwald 2015; Mittler 2017; Kollist et al. 2019; Peck and Mittler 2020). In contrast, some mechanisms take more time to reach their systemic target, and they are known as slow systemic signals (Kollist et al. 2019; Fichman and Mittler 2020). ROS waves are a system that propagates a cell-to-cell signal through the respiratory burst homolog D (RBOHD) protein (Miller et al. 2009). The perception of stress causes the generation of ROS, which accumulates in the apoplast and is transmitted to neighboring cells, causing the signal to spread throughout the entire plant (Mittler 2017; Peck and Mittler 2020). Therefore, the generation of ROS is essential in the stress response, as it acts as a signaling mechanism, although the antioxidant machinery has to be tightly tuned, since the maintenance of the cellular redox state homeostasis will highly depend on it.

1.6.4. Carbohydrate metabolism

During ripening, the smell, flavor and color of the fruits are modified. This change in flavor is due, in part, to the accumulation of sugars as a result of the ripening process (Zhu et al. 2013). These sugars, in the case of the genus *Lycopersicon*, are mainly sucrose and/or fructose, depending on the species studied (Ohyama et al. 1995), and it is known that under stress conditions, sucrose also accumulates even more notably than other soluble sugars.

Sugars metabolism can be studied in a generalized manner as a distribution of carbon in different routes according to the source-sink relationship: we consider the beginning of sugar metabolism on the Calvin-Benson-Bassham cycle, also known as the reductive pentose phosphate pathway. Within this cycle, carbon fixation is achieved in the chloroplasts of photosynthetic organs by reducing CO_2 to triose phosphate. Among these trioses, GA3P (glyceraldehyde 3-phosphate) and DHAP (dihydroxyacetone phosphate) can give rise to starch, or leave the cytosol through a specific transporter associated with an inorganic phosphate antiporter (Neuhaus and Wagner 2000) to be later used according to the plant needs (Biel and Fomina 2015), such as synthesis of other sugars, synthesis of the cell wall, synthesis of reducing power or transport to sink organs and degradation.

The main form of the sugars transported from producing organs (source) to sink organs is sucrose, which is degraded to glucose and fructose by different enzymes and re-synthesized in the vacuole, apoplast or cytosol (Qin et al. 2016).

In plants, carbohydrates are involved in a large number of physiological functions, such as cellulose synthesis, which is part of the structural cell wall components, gum and mucilages synthesis, which prevent cells from drying out, and constitute an essential part of other cellular components (Baxter et al. 2005). In the last few decades, carbohydrates were described as important compounds in cell signaling, regulating the expression of a multitude of genes (Koch 1996) and also regulating several important aspects of plant development. Thus, for example, in Arabidopsis seedlings, high levels of carbohydrates in the growth medium inhibited hypocotyl elongation in the dark (Jang et al. 1997), and light-induced opening of cotyledons (Dijkwel et al. 1997; Jang et al. 1997). Additionally, flowering also appears to be regulated by carbohydrates. There is a large amount of evidence suggesting that sucrose promotes flowering in most of the species studied (Bernier et al. 1993). Furthermore, carbohydrates are involved in the tolerance mechanisms related to pathogen attack and in the processes related to cell division. In addition, some specific properties have been attributed to carbohydrates, such as the ability to retain water in periods of desiccation and to confer plasticity to the membranes (Bohnert et al. 1995).

Carbohydrates are one of the largest constituents of plants and represent the largest group within the organic compounds. Besides the functions mentioned before, sugars are important regulatory compounds that mediate, regulate and facilitate important physiological processes such as photosynthesis, flowering, senescence or seeds formation. In addition, sugars also regulate a large number of processes generated under abiotic stress conditions. Their main functions against abiotic stresses reside in their role as osmoprotectans, facilitating the osmotic adjustment, storage, radicals detoxification and stabilization of the protein structure, such as Rubisco (Pommerrenig et al. 2018). Soluble sugars (glucose, fructose, sucrose) accumulate under salt stress to facilitate, among other functions, the ionic balance in vacuoles (Ashraf and Harris 2004; Parida and Das 2005). Similar to drought conditions, when the temperature is high, there is a reduction in the water potential of the different plant tissues, which leads to the synthesis of sugar-related osmoprotectants such as the normal sugars fructose and sucrose, and some complex sugars such as trehalose and fructans (Ahmad et al. 2020). Thus, sugars are capable to transform their inner structure into a gel, thus preventing the cell from collapsing and thereby improving membrane functions, and stabilizing proteins by forming hydrogen bonds between their hydroxyl groups and polar protein residues (Singh et al. 2015b; Ahmad et al. 2020). Specifically, the abiotic stress-related loss of water what occurs in plant tissues, leading to the accumulation of sucrose to the detriment of other sugars (Magán et al. 2008). Nedjimi (2011) showed that a significant accumulation of sugars for osmotic adjustment occurred in triticale under salinity stress, so the concentration of sugars increased as the salinity in the medium increased. Similar results were obtained by Morant-Manceau et al. (2004) who demonstrated that the concentration of sugars changed in response to salinity stress in *Triticum dicoccum*. Homeostasis in general, including carbohydrate biosynthesis and compartmentalization, is affected by high temperatures, compromising all plant tissues (Maestri et al. 2002). Heat modifies the activities of the enzymes involved in starch accumulation and sucrose synthesis, by reducing the expression of specific genes involved in plant carbohydrate metabolism (Ruan et al. 2010). Some studies suggested a high availability of carbohydrates under heat stress (for example sucrose and glucose), which may represent a very important physiological characteristic associated with tolerance to high temperature (Liu and Huang 2000).

This Introduction highlights the importance and involvement of these metabolisms in abiotic stress tolerance. In addition, the high specificity of each stress condition and its possible combinations, together with the expected climate forecasts for the coming years, make the study of its effects on plants of agronomic interest and under field conditions urgent.

CHAPTER II:

JUSTIFICATION AND OBJECTIVES

2. Chapter II. Justification and objectives

One of the most important problems that limit the sustained development of the agricultural sector in the Region of Murcia is the scarcity of water resources, as a result of its low annual rainfall defined by its semi-arid climate. This water shortage, combined with the low-quality water available for irrigation, which contains a high concentration of many salts potentially toxic for plant development, leads to a decrease in crop productivity.

The tomato is one of the most important fruits and vegetable products in the Mediterranean basin. Much of the tomato production is carried out within greenhouses, where new cultivation systems such as hydroponic substrates (i.e rockwool, perlite, coconut fiber, among others) have been developed, which together with the development of new technologies related to climate management and fertigation, have strongly helped to improve our crop productivity.

Among the factors that most negatively influence tomato productivity in southeastern Spain are abiotic stresses, such as salinity, high temperatures, drought, high light, etc. Most of the traditional studies conducted in plant responses to abiotic stress only focused on the plant response mechanisms to a single stress application, although in the last decade, abiotic stresses applied in combination is gaining importance. Since most of the agricultural areas affected by salinity are located in arid or semi-arid regions, the influence of salinity stress tends to be aggravated by the simultaneous action of xerothermic factors, such as high temperatures. Because climate change is likely to increase the exposure of many different crops to stress combinations, it is important to understand how the interaction of different stresses affect plant growth, yield, and survival. Unraveling the complexities of plant responses to stress combination could therefore facilitate the development of climate-resilient crops, improving global food production and securing our future food supply

Therefore, as a consequence of the above, the following objectives have been set in this Thesis.

2.1. Main objective

The general objective of this Thesis is to unravel the physiological, biochemical and molecular responses of tomato plants to the combination of salinity and heat through the use of commercial tomato plants and recombinant tomato inbred lines (RILs) characterized for their different sensitivity to these combined stresses. The research intends to integrate the results obtained into different known metabolic pathways to obtained complete and detailed information on the specific mechanisms involved in the development of plant tolerance to the combination of salinity and heat, which are two of the most devastating abiotic stresses in agricultural production in southeastern Spain.

2.2. Specific objectives

I. Study the N assimilation pathway to better understand how this essential metabolic pathway can be affected by the specific combination of salinity and high temperature in plants with different degrees of tolerance to these conditions.

II. To elucidate how the combination of salinity and high temperatures affect the metabolism and the accumulation of sugars in tomato fruits, as well as their relevance in the tolerance of the plant and in the final quality traits of the fruit.

III. To establish the possible relationships between signaling mechanisms involved in tolerance to the combination of salinity and high temperature in a commercial tomato variety

RESULTS

CHAPTER III

3. Chapter III. Results

Using Tomato Recombinant Lines to Improve Plant Tolerance to Stress Combination Through a More Efficient Nitrogen Metabolism

Lopez-Delacalle M, Camejo DM, García-Martí M, Nortes PA, Nieves-Cordones M, Martínez V, Rubio F, Mittler R, Rivero RM

Front. Plant Sci., 17 January 2020 | doi./10.3389/fpls.2019.01702

The development of plant varieties with a better nitrogen use efficiency (NUE) is a means for modern agriculture to decrease environmental pollution due to an excess of nitrate and to maintain a sufficient net income. However, the optimum environmental conditions for agriculture will tend to be more adverse in the coming years, with increases in temperatures, water scarcity, and salinity being the most important productivity constrains for plants. NUE is inherently a complex trait, as each step, including N uptake, translocation, assimilation, and remobilization, is governed by multiple interacting genetic and environmental factors. In this study, two recombinant inbred lines (RIL-66 and RIL-76) from a cross between *Solanum lycopersicum* and *Solanum pimpinellifoilum* with different degree of tolerance to the combination of salinity and heat were subjected to a physiological, ionomic, amino acid profile, and gene expression study to better understand how nitrogen metabolism is affected in tolerant plants as compared to sensitive ones. The ionomics results showed a different profile between the two RILs, with K^+ and Mg^{2+} being significantly lower in RIL-66 (low tolerant) as compared to RIL-76 (high tolerant) under salinity and heat combination. No differences were shown between the two RILs in N total content; however, N-NO_3^- was significantly higher in RIL-66, whereas N-N_{org} was lower as compared to the other genotype, which could be correlated with its tolerance to the combination of salinity and heat. Total proteins and total amino acid concentration were significantly higher in RIL-76 as compared to the sensitive recombinant line under these conditions. Glutamate, but more importantly glutamine, was also highly synthesized and accumulated in RIL-76 under the combination of salinity and heat,

which was in agreement with the upregulation of the nitrogen metabolism related transcripts studied (*SlNR*, *SlNiR*, *SlGDH*, *SlGLT1*, *SlNRT1.2*, *SlAMT1*, and *SlAMT2*). This study emphasized the importance of studying abiotic stress in combination and how recombinant material with different degrees of tolerance can be highly important for the improvement of nitrogen use efficiency in horticultural plants through the targeting of N-related markers.

CHAPTER IV

4. Chapter IV. Results

Deciphering fruit sugar transport and metabolism from tolerant and sensitive tomato plants subjected to simulated field conditions

Lopez-Delacalle M, Camejo D, Garcia-Marti M, Lopez-Ramal MJ, Nortes PA, Martinez V, Rivero RM

Physiol Plant, 05 February 2021 | doi.org/10.1111/ppl.13355

In the current state of climate change, we must assume that abiotic stresses act together under natural field conditions, these will increase in the coming years. Therefore, in this report we investigated how sugar metabolism was affected under simulated field conditions, where plants faced high ambient temperatures and a low-quality water irrigation. Our studies were carried out on fruits of two tomato recombinant lines, a tolerant and a sensitive one exposed to the combination of heat and salinity. Two ripening stages (mature green and red ripe fruits) were used in our analyzes, where the gene expression levels of the main biosynthetic genes and transporters, enzymatic activities and compounds related to the synthesis, accumulation, and degradation of sugars in plants were analyzed. The tolerant line showed highly significant differences in red ripe fruits in comparison to the sensitive one under the simulated field conditions (35°C + 60 mM NaCl), with an overexpression of the genes *SlFBP*, *SlSPS*, *SlSUS3*, and *SlNi*. These expression patterns correlated with a higher activity of the enzymes FBP, SPS, SUS3, AI, and G6PDH, which resulted in the accumulation of fructose, glucose and UDP-glucose. Our results showed the advantage of using tomato recombinant lines for rescuing important traits, such as the resistance to some abiotic stresses, and for the identification of important molecular and metabolic markers that could be used to determine fruit quality in green or red maturity stages under detrimental environmental field conditions.

CHAPTER V

5. Chapter V. Results

Synchronization of proline, ascorbate and oxidative stress pathways under the combination of salinity and heat in tomato plants.

Lopez-Delacalle M, Silva CJ, Mestre TC, Martinez V, Blanco-Ulate B, Rivero RM

Environ Exp Bot, March 2021 | doi.org/10.1016/j.envexpbot.2020.104351

Adverse environmental conditions have a devastating impact on plant productivity. In nature, multiple abiotic stresses occur simultaneously, and plants have evolved unique responses to cope against this combination of stresses. Here, we coupled genome-wide transcriptional profiling and untargeted metabolomics with physiological and biochemical analyses to characterize the effect of salinity and heat applied in combination on the metabolism of tomato plants. Our results demonstrate that this combination of stresses causes a unique reprogramming of metabolic pathways, including changes in the expression of 1388 genes and the accumulation of 568 molecular features. Pathway enrichment analysis of transcript and metabolite data indicated that the proline and ascorbate pathways act synchronously to maintain cellular redox homeostasis, which was supported by measurements of enzymatic activity and oxidative stress markers. We also identified key transcription factors from the basic Leucine Zipper Domain (bZIP), Zinc Finger Cysteine-2/Histidine-2 (C2H2) and Trihelix families that are likely regulators of the identified up-regulated genes under salinity + heat combination. Our results expand the current understanding of how plants acclimate to environmental stresses in combination and unveil the synergy between key cellular metabolic pathways for effective ROS detoxification. Our study opens the door to elucidating the different signaling mechanisms for stress tolerance.

CHAPTER VI:

DISCUSSION

6. Chapter VI. Discussion

6.1. Plant material

Tomato is one of the most important vegetables, and is produced in 144 countries around the world (http://faostat.fao.org), due to the fundamental role it plays in human diet and nutrition (Atkinson et al. 2011).

Semi-arid regions, such as the Mediterranean area, suffer from a shortage of good quality water, encouraging the use of saline water from aquifers for irrigation, which contain excessive concentrations of soluble salts, mainly chlorides (3-6 dS m^{-1}) (Fernández-García et al. 2004). In these areas, in addition, the influence of saline stress is aggravated in most cases by the simultaneous action of other factors, such as high temperatures for long periods, which causes the damage produced by these combined stresses on crop yields, to be more devastating than when stresses occur separately (Mittler 2006).

This doctoral Thesis project was carried out on tomato plants with the consecution of three independent experimental studies.

The first and second experiments were carried out with a unique plant material available based on an agreement between Professor Francisco Perez-Alfocea (CEBAS-CSIC) and The World Vegetable Center (AVRDC). This plant material consisted of an inbred seed population of 84 lines resulting from the cross between *Solanum lycopersicum* L. CLN2498E x *S. pimpinellifolium* L. LA1579. Based on the recent publications from our research group, which showed that the plant response to the combination of salinity and heat was very specific and that it could not be deduced only from the plant responses obtained with the application of single stresses, the use of this inbred population in our studies was very novel.

The advantages of having a tomato RIL population for the consecution of our different experiments led us to select contrasting lines with different degrees of tolerance to the combination of salinity and high temperature. Since abiotic stress combination originates a specific plant response, the use of this plant material allowed us to identify plant metabolic pathways, genes, proteins and metabolites that were specifically induced or repressed in the tolerant line with respect to the sensitive one, contributing to the increase in knowledge about these abiotic stress interactions.

Before the initiation of our different experimental studies, a preliminary experiment was carried out with the entire RIL population (a total of 84 RILs) with the objective of selecting a set of 12 contrasting lines with different degrees of tolerance (from the lowest to the highest) to the combination of salinity and heat. In consecutive experiments, 5 lines and lately 2 lines (a very sensitive and a high tolerant line to the combination of salinity and heat) were used to narrow our research and to obtain putative specific pathways that might be implied in the development of this tolerance (Chapter III, Figure 1).

Based on vegetative growth and development, the RIL-76 line was identified as the tolerant one, and the RIL-66 and RIL-18 lines were identified as the most sensitive ones (Lopez-Delacalle et al. 2020, 2021a).

Finally, with the aim of identifying the putative molecular markers involved in plant tolerance to the combination of salinity and heat and to establish the possible relationships between them, a third experimental study was carried out in a commercial tomato plant (*Solanum lycopersicum* L. cv Boludo), which was kindly provided by Monsanto (Torre Pacheco, Murcia, Spain). This commercial variety was selected as it is highly appreciated by the consumer, as it has a uniform size, good firmness, shape and color and a long postharvest period.

It is well known that salinity negatively affects plant growth and crop production (Cuartero and Fernández-Muñoz 1998). Normally, this problem occurs in areas with low rainfall and characterized by a semi-arid climate, as we find in many of the agricultural lands worldwide, and specifically in southeastern Spain. Sometimes in these areas, the problem of salinity found in the irrigation waters is increased by periods of high temperatures above 40ºC that crops have to withstand during their development. Global warming and climate change are alarmingly increasing the frequency and intensity of different abiotic stresses, such as droughts, heat waves, cold snaps and flooding, negatively impacting crop yields and causing food shortages. Although we are trained to study the impact of individual stress conditions on plants in laboratories, in the field many stresses, pollutants, and pests could simultaneously or sequentially impact plants, causing conditions of stress combination. Because climate change is expected to increase the frequency and intensity of such stress combination events, a concentrated effort is needed to study how stress combination affects crops. This need is especially critical since many studies have shown that the response of plants to stress combinations is unique and cannot be predicted from simply studying each of the different stresses that are part of the stress combination. Therefore, this Thesis was designed to gain knowledge and cover some of the unknown gaps in this field of study.

6.2. N metabolism and stress combination

Nutrition plays a fundamental role against stress, both in crop yield and in fruit quality. Among the nutrients that plants need for proper growth and development, nitrogen (N) is perhaps the most important one. Plants use N to synthetize important molecules for their development, such as amino acids, proteins, nucleic acids or chlorophyll. The involvement of the N assimilation pathway in plant growth and development, together with the need for reducing N-fertilizers application in the field by increasing plant nitrogen use efficiency (NUE), makes the study of this pathway highly interesting to agriculture. Most plants absorb N in the form of NO_3^-, which later becomes NH_4^+ to be incorporated into amino acids during the nitrogen assimilation process (Ferrario-Méry et al. 1998; Hoshida et al. 2000; Debouba et al. 2007). These absorption and assimilation processes have been shown to be affected by different abiotic stresses such as heat, salinity or drought (Esposito 2016; Ren et al. 2017; Ashraf et al. 2018).

6.2.1. Relationship between CO_2 assimilation and N metabolism

From an agronomic point of view, one of the most important physiological parameters to take into account when measuring plant growth is photosynthesis. To determine the physiological response of plants to any stress combination during the vegetative growth phase, changes in CO_2 assimilation, transpiration rate, stomatal conductance, electronic transport rate (ETR), maximum quantum efficiency rate of yield (Fv’/Fm’) and PSII efficiency (ϕPSII) were selected and studied as the most characteristics photosynthetic-related parameters.

Our results showed that tomato growth and its photosynthetic-related parameters were significantly affected in the sensitive RIL-66 line with respect to the tolerant RIL-76 one. Specifically, under both salinity, and salinity and heat combined treatments, the sensitive line RIL-66 showed a significant decrease in its biomass (Chapter III, Figure 2). Similarly, under salinity conditions, RIL-66 showed a significant inhibition of all the photosynthetic and chlorophyll fluorescence-related parameters, in agreement with that previously found by other authors (Yu et al. 2010; Ashraf et al. 2018; Naliwajski and Skłodowska 2018). However, in this sensitive RIL-66 line under salinity and heat conditions applied in combination, only the Fv'/Fm' and the CO_2 assimilation rate were inhibited (Chapter III, Figure 3), with the rest of the parameters showing values very similar to those found under control conditions. This could indicate that the inhibition produced in the photosynthetic parameters of the sensitive line was independent of the stomatal limitations, which may be due to a downstream inhibition of Rubisco or the use of triose phosphate (Rivero et al. 2010). On the other hand, the increase in stomatal conductance, ETR and transpiration rate shown by the tolerant line (RIL-76) may indicate that the photosynthetic sensitivity to salinity and salinity and heat observed in the RIL-66 line may be related to enzymatic inhibition of photosynthesis (Rivero et al. 2010; Zhou et al. 2011; Wang et al. 2018).

6.2.2. Nutritional status, different N forms and soluble proteins concentration under stress combination

Abiotic stresses have been shown to induce changes in the absorption and assimilation of different nutrients. Numerous studies showed that crops grown under salinity or high temperature conditions often showed a nutritional imbalance, due to direct inhibition of nutrient absorption, or to a direct competition in the absorption of some nutrients with others (Cakmak 2005). One of the best-known toxic effects derived from saline stress and that directly affects productivity is the accumulation of Na^+ and Cl^- ions within the plant cells (Ashraf and Harris 2004; Gouiaa and Khoudi 2015). The accumulation of these ions can induce an ionic imbalance due to the blocking of the absorption of other important ions such as K^+ and Ca^{2+} (Plaza et al. 2017), causing visual deficiency symptoms of these nutrients (Blasco et al. 2013). Our results show that under salinity and salinity and heat combination, the sensitive line (RIL-66) showed a decrease in the leaf concentration of K^+ and Mg^{2+} ions, while the tolerant line (RIL-76) only showed a slight decrease in the concentration of the Ca^{2+} under the combination of salinity and heat and K^+ under salinity stress alone (Chapter III, Figure 4). This nutritional imbalance could be induced in our plants by the differential absorption and accumulation of Na^+ that takes place when they are subjected to salinity and salinity and heat stress in combination (Rus et al. 2004; Wang et al. 2013).

On the other hand, it has been shown that the accumulation of NO_3^- in the edible parts of the plant is unhealthy for human consumption, and can lead to the appearance of tumors and cancers (Dutt et al. 1987; Du et al. 2007). Both RIL lines showed a reduction in the concentration of NO_3^- under any abiotic stress assayed, except for line RIL-66 grown under salinity stress alone, where no significant differences were observed with respect to the control plants (Chapter III, Figure 4). These results may be derived from the known existing antagonism between Na^+ and NO_3^- (Yildirim et al. 2009) or, also, it may indicate a positive effect on the assimilation of NO_3^- into other nitrogen forms, such as amino acids or proteins. To confirm this hypothesis, organic N and total N were measured. Our results showed that N absorption and accumulation were negatively affected in the sensitive line (RIL-66) as compared to the tolerant line (RIL-76) and to control plants under salinity and salinity and heat stress in combination. Thus, under salinity, total N, but specifically organic N, was significantly reduced in the sensitive line (RIL-66), which was more evident when salinity and heat were combined. This reduction was translated into a lower concentration of soluble proteins but not into the total amino acid concentration as compared to control plants. In any case, the tolerant line RIL-76 showed a higher organic N concentration (i.e. soluble proteins and total amino acids) than RIL-66 under any stress assayed and similar to control plants, which can be translated into a better NUE under stress for these plants.

6.2.3. Transcriptional regulation of the N metabolism-related proteins

The differences shown in the accumulation of soluble proteins and amino acids under stress conditions in the different recombinant lines studied have highlighted the importance of studying N amino acid-related assimilation. The total amino acids concentration increased significantly in the tolerant line (RIL-76) with respect to the sensitive line (RIL-66) under all stress conditions, but was especially increased (about 60% higher) under the combination of salinity and heat (Chapter III, Figure 5). These important differences indicate that the combination of salinity and heat induced a specific response in the amino acid accumulation pattern. Proline (Pro) was the amino acid which most significantly accumulated in all the stress conditions assayed with respect to control plants. Pro was previously described as an organic compound rich in N, with a low molecular weight and high solubility (Rivero et al. 2004; Hayat et al. 2012), which makes it non-toxic to cells when it is accumulated in high concentrations under many abiotic stresses (Kaur and Gupta 2018; Siddique et al. 2018; Forlani et al. 2019). Under these conditions, Pro accumulates in stressed cells and prevents the associated osmotic stress, by protecting the cell plasma membrane and aiding in ROS detoxification and protein stabilization (Bohnert and Jensen 1996; Yancey 2005; Taunk et al. 2019; Zulfiqar et al. 2019). This osmoprotective effect could explain the general Pro accumulation observed in both recombinant tomato lines subjected to any abiotic stress assayed. The accumulation of osmoprotectants plays many important functions in the adaptation of cells to various environmental stresses (Jogawat 2019; Kido et al. 2019; Zulfiqar et al. 2019) and depending on the plant species and the type of stress studied, plants preferentially accumulate some osmoprotectors over others. In this case, the amino acid profile showed that Pro was the preferred osmoprotectant accumulated in both recombinant lines.

Concerning the amino acid profile obtained under the combination of salinity and heat, it was interesting to observe that most of the amino acids showed an opposite accumulation pattern in the sensitive line with respect to the tolerant one. This led us to identify possible data that could help breeders create varieties with greater tolerance to this specific abiotic stress combination. In this sense, the significant accumulation observed in the tolerant line RIL-76 of the amino acids γ-aminobutyric acid (GABA), valine (Val), asparagine (Asn), isoleucine (Ile) and glutamine (Gln) under the combination of salinity and heat indicated the specificity of this stress interaction and the importance of these amino acids in the tolerance to this stress combination (Chapter III, Figure 5). In agreement with our results, Jin et al. (Jin et al. 2016) demonstrated that when *P. Oleracea* L. plants were subjected to drought and heat stress conditions, a pattern of amino acid accumulation specific to this stress combination was produced, specifically Gln, tyrosine (Tyr), Val and tryptophan (Trp). These authors indicated that this accumulation was due to a process of cellular osmotic adjustment to maintain the leaf turgor under stressful conditions. Therefore, we hypothesized that the tolerant line RIL-76 could be varying its amino acid accumulation pattern specifically to cope with the loss of turgor that takes place under salinity and heat conditions acting in combination.

The N assimilation process comprises the synthesis of certain amino acids, which can take place in the cytoplasm or other cellular compartments, such as the plastids or the chloroplast (Krapp 2015). These amino acids can be used for certain cell functions or transferred to other cell compartments for other purposes. The

GS/GOGAT cycle allows the synthesis of amino acids with the use of NH_4^+ and C skeletons. Previous studies showed that salinity stress caused negative alterations in NO_3^- assimilation by roots (Flores et al. 2004; Debouba et al. 2006, 2007; Ehlting et al. 2007). A significant decrease in the activity of the NR enzyme has also been found for tomato (Debouba et al. 2007), leaves and roots of beans (Gouia et al. 1994), sugar beet (Ghoulam et al. 2002) and maize leaves (Abd-El Baki et al. 2000) subjected to different concentrations of NaCl. Contradictorily, other authors have described a stimulatory effect on NR activity under salinity stress on tomato roots (Debouba et al. 2007), bean seeds (Misra and Dwivedi 1990) and soybean roots (Bourgeais-chaillou et al. 1992). Our experiments showed a decrease in NR activity in both the sensitive and tolerant lines under salinity conditions. This decrease in activity was induced at the transcriptomic level since the gene that codes for this enzyme (*SlNR*) was downregulated under salinity as well (Chapter III, Figure 6). Flores et al. (Flores et al. 2004) suggested that a reduction in the uptake of NO_3^- under salinity stress for more than 24 h could cause a decrease in the activity of NR. In this sense, several studies showed that a high concentration of NaCl reduces the absorption of NO_3^- in tomato (Flores et al. 2004), tobacco (Ruiz et al. 2005), wheat (Botella et al. 1997) and bean (Gouia et al. 1994). In addition, an increase in temperature has also been observed to limit NO_3^- absorption (Ito et al. 2009; Ercoli et al. 2010). In our experiments, we have found that in the tolerant line RIL-76, the leaves' NO_3^- concentration significantly decreased under all the stress conditions assayed (Chapter III, Figure 4). However, for the sensitive line RIL-66, the NO_3^- concentration only decreased when the heat treatment was applied individually (Chapter III, Figure 4). Therefore, according to Flores et al. (Flores et al. 2004), a possible hypothesis that could explain the reduction in the activity of the NR enzyme observed in our plants could be the reduction in NO_3^- uptake of our plants with the consequent low presence of NO_3^- in tissues, which decreases the NR related genes expression. Also, in agreement with our results, other authors suggested that salinity causes a decrease in the activity of several enzymes involved in nitrogen assimilation (Khan and Srivastava 1998; Chakrabarti and Mukherji 2003; Debouba et al. 2006, 2007). In *Brassica Juncea*, salinity stress has been shown to reduce the activity of the enzymes NR, NiR, GS, GOGAT and GDH (Nathawat et al. 2005; Siddiqui et al. 2009).

However, on contrary, under salinity conditions, the alteration in NR activity has been linked to an increase in the activation state of this enzyme in roots, and a decrease in the pool of phosphorylated enzymes in the cytosol (Di Gioia et al. 2017). Therefore, the decrease in cellular pH induced by salinity conditions can lead to an increase in the enzymatic activity of NR in the roots, due to the de-phosphorylation of the NR protein. Huber et al. (Huber et al. 1994) showed that an increase in the growth media of saline concentration in spinach leaves caused an increase in NR activity. Furthermore, these authors suggested that this stimulation may be due to the presence of salts and the consequent effect of high ionic strength.

The activity of the enzymes related to N assimilation into amino acids, such as GOGAT, GS and GDH, showed a different behavior depending on the RIL line studied (sensitive or tolerant) and the stress condition applied. At the transcriptional level, GOGAT (*SlGLT1*) was inhibited in the sensitive line and overexpressed in the tolerant line under the combination of salinity and heat (Chapter III, Figure 6). GS (*SlGTS1*, cytoplasmic), was found inhibited in RIL-76 under the combination of salinity and heat, while GDH (*SlGDH*) was overexpressed in both the sensitive and tolerant line under this stress combination. From the comparison between the transcriptomic and the enzymatic activities results, it could be deduced that under the combination of salinity and heat, GOGAT and GS were regulated at the transcriptional level, while GDH experienced post-transcriptional modifications, as its activity was lower than expected based on the expression of its gene (Chapter III, Figure 6). Ashraf et al. (Ashraf et al. 2018), suggested that under stress conditions, GS may have a lower access to glutamate because it is being consumed in the synthesis of osmolytes such as Pro. Therefore, as previously reported by Ashraf et al. (2018) the low access to glutamate, together with the low absorption of NO_3^- and the high level of Pro accumulation observed in our plants, as well as the osmotic changes induced by salinity, could have promoted the alterations observed in the activities of the N-assimilation related enzymes.

The most important parameters considered in the past for the selection of varieties of agronomic interest were exclusively the yield and the quality of the harvest. The new scenarios posed by climate change, together with the molecular tools that currently exist, allow us to select varieties by also considering tolerance to new environmental conditions. Our results can serve as the basis for the identification of stress tolerance parameters that also help to make responsible and efficient use of nitrogen without damaging the environment.

6.3. Sugar metabolism and stress combination

Tomato plants are considered moderately sensitive to salinity since growth and crop yield begin to decrease when the electrical conductivity of the nutrient solution with which it is watered exceeds 2.5-4.0 dS m^{-1} (Rosadi et al. 2014). This level of salinity improves the quality of the fruits, as the cells tend to accumulate total soluble solids and antioxidant compounds to a greater extent to cope with these stress conditions (Rodríguez-Ortega et al. 2019).

Given to their not only involvement as osmoprotectors and as secondary messengers under stress conditions, but also to their importance as a fruit quality parameter, the study of sugar metabolism under certain extreme conditions is of great interest for agriculture (Aslam et al. 2019; Gao et al. 2019b). Some important quality attributes to consider when processing harvested tomatoes are sugar content or soluble solids (°Bx), pH, titratable acidity, viscosity and color (Beckles 2012). About half of the soluble solids are reducing sugars, mostly fructose and glucose (Beckles 2012; García-Cañas et al. 2012). The rest of soluble solids are made up of organic acids (citric and malic), amino acids, proteins, lipids, minerals, pectic substances, cellulose and hemicellulose (Davies et al. 2009; Beckles 2012). Since the tomato flavor partially depends on the proportion of soluble solids it contains, the concentration of glucose and fructose**,** as well as their synthesis pathways, are essential parameters to take into account to evaluate their quality.

6.3.1. Photosynthetic parameters and their relationship with sugar metabolism

Recently, it was shown that plants that are more tolerant to high temperatures maintain both net photosynthesis and stomatal conductance at higher values than those which are less tolerant, and this can be considered as a physiological marker for the selection of tolerant species and for the improvement of the crop production (Poudyal et al. 2019). To study and improve crop yield and to delve into the existing knowledge, it is of special interest to conduct experiments under real field conditions. Therefore, sugar synthesis and accumulation was studied in the present thesis in tomatoes lines from a RIL population which were sensitive and tolerant to the combination of salinity and heat. In this experiment, the plants were subjected to simulated field conditions, with a specific growth temperature of 35ºC and salinity in their irrigation water of 60mM NaCl, conditions that simulate agriculture in Southeastern Spain. Photosynthetic parameters were measured in the leaves of the previously described plants at two different time points: T1, time where their fruits were at the mature green stage, and T2, time where their fruits were at the red ripe stage (Chapter IV, Figure 1).

The photosynthetic parameters results obtained in this work at the ripening stage when the fruits were still green showed that the application of simulated field conditions caused inhibition of photosynthesis (Chapter IV, Figure 1). However, as the treatments progressed, the acclimatization of the plants was observed, with no significant differences in the data obtained for the CO_2 assimilation when comparing the stressed plants with the controls. This suggests that the alterations shown later in the synthesis and accumulation of carbohydrates in the same plants may not be due to photosynthetic alterations per se but to a downstream differential regulation in the use of triose phosphate (Rivero et al. 2010). Contrary to the results found in this experiment, Martinez et al. (2018) observed that when commercial tomato plants were subjected to the combination of salinity and heat, a photosynthetic inhibition took place with a consequent decrease in plant growth, probably because in this study done by Martinez et al. (2018) the photosynthetic parameters were evaluated in different stage of vegetative development.

6.3.2. Synthesis and accumulation of sugars under stress combination

As previously reported, plants respond to heat or saline environments through multiple physiological, metabolic, and molecular mechanisms. These mechanisms are interconnected in the effort of the plant to initiate specific responses that lead to the acclimation of the plant to extreme environments (Conde et al. 2011). Furthermore, combined stress can affect plants differently depending on their stage of development. Germination, vegetative growth, flowering or reproduction can be affected differently by the same stress, and, on the other hand, it can also affect the different tissues of the plant differently (Mittler 2006). In this experiment, it was studied how simulated field conditions composed by the combination of salinity and heat affected the fruit sugar synthesis and accumulation. These studies were conducted at gene, enzyme and metabolite levels in mature green and red ripe tomato fruits. Sugars balance and accumulation is an essential trait for the final organoleptic characteristics of tomato fruits. Sugars will also contribute to maintain cellular homeostasis. Although various scientific assays analyzed the final concentration of fruit sugar and showed that its synthesis (in leaves) was affected, it seems interesting to study how this pathway takes place directly in the fruit and how this process is affected by simulated field conditions.

When analyzing the results obtained, it was observed that a prolonged exposure to 60 mM of NaCl (this experiment was 4 months long), caused significant differences in the sugar synthesis and accumulation pathway between the sensitive and tolerant lines, directly related to sugar-related gene expression and the activity of the enzymes coded by these genes. If we initially look at the pentose phosphate cycle, which takes place in the chloroplast, it is of special interest to know the regulation of the glucose-6-phosphate dehydrogenase (G6PDH) enzyme, not only for its role in the pentose-phosphate cycle, but also for its involvement in the detoxification and accumulation of ROS, by-products of the abiotic stress (Martinez et al. 2018; García-Martí et al. 2019). Then, the overexpression of the gene coding for this enzyme would be expected under stress conditions. However, our experiments showed that under simulated field conditions, the red fruit of the sensitive line and the green fruits of the tolerant line significantly decreased their expression with respect to the control. On the other hand, the green fruit of the sensitive line and the red fruits of the tolerant line did not show any significant alterations in the expression of the *SlG6PDH* gene with respect to the control conditions (Chapter IV, Figure 3). Wenderoth et al. (1997) reported that this behavior may be due to cysteine-mediated redox inactivation under light conditions. This inactivation can lead to an inhibition of the synthesis of NADPH, a powerful reducing agent that reduces oxidized glutathione (Landi et al. 2016), leaving the plant more exposed to oxidative damage caused by stress. These processes, among others, can help us understand the deterioration shown in plants under the stress conditions to which they are subjected, such as the loss of biomass or the size and number of fruits (data not shown).

Through the Calvin Cycle, which takes place in the chloroplast, CO_2 is fixed as triose phosphate which, depending on cellular needs, can be transported to the cytosol and used for plant growth or converted into starch for energy reserve (Fliege et al. 1978). In this sense, Reuscher et al. (2014) showed that plastidic glucose translocators proteins (pGlcT) can export starch decomposition products. Considering this, the tolerant line RIL-76 showed an overexpression of the glucose transporter (*SlGLCT1)* gene in all maturation stages (green and red fruits) under stress conditions, while the sensitive line showed the opposite behavior (Chapter IV, Figure 3). This may help explain that in the tolerant line, the starch degradation products were being transported more efficiently to the cytosol, a process that may be partly responsible for a higher final accumulation of sugars in the tolerant line.

The first reaction studied concerning the synthesis of sucrose is carried out by the cytosolic fructose 1,6-bisphosphatase (FBPase). The expression of the *SlFBPase* gene was overexpressed in the tolerant line in the two maturation stages studied (green and red fruits) and under stress conditions. On the contrary, all the fruits from the sensitive line showed an inhibition of the expression of this gene (Chapter IV, Figure 3). The high activity found later in the tolerant line for the enzyme coded by this gene may respond to the need of the plant to obtain energy for proper fruit growth and development (Chapter IV, Figure 4). It has been shown that in tomato, there is a direct relationship between the reduction of the FBPase enzyme activity and the size of the fruit, which can be around 20% (Obiadalla-Ali et al. 2004). Therefore, the larger size of the fruits from the tolerant line could be related, among other factors, to the overexpression of the *SlFBPase* gene and the corresponding activity of the FBPase enzyme.

If we follow the pathway downstream, fructose 6-bisphosphate can be converted to fructose 2,6-bisphosphate by fructokinase 2 (FK2) or continue on to glycolysis (Martinez-barajas 1996). Exclusively considering the expression of the *SlFK2* gene, it was downregulated in both lines studied under stress conditions (Chapter IV, Figure 3). However, if we look at the enzymatic activity, we find that the activity was higher under stress conditions and in green fruits, for the sensitive line, which might be the cause for the accumulation of fructose 2,6-bisphosphate observed in this line (Chapter IV, Figure 4). In the tolerant line, the activity of FK2 is inhibited when the activity of CyFBPase is overexpressed, which indicated that sugars are being forced to continue to the glycolysis pathway or to sucrose synthesis (Rolland et al. 2006).

Our results indicate that UDP-glucose was accumulated in low concentrations in both lines studied and for both treatments (Chapter IV, Figure 2). As previously reported by Lunn and MacRae (2003), this was possibly because instead of accumulating, it was used as a substrate to obtain other sugars with enzymes such as SPS, responsible for the synthesis of sucrose phosphate, which might explain the level of this sugar found in our experiments.

Considering the activity of the SPS enzyme, it was found that in fruits collected at both maturation stages (green and red), its activity was always significantly higher in the tolerant line. Similar results were found by Zhang et al.

(2012) for tomato plants grown under high temperatures (35ºC) for 40 days. Rivero et al. (2014) also found that under conditions of stress due to salinity and heat, the expression of the genes *SlFK2* and *SlSUS3* was higher than that found under control conditions, which corroborates that these enzymes could be related to stress tolerance and also confirms our results.

The tolerant line showed an overexpression of the *SlSUS3* gene in red fruits of plants grown under stress conditions, a result that was positively correlated with the activity of the protein. The sensitive line showed the opposite behavior, where this gene was downregulated under the same conditions (Chapter IV, Figure 3). The SUS3 enzyme is responsible for converting sucrose into UDP-glucose, which can be later used for starch biosynthesis (Bahaji et al. 2014). This might explain that the red fruits of the tolerant line subjected to stress showed low levels of sucrose and high levels of UDP-glucose.

Taking into account sucrose content under stress conditions, the green fruits from the tolerant line showed a higher concentration of this sugar than the sensitive one, but this sugar had an opposite behavior when the fruits ripened and were red (Chapter IV, Figure 2). This can be explained by the activity of the neutral (NI) and acid (AI) invertase enzymes, which are responsible for degrading sucrose in different organelles after being synthesized (Ruan et al. 2010; Li et al. 2012). The red fruits of the tolerant line subjected to simulated field conditions showed a significant increase in the enzymatic activity of AI, coinciding with a lower content of sucrose. In addition, and in accordance with the results, fructose and glucose were highly accumulated in these fruits due to the activity of the invertase enzymes (Chapter IV, Figures 2 and 4). Similar results were found by other authors in tomato fruits (Rosales et al. 2007) and leaves (Zhang et al. 2012). In an experiment with two tomato lines, one sensitive and the other tolerant to heat stress, Li et al. (2012) found that AI activity was significantly higher in the tolerant plant with respect to the sensitive one. The concentration of fructose and glucose is partially justified in the tolerant line by the enzymatic activity of the invertases; however, it can be also explained by the overexpression observed for the *SlTMT1* gene, whose protein is responsible for transporting sugars within the vacuole (Schulz et al. 2011; Reuscher et al. 2014).

In general, the tolerant line accumulated more sugars than the sensitive line under simulated field conditions and in red fruits. Similarly, Klann et al. (1993) found higher concentrations of soluble sugars in the wild tomato species *Lycopersicon chmirlewskii* than in the domesticated species *Lycopersicon esculentum*. Therefore, the results found in the tolerant RIL may be due to the presence in the genome of traits from the wild parent.

6.4. Synchronization of possible physiological, metabolic and molecular mechanisms under abiotic stress combination in a tomato commercial variety.

Given the relevance of the findings made previously in the RILs population, we decided to study what occurred in a commercial variety (*Solanum lycopersicum* L. cv Boludo, Monsanto) under the same treatments, to validate our previous results and complement them with new observations. In this context, a new experiment was carried out, trying to give it a broad meaning, which could provide solutions to the problems found in today's agriculture. In this case, instead of focusing on a single metabolic pathway, as previously done with the RILs, an untargeted metabolomics study and an RNA sequencing study were carried out and complemented with some physiological and biochemical studies. The data from all these analyses were subsequently integrated, and some interesting results were found which could be transferred to current agricultural practices.

6.4.1. Photosynthetic- and oxidative-related markers

As our results demonstrate, the primary physiological response of tomato plants to individually applied salinity or high temperatures was consistent with several previously-published works (Romero-Aranda et al. 2001; Camejo et al. 2005; Wahid et al. 2007). However, when the two stresses were applied together, the physiological response observed was not deducible from the single stress experiments. From the physiological point of view, the combination of salinity and heat affected the plant biomass production to a lesser extent than the salinity applied individually, as previously published by our research group (Rivero et al. 2014; Martinez et al. 2018; García-Martí et al. 2019). The growth data were consistent with the data obtained from the different photosynthetic parameters analyzed and those related to oxidative metabolism. In this sense, the plants subjected to the combination of heat and salinity also showed a better photosynthetic performance and lower cellular oxidation if we compared them with those under salinity applied individually (Chapter V, Figures 1 and 5). A good balance between these two physiological processes is of great importance for plants to survive abiotic stresses (Considine and Foyer 2014; Woehle et al. 2017). It should be noted that under salinity, a greater accumulation of ROS was detected as compared to other treatments (heat or the stress combination). This large accumulation led to protein oxidation, damage to the membranes, and a lower cellular antioxidant capacity. These oxidative processes could be the cause of the photosynthetic inhibition observed and thus, the concomitant reduction in biomass production and growth. Previously, Rivero et al. (2014) reported similar results, showing that under heat conditions, both photosynthetic and oxidative markers were similar to those obtained in plants grown under control conditions, and plants grown under the combination of salinity and heat showed a better performance than those grown under salinity applied as a single stress.

The alterations caused by a certain type of stress on the photosynthetic parameters are good indicators of the damage that this stress causes in plants, as growth, production and photosynthesis are closely interconnected. Any restrictions to photosynthesis limits the growth of the plant and, ultimately, its production and quality (Kocal et al. 2008; Tomaz et al. 2010). However, and as observed in our results, salinity and high temperatures tended to affect photosynthetic processes differently. In salt-treated plants, the main aspects that inhibited photosynthesis were stomatal closure caused by the reduction in cellular water content, and the reduction of assimilate transport (Hare et al., 1998). As a consequence, the accumulation of glucose, fructose and mainly sucrose in the leaves could be drastically reduced (Epstein 1979).

On the other hand, high temperatures mainly affect the biochemical reactions of photosynthesis, and depending on the duration and intensity, high temperatures can irreversibly damage Rubisco, oxygen evolution complexes, the ultrastructure of chloroplasts, thylakoid membranes and PSII reaction centers (Havaux 1993; Allakhverdiev et al. 2003; Chen et al. 2012). As shown in our results, salinity stress led to a significant inhibition of photosynthesis as compared to high temperatures, due to a significant decrease in CO_2 assimilation, transpiration rate and stomatal conductance (Chapter V, Figure 1). Stomatal closure, which directly affects CO_2 assimilation and stomatal conductance and takes place under salinity conditions, may be due to the osmotic stress caused by NaCl. Maintaining a high transpiration rate is important for plants that grow under stress from high temperatures, as this helps them to cope with the increase in leaf temperature and at the same time protects their photosystems (Ilan et al. 1995).

Our results suggest that when salinity is applied in combination with high temperatures, this second stress may confer some advantages over salinity applied individually. This is achieved through an increase in the transpiration rate, which causes a decrease in damage to the photosynthetic apparatus, and which has a direct effect on an increase in the CO_2 assimilation rate and, finally, the growth rate with respect to plants grown only with salinity. In this sense, under stress combination conditions we also found a lower accumulation of ROS, accompanied by less damage to proteins and membranes and helping to maintain the antioxidant capacity above 60%, as compared to plants subjected only to salinity.

6.4.2. Effect of stress combination on metabolites, genes and related enzymatic activities

Previously, we showed that under the combination of salinity and heat detoxification seemed to be more effective than under a single salinity stress.

Interestingly, our results showed that under the combination of salinity and heat, important pathways involved in plant stress tolerance were activated, such as proline and ascorbate metabolism. Furthermore, we demonstrated a synchronization between both pathways mentioned and their interconnection with oxidative metabolism to counteract the negative effects induced by the abiotic stress combination.

This synchronization was not found in plants subjected to these stresses applied individually, which highlights the importance of our study. Proline aids in stress tolerance by protecting against cell damage due to its role as an osmoprotectant and by acting as a direct ROS scavenger (Hossain 2014, Rejeb 2014). Although proline metabolism was activated under the combination of salinity and heat, proline was not significantly accumulated as compared to control plants (as shown in our previous study with RILs), but the derivatives 4-hydroxyproline and L-glutamate-5-semialdehyde were significantly accumulated. Some studies have shown that under stress conditions, proline is oxidized to obtain energy in the form of ATP and Nicotinamide-Adenine Dinucleotide Phosphate (NADPH), thanks to the action of the Proline Dehydrogenase (PRODH) enzyme (Verbruggen and Hermans 2008; Jaspers et al. 2010; Zhang and Becker 2015). In turn, this oxidative process increases ROS formation and activates a molecular stress signaling cascade (Jaspers and Kangasjärvi 2010), which associates proline metabolism to oxidative stress as response mechanisms. Our results showed a relationship between proline catalysis and the generation of ROS, which causes an overexpression of important genes related to the ascorbate/glutathione pathway. Consequently, ASC metabolism is also overexpressed to keep oxidative metabolism active, as ASC is the main substrate for this pathway. With these evidences, this experiment intended to interconnect the oxidation of proline with glutathione redox homeostasis for an efficient detoxification of ROS (Chapter V, Figure 4). Tang and Pag (2016), demonstrated that in infected human cells, the catabolism of proline to P5C was induced thanks to the action of PRODH and PROC, to maintain ROS homeostasis. In plants, it is still necessary to deepen the knowledge of this mechanism, but Qamar et al. (2015) proposed it as a response mechanism in *Arabidopsis thaliana* attacked by pathogens. In a previous study by our research group (Rivero et al. 2014), it was shown that under the combination of salinity and high temperature, PRODH and PROC accumulated significantly at the gene and protein levels. This did not occur under salinity conditions, where the accumulation of proline was favored. This study provides evidence that proline oxidation might act as a signaling mechanism for ROS, but more studies are needed to confirm this hypothesis.

Plants have developed a strong antioxidant system to prevent ROS-related damage, consisting of several enzymes (SOD, CAT, and the enzymes of the ascorbate-glutathione cycle) and low molecular weight antioxidant compounds, such as ascorbic acid and glutathione, among others (Noctor and Foyer 1998; Foyer and Noctor 2005). Glutathione (GSH) is one of the main cellular antioxidant compounds. GSH is involved in several important functions related to cellular protection against most environmental stresses, as well as in the direct neutralization of free radicals and reactive oxygen compounds, in the regulation of the nitric oxide cycle, in the synthesis and repair of DNA, in the synthesis of proteins, in the transport of amino acids and the direct activation of enzymes (Noctor and Foyer 1998; Foyer and Noctor 2005; Sharma and Dietz 2009), among others. GSH is also involved in the formation of phytochelatins, and together with its oxidized form (GSSG) it completes the redox couple (GSH/GSSG) necessary for the maintenance of cell homeostasis and plant signaling systems (Rauser 1995; Clemens 2006; Srivalli and Khanna-Chopra 2009). This leads us to suggest that the GSH/GSSG ratio, an indicator of the redox balance of the cell, could be involved in the perception of ROS (Aon et al. 2010; Suzuki et al. 2012).

ASC, as commented previously for GSH, is an antioxidant compound with a strong involvement in oxidative metabolism through the Halliwell-Asada cycle. The activity of the main enzymes involved in this cycle were measured to confirm the overexpression of oxidative metabolism at the protein level (Chapter V, Figure 4). Several authors have previously confirmed the activation of oxidative metabolism under salinity and heat conditions (Rivero et al. 2014; Martinez et al. 2018; García-Martí et al. 2019). In this sense, all the results found in our study at the gene, protein and metabolite levels were in agreement, corroborating the efficient detoxification of H_2O_2 and lipid recovery from oxidation thanks to PhGPX enzymatic activity under these particular conditions. This evidence helps explain the better state of plants subjected to the combination of stresses as compared to those that were subjected only to salinity stress.

6.4.3. Regulation induced by the combination of salinity and heat: identification of specific transcription factors

Transcription factors (TFs) are proteins that bind to specific DNA sequences, thus controlling the transcription of genetic information from DNA to messenger RNA. Transcription factors may do this alone, but more commonly in synchronization with other protein complexes by promoting (as an activator) or silencing (as a repressor) the recruitment of RNA polymerase to specific genes.

In recent years, some authors have demonstrated the involvement of TFs in the stress response. Zandalinas et al. (2020a) have shown in *Arabidopsis thaliana*, that the exposure to different combinations of stresses caused a unique transcriptional profile especifically regulated by different families of TFs. In this study (Lopez-Delacalle et al. 2021b), we showed that the TFs families MYB, bZYP and bHLH were differentially expressed. Furthermore, unique to the stress combination experimental conditions, several genes of the TF C2H2 or Trihelix families were differentially expressed (Chapter V, Figure 6). Many of these TF families have been associated to cell division or various physiological processes, but also in response to different abiotic stresses (Kaplan-Levy et al. 2014; Agarwal et al. 2019). Kaplan-Levy et al. (2014) showed that the TF family Trihelix is involved in the response to the combined stress of salinity and pathogens, acting on stomata opening, trichome development and abscission of the seed. The bZIP family has been reported to be associated to the productivity of plants and the mitigation of the adverse effects caused by stress (Agarwal et al. 2019). In this same sense, Kiełbowicz-Matuk (2012) demonstrated the involvement of the TF C2H2-type zinc finger family in the response to abiotic stress and the transduction of the hormonal signal. The C2H2-type zinc finger family has been studied mainly in *Arabidopsis thaliana*. Among the few studies on its involvement in tomato, its role in the regulation of some genes related to heat stress has been reported (Hu et al. 2019). Of special interest is the relationship between the overexpressed TFs, aligned with the promoter regions of many of the genes studied in this work, with those that belong to ASC, proline, and oxidative metabolism.

In summary, this work shows the interconnection between ASC, proline and oxidative metabolism in acclimatization to the specific combination of salinity and heat, which allows the maintenance of cellular homeostasis and an adequate redox balance, thanks to the signaling mechanism triggered.

CHAPTER VII:

CONCLUSIONS

7. Chapter VII. Conclusions

I. The tolerant tomato line, RIL-76, under the combination of salinity and heat stress, showed greater growth compared to the sensitive lines, RIL-66 and RIL-18, subjected to the same conditions.

II. Under the combination of salinity and heat stress, a decrease in the content of K^+, Mg^{2+}, total N and organic N was induced in the sensitive line, RIL-66, compared to its control. However, this decrease did not occur in the tolerant line RIL-76, which maintained control levels.

III. The tolerant line, RIL-76, under the combination of salinity and heat, increased the efficiency of nitrogen metabolism at transcriptional and post-transcriptional levels. Specifically, the tolerant line RIL-76 showed an increase in the enzymatic activity of NR, GOGAT and GDH that could be associated to the levels of Gln, Glu, Asn, GABA, Val, Pro and Isoleu found under the combination of these stresses.

IV. The tolerant line, RIL-76, presented a higher enzymatic activity of FBPase, SPS, SUS3, G6PDH and AI compared to the sensitive line, RIL-18 under field conditions.

V. The tolerant line, RIL-76 presented a higher concentration of the sugars fructose, glucose and UDP-glucose in tomato fruits under the combination of salinity and heat in field conditions as compared to the sensitive line RIL-18.

VI. The tolerant line RIL-76 presented an overexpression of the genes that code for the sugar transporters *SlGLCT, SlINT4, SlSUT4, SlTMT1* and *SlVGT1* under stress conditions in the field, which was not observed in the sensitive line RIL-18.

VII. The commercial variety Boludo showed an increase in photosynthetic parameters under the combination of salinity and heat compared to those plants that were subjected only to the salinity condition.

VIII. Salinity and heat applied in combination showed a unique and specific transcriptomic profile with a total of 1388 differentially expressed genes, which did not show alterations under the salinity or heat conditions applied individually.

IX. The combination of salinity and heat induced a specific metabolic profile with a differential and significant concentration of 568 specifically accumulated molecular compounds that did not show alterations under these stress conditions applied individually.

X. The integration of metabolomic and transcriptomic data into known biosynthetic pathways under the combination of salinity and heat revealed a synchronization between the ascorbate and proline pathways to maintain ROS cell homeostasis.

XI. The response to the combination of salinity and heat of commercial tomato plants was associated with the positive regulation of specific transcription factors included in the bZIP, C2H2 and Trihelix families.

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