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Estudio comparativo del efecto del estrés salino en plantas de tomate. Influencia de la variabilidad genotípica

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ÍNDICE

RESUMEN/SUMMARY

Uno de los mayores retos de la agricultura mundial a día de hoy es cubrir las necesidades de producción de alimentos a nivel mundial, problema que crece cada año debido al crecimiento poblacional. Una de las soluciones es incrementar el porcentaje de tierras de cultivo disponibles para satisfacer esta demanda. En este sentido, la FAO ha registrado un aumento del porcentaje de tierras de cultivo en los últimos años. Sin embargo, no todas las tierras de cultivo presentan unas condiciones adecuadas para cultivar en ellas. Efectivamente, muchos suelos cultivables suelen presentar elevadas concentraciones de sales solubles, como el NaCl, que son perjudiciales para el desarrollo óptimo de muchos cultivos agrícolas. La salinidad está causada principalmente por esta acumulación excesiva de sales en el suelo, afectando al crecimiento y desarrollo de las plantas. El NaCl es la sal más soluble y extendida que provoca estrés salino, por lo que algunas plantas presentan mecanismos de tolerancia, aunque estos a veces no resultan lo suficientemente eficientes generando deficiencias nutricionales, alteración del metabolismo y otros daños.

La salinización de los cultivos puede darse por muchos factores, entre ellos la salinización por riego. Se estima que en torno a un tercio de la superficie mundial de las tierras agrícolas con riego están afectadas por la salinidad, y se prevé que aumente el porcentaje en el futuro. Esto es especialmente grave, ya que la superficie agrícola con regadío genera más de un tercio de la producción mundial a pesar de que constituye solo un 15% de la superficie cultivable mundial.

Por todo esto, es necesario comprender en profundidad como la salinidad afecta al metabolismo de las plantas y que posibles mecanismos de defensa pueden presentar las plantas. Conociendo estos mecanismos se podrán generar y/o seleccionar variedades más tolerantes ante este tipo de estrés. Por ello, el objetivo general de la tesis fue conocer mejor el efecto toxico del estrés salino en el metabolismo y la fisiología de dos genotipos de tomate comerciales (*Solanum Lycopersicum* L.) (Grand Brix y Marmande RAF), con el fin de arrojar una visión global e interconectada de los mismos, atendiendo también a las posibles diferencias genotípicas. El establecimiento de un amplio conocimiento en este sentido podría sentar las bases para la generación o mejora de genotipos más tolerantes/resistentes al estrés salino.

Para lograr dichos objetivos se llevaron a cabo experimentos donde se mostró como el efecto toxico del estrés salino afecta a diferentes rutas y procesos metabólicos de los genotipos de tomate seleccionados. Estos experimentos fueron agrupados y divididos en cuatro capítulos, además, dentro de estos capítulos se trató de describir los procesos fisiológicos clave donde podría residir la tolerancia al estrés salino, dejando puertas abiertas para la generación de nuevos genotipos más tolerantes a este tipo de estrés. Aunque ambos genotipos sobrevivieron al efecto toxico del estrés salino, estos presentaron algunas alteraciones en su metabolismo. A lo largo de la presente tesis doctoral se desarrolla con detalle como algunas de estas alteraciones podrían mejorar la tolerancia al estrés salino y como otras son consecuencia del efecto toxico de este estrés.

En el capítulo 1 se llevaron a cabo ensayos para comprobar el efecto de la salinidad en la generación de estrés oxidativo en las plantas (como la generación de especies reactivas de oxígeno (ROS)). Complementariamente, se estudiaron los mecanismos antioxidantes enzimáticos como la superóxido dismutasa (SOD) o la catalasa (CAT), y los no enzimáticos como el ascorbato o el glutatión que poseen las plantas para defenderse ante el estrés oxidativo. Además, en este mismo capítulo se muestra el efecto de la salinidad sobre el perfil fitohormonal y como la alteración en los niveles de las diferentes hormonas puede afectar a diferentes rutas y procesos metabólicos.

En el capítulo 2 nos centramos en el ciclo de los ácidos tricarboxilicos (TCA), para ello se estudiaron enzimas como la citrato sintasa (CS) o la malato deshidrogenasa (MDH), y los ácidos orgánicos malato, citrato y oxalacetato. En este capítulo comprobaremos como este ciclo es de vital importancia para las plantas en los procesos de resistencia a la salinidad generando energía, poder reductor, ácidos orgánicos y precursores de aminoácidos (Aas).

En el capítulo 3 se estudiaron compuestos osmoprotectores como la prolina o la glicina betaina (GB), los cuales compuestos protegen las estructuras celulares y los procesos metabólicos del estrés oxidativo generado por el estrés salino. Para comprender mejor el proceso que rodea a estos compuestos se estudiaron sus enzimas de síntesis como la pirrolina-5-carboxilato sintasa (P5CS) o la betaina aldehído deshidrogenasa (BADH), y enzimas para su degradación como la prolina deshidrogenasa (PDH).

Por último, en el capítulo 4 estudiamos uno de los procesos metabólicos más importantes para las plantas, la asimilación del nitrógeno (N), proceso que genera compuestos nitrogenados esenciales para las plantas. En este capítulo nos centramos en procesos como la principal vía de asimilación de N (el ciclo glutamina sintetasa (GS)/ glutamato sintasa (GOGAT)) o la fotorrespiración (analizando enzimas como la glioxilato oxidasa (GO) o la glioxilato aminotransferasa (GGAT)).

Además de estudiar estos procesos fisiológicos, y con el fin de evaluar el grado de tolerancia al estrés salino se utilizaron parámetros como la biomasa, la tasa de crecimiento relativa (TCR), o la concentración de Na^+. En este sentido, y tal como se describe en los diferentes capítulos de esta tesis doctoral el genotipo Grand Brix presento mayor tolerancia al estrés salino que el genotipo Marmande RAF, lo que se vio reflejado en la biomasa y en los valores de TCR. Ambos parámetros presentaron una menor disminución en el tratamiento salino en el genotipo Grand Brix. Sin embargo, la concentración de Na^+ fue mayor en este genotipo. A este respecto, en el capítulo 2 se describe como el genotipo Grand Brix pudiera estar compartimentalizando el exceso de Na^+ en las vacuolas como oxalato-Na^+, evitando de esta forma su efecto citotóxico.

Bajo condiciones de estrés salino la generación de ROS y el perfil fitohormonal se ven alterados, y estos a su vez interfieren en el metabolismo de la planta. Es por esto que en el capítulo 1 se describe con más detalle la influencia del estrés oxidativo y las fitohormonas sobre el metabolismo de las plantas. Grand Brix

mostró niveles más bajos de las principales ROS (O_2^- y H_2O_2) y de indicadores de daño oxidativo como el malondialdehido (MDA), lo que pudo favorecer que los procesos metabólicos y estructuras celulares estuvieran menos afectadas mejorando la tolerancia al estrés salino de forma directa e indirecta. No obstante, en el estudio de los diferentes sistemas antioxidantes no hubo una clara diferencia entre genotipos, siendo por ejemplo la actividad SOD inducida de forma similar bajo estrés salino para ambos genotipos, mientras que la actividad CAT mostró un mayor incremento en Marmande RAF en condiciones de salinidad. Sin embargo, la concentración de ascorbato fue mayor en el genotipo Grand Brix, más tolerante a la salinidad. Con respecto a las fitohormonas, uno de los muchos procesos metabólicos en los que tienen influencia es la generación y detoxificación de ROS. Pudimos observar en el genotipo Grand Brix un aumento significativo de algunas fitohormonas como el ácido abscísico (ABA), ácido indolacético (AIA), ácido salicílico (SA) y una disminución de aminociclopropano carboxílico (ACC), precursor del etileno. Los cambios en la concentración de estas fitohormonas podrían estar generando una mayor tolerancia al estrés salino en este genotipo, ya que estas fitohormonas regulan procesos como el desarrollo y crecimiento de las plantas, la senescencia o el cierre estomático, además de favorecer el aumento de la actividad de algunas enzimas como las relacionadas con la asimilación del N.

Otro proceso metabólico de gran importancia para las plantas es el ciclo TCA, ya que interviene en la producción de energía, ácidos organicos y formación de precursores para la formación de Aas. En condiciones de estrés salino los ácidos orgánicos generados en este ciclo mejoran los desbalances osmóticos

provocados por los iones Na^+ y Cl^-, y previenen el daño oxidativo. Por ello en el capítulo 2 se estudió el efecto toxico de la salinidad sobre este proceso metabólico. En nuestro estudio el efecto toxico del estrés salino fue menos perjudicial en el genotipo Grand Brix, el cual mostró un incremento de todas las actividades enzimáticas que intervienen en este ciclo y de las concentraciones de los ácidos orgánicos malato y citrato. Esto podría ayudar al mantenimiento energético y mejorar la regulación osmotica, procesos que podrían ser clave en la tolerancia a la salinidad. Por otro lado, una mayor actividad enzimática del ciclo TCA podría estar proporcionando precursores para la asimilación de N en Grand Brix como el α-cetoglutarato (α-KG) y generar así compuestos nitrogenados relacionados con la resistencia al estrés salino.

En este sentido las rutas metabólicas para la generación de los compuestos nitrogenados prolina y GB han sido también estudiadas por su implicación en la tolerancia ante estreses abióticos como el estrés salino. La presencia de estos compuestos ayudan a la prevención del estrés osmotico en plantas, contrarrestando el efecto citotóxico de algunos iones como el Na^+ y el Cl^- en el caso del estrés salino. Por ello, en el capítulo 3 se describe la influencia de la prolina y la GB sobre la tolerancia a la salinidad. En este caso ambos genotipos acumularon prolina bajo condiciones de estrés salino. Sin embargo, la vía de síntesis de este Aa fue diferente en ambos genotipos. Pudimos observar que la vía principal de síntesis de prolina (P5CS) no presentó un incremento significativo en ambos genotipos, lo que no se corresponde con la acumulación de prolina que si encontramos en los mismos. Sin embargo, en el genotipo Grand Brix se observó el incremento de la enzima Ornitina-δ-aminotransferasa (OAT),

la cual es una vía secundaria para la generación de prolina que podría verse favorecida en plantas bajo estrés abiótico como el estrés salino. Por otro lado, la principal enzima para la degradación de prolina (PDH) presento una disminución de su actividad para ambos genotipos, sin embargo, esta reducción fue menor en el genotipo Marmande RAF degradándose así mas prolina en este genotipo. Por tanto, la acumulación de prolina en el genotipo Grand Brix viene proporcionada por una síntesis a través de esta vía secundaria y una menor actividad de la PDH, mientras que en el genotipo Marmande RAF la acumulación de prolina podría estar generada por una degradación proteica a consecuencia del estrés salino.

En el capítulo 4 se estudia el efecto toxico de la salinidad sobre la asimilación del N, uno de los procesos metabólicos principales en las plantas, ya que genera compuestos nitrogenados, como los Aa, esenciales para el crecimiento y desarrollo de las plantas. Además, la ruta de asimilación de N genera precursores/sustratos para otras rutas o procesos metabólicos como oxalacetato para el ciclo TCA o glutamato para la síntesis de prolina, mejorando no solo la biosíntesis de proteínas sino la de compuestos osmoprotectores, formación de energía en forma de ATP y poder reductor. En el genotipo Marmande RAF el efecto toxico del estrés salino no altero la actividad enzimática de la nitrato reductasa (NR), sin embargo en líneas generales provoco una disminución de las actividades enzimáticas en este genotipo, tanto en el ciclo GS/GOGAT (principal ruta de asimilación del NH_4^+) como en la fotorrespiración, disminuyendo la tasa de asimilación de N y obteniendo una menor concentración de N total en la planta.

En líneas generales el genotipo Grand Brix presentó una menor concentración de los principales ROS, una menor concentración de MDA y un mejor perfil fitohormonal, lo que favorece que los procesos metabólicos no se vean tan afectados por el estrés salino. Además, Grand Brix presento una mayor actividad de las enzimas implicadas en el ciclo TCA pudiéndose generar así más energía y poder reductor los cuales podrían ser usados en diferentes procesos metabólicos (como la asimilación del N o la generación de compuestos osmoprotectores). Por último, una mayor actividad en las principales enzimas de asimilación de N proporcionara en el genotipo Grand Brix una constante tasa de generación de proteínas lo cual es fundamental para el correcto desarrollo de las plantas.

En definitiva, la comprensión del metabolismo de la planta, como se interrelacionan los procesos metabólicos y como estos pueden mejorar la tolerancia al estrés salino es fundamental para poder generar genotipos más resistentes a dicho estrés. En este trabajo de tesis doctoral se ha avanzado en este conocimiento, mostrando una visión global de los procesos metabólicos estudiados en ambos genotipos, dejando abiertas líneas de trabajo para la generación de genotipos más resistentes.

Summary

One of the biggest challenges of world agriculture today is to take care of the need for food production worldwide, this problem grows every year due to population growth. One of the solutions is to increase the percentage of available farmland to satisfy this demand. In this sense, FAO has registered an increase in the percentage of farmland in recent years. However, not all farmland has adequate conditions to be cultivated. Many cultivable soils usually have high concentrations of soluble salts, such as NaCl, which are detrimental to the optimal development of many agricultural crops. Salinity is mainly caused by this excessive accumulation of salts in the soil, affecting the growth and development of plants. NaCl is the most soluble and widespread salt that causes saline stress, so some plants have tolerance mechanisms. However, sometimes they are not enough efficient, it generates nutritional deficiencies, impaired metabolism and other damage.

The salinization of crops can occur due to many factors, including salinization by irrigation. It is estimated that around one-third of the world's area of irrigated agricultural land is affected by salinity, and the percentage is expected to increase in the future. This is especially serious since irrigated agricultural land generates more than a third of world production despite the fact that it constitutes only 15% of the world's arable land.

For all this, it is necessary to understand in depth how salinity affects the metabolism of plants and the possible defense mechanisms plants can present.

Knowing these mechanisms more tolerant varieties can be generated and/or selected for this stress. Therefore, the general objective of this thesis was to know better the toxic effect of saline stress on the metabolism and physiology of two commercial tomato genotypes (*Solanum Lycopersicum* L.; Grand Brix and Marmande RAF), throwing a global and interconnected vision of them, also attending to Possible genotypic differences. The establishment of extensive knowledge in this regard could be essential for the generation or improvement of more tolerant/resistant genotypes to saline stress. Although both genotypes survived the toxic effect of saline stress, these presented some alterations in their metabolism. Throughout this doctoral thesis, it's developed in detail how some of these alterations could improve tolerance to saline stress and how others are a consequence of the toxic effect of this stress.

To achieve these objectives, experiments were carried out where it was shown how the toxic effect of saline stress affects different pathways and metabolic processes of our selected tomato genotypes. These experiments were grouped and divided into four chapters, in addition, within these chapters we tried to describe the key physiological processes where tolerance to saline stress could reside, leaving open doors for the new genotypes generation more tolerant to this type of stress.

Chapter 1 tests were carried out to verify the salinity effect on the oxidative stress generation in plants (such as the reactive oxygen species generation (ROS)). In addition, enzymatic antioxidant mechanisms such as superoxide dismutase

(SOD) or catalase (CAT) and non-enzymatic mechanisms such as ascorbate or glutathione were studied, the plants possess these defenses against oxidative stress. In addition, this same chapter shows the salinity effect on the phytohormonal profile and how the levels alteration in the different phytohormons could be affects different pathways and metabolic processes.

In Chapter 2 we focus on the tricarboxylic acids cycle (TCA), for this, we studied enzymes such as citrate synthase (CS) or malate dehydrogenase (MDH) and organic acids malate, citrate and oxaloacetate. In this chapter check how this cycle is really important for plants in salt resistant process, generating energy, reducing power, organic acids and amino acid precursors (Aas).

In Chapter 3, osmoprotective compounds such as proline or glycine betaine (GB) were studied, these compounds protect cellular structures and metabolic processes from oxidative stress generated by saline stress. To better understand the process surrounding these compounds, their synthesis enzymes such as pyrroline-5-carboxylate synthase (P5CS) or betaine aldehyde dehydrogenase (BADH) and enzymes for degradation such as proline dehydrogenase (PDH) were studied.

Finally, in Chapter 4 we study one of the most important metabolic processes for plants, the nitrogen (N) assimilation, this generates nitrogen compounds essential for plants. In this chapter, we focus on processes such as the main N assimilation pathway (the glutamine synthetase (GS)/glutamate synthase

(GOGAT) cycle) or photorespiration (analyzing enzymes such as glyoxylate oxidase (GO) or glyoxylate aminotransferase (GGAT)).

Also, study these physiological processes and to assess the tolerance degree to saline stress, parameters such as biomass, relative growth rate (RGR), or Na^+ concentration were used. In this sense, and as described in the different Chapters of this doctoral thesis, the Grand Brix genotype showed a greater saline stress tolerance than the Marmande RAF genotype, which was reflected in biomass and RGR values. Both parameters showed a smaller decrease in saline treatment in the Grand Brix genotype. However, the Na^+ concentration was higher in this genotype. Chapter 2 describes how the Grand Brix genotype could be compartmentalizing excess Na^+ in vacuoles as oxalate-Na^+, thus avoiding its cytotoxic effect.

Under saline stress conditions, the ROS generation and the phytohormonal profile are altered, and these interfere with the plant metabolism. This is why in Chapter 1 the oxidative stress and phytohormones influence on plant metabolism is described in more detail. Grand Brix showed lower levels of the main ROS (O_2^- and H_2O_2) and oxidative damage indicators such as malondialdehyde (MDA), which could favor metabolic processes and cellular structures being less affected by improving saline stress tolerance directly and indirectly. However, the antioxidant systems study there was no clear difference between genotypes, SOD activity was similarly induced under saline stress for both genotypes, while CAT activity showed a greater increase in Marmande RAF under salt conditions.

However, ascorbate concentration was higher in Grand Brix genotype, most salt tolerant. regarding phytohormones, they have an influence on the ROS generation and detoxification. We were able to observe in the Grand Brix genotype a significant increase in some phytohormones such as abscisic acid (ABA), indolacetic acid (IAA), salicylic acid (SA) and a decrease in carboxylic aminocyclopropane (ACC), a precursor to ethylene. Changes in the concentration of these phytohormones could be generating a greater tolerance to saline stress in this genotype, since these phytohormones regulate processes such as the development and growth of plants, senescence or stomatic closure, in addition to favoring the increase in activity of some enzymes such as enzymes for the assimilation of N.

Another metabolic process of great importance for plants is the TCA cycle, since it is involved in the energy production, organic acids and precursors formation for the Aas generation. Under saline stress conditions, the organic acids generated in this cycle improve the osmotic imbalances caused by the Na^+ and Cl^- ions and prevent oxidative damage. Therefore, chapter 2 studied the toxic effect of salinity on this metabolic process. In our study, the toxic effect of saline stress was less harmful in the Grand Brix genotype, which showed an increase in all the enzymatic activities involved in this cycle and in the organic acids malate and citrate concentrations. This could help to energy maintain and improve osmotic regulation, processes that could be key in salinity tolerance. On the other hand, the greater enzymatic activity of the TCA cycle could be providing precursors for the N assimilation in Grand Brix such as α-ketoglutarate (α-KG) and thus generate nitrogen compounds related to saline stress resistance.

In this sense, the metabolic pathways for the proline and GB generation have also been studied for their involvement in tolerance to abiotic stresses such as saline stress. This is because these compounds help prevent osmotic stress in plants, counteracting the cytotoxic effect of some ions such as Na^+ and Cl^- in the case of saline stress. Therefore, chapter 3 describes the proline and GB effect on salinity tolerance. In this case both genotypes accumulated proline under saline stress conditions, however, the synthesis pathway of this Aa was different in both genotypes. We could observe that the main pathway of proline synthesis (P5CS) did not show a significant increase in both genotypes, which does not correspond to the accumulation of proline that we find in them. However, in the Grand Brix genotype the increase of the enzyme Ornithine-δ-aminotransferase (OAT) was observed, which is a secondary pathway for the proline generation that could be favored in plants under abiotic stress such as saline stress. On the other hand, the main enzyme for proline degradation (PDH) showed a decrease in its activity for both genotypes, however, this reduction was lower in the Marmande RAF genotype thus degrading more proline in this genotype. Therefore, the proline accumulation in the Grand Brix genotype is provided by a synthesis through this secondary pathway and lower activity of the PDH, while in the Marmande RAF genotype the proline accumulation could be generated by a protein degradation to a consequence of saline stress.

Chapter 4 we study the salinity toxic effect on the N assimilation, one of the main metabolic processes in plants, as it generates nitrogen compounds, such as Aa, essential for plant growth and development. Also, the N assimilation pathway generates precursors/substrates for other pathways or metabolic processes such

as oxaloacetate for the TCA cycle or glutamate for proline synthesis, improving the protein biosynthesis, osmoprotective compounds, formation of ATP energy and power reducer. In the Marmande RAF genotype the toxic effect of saline stress does not alter the enzymatic activity of nitrate reductase (NR), however in general terms it caused a decrease in the enzymatic activities in this genotype, both in the GS/GOGAT cycle (main route of NH_4^+ assimilation) and in photorespiration, reducing the N assimilation rate and obtaining a lower concentration of total N in the plant.

In general, the Grand Brix genotype showed a lower concentration of the main ROS, a lower MDA concentration and a better phytohormonal profile, which favors that the metabolic processes are not so affected by saline stress. In addition, Grand Brix presented a greater enzymes activity involved in the TCA cycle could be generated more energy and reducing power, which will be used in different metabolic processes (such as the N assimilation or the generation of the osmoprotective compound). Finally, greater activity of the main N assimilation enzymes would provide a constant protein generation rate in Grand Brix genotype which is essential for the proper plants development.

In short, understanding the plant metabolism, how metabolic processes are interrelated and how they can improve saline stress tolerance is essential to generate genotypes more resistant to such stress. This doctoral thesis work has advanced in this knowledge, showing a global vision of the metabolic processes

studied in both genotypes, leaving open lines of work for the generation of more resistant genotypes.

INTRODUCCIÓN GENERAL

1. Concepto de estrés salino en los cultivos

El estrés salino para los cultivos se pude definir como todo aquel suelo o medio de cultivo que presente altas concentraciones de sales solubles, como el cloruro de sodio (NaCl) (la sal más soluble), magnesio o calcio, y un porcentaje de sodio intercabiable (PSI) >15% (Munns 2009; Sheng et al. 2010). Sin embargo, podemos observar una acumulación en los suelos de diferentes tipos de sales solubles y un PSI que puede ser variable, dando como resultado diferentes "tipos de suelos" definidos como: suelos salinos, sódicos, alcalinos o salino-sódicos (Vargas et al. 2018).

Muchos autores indican que un suelo es salino cuando su conductividad eléctrica (EC) >4 dS m^{-1} (~36 mM NaCl) medido a 25ºC (Chinnusamy et al. 2005; Wicke et al. 2011). Sin embargo, más recientemente Vargas et al. (2018) publicaron en la Organización Mundial de las Naciones Unidas (FAO), que un suelo podría ser considerado afectado por estrés salino con una EC >2 dS m^{-1} (~18 mM NaCl) medido a 25ºC (Vargas et al. 2018).

En la introducción de esta tesis doctoral nos centraremos en el estrés salino causado por NaCl, cuyas características, según Vargas et al (2018), son:

- Un PSI >15% capaz de interferir con el crecimiento de la mayoría de las plantas de cultivo.
- Un contenido apreciable de sales solubles con una EC >2 dS m^{-1} (a 25°C).
- Un pH generalmente ≤8.5 en el suelo saturado.

2. Estrés salino como problema en la agricultura mundial

Debido al crecimiento poblacional las necesidades de producción de alimentos a nivel mundial crecen cada año. Una de las soluciones es incrementar el porcentaje de tierras de cultivo disponibles para satisfacer esta demanda. En este sentido la FAO (1990-2016) ha registrado un aumento del porcentaje de tierras de cultivo en los últimos años (Imagen 1).

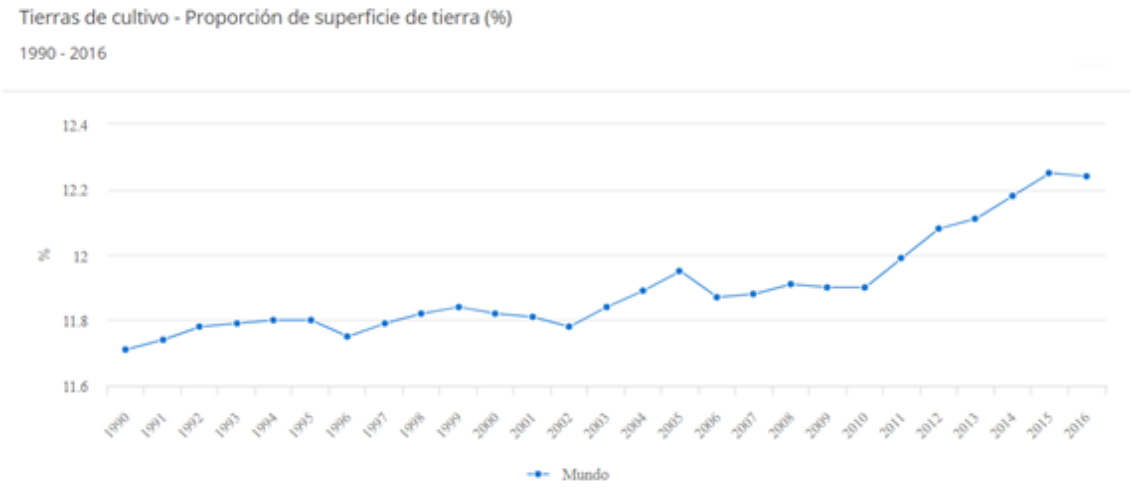

Imagen 1: Representación gráfica del incremento en el porcentaje de tierras de cultivo en el mundo según la FAO para los años 1990-2016 (FAO 1990-2016).

Sin embargo, no todas las tierras de cultivo presentan unas condiciones adecuadas para cultivar en ellas (FAO et al. 2012). Uno de los problemas que suelen presentar son las elevadas concentraciones de sales solubles, como el NaCl, que son perjudiciales para el desarrollo óptimo de muchos cultivos agrícolas (Imagen 2). Por ejemplo, en España según la base de datos de Harmonized World Soil Database (HWSD), prácticamente todo el estrés salino presente muestra una EC >8 dS m^{-1} y un PSI>15% (Imagen 3).

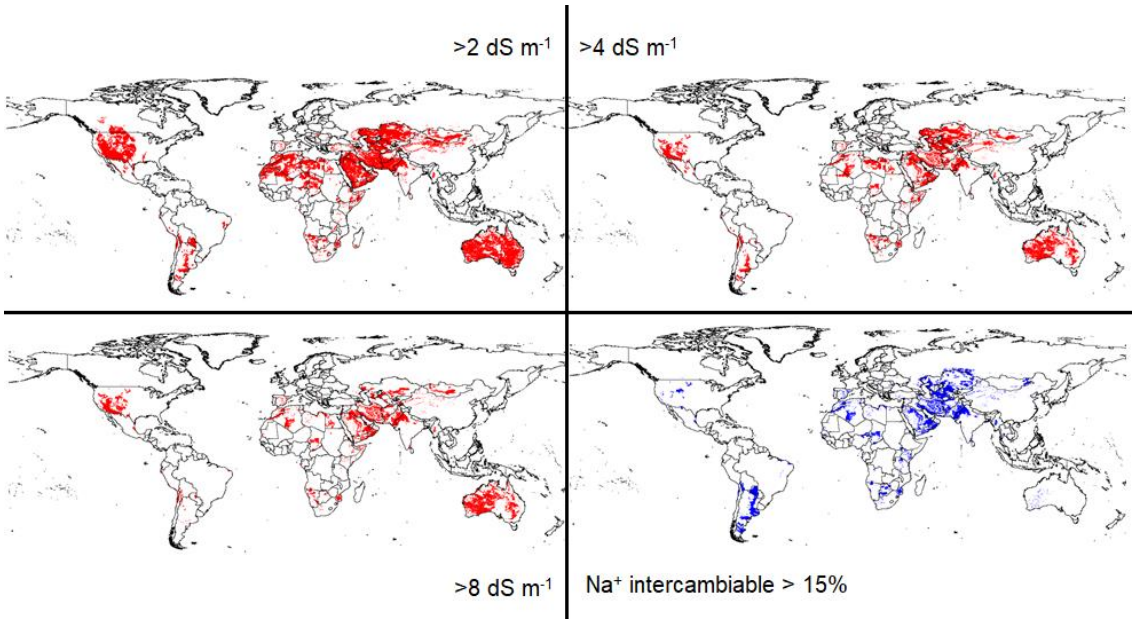

Imagen 2: Representación visual de la distribución mundial de los suelos afectados por el estrés salino. En rojo se representan los suelos con una conductividad >2 dS m^{-1}, >4 dS m^{-1} y >8 dS m^{-1} tanto en superficie (0-30 cm) como en el subsuelo (30-100cm). En azul se representan aquellos suelos con un PSI >15% tanto en superficie (0-30 cm) como en el subsuelo (30-100cm). Datos extraidos de Harmonized World Soil Database (HWSD) (version 1.2), (Consultado 17.06.19)

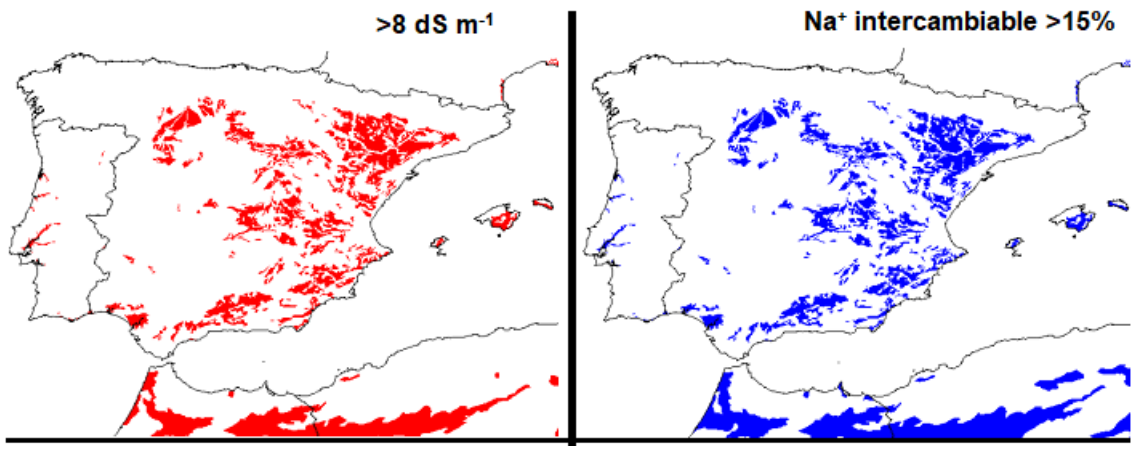

Imagen 3: Representación visual de la distribución de los suelos afectados por el estrés salino en españa. En rojo se representan los suelos con una conductividad >8 dS m^{-1} tanto en superficie (0-30 cm) como en el subsuelo (30-100cm). En azul se representan aquellos suelos con un PSI >15% tanto en superficie (0-30 cm) como en el subsuelo (30-100cm). Datos extraidos de Harmonized World Soil Database (HWSD) (version 1.2), (consultado 17.06.19)

La salinización de los cultivos puede darse por muchos factores, entre ellos la salinización por riego. Se estima que en torno a un tercio de la superficie mundial de las tierras agrícolas con riego están afectadas por la salinidad, y se prevé que aumente el porcentaje en el futuro (Mateo-Sagasta y Burke 2010; Abbas et al. 2013). Esto es especialmente grave, ya que la superficie agrícola con regadío genera más de un tercio de la producción mundial a pesar de que constituye solo un 15% de la superficie cultivable mundial (Munns y Tester 2008).

3 Factores que influyen en la salinidad de los cultivos

Existen ciertas condiciones ambientales y geográficas que precondicionan que un suelo sea salino, factores como: degradación de las rocas, actividad volcánica, aguas de superficie o subterráneas, sales marinas transportadas por el viento o la lluvia, un drenaje limitado o un balance negativo de agua (Bosco et al. 2008; Munns y Tester 2008; Munns 2009).

Sin embargo, también existen factores humanos que favorecen la salinización de los suelos, como el uso de aguas de riego con un alto contenido en NaCl o el uso excesivo de fertilizantes en agricultura intensiva (Bosco et al. 2008). El riego excesivo puede elevar los niveles freáticos de acuíferos salinos, y esto puede aumentar la infiltración de aguas subterráneas salinas en cursos de agua y aumentar su salinización. La intrusión de agua de mar en los acuíferos es otra causa importante de la salinización de los recursos hídricos en las zonas costeras. Esta intrusión es con frecuencia el resultado de extracciones excesivas de agua subterránea para la agricultura (Feng et al. 2017). La industrialización

es otro problema que saliniza el agua de los ríos con la descarga de agua salina provenientes de industrias y actividades mineras (Mateo-Sagasta y Burke 2010).

4 Efecto toxico del estrés salino y mecanismos de respuesta en plantas

La salinidad como hemos explicado previamente está causada principalmente por una acumulación excesiva de sales en el suelo, afectando al crecimiento y desarrollo de las plantas. El NaCl es la sal más soluble y extendida que provoca estrés salino a las plantas, por lo que algunas plantas presentan mecanismos de tolerancia. Sin embargo, a veces no resultan lo suficientemente eficientes generando deficiencias nutricionales, alteración del metabolismo y otros daños (Munns y Tester 2008; Acosta-Motos et al. 2017).

4.1 Estrés osmótico

El primer efecto del estrés salino sobre la planta es un estrés osmótico. Este esta generado por la acumulación excesiva de sales en el suelo, que ya comienza a repercutir en la planta incluso antes de absorberlas afectando al crecimiento de la misma (Acosta-Motos et al. 2017). El estrés osmótico generado provoca un menor crecimiento en las hojas, una emergencia más lenta de hojas nuevas y las yemas laterales se desarrollan más lentamente o permanecen inactivas, por lo que se forman menos ramas laterales (Munns y Tester 2008). Curiosamente esta reducción del crecimiento es más severa en hojas que en raíz, al igual que pasa con el estrés hídrico. Este hecho se debe a que una reducción del área foliar se traduce en una menor tasa de absorción de agua evitando de este modo

la absorción de sales, lo cual puede provocar estrés hídrico indirecto (Munns y Tester 2008).

Las plantas poseen mecanismos de defensa, frente a este estrés osmótico, como la acumulación de solutos osmocompatibles en la célula para compensar la presión osmótica, y para permitir que los intercambios a través de los transportadores sean posibles. Algunos de estos solutos osmocompatibles son por ejemplo la prolina o la glicina betaina (GB). No obstante, la acumulación de estos solutos es además una estrategia de tolerancia en sí misma, ya que evita los daños por estrés osmótico (Liang et al. 2018). Sin embargo, las rutas metabólicas que regulan la síntesis y degradación de prolina y GB también se ven afectadas por el efecto del estrés salino (Ashraf y Foolad 2007; Mansour y Ali 2017; Per et al. 2017).

La prolina puede ser sintetizada a partir del glutamato o de la ornitina (Imagen 4), pudiendo funcionar ambas vías al mismo tiempo. Sin embargo, la vía de síntesis a partir del glutamato se considera la vía principal de síntesis de prolina. La síntesis de prolina puede darse en el cloroplasto, el citosol o la mitocondria, aunque el citosol es el principal medio donde se lleva a cabo la síntesis (Per et al. 2017). La prolina sintetizada desde glutamato es sintetizada en dos reacciones sucesivas llevadas a cabo por la pirrolina-5-carboxilato sintasa (P5CS) y la pirrolina-5-carboxilato reductasa (P5CR) (Imagen 4). La P5CS genera pirrolina-5-carboxilato (P5C) a partir del glutamato, pudiendo pasar el P5C de forma espontánea a glutamico-5-semialdehido (GSA) (Imagen 4).

Finalmente, estos dos compuestos serán reducidos a prolina por la P5CR (Mansour y Ali 2017; Per et al. 2017). En la vía de la ornitina, esta es transaminada a P5C por la Ornitina-δ-aminotransferasa (OAT) entrando en el ciclo de síntesis de prolina (Imagen 4). En las diferentes estrategias de regulación de la concentración prolina es importante destacar la función de la prolina deshidrogenasa (PDH) la cual degrada prolina hasta P5C (Imagen 4) y es regulada por la propia concentración de prolina (Mansour y Ali 2017; Per et al. 2017).

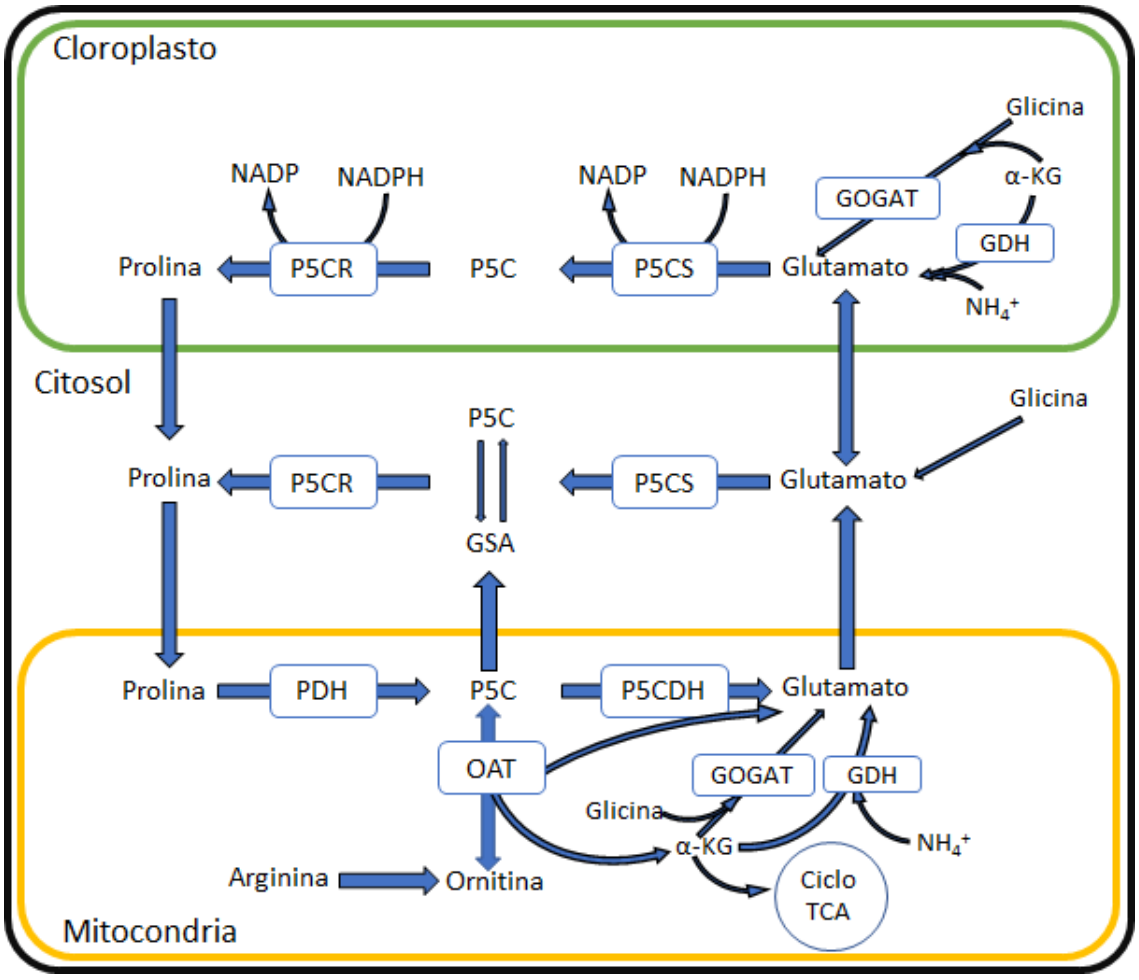

Imagen 4: Representación esquemática de las rutas de síntesis y degradación de prolina. Alfa-cetoglutarato (α-KG), Pirrolina-5-carboxilato deshidrogenasa (P5CDH), Glutamato sintasa (GOGAT), Glutamato deshidrogenasa (GDH).

La síntesis de GB se genera en el cloroplasto mediante la enzima betaina aldehído deshidrogenasa (BADH), que convierte la betaina aldehído en GB usando como cofactor la oxidación de una molecula de NADH (Fitzgerald et al. 2009; Hussain Wani et al. 2013).

Alrededor de la acumulación de estos solutos osmocompatibles existe una gran controversia. Hay autores que apoyan la teoría de que estos se acumulan en un intento por reducir el estrés osmotico generado por el estrés salino en las plantas. En este sentido, autores como Sarabi et al. (2017) mostraron en diferentes genotipos de *Cucumis melo* L. un incremento de la prolina en los genotipos más tolerantes. En el caso de la GB, autores como Chen y Murata (2011) describen su acumulación en las plantas bajo estrés salino mejorando la tolerancia de las misma a dicho estrés.

Sin embargo, otros autores defienden la idea de que estos compuestos son moleculas indicadoras de estrés y que su acumulación no favorece la tolerancia al estrés salino. Autores como Hannachi y Van Labeke (2018), en un estudio con diferentes cultivares de *Solanum melongena* L., mostraron un menor incremento de la prolina en los cultivares más tolerantes. En este mismo sentido, autores como Heuer (2003) mostraron un efecto negativo de la aplicación de prolina y GB exógenas sobre el crecimiento de plantas de tomate sometidas a estrés salino.

Por tanto, el daño osmótico derivado de la salinidad y sus posibles mecanismos de tolerancia han de ser estudiados en profundidad. Ya que uno de los principales daños del estrés salino es la alteración del potencial osmótico, algunos autores creen que la tolerancia a la salinidad puede residir en una mayor actividad de las enzimas de síntesis de estos solutos osmoprotectores combinada con una menor actividad de las enzimas para su degradacion (Munns y Tester 2008; Liang et al. 2018).

4.2 Estrés iónico

Si la acumulación de sales en el suelo perdura en el tiempo la planta empezara a absorber los iones de estas sales incrementando de esta manera los daños por estrés salino. El mecanismo por el cual las plantas bajo estrés salino absorben sodio (Na^+) y el cloruro (Cl^-) es un mecanismo muy complejo. Tal como se observa en la imagen 5. la absorción ocurre via simplastica y apoplastica, proceso de absorción en el cual están implicados más de una docena de transportadores diferentes (Isayenkov y Maathuis 2019).

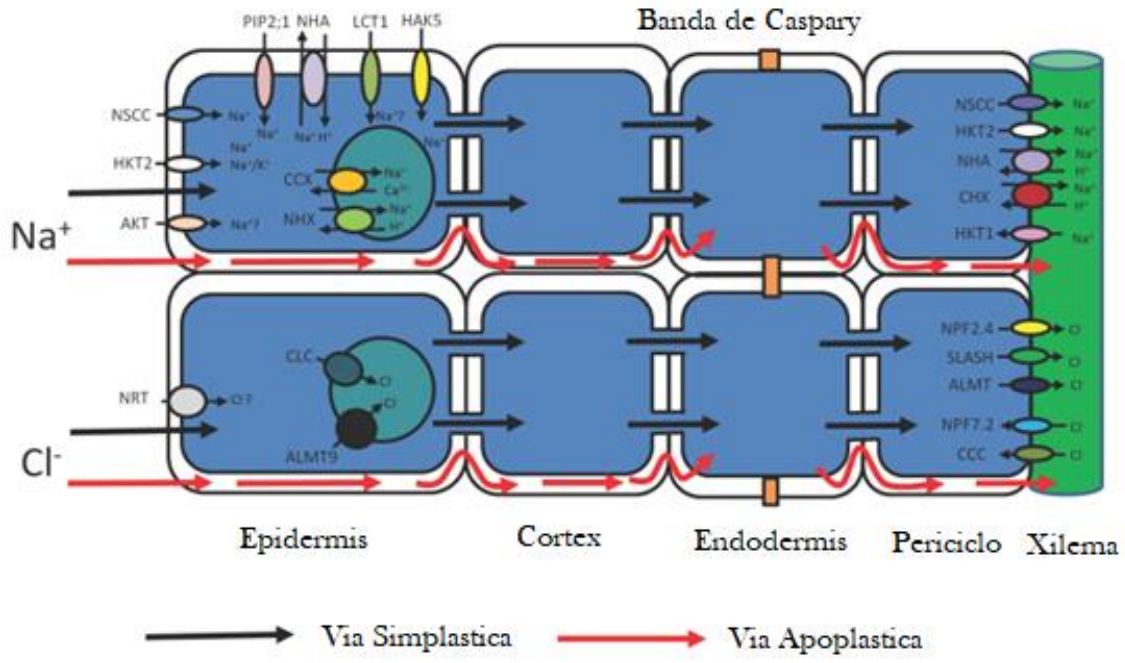

Imagen 5: Adaptación de Isayenkov y Maathuis (2019). Representación grafica del mecanismo de entrada del Na^+ y Cl^- en las plantas.

Como ya hemos indicado la absorción excesiva de Na^+ y Cl^- también genera una toxicidad en si misma alterando el metabolismo y la ionómica de la planta (Acosta-Motos et al. 2017). Por lo general, un exceso en la absorción de estos iones provoca una reducción en la absorción y una alteración en la distribución de otros nutrientes como el potasio (K^+), calcio (Ca^{2+}), nitratos (NO_3^-) o fosfato (PO_4^-) (Acosta-Motos et al. 2017; Liang et al. 2018; Isayenkov y Maathuis 2019). Con el fin de evitar estas alteraciones metabólicas y el efecto toxico de la acumulación de Na^+ y Cl^- algunas plantas han desarrollado estrategias de defensa como:

- La exclusión, donde el Na^+ y el Cl^- son expulsados directamente a la rizosfera a través de canales iónicos específicos, evitando de esta manera el estrés osmótico e ionico generado por la absorción de estos iones (Wu 2018).

- Acumulación de K^+ y su concentración relativa con respecto al Na^+ puede constituir, en si mismo, un mecanismo de defensa ante el estrés salino (Munns y Tester 2008), lo que se debe a las similares características bioquímicas del K^+ y el Na^+. El K^+ puede competir por la entrada de Na^+ en la raíz y en las células, por lo que si la planta logra mantener un ratio Na^+/K^+ bajo podría mejorar su tolerancia al estrés (Liang et al. 2018; Isayenkov y Maathuis 2019).

- Compartimentalización, que constituye otro importante y común mecanismo de tolerancia, donde los iones Na^+ son recluidos en el interior de las vacuolas mediante antiportadores Na^+/H^+ evitando así las alteraciones osmóticas y bioquímicas que genera (Acosta-Motos et al. 2017; Liang et al. 2018; Isayenkov y Maathuis 2019). Sin embargo, para la compartimentalización se requiere un incremento consecuente de la presión osmótica en el citosol, lo que puede darse por ejemplo mediante una acumulación de K^+ o compuestos osmocompatibles (Munns y Tester 2008).

4.3 Estrés oxidativo y sistemas antioxidantes

Si el estrés salino se mantiene en el tiempo se empiezan a generar otros problemas en las plantas. En las hojas adultas empiezan a acumularse sales que

la planta no puede diluir lo que provoca desajustes en el metabolismo de la planta que da lugar por lo general a la formación de especies reactivas de oxígeno (ROS), y a la aparición de daño y muerte celular (Acosta-Motos et al. 2017; Isayenkov y Maathuis 2019). Además, si la tasa de crecimiento de hojas jovenes es inferior a la perdida de hojas adultas, la maquinaria fotosintética no puede abastecer de carbohidratos a las hojas jóvenes reduciendo aún más el ratio de crecimiento (Munns y Tester 2008).

La reducción de la capacidad fotosintética generada por el estrés salino es la principal causa de generación de ROS, que se acumulan en las hojas provocando un daño oxidativo a estructuras celulares, compuestos y alterando el metabolismo (Acosta-Motos et al. 2017; Liang et al. 2018). Además de la reducción de la capacidad fotosintética, el estrés salino provoca el cierre de los estomas, entre otras causas por una reducción en la concentración de K^+. El cierre estomático reduce el flujo de CO_2 en las hojas y su asimilación en el ciclo de Calvin, esto causa que los electrones procedentes de la fotolisis del H_2O no encuentren el aceptor endógeno de e^- $NADP^+$ derivado de la fijación del CO_2 lo que conlleva a que estos electrones pueden unirse a moléculas de O_2 libre, generando de esta manera ROS (Imagen 6) (Cakmak 2005).

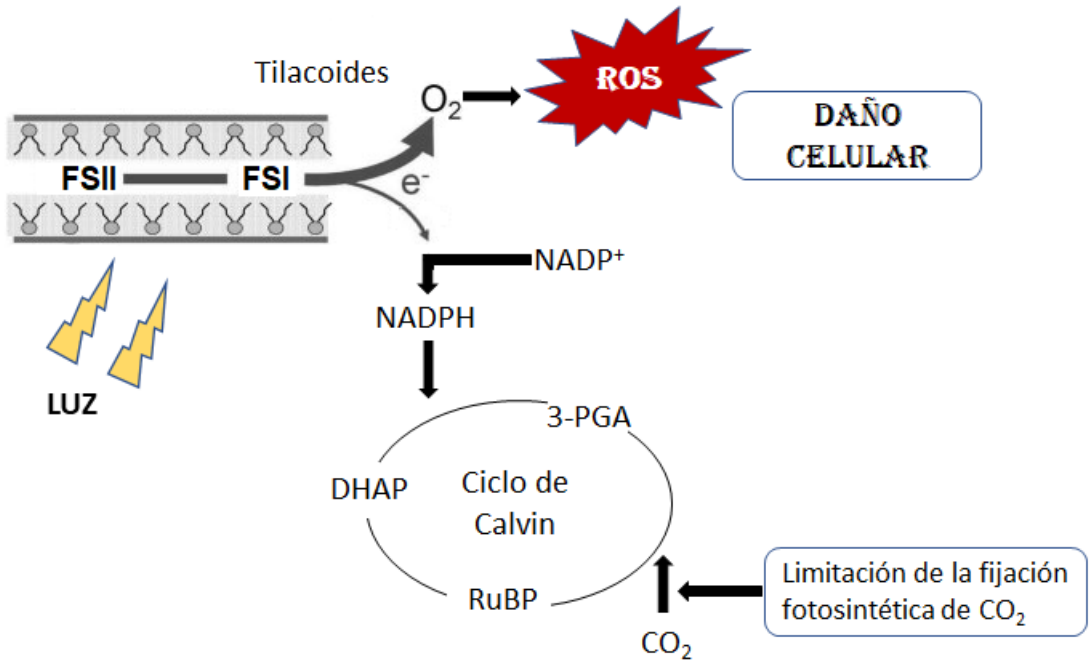

Imagen 6: Adaptación de Cakmak (2005). Representación esquemática de la generación de ROS debido a la limitación de CO_2 proveniente de la fotosíntesis de la plantas.

Los ROS generados bajo condiciones de estrés salino en plantas han de ser eliminados para evitar el daño oxidativo a estructuras celulares y procesos bioquímicos. Los ROS reaccionan con las membranas celulares especialmente en las membranas de los tilacoides que son ricas en ácidos grasos insaturados. La peroxidación de lípidos produce una ruptura de éstos, lo cual afecta a su función en la membrana, causando pérdida de fluidez, rotura de los enlaces lipídicos, e inactivación de enzimas de membrana (Miyake et al. 2005).

Para evitar el daño oxidativo las plantas disponen de mecanismos de detoxificación de ROS. Pueden dividirse por un lado en sistemas enzimáticos, constituidos por enzimas como la superóxido dismutasa (SOD), catalasa (CAT), ascorbato peroxidasa (APX) y glutation reductasa (GR). Por otro lado, las plantas

también presentan, sistemas antioxidantes no enzimáticos formados por compuestos antioxidantes como los compuestos fenolicos (fenoles, flavonoides, carotenoides…), ácido ascórbico (AsA) y glutation (GSH) (Shalata et al. 2001; Reddy et al. 2004). El efecto del estrés salino en plantas no solo genera ROS, sino que también afecta al metabolismo antioxidante enzimático y no enzimático, ya que unido a este incremento de ROS los sistemas antioxidantes tienden a incrementar la actividad de sus enzimas y a generar más compuestos antioxidantes (Singh et al. 2017; Liang et al. 2018).

La SOD constituye la primera línea de defensa y provoca la dismutacion del $O_2^{\cdot -}$ (ion superóxido) en H_2O_2 (peróxido de hidrogeno) (Imagen 7) parte del cual es neutralizado por la CAT (Imagen 7). Esta enzima en hojas se encuentra exclusivamente en los peroxisomas que son los encargados de la detoxificación mediante una reacción peroxidasa siendo el donador de e^- para esa reacción el AsA (Arora et al. 2002). Por otro lado, el AsA está presente a elevadas concentraciones en los cloroplastos, citosol, vacuolas y espacio apoplástico de las células de la hoja, y tiene un papel fundamental en la neutralización del H_2O_2. El AsA se oxida hasta monodeshidroascorbato (MDHA), mediante la APX para detoxificar el H_2O_2 y es regenerado por dos enzimas, la monodehidroascorbato reductasa (MDHAR) que utiliza NADPH como donador de electrones y la dehidroascorbato reductasa (DHAR) que utiliza dos moléculas de GSH (Arora *et al.* 2002) (Imagen 7). El GSH es el compuesto tiolico de bajo peso molecular mayoritario en plantas, cuya función es la regeneración del AsA mediante la DHAR. En esta regeneración del AsA, el GSH se oxida pasando a glutatión disulfuro (GSSG) (Imagen 7). El GSH es regenerado por la GR, en una reacción

NADPH-dependiente (Arora et al. 2002) (Imagen 1.7). Además, el GSH por si solo puede neutralizar el oxígeno singlete y los radicales hidroxilo (otros ROS dañinos para las plantas).

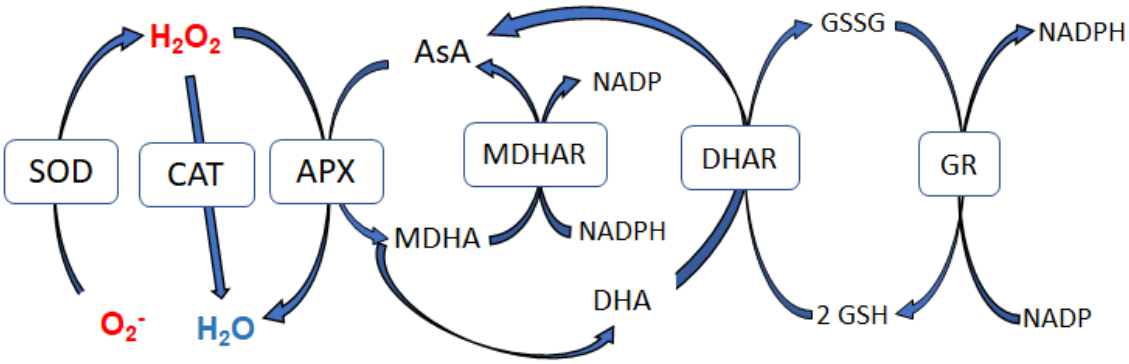

Imagen 7: Representación esquemática del ciclo de Foyer-Halliwell-Asada para la detoxificación de ROS, sistemas enzimáticos y no enzimáticos.

Otro sistema antioxidante no enzimático es la acumulación de compuestos fenólicos (fenoles y flavonoides). Los fenoles pueden actuar como un filtro de absorción de la radiación y limitan la excitación de las clorofilas durante las condiciones desfavorables, protegiendo y conservando así el aparato fotosintético y pudiendo actuar además como antioxidantes. Entre sus mecanismos de acción antioxidante se encuentran (Harbone y Willians 2000):

- La supresión de la formación de especies reactivas por inhibición de enzimas.
- El secuestro de las ROS, evitando el daño oxidativo.
- La sobre-regulación o protección de los sistemas antioxidantes.

Algunos autores como Munns y Tester (2008) han sugerido que la tolerancia al estrés salino se puede encontrar precisamente en la generación de variedades con una notable mejora en la capacidad para detoxificar ROS.

5 Efecto toxico del estrés salino en la fisiología de las plantas

A continuación, describimos los diferentes efectos por los cuales el exceso de NaCl resulta toxico en las plantas y los diferentes procesos fisiológicos que se ven afectados por este estrés abiótico.

5.1 Asimilacion del nitrogeno

La asimilación del nitrógeno (N) es uno de los procesos metabolicos mas importantes de las plantas ya que genera compuestos nitrogenados, como los aminoácidos (Aa), esenciales para el crecimiento y desarrollo de las plantas. En este sentido el N como nutriente es esencial para las plantas, presentando estas un alto requerimiento del mismo siendo la deficiencia de N un factor limitante muy importante (Sánchez-Rodríguez et al. 2011). Las fuentes principales de N que absorben las plantas son NO_3^- y amonio (NH_4^+). El estrés salino afecta a la captación de nutrientes, como hemos visto en el apartado 4.2. En el caso del N, el Na^+ afecta a la absorción de NH_4^+ y el Cl^- a los NO_3^- generando alteraciones iónicas en las plantas (Ashraf et al. 2018).

Después de que los compuestos nitrogenados sean captados por las raíces los NO_3^- pueden ser reducidos, en la raíz o en las hojas, hasta NH_4^+. Esta reducción

es llevada a cabo por dos enzimas (Imagen 8), la nitrato reductasa (NR) y la nitrito reductasa (NiR) (Lea y Azevedo 2007). Es bien conocido que estas enzimas representan un punto de control en la asimilación de NO_3^- y pueden ser un factor limitante, además están reguladas por Aa y aminas como la glutamina que ejerce un efecto negativo en la NR (Causin 1996). Además de esta regulación interna, la presencia de diversos estreses, como el estrés salino puede afectar el proceso de reducción de NO_3^-. Debouba et al. (2007) mostraron una disminución de las actividades NR y NiR en plantas de tomate sometidas a estrés salino, acompañado de una disminución en la concentración de NO_3^-.

Las plantas generan los compuestos nitrogenados necesarios para su crecimiento y desarrollo a partir del NH_4^+ (Imagen 1.8). Este puede provenir de la captación directa, la reducción de NO_3^-, degradación de compuestos nitrogenados o de la fotorrespiración. El NH_4^+ es asimilado principalmente por el ciclo glutamina sintetasa (GS)/glutamato sintasa (GOGAT) hasta Aa y proteínas. Además, también existe una vía alternativa en la que participa la glutamato deshidrogenasa (GDH) (Ghanem et al. 2011). Si observamos el ciclo (Imagen 8) parece que el NH_4^+ es un eje central, sin embargo, altas concentraciones de NH_4^+ pueden provocar toxicidad. Autores como Surabhi et al. (2008) observaron, en plantas sometidas a estrés salino un incremento en la actividad del ciclo GS/GOGAT con el fin de reducir el efecto toxico del NH_4^+.

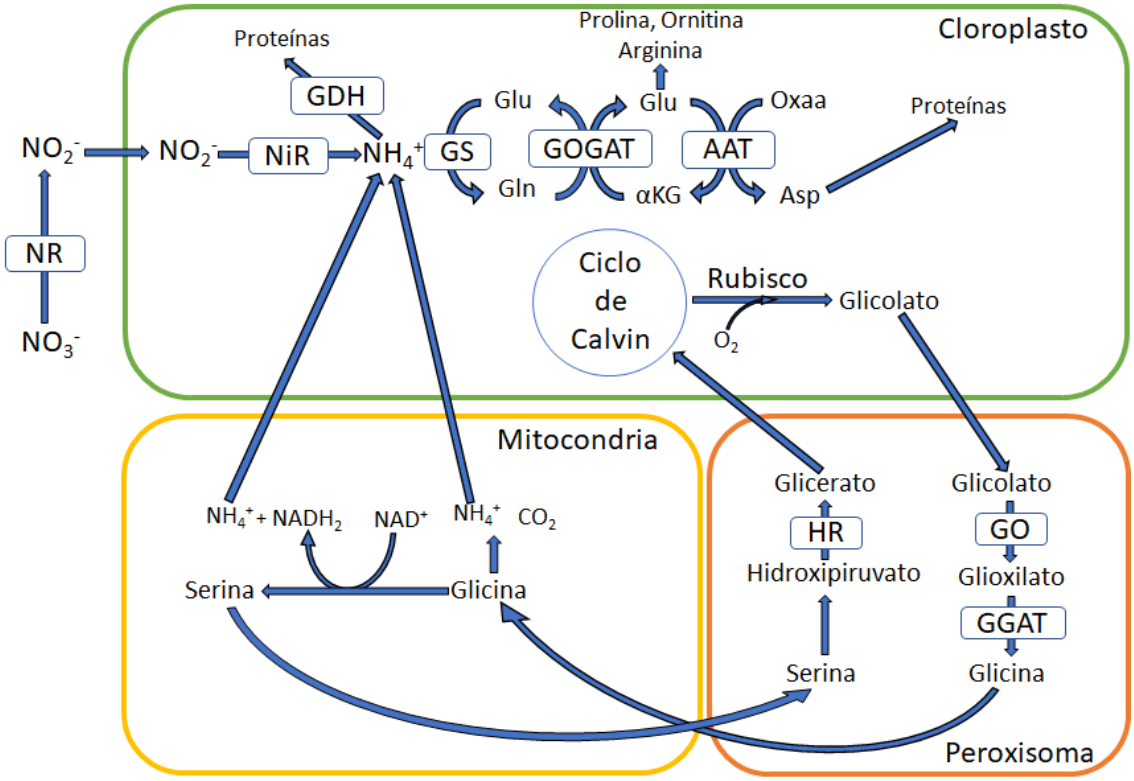

Imagen 8: Representación esquemática de la asimilación de N y la fotorrespiración. Alfa-cetoglutarato (α-KG), Glutamato (Glu), Glicina (Gln), Oxalacetato (Oxaa), Aspartato (Asp), Aspartato Aminotransferasa (AAT), Hidroxipiruvato Reductasa (HR), Glioxilato Oxidasa (GO), Glioxilato Aminotransferasa (GGAT).

Bajo estrés salino, las proteínas generadas por la asimilación del N podrían estar en un continuo estado de renovación (Surabhi et al. 2008). Esto es debido a un mecanismo de defensa de las plantas ante el estrés osmótico generado por el estrés salino. En este sentido, las proteínas generadas a través de la asimilación del nitrógeno pueden degradarse formando Aa como la prolina, GB, arginina o ornitina, los cuales mejoran el equilibrio osmótico en las células. En este sentido la acumulación de Aa es considerado como un indicador de la tolerancia al estrés salino (Ashraf y Foolad 2007).

5.2 Ciclo de los ácidos tricarboxilicos

La salinidad también afecta al ciclo de los ácidos tricarboxilicos (TCA) el cual es de gran importancia para las plantas. El ciclo TCA se encuentra estrechamente relacionado con la fotosíntesis y produce ácidos orgánicos, como el malato, citrato u oxalacetato, precursores de aminoácidos (Aa), poder reductor, energía en forma de ATP o GTP (Imagen 9), y ayuda a la captación de nutrientes (Ryan et al. 2001; Millar et al. 2011). La primera enzima del ciclo es la piruvato deshidrogenasa, la cual convierte el piruvato en Acetil-CoA. Este Acetil-CoA junto con el oxalacetato (OAA) es convertido a citrato por la citrato sintasa (CS) (Imagen 9), siendo esta conversión considerada el primer paso del ciclo TCA (Millar et al. 2011; Araujo et al. 2012). Desde el citrato una serie de enzimas como la isocitrato deshidrogenasa (ICDH), fumarasa o malato deshidrogenasa (MDH) generan conversiones consecutivas cerrando el ciclo desde el citrato hasta el OAA (Imagen 9). El ciclo TCA en sus diferentes etapas genera poder reductor en forma de NADH y sustratos para procesos metabolicos tan importantes como la asimilacion del carbono (CO_2) o del N (α-cetoglutarato, α-CK), con lo cual es vital para las plantas mantener la eficiencia del ciclo TCA (Araujo et al. 2012).

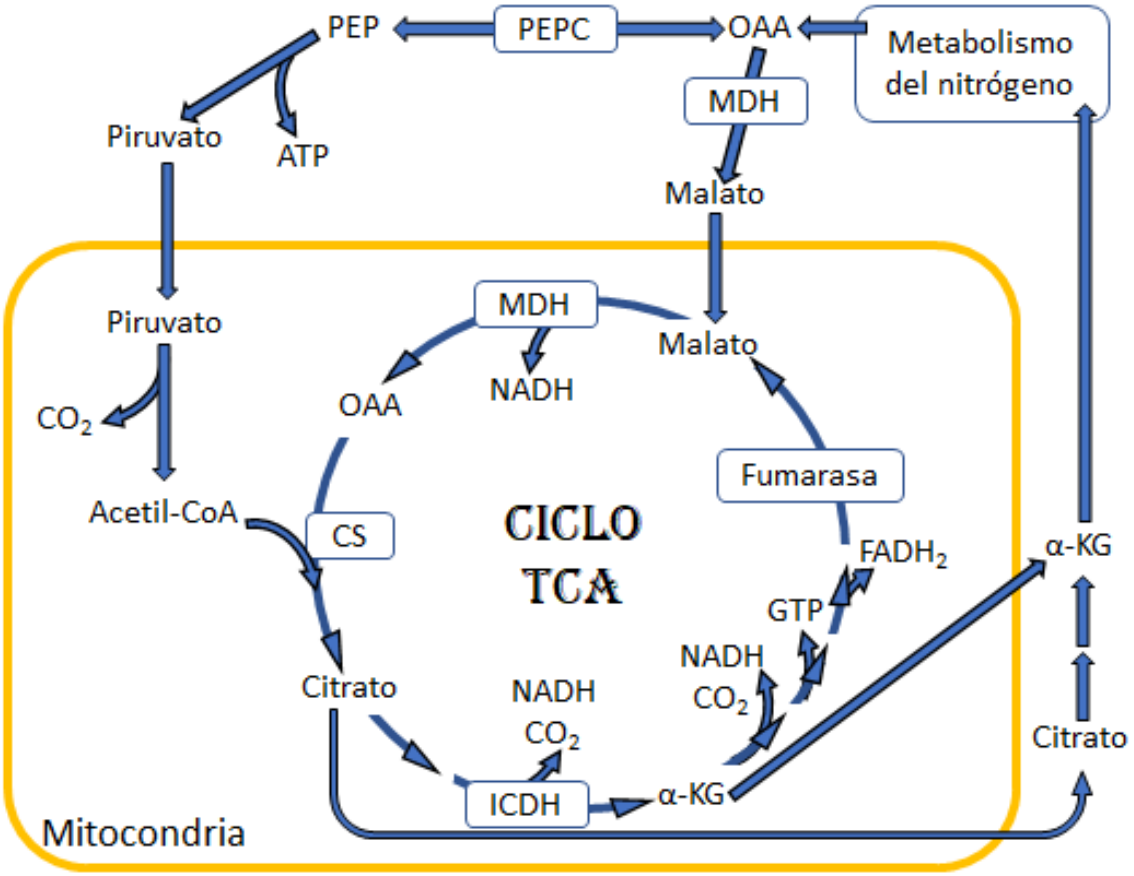

Imagen 9: Representación esquemática del ciclo TCA. Fosfoenol piruvato carboxilasa (PEPC), Fosfoenol piruvato (PEP), Alfa-cetoglutarato (α-KG).

Sin embargo, en este ciclo algunas etapas son sensibles al estrés oxidativo como la conversión de piruvato a Acetil-CoA, de citrato a isocitrato y la conversión de α-KG a succinil-CoA, hecho que se agrava con el estrés salino ya que este genera ROS (Millar et al. 2011; Araujo et al. 2012). En este sentido, autores como Richter et al. (2015) describen como el efecto del estrés salino afecta al ciclo TCA alterando la asimilación del carbono, reduciendo el crecimiento y desarrollo de las plantas.

Además, los ácidos orgánicos parecen jugar un papel bastante importante en la tolerancia al estrés salino. Algunos autores muestran como los diferentes ácidos

orgánicos potencian la acumulación de otros compuestos que generan tolerancia al estrés salino (Saito et al. 2008) o pueden generar tolerancia a la toxicidad iónica como ocurre en el caso del Al^{3+} mediante quelación (Ryan et al. 2001). Con respecto a la quelación, es posible que ácidos orgánicos como el oxalato puedan formar cristales de oxalato sódico, el cual podría almacenarse en las vacuolas evitando el efecto citotoxico del Na^+ (Fu et al. 2014a; Fu et al. 2014b). De hecho, es conocida la capacidad del oxalato para formar cristales de oxalato cálcico que se pueden acumular en las vacuolas (Nakata 2003).

Mantener un correcto funcionamiento del ciclo TCA es importante no solo por los precursores que genera, sino porque también es una gran fuente de energía. Por ejemplo, en las raíces una menor alteración en el ciclo TCA en plantas sometidas a estrés salino puede generar una mayor tolerancia a este estrés. Esto es debido a que la generación de ATP por el ciclo TCA ayuda a la correcta captación de nutrientes (Millar et al. 2011). No obstante, junto a esta captación de nutrientes también puede ir asociada una mayor captación de Na^+, en este sentido, autores como Millar et al. (2011) describen mayores ratios Na^+/K^+ en las raíces, van acompañados de menores concentraciones de Na^+ en hojas, reduciendo así las alteraciones metabólicas generadas por el Na^+.

5.3 Alteración del perfil fitohormonal

Como hemos comentado anteriormente, la acumulación de Na^+ y Cl^- puede afectar alterando o inhibiendo procesos metabólicos en las plantas. En este

sentido, afecta a procesos tan importantes como la síntesis y función de las fitohormonas.

Las fitohormonas son moléculas que, a pesar de presentar una concentración muy baja, son capaces de regular una gran variedad de procesos en las plantas, de ahí su importancia. Estas actúan como mensajeros químicos para la comunicación celular en plantas superiores. Las fitohormonas regulan además la respuesta tanto a estímulos internos como externos, y juegan un papel fundamental coordinando señales de transducción de las rutas metabólicas durante el estrés salino (Wani et al. 2016). Si la regulación de estas fitohormonas se ve afectada de forma negativa esto puede influir en el correcto desarrollo y crecimiento de las plantas, por ejemplo, retrasando el crecimiento de las hojas (Acosta-Motos et al. 2017). Las diferentes fitohormonas suelen clasificarse en fitohormonas de estrés y fitohormonas de crecimiento, siendo las principales: Acido abscísico (ABA) y Etileno (ET), y Auxinas (AIA) Citoquininas (CKs) y Giberelinas (GAs) respectivamente (Wani et al. 2016).

Entre las diferentes funciones de estas fitohormonas el ABA es considerado un mensajero esencial en las respuestas al estrés, modificando la expresión de genes o favorececiendo el cierre estomático para evitar la pérdida de agua, lo que algunos autores señalan como un mecanismo de tolerancia (Ryu y Cho 2015). Sin embargo, como hemos visto en la sección 4.3 esta acción influye también en la capacidad fotosintética de la planta al disminuir la cantidad de CO_2 disponible pudiendo generar de esta manera ROS.

El ET se relaciona con los procesos de senescencia de las plantas y la salinidad afecta a el contenido endógeno de ET aumentando su concentración dentro de la planta, favoreciendo la abscisión de los pétalos, hojas y frutos (Wani et al. 2016).

Las AIA han sido estudiadas ampliamente debido a su papel en el desarrollo y crecimiento de las plantas bajo estreses abióticos. Concretamente en estrés salino parece que muchos genes de respuesta a la defensa de este estrés están regulados por AIA (Wani et al. 2016). La concentración de AIA esta relacionada con la senescencia y el ET, de forma que una alta concentración de AIA retrasa la senescencia por lo que el equilibrio de estas hormonas es importante para el correcto desarrollo y crecimiento de las plantas. En este sentido autores como Ghanem et al. (2008) observaron en plantas de tomate sometidas a estrés salino que una baja concentración de AIA podría estimular la senescencia. Por otra parte, al igual que el ABA las AIA también están relacionadas con el metabolismo oxidativo, pudiendo ayudar a la detoxificación de ROS (Noctor et al. 2015).

Las CKs y las GAs podrían ser consideradas hormonas antagonistas del ABA y el ET, ya que favorecen la germinación de semillas, la dominancia apical y en definitiva la expansión celular y crecimiento de las plantas (Wani et al. 2016). Algunos autores señalan una disminución de las GAs y un aumento del ABA como consecuencia de la acumulación de Na^+ y Cl^-, que retrasaría el crecimiento foliar (Munns y Tester 2008). Las CKs y las GAs también estarían relacionadas con el metabolismo antioxidante, ejerciendo una regulación sobre las enzimas

de detoxificación y disminuyendo el estrés oxidativo en las plantas (Pogány et al. 2004; Iqbal y Ashraf 2013).

Las plantas también presentan otras hormonas como el ácido jasmonico (JA) o el ácido salicílico (SA) aunque estas hormonas están más relacionadas con la defensa ante estreses bióticos provocadas por patógenos. Sin embargo, también se ha relacionado al JA con la reducción de los daños provocados por la salinidad, ayudando a mantener el cociente fotosintético y el crecimiento en plantas (Dar et al. 2015).

6 Perspectivas de futuro para reducir el efecto del estrés salino en plantas

Debido a todo lo anteriormente descrito, es necesario mejorar el conocimiento existente sobre cómo son y donde residen los mecanismos de tolerancia al estrés salino en las plantas. De este modo podremos generar variedades más productivas y tolerantes frente a este tipo de estrés.

La generación de variedades mas resistentes, productivas o simplemente con un carácter fenotípico deseable se lleva realizando, con más o menos éxito, desde que los humanos formaron asentamientos mediante lo que se conoce como *breeding* clásico (Acquaah 2017). El *breeding* clásico se trata de realizar cruzamientos entre variedades “elite” (con características de alto interés agronómico) con variedades silvestres (normalmente mas resistentes a plagas o condiciones ambientales) tratando de transferir sus características de unas a

otras. Sin embargo, el tiempo necesario para obtener resultados satisfactorios en el *Breeding* clásico es de 5 a 7 años (Bergougnoux 2014; Acquaah 2017).

Por esta razón, el *Breeding* clásico esta siendo sutituido por la ingeniería genética, la cual puede ser una solución para aumentar la tolerancia al estrés salino en las plantas (Liang et al. 2018). Una de las estrategias que se intenta mejorar para conseguir variedades mas resistentes a la salinidad es la capacidad de las plantas para compartimentalizar o excluir el Na^+ (Munns y Tester 2008; Acosta-Motos et al. 2017). Un ejemplo de esto lo mostraron Chinnusamy et al. (2005) en plantas con una sobreexpresión en los genes de los transportadores NHX1 y SOS1, cuya función es evitar la acumulación de Na^+ libre en las células. Estas plantas pueden llegar a producir frutos sin desperfectos apreciables bajo unas condiciones de salinidad de 200 mM (20 dS m^{-1}), mientras que para las plantas no modificadas genéticamente es letal.

Otros autores apuntan a la acumulación de solutos osmocompatibles para mejorar la tolerancia a la salinidad (Liang et al. 2018). Por otro lado, autores como Ashraf y Akram (2009) también señalan a la mejora de la producción de compuestos antioxidantes. Otra alternativa la muestran Acosta-Motos et al. (2017) quienes sugieren una mejora en la regulación hormonal para la tolerancia a la salinidad.

Como hemos visto el escenario es bastante amplio y se nos plantean multitud de opciones donde podría residir la tolerancia a la salinidad. Generando variedades

más tolerantes se podrá intentar aportar una solución a un problema cada vez más presente e importante como es el estrés salino, el cual amenaza la sostenibilidad de los cultivos. Por tanto, si identificamos los procesos fisiológicos clave donde puede residir la tolerancia a la salinidad tendremos objetivos más claros donde actuar.

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JUSTIFICACIÓN Y OBJETIVOS

1 ¿Por qué el tomate como planta de estudio?

El cultivo de tomate es uno de los cultivos más extendidos y de mayor valor económico que no ha parado de crecer en los últimos años (Imagen 1). Los datos de la FAO registran que aproximadamente cada 10 años el crecimiento de la producción de tomate aumenta en torno a 50 millones de toneladas (Mt), y el área de cultivo dedicado a estos crece aproximadamente 1 millón de ha (Mha), a nivel mundial (FAO 1994-2017).

Imagen 1: Representación gráfica del incremento de la producción y área cultivada de tomates frescos en el mundo según la FAO para los años 1994-2017 (FAO 1994-2017).

Según los últimos datos proporcionados por el servicio de estadisticas de la Organización de las Naciones Unidas para la Alimentación y la Agriculcura para 2017 (FAOSTAT), el área cultivada de tomate a nivel mundial es de casi 5 Mha, teniendo una producción en la misma de aproximadamente 182 Mt (FAO 1994-2017).

En España los datos recogidos por el anuario de estadística agraria reflejan una producción de tomate de alrededor de 5.2 Mt en una superficie cultivable de aproximadamente 61 mil ha, siendo Andalucía la principal productora de tomate seguida de Extremadura (Tabla 1).

SUPERFICIES Y PRODUCCIONES DE CULTIVOS

7.6.27.3. HORTALIZAS DE FRUTO-TOMATE: Análisis provincial de superficie, rendimiento y producción, 2017

Provincias y Comunidades Autónomas	Superficie (hectáreas)				Rendimiento (kg/ha)			Producción (toneladas)
	Secano	Regadío		Total	Secano	Regadío		
		Aire libre	Protegido			Aire libre	Protegido	
GALICIA	–	231	912	1.143	–	57.258	90.283	95.563
P. DE ASTURIAS	62	35	45	142	14.000	25.000	40.000	3.543
CANTABRIA	7	–	10	17	15.000	–	70.000	805
PAÍS VASCO	77	137	75	289	8.418	18.047	50.068	6.875
NAVARRA	–	2.017	42	2.059	–	71.260	73.730	146.828
LA RIOJA	–	218	19	237	–	77.000	106.000	18.800
ARAGÓN	1	692	11	704	15.000	79.286	162.191	56.666
CATALUÑA	62	781	198	1.041	8.081	35.554	91.939	46.473
BALEARES	–	320	47	367	–	27.600	39.930	10.709
CASTILLA Y LEÓN	–	90	25	115	–	37.770	63.044	4.976
MADRID	–	21	33	54	–	52.000	120.000	5.052
CASTILLA-LA MANCHA	53	1.048	35	1.136	4.758	73.886	150.000	82.935
C. VALENCIANA	28	741	490	1.259	7.107	34.758	101.488	75.684
R. DE MURCIA	–	123	2.353	2.476	–	28.503	95.739	228.780
EXTREMADURA	–	24.083	7	24.090	–	86.155	120.000	2.075.704
ANDALUCÍA	202	10.579	14.053	24.834	18.282	79.219	98.785	2.229.975
CANARIAS	4	111	774	889	30.625	43.910	89.278	74.098
ESPAÑA	496	41.227	19.129	60.852	12.911	79.985	97.209	5.163.466

Tabla 1: Producción y area cultivada de tomates frescos en las diferentes provincias y comunidades autónomas de España. Datos proporcionados por el anuario de estadística agraria. https://www.mapa.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/2018/default.aspx?parte=3&capitulo=07&grupo=6&seccion=27 (consultado 26/06/2019).

A parte de su interés económico el tomate posee características agronómicas que la convierten en una adecuada planta de experimentación. Algunas de estas características más destacables son: 1) la posibilidad de cultivar tomate en diferentes condiciones, lo que permite comprender la adaptabilidad del tomate a diferentes estreses abióticos, como por ejemplo el estrés salino, 2) su ciclo de

vida relativamente corto, 3) su insensibilidad al fotoperíodo, es decir, la capacidad de floración, y posteriormente la producción de semillas independientemente de las condiciones de la duración del día, 4) la facilidad de polinización controlada e hibridación, 5) la simplicidad de su genética con un genoma relativamente pequeño (aproximadamente 900 Mb) y la falta de duplicación de genes, y 6) su capacidad para propagarse asexualmente por injerto, o para regenerar plantas enteras de diferentes partes de la planta (Bergougnoux 2014).

2 Objetivos

Como hemos descrito en la introducción la salinidad es un problema que afecta al crecimiento y desarrollo de los cultivos, que está creciendo día a día. Por ello, es necesario comprender en profundidad como la salinidad afecta al metabolismo de las plantas y que posibles mecanismos de defensa pueden presentar las plantas. Conociendo esto, se podrán generar variedades más tolerantes ante este tipo de estrés. Por ello el objetivo general de la tesis fue: Conocer mejor el efecto toxico del estrés salino en el metabolismo y la fisiología de los genotipos seleccionados, arrojando una visión global e interconectada de los mismos, atendiendo también a las posibles diferencias genotípicas. El establecimiento de un amplio conocimiento en este sentido podría sentar las bases para la generación o mejora de genotipos más tolerantes/resistentes al estrés salino.

Así, el objetivo general de esta tesis doctoral se subdivide en 2 objetivos que son:

Objetivo 1: Comprobar el efecto toxico de la salinidad en la ionómica y la fisiología de los genotipos estudiados e identificar los posibles procesos metabólicos y/o compuestos que desarrollen un papel en la resistencia/tolerancia al estrés salino.

Objetivo 2: Comprobar la influencia de la variabilidad genotípica en el grado de resistencia/tolerancia al estrés salino.

2.3 Bibliografia

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MATERIAL Y MÉTODOS GENERAL

A continuación, se expone la parte común del material y métodos que se han usado para esta tesis doctoral. Información más especifica de describe en los materiales y métodos de cada capítulo.

1 Material vegetal

Las semillas de *Solanum lycopersicum* L., genotipos Grand Brix y Marmande RAF, fueron germinadas y crecidas durante 30 dias en alveolos de 3 cm × 3 cm × 10 cm llenos de una mezcla de perlita-vermiculita como sustrato. Las semillas fueron proporcionadas por el vivero Saliplant S.L., España. Las semillas fueron colocadas en bancos de un invernadero experimental localizado en el sur de España (Saliplant S.L., Motril, Granada). Después de 30 días, las plantulas fueron transferidas a una cámara de cultivo en el departamento de Fisiología Vegetal de la Universidad de Granada. Las plantulas crecieron bajo condiciones ambientales controladas: humedad relativa 60-80%; temperatura día/noche de 28/19 ºC respectivamente; 16/8h de fotoperiodo a una densidad de flujo fotosintético de fotones (PPFD) de 350 μmol $m^{-2}s^{-1}$ (medido en el punto alto de las plantulas con 190 SB quantum sensor, LI-CORInc., Lincoln, Nebraska, USA). Bajo estas condiciones las plantas crecieron en cultivo hidropónico en bandejas ligeras de polipropileno (60 cm de ancho, 60 cm de largo y 7 cm de altura) con una capacidad de 8 plantas por bandeja. A lo largo del experimento, las plantas fueron tratadas con una solución nutritiva completa compuesta por: 4 mM KNO_3, 3 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2 mM $MgSO_4{\cdot}7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4{\cdot}2H_2O$, 2 µM $MnCl_2{\cdot}4H_2O$, 1 µM $ZnSO_4{\cdot}7H_2O$, 0.25 µM $CuSO_4{\cdot}5H_2O$, 0.1 µM $Na_2MoO_4{\cdot}2H_2O$, 5 µM Fe-quelato (Sequestrene; 138 FeG100) y 50 µM

H_3BO_3. Esta solución nutritiva presentó un pH de 5.5-6.0 y fue renovada cada 3 dias.

2 Diseño experimental y tratamientos

El tratamiento de estrés salino empezó a los 38 dias después de la germinación y fue mantenido durante 15 dias para ambos genotipos. El tratamiento control recibió una solución nutritiva completa, mientras el tratamiento salino recibió junto con la solución nutritiva completa una concentración de 100 mM de NaCl. El diseño experimental fue un bloque completamente aleatorizado con 2 tratamientos por genotipo de tomate, 8 plantas por tratamiento y 3 réplicas por tratamiento.

3 Muestreo vegetal y determinación de la Tasa de Crecimiento Relativa (TCR)

Las plantas de cada tratamiento fueron muestreadas a los 53 días después de la germinación y el material vegetal fue dividido en raíces y hojas. El material vegetal se lavó con agua destilada, se secó con papel de filtro y fue pesado, obteniéndose el peso fresco (FW). Esta medida se utilizaría posteriormente para la determinación de la biomasa fresca. La mitad de las hojas de cada tratamiento fueron congeladas a -30ºC para los posteriores ensayos bioquímicos y la otra mitad de las hojas fueron liofilizadas durante 48h obteniendo el peso seco (DW) y posteriormente almacenadas para el posterior análisis de la concentración de nutrientes y otros compuestos.

Para determinar la TCR las hojas de 3 plantas por tratamiento y repetición fueron muestreadas en el día 38 después de la germinación, inmediatamente antes de aplicar el estrés salino (Ti). Las hojas fueron liofilizadas durante 48h y se obtuvo el peso seco (DW) que fue recogido como gramos por planta, siguiendo el procedimiento descrito en el párrafo anterior. A los 53 días después de la germinación (15 días de tratamiento) se repitió en proceso con la mitad de las plantas restantes por tratamiento (Tf), como de describió en el párrafo anterior. La TRC fue calculada a partir del incremento en el peso seco desde el muestro inicial hasta el muestreo final después del tratamiento salino, usando la ecuación TCR = (ln DWf – ln DWi)/(Tf – Ti). Donde T es el tiempo y los subíndices denotan el muestreo final e inicial.

4 Análisis estadístico

Todos los análisis fueron repetidos por triplicado y los resultados fueron evaluados estadísticamente usando un análisis de varianza ANOVA simple con un 95% de intervalo de confianza. Se realizo un análisis ANOVA multivariante para determinar si el tratamiento salino y el genotipo afectaron de forma significativa a los resultados. Las diferencias entre las medias de los tratamientos fueron comparadas usando el test de diferencias mínimas de Fisher (LSD) con un 95% de nivel de probabilidad. Los niveles de significancia se expresaron como: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS no significativo. El software estadístico usado fue Statgraphics Centurion.

CAPÍTULO 1

Estudio del perfil fitohormonal y metabolismo oxidativo como procesos clave para la identificacion de la respuesta a la salinidad en genotipos comerciales de tomate

Alejandro de la Torre-González, Eloy Navarro-León, Alfonso Albacete, Begoña Blasco, Juan M. Ruiz

Resumen

El cambio climático, la agricultura intensiva y la peor calidad del agua induce estrés abiótico en plantas. Entre esos factores, el estrés salino es un factor limitante para el crecimiento de las plantas. Por lo tanto, el propósito de este estudio fue analizar el papel de las fitohormonas y el metabolismo oxidativo en respuesta al estrés salino en dos genotipos de tomate cv. Grand Brix y cv. Marmande RAF. El estrés salino reduce la biomasa y la tasa de crecimiento relativa (TCR) en ambos genotipos, siendo este efecto mayor en el cv. Marmande RAF. Estos resultados, junto con el principal indicador de estrés oxidativo, el $O_2^{\cdot -}$, indican que cv. Marmande RAF es más sensible al estrés salino. Grand Brix mostró menos estrés oxidativo, debido a una mayor detoxificación del $O_2^{\cdot -}$, por inducción de la actividad enzimática SOD y una mayor capacidad antioxidante. Por otro lado, comprobamos que el genotipo Grand Brix presentó un mejor perfil fitohormonal adaptado a la resistencia al estrés salino, ya que observamos una acumulación de las fitohormonas IAA, GA_4 y CKs. La acumulación de estas fitohormonas podría ser beneficioso contra el estrés oxidativo y explicar la diferencia entre la resistencia y la sensibilidad al estrés salino. Ademas, una menor concentración de ACC, precursor de etileno, combinado con una mayor desintoxicación de $O_2^{\cdot -}$ en el cv. Grand Brix podría desempeñar un papel fundamental en la tolerancia al estrés salino. Finalmente,

un aumento en los niveles de ABA en Grand Brix promueve un mejor cierre estomático, un mejor control de la fotosíntesis y una menor tasa de pérdida de agua. Estos datos podrían ser esenciales para seleccionar plantas con mayor resistencia al estrés salino.

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Study of phytohormone profile and oxidative metabolism as key process to identification of salinity response in tomato commercial genotypes.

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1 Abstract

Climatic change, intensive agriculture, and worsening water quality induce abiotic stress conditions for plants. Among these factors, salinity stress is a limit factor for plant growth. Therefore, the purpose of this study was to analyze the phytohormones role and oxidative metabolism in response to salt stress of two genotypes of tomato cv. Grand Brix and cv. Marmande RAF, the crops was carried out in a growth chamber. Salinity stress reduces biomass and relative growth rate (RGR) in both genotypes, this effect being greater in cv. Marmande RAF. These results, together with main stress indicator response, the $O_2^{\cdot -}$, indicate that cv. Marmande RAF is more sensitive to Saline stress. Grand Brix showed less oxidative stress, because it presented greater detoxification of the $O_2^{\cdot -}$, due to SOD enzyme activity induction and greater antioxidant capacity. Furthermore, Grand Brix has a better hormonal profile adapted to salt stress resistance, the accumulation of IAA, GA_4 and CKs and their beneficial role against oxidative stress could make the difference between resistance and sensitivity to salt stress. On the other hand, a lower ACC concentration, ethylene precursor, combined with a greater $O_2^{\cdot -}$ detoxification in the cv. Grand Brix could

play a fundamental role in tolerance to saline stress. Besides, an increase in ABA levels promotes better stomatal closure, better photosynthesis control and a lower rate of water loss. This data could be essential to select plants with greater resistance to saline stress.

Keywords: Salt stress; Oxidative metabolism; Phytohormones; *Solanum lycopersicum* L.

2 Introduction

Tomato is a crop with the greatest economic importance in the world. According to the FAO, in 2014 roughly 4,888,880 tonnes of tomato were produced only in Spain, cultivated on 54,750 Ha. A great part of this cultivation area is affected by salinity stress. In particular, salinity stress causes a reduction in the quantity and quality of crop production (Saito et al. 2008). Currently, the main challenge of world agriculture is to sustain the continuously growing global population, and this becomes more difficult due to climatic change, as this imposes further abiotic stress. Coming years, several factors could exacerbate this situation, such as intensive agriculture and the use of poorer quality water. Therefore, it is great importance to ascertain the impact of saline stress in tomato cultivation. Greatly limiting crop yield in semiarid and arid regions, salinity affect roughly 397 million Ha of soils in the world (Gong et al. 2013). This is particularly true in the Mediterranean area, where cultivation tends to occupy small fields often with crops of high quality and commercial value, such as the tomato (Lynch and Clair 2004). Growth conditions under salinity stress, trigger osmotic and ionic imbalances, prompt oxidative stress, and upset the plant's metabolism.

The capacity of the plant to tolerate salinity is determined by multiple biochemical and physiological mechanisms, in particular by controlling the generation of reactive oxygen species (ROS) and readjusting the cell redox state (Gong et al. 2013). ROS negatively affect biological structures, provoking DNA damage, protein and amino acid oxidation, and lipid peroxidation (Asada 1999). The ROS generated under stress conditions in plants should be eliminated and, for this purpose, plants have mechanisms to detoxify ROS. These can be classified as enzymatic or non-enzymatic. In addition, phytohormones also are related to ROS generation/detoxification processes.

Plant hormones are structurally diverse compounds involved in multiple processes. Phytohormones thus have a vital role in mediating plant response to abiotic stress, the enzymes play a role in the regulation of oxidative stress. (Fahad et al. 2015). It has been observed that in tomato plants under salt stress a decrease in IAA concentration. A low IAA content could stimulate senescence, this compound has been generally seen as a senescence-retarding factor (Ghanem et al. 2008). The beneficial effect of auxins on the prevention of damage caused by oxidative stress has been known for some years (Noctor et al. 2015). The auxins can help detoxify ROS, this is observed in plants with decreased catalase (CAT) activity (Noctor et al. 2015). Other authors point out how ROS alter gradients and auxin signaling (Raja et al. 2017).

Cytokinins (CKs) such as trans-zeatine (tZ) and isopentenyl adenine (iP), are also known to alleviate the adverse effects of salinity on plant growth (Fahad et al.

2015). It has been observed that an increase of the CKs can decrease the damages caused by the ROS, this can help you to be more tolerant of stress (Pogány et al. 2004). Ghanem et al. (2011) underwent two varieties of tomato to salt stress and they showed that the concentration of CKs in the plants increase in the leaves in saline treatment. These authors concluded that a greater accumulation of CKs, could improve the resistance to salt stress by delay leaf senescence, which would improve maintaining stomatal conductance.

A rapid accumulation of Gibberellins (GAs) is characteristic of plants exposed to abiotic stresses, and this phytohormone can impart stress tolerance, including salinity. Under abiotic stress GAs regulates metabolic processes such as sugar signaling and antioxidative enzymes. Likewise, they act in stress response since these hormones are antagonistic with respect to abscisic acid (ABA) (Iqbal and Ashraf 2013).

ABA is known as a hormone that increases their concentration in stressed plants and is key to coordinate stress responses. In maize plants subjected to salt stress, the most resistant hybrid to salt stress had higher ABA concentration. These authors describe how increasing ABA could benefit to plants under salt stress conditions, inducing a lower rate of transpiration, so the tissues accumulate fewer Na^+ (Zörba et al. 2013). ABA is also closely related to H_2O_2 and Ca^{2+} ion, both of which interact in the stomatal closure/opening process. In addition, H_2O_2 stimulates the accumulation of ABA. Finally, the accumulation of H_2O_2 stimulates

the accumulation of ABA, this ABA promotes the stomatal closure thus avoiding the loss of water that can cause saline stress (Mittler and blumwald 2015).

Ethylene interacts with other hormones (GA and ABA) in homeostasis processes (Rzewuski and Sauter 2008). It accumulates alongside ROS. The ethylene and $O_2^{\cdot -}$ in plants under abiotic stress are the main cause of programmed cell death. The ethylene and $O_2^{\cdot -}$ in plants under abiotic stress behaves as an indicator of stress. An increase in the hormone jasmonic (JA) can lead to an increase in ethylene levels (Overmyer et al. 2003).

Other two phytohormones relevant in stress response are JA and salicylic acids (SA). JA is commonly associated with stress by pathogens. However, pretreatment with JA diminished the inhibitory effect of high salt concentration on growth and photosynthesis in barley. However, there is no information about how salinity affects endogenous JA levels in natural plant. SA has antagonistic effects on JA by preventing its accumulation in injury response. On the other hand, SA is usually associated with the chemical defense of plants against microbes and herbivores (Singh and Gautam 2013). The role of hormones in defense against abiotic stress and ROS is becoming clearer. In spite of this, more studies are necessary to understand this process well.

In this context, considering the importance of phytohormones and role oxidative metabolism in plant resistance to saline stress, we investigate here the response of oxidative metabolic process and phytohormones concentrations in two tomato

genotypes submitted to salinity stress. The aim is to determine whether the oxidative metabolism and hormonal profile are determinant to define the cultivar with the strongest tolerance to saline stress. Also, understand how different hormones and oxidative metabolism are related with salt stress tolerance.

3 Materials and Methods

3.1 Plant material and treatments

Seeds of *Solanum lycopersicum* cv. Gran brix and *Solanum lycopersicum* cv. Marmande RAF (Saliplant S.L., Spain) were germinated and grown for 30 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were placed on benches in an experimental greenhouse located in Southern Spain (Saliplant S.L., Motril, Granada). After 30 days, the seedlings were transferred to a growth chamber (Department of plant physiology, University of Granada) under the following controlled environmental conditions: Relative humidity 60-80%; Day/night temperatures 28/19 °C; 16/8 h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol $m^{-2}s^{-1}$ (measured at the top of the seedlings with a 190 SB quantum sensor, LI-CORInc., Lincoln, Nebraska, USA). Under these conditions, the plants were grown in hydroponic cultivation in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) of 3 L volume, 8 plants/tray. Throughout the experiment the plants were treated with a growth solution made up of 4 mM KNO_3, 3 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2 mM $MgSO_4{\cdot}7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4{\cdot}2H_2O$, 2 µM $MnCl_2{\cdot}4H_2O$, 1 µM $ZnSO_4{\cdot}7H_2O$, 0.25 µM $CuSO_4{\cdot}5H_2O$,

0.1 µM $Na_2MoO_4 \cdot 2H_2O$, 5 µM Fe-chelate (Sequestrene; 138 FeG100) and 50 µM H_3BO_3, pH 5.5–6.0.

3.2 Experimental design

Treatment of saline stress started 38 days after germination, this treatment was maintained for 15 days. The control treatment received the growth solution, described in section 2.1, this solution was renewed every three days. Saline treatment received the growth solution plus 100 mM NaCl, this solution was renewed every three days. The experimental design was a randomized complete block with two treatments, 8 plants per treatment and with 3 replications per treatment (n = 9).

3.3 Plant sampling and determination of the relative growth rate (RGR)

Plants of each treatment (53 days after germination) were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of the leaves from each treatment were frozen at −30 °C for further work and biochemical assays and the other half of the plant material was lyophilised for 48h to obtain the dry weight (DW) and the subsequent analysis of the concentrations of nutrients. To determine the relative leaf growth rate (RGR), leaves from three plants per cultivar were sampled on day 38 after germination, immediately before starting the stress treatment (Ti). The leaves were dried in a forced-air oven at 70 °C for 24 h, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled 53 days after germination (15 days of treatments, Tf). The relative growth rate was calculated

from the increase in leaf DW at the beginning and at the end of the saline-stress treatment, using the equation RGR = (ln DWf − ln DWi)/(Tf − Ti) where T is the time and the subscripts denote the final and initial sampling.

3.4 Determination of the concentration of promoters and indicators of oxidative stress (MDA, H_2O_2 and $O_2^{\cdot -}$), lipoxygenase (LOX) activity

For the extraction of MDA, 0.1 g of frozen leaf material was grounded, in 1 mL of buffer 50 mM (0.07% of $NaH_2PO_4 \cdot 2H_2O$ and 1.6% of $Na_2HPO_4 \cdot 12H_2O$). The extract was centrifuged to 20000 g for 25 min. Subsequently, an aliquot of supernatant was mixed in test tubes with 4 mL of trichloroacetic acid 20% containing 0.5% of thiobarbituric acid. The resulting mixture was heated to 95°C for 30 minutes. Then it was rapidly cooled in an ice bath. The absorbance of the supernatant was measured at 532 nm. The value for the non-specific absorption at 600 nm was subtracted from the reading obtained at 532 nm, (Fu and Huang 2001).

Leaf H_2O_2 concentration was measured colorimetrically according to Mukherjee and Choudhuri (1983). 0.1 g of frozen leaf material was grounded in cold acetone. An aliquot of 1 mL of the extract was mixed with 200 µL of 0.1% titanium dioxide H_2SO_4 to 20% (v:v) and the mixture was centrifuged at 6000 g for 15 minutes. The intensity of the yellow colour of the supernatant was measured at 415 nm. The concentration of H_2O_2 was calculated from a standard curve of H_2O_2.

The $O_2^{\cdot -}$ concentration in the leaves was measured colorimetrically according to Barrameda-Medina et al. (2014). 0.1 g of frozen leaf material was grounded and 300 µL of phosphate buffer 50 mM was added. The mixture was centrifuged at 10000 g for 15 min. From the mixture, 250µL of the supernatant were caught. Then, buffer phosphate 50 mM and 250 µL of hydroxylamine 10 mM were added to it. The mix was incubated for 20 minutes at 25ºC. Subsequently, 60 µL of the supernatant were caught and 180 µL of sulfonyl acid 17 mM and 180 µL of α-1-Naphthylamine 7 mM were added and the mixture was incubated for 1h at room temperature. When the incubation was finished, the colour intensity was measured at 530 nm. The $O_2^{\cdot -}$ concentration was calculated from a standard curve of $O_2^{\cdot -}$.

The LOX activity was measured according to Minguez-Mosquera et al. (1993), A weighed sample was triturated with phosphate buffer 50 mM pH 7 until homogenized. The triturate was centrifuged at 7000 g, and the supernatant used as crude enzymatic extract. All operations were carried out in an ice bath. The absorbance of the sample was measured at a wavelength of 234 nm.

3.5 Determination of the activity of antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), CAT, glutathione reductase (GR) and glutathione peroxidase (GSHPx)

The SOD activity was measured in accordance with Yu et al. (1998), by means of a test based on the inhibition of the Nitro photochemical reduction tetrazolium (NBT). The absorbance of the sample was measured at a wavelength of 560 nm.

The SOD activity was expressed in units (U) min^{-1} mg^{-1} protein, where one unit corresponds to the amount of the enzyme required to cause inhibition of 50% of the reduction of the NTB.

CAT activity was determined as described by Badiani et al. (1990), through the analysis of the consumption of H_2O_2 (39.4 molar extinction coefficient $mM^{-1}cm^{-1}$) at a wavelength of 240 nm for 3 minutes.

The assay of enzymes APX and GR was performed according to Rao et al. (1996). The APX activity was determined by recording the change in absorbance at 290 nm for 3 minutes of a reaction mixture containing buffer potassium phosphate 100 mM (pH 7.5), 0.5 mM of AsA, 0.2 mM H_2O_2 and 0.75 mL of enzyme extract. On the other hand, the GR activity was measured following the oxidation of NADPH at 340 nm for 3 minutes in a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 2 mM Na_2-EDTA, 0.2 mM NADPH, 0.5 mM GSSG and 0.75 mL of enzyme extract.

GSHPx activity was determined as described by Edwards *et al.* 1996, through the analysis of the consumption of H_2O_2 (39.4 molar extinction coefficient $mM^{-1}cm^{-1}$) at a wavelength of 240 nm for 3 minutes.

3.6 Determination of the concentration of protein in the plant extracts

The concentration of proteins in the enzyme extracts was determined by the method of Bradford (1976), using serum-albumin as standard.

3.7 Determination of the concentration of non-enzymatic antioxidant systems ascorbic acid (AsA) and glutathione (GSH)

For the extraction and quantification of the AsA reduced the method followed was described by (Law et al 1992). This method is based on the reduction of Fe^{3+} to Fe^{2+} by AsA in acid solution. 0.5 g frozen leaf material was ground in 5 mL of metaphosphoric acid 5% (w/v) and was subsequently centrifuged at 16000 g for 15 minutes. After 0.2 mL of supernatant was added to a tube with 0.5 mL buffer phosphate sodium 150 mM (pH 7.5) and 0.1 mL of distilled water. The mixture was shaken and incubated at room temperature and in darkness for 10 minutes. Then, 0.1 mL of N-etilmaleimida 0.5% (w/v), 0.4 mL of orthophosphoric acid to 44% (v/v), 0.4 mL of 2, 2´-bipiridil to 4% (w/v) in ethanol 70% and 0.2 mL of $FeCl_3$ to 3% (w/v) were added. Then the test tubes were shaken and incubated at 40°C and in darkness for 40 minutes. Finally, the absorbance measured at 525 nm against a standard curve of AsA followed the same procedure above.

For the extraction and quantification of the GSH reduced, the method followed was described by Gronwald et al. (1987). 0.2 g frozen leaf material was ground in 1 mL of HCl 0.2 M and was centrifuged at 16000 g for 10 minutes. Then 500 µl of the supernatant was caught and 500 µl of sodium phosphate buffer (pH 7.5) was added. An aliquot of 25 µl was extracted and 90 µl sodium phosphate buffer,

10 µl EDTA 10 mM, 10 µl of NADPH 10 mM, 10 µl DTNB 6 mM, 35 µl of distilled water and 10 µl of GR 10 UD/mL were added. The GSH Reduced concentration was measured at 412nm.

3.8 Determination of the antioxidants test

The TEAC and FRAP assay was determined as described Benzie and Strain 1996 the absorbances were measured at 734 nm and 593 nm respectively.

The DPPH and Reducing-power assay were determined as described by Hsu *et al.* 2004 the absorbances were measured at 517 nm and 700 nm respectively.

3.9 Hormone extraction and analysis

IAA, GA4, CKs (tZ and iP), ethylene precursor ACC, ABA, SA and JA concentrations were analysed as in Ghanem et al. (2008) with some modifications. The GA1, GA3 and Riboside concentrations were analysed too, these hormones concentrations were not found in both genotypes, data not revealed. Briefly, 30 mg of homogenized dry material was dropped in 0.5 ml of cold (-20°C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20000 g, 15 min) and re-extracted for 30 min at 4ºC in an additional 0.5 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and part of plant pigments and evaporated at 40ºC under vacuum either to near dryness or until the organic solvent was removed. The

residue was dissolved in 1 ml methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through Millex nylon membrane filters 13 mm diameter of 0.22 µm pore size (Millipore, Bedford, MA, USA). Next, 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using heated electrospray ionization (HESI) interface. The mass spectra were determined using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each component analysed (1, 10, 50, and 100 µg l-1) and corrected for 10 µg l-1 deuterated internal standards. Recovery percentages ranged between 92 and 95%.

3.10 Determination of Na^+ and K^+ ions

The samples were mineralized by wet digestion according to Wolf (1982). To carry this out, 0.2 g of freeze-dried leaves were ground and mineralized with 98% H_2SO_4 and H_2O_2 to 30% at 300 °C. K^+ and Na^+ were analyzed by ICP-OES.

3.11 Statistical analysis

All analyses were repeated in triplicate and the results were evaluated statistically using an analysis of variance ANOVA simple with a 95% confidence interval. The differences between the treatments means were compared using the test of the minor differences of Fisher (LSD) at a 95% probability level. Significance levels were expressed as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS not significant.

4 Results

4.1 Foliar biomass, RGR, Na, and K concentration and Na/K ratio

Plant growth was determined by the foliar biomass and RGR. Of the two genotypes, the one more negatively influenced by salinity stress was cv. Marmande RAF, showing a sharp reduction in both parameters. Nevertheless, cv. Grand Brix also presented a reduction in salinity stress treatment, but this reduction was less pronounced (Table 1).

Our results showed an accumulation of the Na^+ ion in the salinity treatment in comparison to control in both genotypes (Table 2). For the K^+ ion, the salinity treatment resulted in a decline in concentration in both genotypes (Table 2). As with the Na^+ ion, the Na/K ratio reached its highest value in the salinity treatment (Table 2). Finally, the comparison between these two genotypes revealed that the cv. Grand Brix reached higher values for the three above-mentioned parameters (Table 2).

Table 1: Effect of saline treatment on the foliar biomass and RGR in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Biomass (g^{-1} FW)		RGR (g g^{-1} day^{-1})	
	Grand Brix	Marmande	Grand Brix	Marmande
Control	8.55±0.04[a]†	8.47±0.13[a]	0.14±0.00[a]	0.14±0.01[a]
Salinity	5.83±0.13[b]	4.66±0.13[b]	0.11±0.01[b]	0.09±0.01[b]
P-value	***§	***	***	***
Grand Brix	7.22[a]‡		0.13[a]	
Marmande	6.56[b]		0.11[b]	
P-value	***		*	
$LSD_{0.05}$	0.30		0.01	

Table 2: Effect of saline treatment in the concentration of Na, K and Na/K ratio in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Na (mg g^{-1}DW)		K (mg g^{-1}DW)		Na/K (mg g^{-1}DW)	
	Grand Brix	Marmande	Grand Brix	Grand Brix	Grand Brix	Marmande
Control	3.29±0.28[b]†	2.70±0.01[b]	24.39±0.48[a]	22.33±0.17[a]	0.14±0.01[b]	0.12±0.00[b]
Salinity	18.78±0.07[a]	11.25±0.21[a]	16.34±0.11[b]	15.26±0.19[b]	1.15±0.00[a]	0.74±0.01[a]
P-value	***§	***	***	***	***	***
Grand Brix	11.03[a]‡		20.37[a]		0.65[a]	
Marmande	6.98[b]		18.80[b]		0.43[b]	
P-value	***		***		***	
$LSD_{0.05}$	1.28		0.59		0.07	

†Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).
‡ Values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0,05).
§Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0,05; **P< 0,01 and *** P< 0,001 relative to the control.

4.2 Promoters and indicators of oxidative stress

Our results showed a decline in the O_2^- concentration in the salinity treatment in both genotypes with respect to control (Figure 1A; P<0.001). The foliar H_2O_2 concentration increased in the salinity treatment in Marmande RAF and decreased in Grand Brix (Figure 1B; P<0.05).

The saline treatment affected the genotypes differently in terms of the LOX activity (Figure 1C). This enzymatic activity rose significantly in the salinity treatment in Grand Brix (Figure 1C; $P<0.05$), maintaining without significant changes in Marmande RAF (Figure 1C; $P>0.05$). On the other hand, the MDA concentration increased in saline treatment in both cultivars (Figure 1D; $P<0.001$).

In general, on comparing the two genotypes, we found that Marmande RAF had higher concentrations of O_2^- and H_2O_2 (Figure 1A1; $P<0.001$; Figure 1B; $P<0.05$), whereas LOX activity and MDA concentration did not differ between genotypes (Figure 1C1 and 1D1; $P>0.05$)

4.3 Antioxidant enzyme systems

Our results for SOD showed greater activity for saline treatment in both genotypes with respect to control (Table 5). Meanwhile, Grand Brix presented significantly greater SOD activity than in Marmande RAF (Table 5). On the other hand, the CAT activity fell in salinity treatment for both genotypes (Table 5). The genotypes didn´t register differences for CAT activity (Table 5).

APX and GSHPx enzymes increased in activity with the treatment in both genotypes (Table 5). For these enzymes, Marmande RAF presented greater activity than did Grand Brix (Table 5). The GR enzyme didn't show changes significant in both genotypes (Table 5).

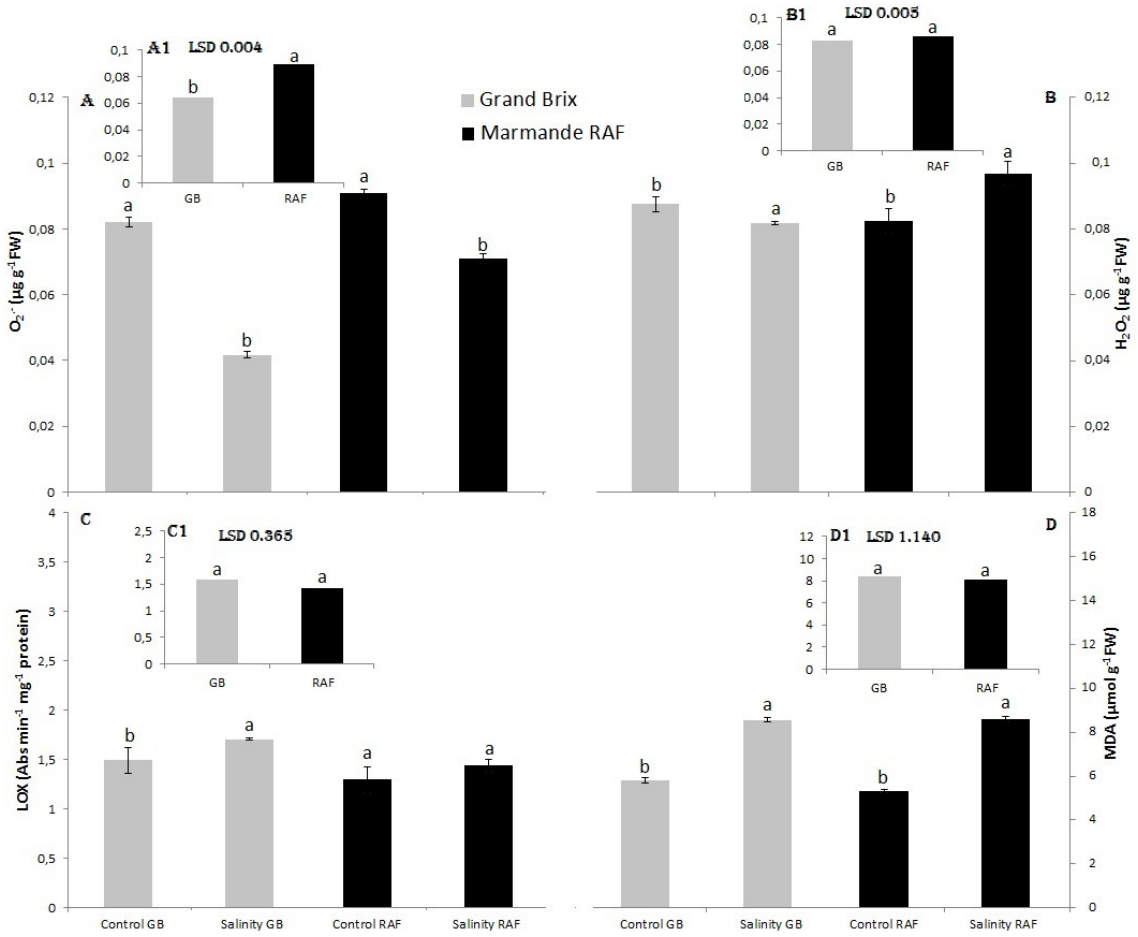

Figure 1: Effect of saline treatment in the concentration of $O_2^{\cdot-}$(µg g^{-1} FW) (A), H_2O_2(µg g^{-1} FW) (B), LOX (Abs min-1 mg-1 protein) (C) and MDA (µmol g^{-1} FW) (D) in plants of Solanum lycopersicum cv. Grand Brix and cv. Marmande RAF. The columns Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1, B1, C1 and D1 Values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0.05). Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

Table 5: Effect of saline treatment in the CAT, SOD, APX, GR and GSHPx activity in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	SOD (U mg^{-1}protein)		CAT (Abs min^{-1} mg^{-1} protein)		APX (Abs min^{-1} mg^{-1} protein)		GR (Abs min^{-1} mg^{-1} protein)		GSHPx(Abs min^{-1} mg^{-1} protein)	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	2.50±0.08^{b}†	1.91±0.04^{b}	0.04±0.00^{a}	0.02±0.00^{a}	0.03±0.00^{b}	0.03±0.00^{b}	0.02±0.00^{a}	0,02±0,00^{a}	0,01±0,00^{b}	0,01±0,00^{b}
Salinity	3.38±0.05^{a}	2.63±0.04^{a}	0.01±0.00^{b}	0.01±0.00^{b}	0.04±0.00^{a}	0.07±0.00^{a}	0.02±0.00^{a}	0,02±0,00^{a}	0,02±0,00^{a}	0,02±0,00^{a}
P-valor	***§	***	***	***	***	***	NS	NS	***	***
Grand Brix	2.85^{a}‡		0.02^{a}		0.05^{b}		0.02^{a}		0.02^{b}	
Marmande	2.05^{b}		0.02^{a}		0.07^{a}		0.02^{a}		0.03^{a}	
P-valor	***		NS		***		NS		***	
$LSD_{0.05}$	0.15		0.00		0.01		0.00		0.00	

†Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).

‡ Values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0,05).

§Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0,05; **P< 0,01 and *** P< 0,001 relative to the control.

4.4 Non-enzymatic antioxidant systems

For a complete assessment of the antioxidant response, an analysis was made of the concentrations of the different forms of AsA and GSH. Both the (ascorbate total) AsA_{tot} concentration and the (ascorbate reduced) AsA_{red} concentrations rose with the salinity treatment for Grand Brix. While in Marmande RAF there parameters hadn't changed significant (Table 6). On the other hand, the ratio AsA_{tot}/AsA_{red} reached its maximum value in the salinity treatment for both genotypes (Table 6). Finally, differences were found between genotypes only for AsA_{tot}, which proved greater in Grand Brix (Table 6).

With respect to the forms of GSH, we found a decline after applying the salinity treatment (Table 7). On comparing the genotypes, we found that the GSH_{tot} concentration was greater in Grand Brix, the ratio GSH_{tot}/GSH_{red} was higher in Marmande RAF, and no significant differences appeared for GSH_{red} (Table 7).

Table 6: Effect of saline and alkaline treatments in the concentration of AsA_{tot}, AsA_{red} and AsA_{tot}/AsA_{red} ratio in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Total AsA (mg g^{-1} FW)		Reduced AsA (mg g^{-1} FW)		AsAtot/AsAred (mg g^{-1} FW)	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	0.09±0.00[b]†	0.10±0.00[a]	0.01±0.00[b]	0.02±0.00[a]	5.88±0.18[b]	7.15±0.09[b]
Salinity	0.12±0.00[a]	0.10±0.00[a]	0.02±0.00[a]	0.02±0.00[a]	7.36±0.21[a]	7.74±0.22[a]
P-valor	***§	NS	***	NS	***	***
Grand Brix	0.11[a]‡		0.03[a]		5.91[a]	
Marmande	0.10[b]		0.03[a]		6.10[a]	
P-valor	**		NS		NS	
$LSD_{0.05}$	0.00		0.00		0.42	

Table 7: Effect of saline and alkaline treatments in the concentration of GSH_{tot}, GSH_{red} and GSH_{tot}/GSH_{red} ratio in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Total GSH (mg g^{-1} FW)		Reduced GSH(mg g^{-1} FW)		GSHtot/GSHred (mg g^{-1} FW)	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	0,27±0,00[a]†	0,25±0,00[a]	0,18±0,00[a]	0,18±0,01[a]	1,47±0,01[a]	1,46±0,01[a]
Salinity	0,12±0,00[b]	0,11±0,00[b]	0,08±0,01[b]	0,08±0,00[b]	1,28±0,01[b]	1,39±0,02[b]
P-valor	***§	***	***	***	***	**
Grand Brix	0,20[a]‡		0,13[a]		1,38[b]	
Marmande	0,18[b]		0,13[a]		1,43[a]	
P-valor	**		NS		***	
$LSD_{0.05}$	0,01		0,01		0,04	

†Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).
‡ Values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0,05).
§Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0,05; **P< 0,01 and *** P< 0,001 relative to the control.

4.5 Antioxidant test

The antioxidant test in general presented increases for the stress treatment in both genotypes (Figure 2), the DPPH test reflecting the most significant differences (Figure 2A; P<0.001). Grand Brix showed the highest levels for the DPPH and TEAC tests (Figure 2A1 and 2D1; P<0.001) while Marmande RAF reached the highest values for FRAP and reducing power (Figure 2B1 and 2C1; P<0.001)

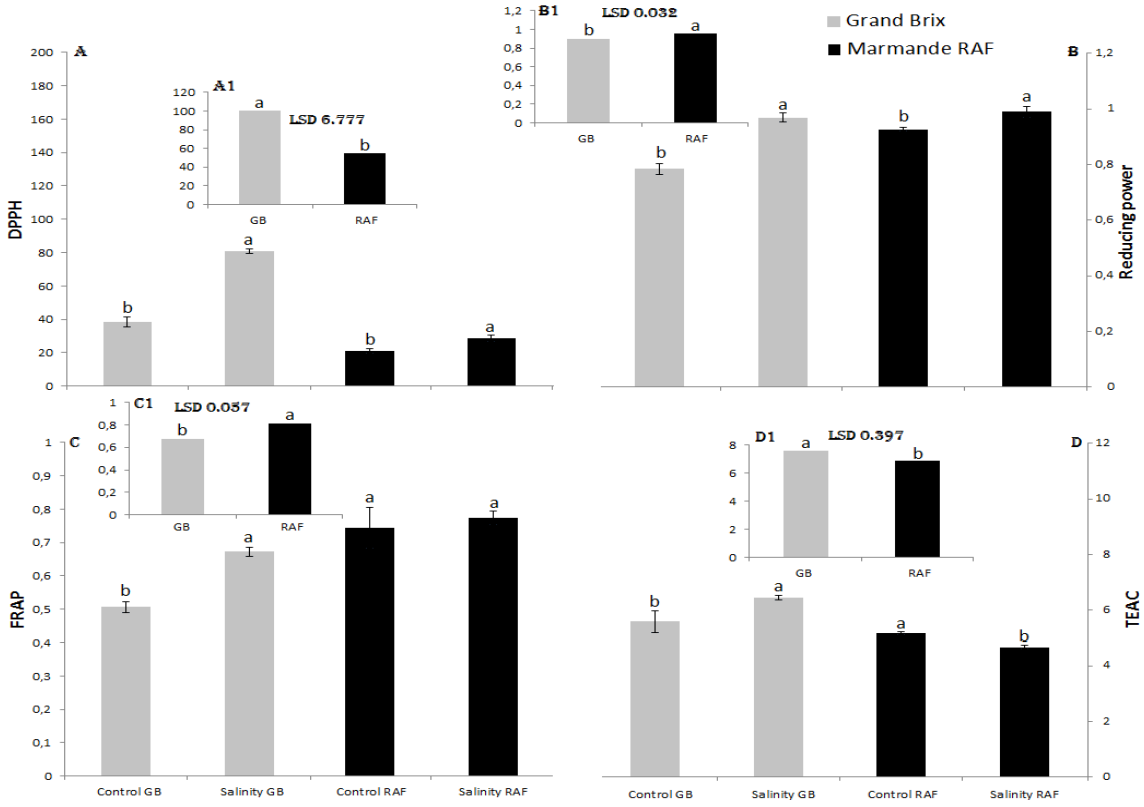

Figure 2: Effect of saline treatment in the antioxidant test DPPH expressed as % g^{-1} FW (A), Reducing power expressed as% of ascorbic acid (1 mM) equivalent activity g^{-1} FW (B), FRAP expressed as mg $Fe(SO_4)$ g^{-1} FW (C) and TEAC expressed as mmol Trolox equivalent (TE) g^{-1} FW. (D) in plants of Solanum lycopersicum cv.Grand Brix and cv.Marmande RAF. The columns Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1, B1, C1 and D1 Values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0.05). Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

4.6 Phytohormone profile

In our experiment, the cv. Grand Brix showed in the saline treatment a clear increase of hormones concentration related to growth (IAA, tZ, iP and GA_4). In the case of GA4 was detected only in the saline treatment (Table 3). In the cv. Marmande RAF, although also registered an increase in tZ and iP hormones concentration in saline treatment, the IAA and GA_4 hormones were not detected (Table 3). On the other hand, the comparative study of genotypes showed that the cv. Marmande RAF presented higher concentrations of the hormones iP and tZ than the cv. Grand Brix (Table 3).

Regarding stress-related hormones the cv. Grand Brix presented a significant increase for the concentration of the hormones ABA and SA while for the hormones ACC and JA concentrations presented a decrease in the saline treatment (Table 4). The cv. Marmande RAF showed an increase in all stress hormones concentration (ABA, ACC, JA and SA) in the saline treatment but the increase in JA concentration was not significant (Table 4). The comparative study revealed that the cv. Grand Brix registered higher concentrations of ACC, JA and SA hormones, whereas Marmande RAF presented a higher ABA concentration (Table 4).

Table 3: Effect of saline treatment in the concentration of indole-3-acetic acid (IAA), Trans-Zeatine (tZ), isopentenyl adenine (iP) and Gibberellin A4 (GA4) in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	IAA (ng g^{-1})		tZ (ng g^{-1})		iP (ng g^{-1})		GA4 (ng g^{-1})	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	0.86 ± 0.14^{b}†	NF	379.72 ± 1.12^{b}	921.31 ± 32.00^{b}	0.28 ± 0.04^{b}	2.46 ± 0.11^{b}	NF	NF
Salinity	1.38 ± 0.08^{a}	NF	715.25 ± 7.98^{a}	1213.41 ± 25.49^{a}	0.74 ± 0.03^{a}	6.87 ± 0.21^{a}	0.26 ± 0.14^{a}	NF
P-value	*§	-	***	**	***	***	-	-
Grand Brix	-		547.48^{b}‡		0.51^{b}		-	
Marmande	-		1067.36^{a}		4.66^{a}		-	
P-value	-		***		***		-	
$LSD_{0.05}$	-		47.38		1.51		-	

Table 4: Effect of saline treatment in the concentration of abscisic acid (ABA), aminocyclopropane-1-carboxylic acid (ACC), jasmonic acid (JA) and salicylic acid (SA) in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	ABA (ng g^{-1})		ACC (ng g^{-1})		JA (ng g^{-1})		SA (ng g^{-1})	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	369.29 ± 9.47^{b}†	507.74 ± 0.81^{b}	18.55 ± 2.76^{a}	3.70 ± 0.23^{b}	580.12 ± 16.66^{a}	70.87 ± 2.53^{a}	4048.37 ± 40.92^{b}	650.19 ± 10.42^{b}
Salinity	602.56 ± 0.58^{a}	568.02 ± 1.56^{a}	9.33 ± 2.60^{b}	5.56 ± 1.18^{a}	345.03 ± 10.18^{b}	80.39 ± 4.82^{a}	14338.3 ± 99.75^{a}	1797.71 ± 13.82^{a}
P-value	***§	***	**	*	***	NS	***	***
Grand Brix	485.92^{a}‡		13.94^{a}		462.58^{a}		9193.35^{a}	
Marmande	537.88^{a}		4.63^{b}		75.63^{b}		1223.95^{b}	
P-value	NS		**		***		***	
$LSD_{0.05}$	66.03		5.96		94.73		3448.91	

†Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).

‡ Values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0,05).

§Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0,05; **P< 0,01 and *** P< 0,001 relative to the control.

5 Discussion

Biomass and RGR are optimal indicators to evaluate plant stress and thus reflect plant growth (Gong et al. 2013). The reduction of growth under conditions of saline stress is well characterized in plants such as tomato, maize, and alfalfa (Li et al. 2010; Wang et al. 2011; Gong et al. 2014b; Li et al. 2014). In our experiment, both parameters declined in the plants subjected to the salinity treatment. The saline treatment reduced plant biomass by 32% in the cv. Grand Brix and by 52% in the cv. Marmande RAF (Table 1). Like biomass, RGR was reduced in the salinity treatment by 19% for the cv. Grand Brix and by 39% for the cv. Marmande RAF (Table 1). Concerning the negative effect in relation to genotypes, Sánchez-Rodríguez et al. (2010) observed that the most tolerant tomato cultivar lowered its biomass and its RGR levels under water stress. Khaliq et al. (2015) concluded that the most sensitive maize cultivar also registered the lowest biomass under salinity stress. In our study, the greatest loss of biomass and RGR were found in the cv. Marmande RAF. In short, according to these results, we can define the cv. Marmande RAF as the more sensitive genotype of the two regarding salinity stress.

The decline in biomass and RGR under salinity stress, are related to Na^+ accumulation and K^+ deficit, as these alter the basic physiological processes for the plants such as photosynthesis (Li et al. 2010). In our experiment, the salinity treatment resulted in the greatest accumulation of Na^+ (an increase of 470% in the cv. Grand Brix 318% and an increase of in the cv. Marmande RAF; Table 2). Works such as Li et al. (2010) have also reported a greater accumulation of Na^+ in the salinity treatments in alfalfa plants. In addition, works as Gong et al. (2013)

and Wang et al. (2011) in tomato or Li et al. (2014) in rice have found a decline in the concentration of K^+ when the plants were grown with saline treatments. This agrees with our results in which the saline treatment in both genotypes registered the lowest K^+ concentrations (Table 2). Finally, our results a priori appear to indicate that the cv. Grand Brix should be more affected by the salinity treatment since this genotype accumulates more Na^+ and the Na^+/K^+ ratio is greater than the cv. Marmande RAF; however, the growth data indicate that the more sensitive genotype was the cv. Marmande RAF (Table 2). This may be due to the strategies such as Na^+ compartmentalization in the vacuoles or the immobilization of this ion (Li et al. 2010), which could be reinforced in the cv. Grand Brix and therefore might be less affected by Na^+ accumulation than the cv. Marmande RAF.

ROS accumulate under different types of stress and for different reasons, as for example under alteration of the photosynthetic machinery, the excess electrons produced are transferred to O_2 molecules and these produce ROS (e.g. H_2O_2 and O_2^-) (Çakmak 2005). In our experiment O_2^- levels are decreased in saline treatment. In our experiment, the $O_2^{\cdot -}$ was reduced by 42% in Grand Brix and by 22% in Marmande RAF, and H_2O_2 was reduced by 13% in Grand Brix and increased by 36% in Marmande RAF. This could be due to the enzyme SOD, but it has also been shown that hormones such as auxins can help in the detoxification of ROS. Therefore in Grand Brix the increase in the concentration of auxins (Table 3) could help explain this O_2^- reduction (Noctor et al. 2015). The increase in ROS promotes lipid peroxidation, a process that can determine LOX activity and the MDA concentration, which is a subproduct of this peroxidation

(Zhou and Zhao 2004). The increases in ROS and lipid peroxidation have been associated with salinity stress in tomato (Gong et al. 2013; Gong et al. 2014b). In our experiment, we found significant increases, especially in the MDA concentrations (Figure 1D $P<0.001$) under stress conditions. Sánchez-Rodríguez et al. 2010 showed in the cultivars most sensitive to water stress a ROS accumulated and an increased in the MDA concentration. ROS and MDA concentration have been defined as good stress indicators (Gong et al 2014a). So, the Figure 1 corroborates that Marmande RAF is more sensitive to this stress than Grand Brix.

Greater SOD activity under salinity stress has been shown by different authors in tomato plants and in Iris lactea (Wang et al. 2008; Gong et al. 2013; Gong et al. 2014b). Our results coincide with the findings of these authors, and the higher SOD activities in the salinity treatment (Table 4) explain the low O_2^- levels (Figure 1A). Also, it bears highlighting that a comparison of the two genotypes indicates that Grand Brix induces more SOD and is more stress resistant. These results suggest that the detoxification of the ion O_2^- by SOD activity is a key process in the identification of the toxicity of saline stress and in the possible generation or selection of plants with greater resistance to this stress.

The other antioxidant enzymes (CAT, APX, GR, and $GSHP_X$) have also been shown to increase in activity in tomato plants and Iris lactea subjected to this type of ionic stress (Wang et al. 2008; Gong et al. 2013; Gong et al. 2014b). For APX and GSHPx enzymes, our results coincide with the results of these authors (Table

4). However, contrary to our expectations, Marmande RAF presented greater activity for APX and GSHPx enzymes, and this would explain the low H_2O_2 accumulation in these plants. Furthermore, would indicate that the enzymatic detoxification of H_2O_2 is not determinant for assessing the tolerant genotype. In our experiment, the H_2O_2 levels are elevated in the saline treatment (Figure 1) causing damage by oxidative stress. But it has been observed that an increase of CKs can decrease the damages caused by the ROS. Grand Brix presents higher levels of CKs (Table 3), this can help to present greater tolerance to stress (Pogány et al. 2004).

In short, Marmande RAF was affected by the stress treatment (Table 1), which could be due to greater O_2^- accumulation, this ROS could be the main cause of the oxidative stress. Grand Brix registered greater SOD activity (Table 4) and therefore would be protected from the damage caused by this ROS, making it the more tolerant genotype against this type of stress.

The levels of non-enzymatic antioxidants are indicators of the redox state of the plant and responsible in part for resistance to stress. Previous studies show that under salinity stress, the levels of AsA and GSH increase (Gong et al. 2013). In this sense, the concentrations of the different forms of AsA and GSH in our work behave in a different way (Table 5 and 6). AsA is used to detoxify H_2O_2 together with APX activity, and therefore its increases coincide in Grand Brix (Tables 4 and 5). Sánchez-Rodríguez et al. 2010 showed high AsA concentrations in the most resistant cultivar. The decrease in GSH in both genotypes with the

applications of salinity treatment (Table 5) could be due to the GSH is being used for other ends and because other compounds under low molecular weight are being generated that could intervene also in the resistance to this type of stress (Khaliq et al. 2015). In any case, it bears emphasizing that, AsA_{tot} and GSH_{tot} concentrations were higher in Grand Brix, which proved more resistant to saline stress in our work (Tables 5 and 6).

The current literature shows how CAT activity increases under saline stress. However, in our experiment the CAT activity declines (Table 5). This decrease may be due to an enzymatic inhibition, however, the accumulation of H_2O_2 is not very high (Figure 1). It could be because the H_2O_2 detoxification is carried out by other ways such as the APX and GSHPx enzymes or by compounds such as AsA.

These tests measure the capacity to reduce pro-oxidant substances (Schleiser et al. 2002). Some studies with metal toxicity indicate that the values of the antioxidant test rise with the level of stress (Ríos et al. 2008). Grand Brix registered the highest antioxidant capacity (Figure 2), and this finding plus the increase in the antioxidant compounds AsA and GSH could corroborate for this genotype being the more resistant than Marmande RAF to this type of ionic stress.

Previous studies on hormones regulating plant growth showed a reduction of the negative effect of salt stress on germination of seeds treated with exogenous IAA

(Javid et al. 2011). Accordingly, the IAA concentration increase observed in our saline treatment (60% for the cv. Grand Brix) (Table 3) would help cv. Grand Brix to have greater tolerance to salt stress. In addition, it was noted that an IAA concentration increase helps improve osmotic stress. Therefore, as salt stress produces osmotic stress, an increase in IAA concentration would help improve plant homeostasis (Naser and Shani 2016). An improvement in the osmotic state of the plant generated by increased IAA concentration also provides better resistance to salt stress in cv. Grand Brix. In addition, in plants with depressed CAT activity (Table 5) an increase of auxins (Table 3) may help to detoxify ROS, which could explain the greater tolerance of Grand Brix to oxidative stress (Noctor et al. 2015).

Ghanem et al. (2011) underwent two varieties of tomato to salt stress and they showed that the concentration of CKs in the plants increase in the leaves in saline treatment. In our experiment, it was also observed an increase in the concentration of CKs in the saline treatment in both varieties. tZ and iP concentrations increased by a 88% and 160% respectively in the cv. Grand Brix and the concentration of these hormones increased by a 32% and 180% respectively for the cv. Marmande RAF (Table 3). Ghanem et al. (2011) concluded that a greater accumulation of CKs, could improve the resistance to salt stress.

Rapid accumulation of GAs is a characteristic of plants exposed to abiotic stresses, and it is an important phytohormone that can impart stress tolerance

including salinity. In our experiment, the GA_4 was only detected in cv. Grand Brix in saline treatment, while in control plants and in the cv. Marmande RAF was at a too low concentration to be detected (Table 3). Therefore, we assume that there is an increase in GA_4 concentration in this treatment which can increase the resistance to salinity in cv. Grand Brix as above mentioned. Iqbal and Ashraf (2013) submitted two cultivars of wheat to salt stress and an external GAs application. They observed that treatments with GAs subjected to salt stress registered higher biomass than untreated plants. Therefore, they concluded that GAs could reduce the detrimental effect of ABA to be antagonists. Whereby, this suggests that the increase of GA_4 concentration in the saline treatment of cv. Grand Brix could increase its tolerance to salt stress.

In short, the IAA, tZ and GA_4 concentrations hormones increase the resistance to salt stress, maintaining the growth of the plant. The cv. Grand Prix presents the greatest increases in the concentrations of these hormones, being the most resistant to salt stress genotype.

ABA and ethylene hormones have long been regarded as stress hormones with specific roles in regulating tolerance and adaptation to salt stress (Amjad et al. 2014). Amjad et al. (2014) submitted two genotypes of tomato to salt stress, their results showed that ABA concentration increased in both genotypes, by saline treatment, but increased more in the salinity tolerant genotype. For ethylene concentration, both genotypes increased equally in saline treatment. In our experiment ABA concentration increased in the same way, increasing more in the

saline treatment cv. Grand Brix (63% in the cv. Grand Brix and 12% in the cv. Marmande RAF; Table 4). Amjad et al. (2014) also relate a higher ABA concentration with greater efficiency in water use and better stomatal regulation, possibly improving salt stress. ACC (ethylene precursor) concentration decreases by 50% in cv. Grand Brix while increasing 50% for cv. Marmande RAF (Table 4). Sharp and LeNoble (2002) showed as an increase in ethylene concentration reduces plant biomass because it promotes programmed cell death and leaf senescence. These authors also show an interaction between ethylene and ABA, where the ABA inhibits ethylene action and synthesis. In our experiment a lower concentration of ethylene combined with a greater $O_2^{\cdot -}$ detoxification in the cv. Grand Brix could play a fundamental role in tolerance to saline stress (Overmyer et al. 2003). Besides, an increase in ABA levels promotes better stomatal closure and better control of photosynthesis and a lower rate of water loss.

The H_2O_2 stimulates the ABA accumulation (Table 4). This accumulation of ABA promotes stomatal closure thus avoiding the loss of water that can cause saline stress, and generating better osmotic adjustment and therefore better resistance to stress. (Mittler and blumwald 2015).

JA and SA hormones are mainly related to the stress by pathogens, however they have other functions related to abiotic stresses and interact with other hormones. For example, JA is known to favour the accumulation of ABA and CKs (Avalbaev et al. 2016), and also is involved in the ACC synthesis (Dar et al. 2015), also the

JA has a close relationship with the SA. When SA has a very high concentration acts as a negative allosteric effector of JA and this can reduce their synthesis and concentration in the plant (Singh and Gautam 2013). This may be the reason why the JA concentration decreases in the saline treatment cv. Grand Brix (Table 4). SA is also similarly related to increased concentrations of ABA and CKs (Shakirova et al. 2003). Our results agree with those of the authors (Table 3 and 4). On the other hand, SA has been linked to improve the response of plants against oxidative stress, such as caused by salt stress (Singh and Gautam 2013). These results further supports that the cv. Grand Brix is more tolerant to salinity, because the hormone SA concentration increased by 254% in the cv. Grand Brix (Table 4).

For stress-related hormones cv. Grand Brix again has the highest resistance to salt stress. This resistance is given by an increase in hormones concentrations that prevent water loss and contribute to the antioxidant defense (ABA and SA). Also, it has a lower concentration of ACC contributing to reducing senescence.

6 Conclusion

This work leads us to conclude that Na^+ accumulation, the ion K^+ reduction and especially the O_2^- increase could be the main causes of the toxic effects of the stress studied. On the other hand, cv. Grand Brix has a better hormonal profile adapted to salt stress resistance. Hormones related to growth reveal differences between genotypes that could be key for resistance to salt stress. The comparative study of the two genotypes indicated that cv. Grand Brix, despite

accumulating a higher Na^+ concentration than did cv. Marmande RAF, showed less oxidative stress, as it showed greater detoxification of the ion superoxide with an induction of the enzymatic activity SOD and greater antioxidant capacity. Furthermore, the accumulation of IAA, GA_4 and CKs and their beneficial role against oxidative stress could make the difference between resistance and sensitivity to salt stress. On the other hand, the ABA and tZ increase, and ACC decreased concentrations could also mark the resistance in the cv. Grand Brix. In conclusion, our results reveal that O_2^- accumulation could be the key factor determining the toxic effect of saline stress, and therefore its detoxification is essential to develop and/or select plants with stronger resistance to such abiotic stress in plants. Furthermore, the key hormones in the saline stress tolerance could be IAA, tZ, GA_4, ABA and ACC. although would be necessary more specific studies.

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CAPÍTULO 2

Estudio comparativo del efecto toxico de la salinidad en diferentes genotipos comerciales de tomate: metabolismo de los carboxilatos

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Resumen

El tomate es uno de los cultivos de mayor importancia económica en el mundo y el estrés salino provoca una reducción en la cantidad y calidad de la producción de cultivos. Hoy el principal desafío en la agricultura mundial es proveer a una población mundial en continuo crecimiento, y esto se vuelve más difícil debido al cambio climático, ya que esto provoca un mayor estrés abiótico en plantas. El objetivo de este estudio fue determinar la participación del ciclo TCA en la resistencia a la salinidad en dos genotipos de tomate. Encontramos que el cv. Grand Brix mejora la actividad del ciclo del ácido tricarboxílico (TCA) como mecanismo de resistencia al estrés salino, mientras que el cv. Marmande RAF no muestra este mecanismo. Esto provoca una mayor concentración de ácidos orgánicos En el genotipo Grand Brix lo que favoreceria la resistencia al estrés salino. Además, en este trabajo proponemos la hipotesis de la formación de un complejo oxaloacetato-Na como mecanismo que ayude a controlar el exceso de Na^+ en el cv. Grand Brix.

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Comparative study of the toxic effect of salinity in different genotypes of tomato plants: Carboxylates Metabolism

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1 Abstract

Tomato is a crop with the greatest economic importance in the world and salinity stress causes a reduction in the quantity and quality of crop production. Today the main challenge in world agriculture is to sustain the continuously growing global population, and this becomes more difficult due to climatic change, as this imposes further abiotic stress. The aim of this study was to determine the involvement of the TCA cycle in resistance to salinity in two genotypes of tomato. We found that the cv. Grand Brix enhances the tricarboxylic acid (TCA) cycle activity as a mechanism of resistance to salt stress, while the cv. Marmande RAF does not have this mechanism. This causes a greater accumulation of organic acids in Grand Brix which favours even more resistance to salt stress. We propose an idea of how the oxaloacetate could help to control Na^+ excess in the cv. Grand Brix.

Keywords: saline stress, *Solanum licopersicum* L., Organic acid, TCA cycle, citrate, malate, oxaloacetate.

2 Introduction

Tomato is the crop with the greatest economic importance in the world. According to FAO, in 2014 roughly 4,888,880 tonnes of tomato were produced in Spain alone, cultivated on 54,750 Ha. A great part of this cultivation area is affected by salinity stress. In particular, salinity stress causes a reduction in the quantity and quality of crop production (Saito et al. 2008). Currently, the main challenge in world agriculture is to sustain the continuously growing global population, and this becomes more difficult due to climatic change, as this imposes further abiotic stress. In the coming years, several factors could exacerbate this situation, such as the continual spread of intensive agriculture and the use of poorer quality water. Therefore, it is very important to ascertain the impact of saline stress in tomato cultivation. Greatly limiting crop yield in semiarid and arid regions, salinity affects roughly 397 million Ha of soils in the world (Gong et al. 2013). This is particularly true in the Mediterranean area, where cultivation tends to occupy small fields often with high quality and commercial value crops, such as tomato (Lynch and Clair 2004). Organic acid metabolism is of fundamental importance at the cellular level for several biochemical pathways, including energy production, formation of precursors for amino acid biosynthesis and at the whole plant level modulating adaptation to the environment (López-Bucio et al. 2000). Nowadays we have a lot of information about the functioning of carboxylate metabolism, however there are few studies about the relationship between salt stress and the activity of TCA cycle enzymes.

Growth conditions under salinity stress, trigger osmotic and ionic imbalances, prompt oxidative stress, and upset the plant's metabolism. Some molecules are

involved to resolve these imbalances, such as organic acids (Sazzad Hossain and Karl-Josef Dietz 2016). Studies have been made in this regard in tomato plants, but especially these papers have focused on the responses of organic acids in fruits. These studies have generally shown that salinity produces fruits with a higher content of sugars and organic acids, which contribute to improve fruit market quality (Cuartero and Fernández-Muñoz 1999). For example, Saito et al. (2008) observed an increase of organic acids in Solanum lycopersicum fruits under salt stress, after the overexpression of the aconitate synthase enzyme. These authors showed that organic acids accumulation, increases fruit quality, and also favours the accumulation of other compounds such as sugars. However, the study of the TCA cycle importance in salinity resistance in tomato plants has not been addressed.

In recent years, in other species, some researchers have tried to overexpress enzymes of this metabolism in order to improve certain types of stress by the accumulation of organic acids (Ryan et al. 2001). In fact, it has been observed, in tobacco transgenic plants and *Arabidopsis thaliana*, a relationship between overexpression of the citrate synthase (CS) enzyme and citrate increase, the accumulation of organic acids and resistance to Al^{3+} stress (Ryan et al. 2001). Richter et al. 2015 in a study with two corn hybrids concluded that the TCA cycle is severely affected by salt stress since the cycle´s metabolites and carbon catabolism are reduced, it affects plant metabolism. In other species as pea plants or Arabidopsis, Jacoby et al. (2011) showed how an increase of malate dehydrogenase enzyme (MDH) increases salt stress tolerance.

In this context, considering the importance of the organic acids and carboxylate metabolism in plant, and its relationship with resistance to saline stress, we investigate here the response of these metabolic processes in two tomato genotypes submitted to salinity stress. The final aim is to determine whether the organic acids and the TCA cycle enzymes are key to select and/or generate the cultivar with the best tolerance to this type of stress.

3 Material and methods

3.1 Plant material and treatments

Seeds of *Solanum lycopersicum* cv. Gran brix and *Solanum lycopersicum* cv. Marmande Raf (Saliplant S.L., Spain) were germinated and grown for 30 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were placed on benches in an experimental greenhouse located in Southern Spain (Saliplant S.L., Motril, Granada). After 30 days, the seedlings were transferred to a growth chamber (Department of plant physiology, University of Granada) under the following controlled environmental conditions: Relative humidity 60-80%; Day/night temperatures 28/19 °C; 16/8 h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol $m^{-2}s^{-1}$ (measured at the top of the seedlings with a 190 SB quantum sensor, LI-CORInc., Lincoln, Nebraska, USA). Under these conditions, the plants were grown in hydroponic cultivation in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) of 3 L volume, 8 plants/tray. Throughout the experiment the plants were cultured with a growth solution made up of 4 mM KNO_3, 3 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 6 mM KH_2PO_4, 1 mM

$NaH_2PO_4 \cdot 2H_2O$, 2 µM $MnCl_2 \cdot 4H_2O$, 1 µM $ZnSO_4 \cdot 7H_2O$, 0.25 µM $CuSO_4 \cdot 5H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2H_2O$, 5 µM Fe-chelate (Sequestrene; 138 FeG100) and 50 µM H_3BO_3. This solution, with a pH of 5.5–6.0, was renewed every three days.

3.2 Experimental design

Treatment of saline stress started 38 days after germination and was maintained for 15 days. The control treatment received the nutrient solution, while the treatment saline stress received the nutrient solution plus 100 mM NaCl. The experimental design was a randomized complete block with two treatments, 8 plants per treatment and with 3 replications per treatment (n = 9).

3.3 Plant sampling and determination of the relative growth rate (RGR)

Plants of each treatment (53 days after germination) were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of leaves from each treatment were frozen at −30 °C for further work and biochemical assays and the other half of the plant material was lyophilised for 48h to obtain the dry weight (DW) and the subsequent analysis of the concentrations of nutrients. To determine the relative leaf growth rate (RGR), leaves from three plants per cultivar were sampled on day 38 after germination, immediately before starting the stress treatment (Ti). The leaves were dried in a forced-air oven at 70 °C for 24 h, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled 53 days after germination (15 days of treatments, Tf). The relative growth rate was calculated from the increase in leaf DW at the beginning and at the end of the water-stress

treatment, using the equation RGR = (ln DWf − ln DWi)/(Tf − Ti) where T is the time and the subscripts denote the final and initial sampling.

3.4 Carboxylate metabolism

Extracts for measuring enzyme activities were made following the method of Li et al. (2000), modified by grinding 0.1 g of plant material in liquid N with 1 ml of extraction buffer containing 1 mM EDTA-Na, 10% glycerol, 1% Triton X-100, 5 mM DTT and 1% polyvinylpyrrolidone (PVP) in 100 mM Tris–HCl pH 8.0. The slurry was centrifuged for 5 min at 14,700 rpm and 4 °C, and the supernatant was collected and analyzed immediately.

The activities of all enzymes were analyzed in 0.2 ml (final volume) of the media indicated below. The activity of MDH (EC 1.1.1.37) was determined with oxalate as substrate by measuring the decrease in absorbance at 340 nm due to the enzymatic oxidation of NADH (Dannel et al. 1995). The reaction was carried out with 0.1 mM NADH, 0.4 mM oxalate and 46.5 mM Tris–HCl, pH 9.5. The activity of CS (EC 4.1.3.7) was assayed spectrophotometrically according to Srere (1969) by monitoring the reduction of acetyl coenzyme A (CoA) to CoA with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm. The reaction was carried out in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5 mM oxalate and 100 mM Tris–HCl, pH 8.1. ICDH (EC 1.1.1.42) activity was determined by monitoring the reduction of NADP at 340 nm in a reaction mixture containing 3.5 mM MgCl2, 0.41 mM NADP, 0.55 mM isocitrate and 88 mM imidazole buffer pH 8.0. Fumarase (EC 4.2.1.2) was assayed following the increase in optical density at 240 nm due to the

formation of fumarate in 50 mM malate and 100 mM phosphate buffer, pH 7.4 (Bergmeyer et al. 1974). Finally PEPC (EC 4.1.1.31) activity was measured in a coupled enzyme assay with the MDH in 2 mM phosphoenolpyruvate (PEP), 10 mM NaHCO3, 5 mM MgCl2, 0.16 mM NADH and 100 mM of N,N-bis[2-hydroxyethyl]glycine (Bicine)-HCl, pH 8.5 (López-Millán et al. 2001).

3.5 Determination of the concentration of protein in the plant extracts

The concentration of proteins in the enzyme extracts was determined by the method of Bradford (1976), using serum-albumin as standard.

3.6 Concentrations of organic anions by U-HPLC-MS

Malic, citric and oxalic acids were analysed according to Gómez-Romero et al. (2010) with some modifications. Briefly, 75 mg of freeze-dried and ground plant material was dropped in 1 ml of cold (-20°C) extraction mixture of methanol/water/acetic acid (80/19.5/0.5, v/v/v). Solids were separated by centrifugation (20.000 g, 15 min) and re-extracted for 30 min at 4ºC in additional 1 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C_{18} cartridges (SepPak Plus, Waters, USA) to remove interfering lipids and part of plant pigments and evaporated at 40ºC under vacuum to near dryness. The residue was dissolved in 1 ml water/methanol/acetic acid (94.5/5/0.5, v/v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA).

Ten µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. The analytes were separated using a Zorbax SB-C18 HPLC column (5 µm, 150 x 0.5 mm, Agilent Technologies, Santa Clara, CA, USA), maintained at 30 ºC. Mobile phase A, consisting of water/methanol/acetic acid (94.5/5/0.5), and mobile phase B, consisting of water/methanol/acetic acid (10/89.5/0.5), were pumped at a flow rate of 300 µl min^{-1}. The elution programme maintained 100% A for 5 min, then a linear gradient from 0 to 6% B in 10 min, followed by another linear gradient from 6 to 100% B in 5 min, and finally 100% B maintained for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 15 min before each analytical run. The mass spectrometer was operated in the negative mode with a capillary spray voltage of 2500 V. The sheath gas flow rate was set to 35 ml min^{-1} whereas the auxiliary gas was set to a flow rate of 10 ml min^{-1}. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the organic acids, calibration curves were constructed for each analysed component (1, 2.5, 5, and 10 mg l^{-1}).

3.7 Determination of Na^+ and K^+ ions

The samples were mineralized by wet digestion according to Wolf (1982). To carry this out, 0.2 g of freeze-dried leaves were ground and mineralized with 98% H_2SO_4 and H_2O_2 to 30% at 300 °C. K^+ and Na^+ were analyzed by ICP-OES.

3.8 Statistical analysis

All analyses were repeated in triplicate and the results were evaluated statistically using an analysis of variance ANOVA simple with a 95% confidence interval. The differences between the treatments means were compared using the test of the minor differences of Fisher (LSD) at a 95% probability level. Significance levels were expressed as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS not significant.

4 Results

4.1 Foliar biomass, RGR, Na, and K concentration and Na/K ratio

Plant growth was determined by foliar biomass and RGR. Of the two genotypes, the one more negatively influenced by salinity stress was cv. Marmande RAF, showing a sharp reduction in both parameters. Nevertheless, cv. Grand Brix also presented a growth reduction under salinity stress treatment, but this reduction was less pronounced (Table 1).

Table 1: Effect of saline treatment on the foliar biomass and RGR in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Biomass (g^{-1} FW)		RGR (g g^{-1} day^{-1})	
	Grand Brix	Marmande	Grand Brix	Marmande
Control	8.55±0.04[a]†	8.47±0.13[a]	0.14±0.00[a]	0.14±0.01[a]
Salinity	5.83±0.13[b]	4.66±0.13[b]	0.11±0.01[b]	0.09±0.01[b]
P-value	***§	***	***	***
Grand Brix	7.22[a]‡		0.13[a]	
Marmande	6.56[b]		0.11[b]	
P-value	***		*	
$LSD_{0.05}$	0.30		0.01	

† Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05).

‡ Values are means (n = 18) and differences between means were compared using LSD test (P = 0.05).

§ Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

Our results showed an accumulation of Na^+ ion in salinity treatment in comparison to control in both genotypes (Table 2). For K^+ ion, salinity treatment resulted in a decline in concentration in both genotypes (Table 2). As with Na^+ ion, Na/K ratio reached its highest value in plants grown under salinity treatment (Table 2). Finally, the comparison between these two genotypes revealed that Grand Brix reached higher values for the three above-mentioned parameters (Table 2).

Table 2: Effect of saline treatment in the concentration of Na, K and Na/K ratio in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Na (mg g^{-1}DW)		K (mg g^{-1}DW)		Na/K (mg g^{-1}DW)	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	3.29±0.28[b]†	2.70±0.01[b]	24.39±0.48[a]	22.33±0.17[a]	0.14±0.01[b]	0.12±0.00[b]
Salinity	18.78±0.07[a]	11.25±0.21[a]	16.34±0.11[b]	15.26±0.19[b]	1.15±0.00[a]	0.74±0.01[a]
P-value	***§	***	***	***	***	***
Grand Brix	11.03[a]‡		20.37[a]		0.65[a]	
Marmande	6.98[b]		18.80[b]		0.43[b]	
P-value	***		***		***	
$LSD_{0.05}$	1.28		0.59		0.07	

† Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05).

‡ Values are means (n = 18) and differences between means were compared using LSD test (P = 0.05).

§ Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

Table 3: Effect of saline treatment in the CS, ICDH, Fumarase, MDH and PEPC activity in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	CS (ΔAbs mg prot^{-1}min^{-1})		ICDH (ΔAbs mg prot^{-1}h^{-1})		Fumarase (ΔAbs mg prot^{-1}h^{-1})		MDH (ΔAbs mg prot^{-1}min^{-1})		PEPC (ΔAbs mg prot^{-1}min^{-1})	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	0.11±0.00^{b}†	0.10±0.01^{b}	0.18±0.03^{b}	1.94±0.09^{a}	3.60±0.24^{b}	0.84±0.08^{a}	0.16±0.00^{b}	0.23±0.01^{a}	0.04±0.00^{b}	0.11±0.01^{a}
Salinity	0.17±0.01^{a}	0.13±0.01^{a}	0.32±0.02^{a}	0.69±0.04^{b}	5.26±0.11^{a}	0.28±0.04^{b}	0.24±0.00^{a}	0.25±0.00^{a}	0.06±0.01^{a}	0.08±0.00^{b}
P-value	***§	**	***	***	***	***	***	NS	***	***

Grand Brix	0.14^{a}‡	0.25^{b}	4.43^{a}	0.20^{b}	0.05^{b}
Marmande	0.12^{b}	1.31^{a}	0.56^{b}	0.24^{a}	0.10^{a}
P-value	***	***	***	***	***
$LSD_{0.05}$	0.01	0.33	0.58	0.02	0.02

†Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).

‡ Values are means (n = 18) and differences between means were compared using LSD test (P = 0.05).

§Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; **P< 0.01 and *** P< 0.001 relative to the control.

4.2 Carboxylate metabolism

Respecting TCA enzymes, our results showed in the cv. Grand Brix an increase in all enzyme activities in saline treatment (Table-3). The cv. Marmande RAF only showed a significant increase in CS activity. Malate dehydrogenase (MDH) and fumarase enzymes showed a not significant change in their activity in saline treatment, while it was found a significant reduction in phosphoenolpyruvate carboxylase (PEPC) and isocitrate dehydrogenase (ICDH) enzymes activities (Table-3). On the other hand, when comparing between the two genotypes, the cv. Grand Brix only presents greater activity in CS and Fumarase enzymes (Table-3).

4.3 Organic acids concentrations

The results showed that among all the organic acids analysed, malate was the most abundant in both genotypes followed by citrate and oxalate as the least concentrated (Table-4). The cv. Grand Brix showed a significant increase in the malate and citrate concentrations, while Marmande RAF only showed a significant increase in the citrate concentration in saline treatment (Table-4). The malate and oxalate organic acids showed in the cv. Marmande RAF a decrease in the saline treatment. The concentration of all organic acids analysed was higher in the cv. Grand Brix when comparing between both genotypes (Table-4).

Table 4: Effect of saline treatment in the concentration of Malate, Citrate and Oxalacetate in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Citrate (mg g^{-1}DW)		Malate (mg g^{-1} DW)		Oxalate (µg g^{-1}DW)	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	8.38±0.14[b]†	4.23±0.02[b]	14.04±0.24[b]	16.21±0.13[a]	0.40±0.03[a]	0.16±0.00[a]
Salinity	15.86±0.06[a]	4.50±0.06[a]	30.47±0.20[a]	14.36±0.18[b]	0.41±0.01[a]	0.08±0.00[b]
P-value	***§	***	***	***	NS	***

Grand Brix	12.12[a]‡	22.25[a]	0.40[a]
Marmande	4.36[b]	15.28[b]	0.12[b]
P-value	***	**	***
$LSD_{0.05}$	1.64	4.17	0.04

† Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05).
‡ Values are means (n = 18) and differences between means were compared using LSD test (P = 0.05).
§ Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

5 Discussion

5.1 Foliar biomass, RGR, Na and K concentration and Na/K ratio

Biomass and RGR are optimal indicators to evaluate plant stress and thus reflect plant growth (Gong et al. 2013). The reduction of growth under saline stress conditions is well characterized in plants such as tomato, maize, and alfalfa (Li et al. 2010; Wang et al. 2011; Gong et al. 2014; Li et al. 2014). In our experiment, both parameters declined in the plants subjected to the salinity treatment. The saline treatment reduced plant biomass by 32% in Grand Brix and by 52% in Marmande RAF (Table 1). Like biomass, RGR was reduced in the salinity treatment by 19% in the cv. Grand Brix and by 39% in the cv. Marmande RAF (Table 1). Concerning the negative effect in relation to genotypes, Sánchez-Rodríguez et al. (2010) observed that the most tolerant tomato cultivar lowered its biomass and its RGR levels under water stress. Khaliq et al. (2015) concluded that the most sensitive maize cultivar also registered the lowest biomass under

salinity stress. In our study, the greatest loss of biomass and RGR were found in the cv. Marmande RAF. In short, according to these results, we can define the cv. Marmande RAF as the most sensitive genotype of the two regarding salinity stress.

The decline in biomass and RGR under salinity stress could be related to Na^+ accumulation and K^+ deficit, as these could alter the basic physiological processes for the plants such as photosynthesis (Li et al. 2010). In our experiment, the salinity treatment resulted in the greatest accumulation of Na^+ (an increase of 470% in the cv. Grand Brix and an increase of 318% in the cv. Marmande RAF; Table2). Works such as Li et al. (2010) have also reported a greater Na^+ accumulation in the salinity treatments in alfalfa plants. In addition, works as Gong et al. (2013) and Wang et al. (2011) in tomato or Li et al. (2014) in rice have found a decline in K^+ concentration when the plants were grown with saline treatments. This agrees with our results in which the saline treatment in both genotypes registered the lowest K^+ concentrations (Table 2). The reduction of the K^+ levels could be due to the Na^+ occupying the channels of entry of the cations. Since there is so much Na^+, the K^+ would be displaced in this case. In fact, increasing the concentration of K^+ is one of the ways to combat saline stress (Munns and Tester 2008). Finally, our results a priori appear to indicate that Grand Brix should be more affected by the salinity treatment, since this genotype accumulated more Na^+ and its Na/K ratio is greater than in Marmande RAF; however, the growth data indicate that the more sensitive genotype was the cv. Marmande RAF (Table 2). This may be due to the strategies such as Na^+ compartmentalization in the vacuoles or the immobilization of this ion (Li *et al.*

2010; Munns and Gilliham 2015), which could be reinforced in the cv. Grand Brix and therefore might be less affected by Na^+ accumulation than the cv. Marmande RAF.

5.2 Carboxylate metabolism and concentrations of organic acids

There are few studies about how saline stress affects TCA enzyme activities. The published literature shows different results, some of these studies reported that under salt stress the PEPC activity increases in the Common Ice Plant. This causes an increase in the oxaloacetate concentration in the TCA cycle, this increase could enhance the TCA cycle and more organic acids would be accumulated, thus increasing resistance to salt stress (Cushman et al. 1989). Jacoby et al (2011) in Arabidopsis plants subjected to salt stress showed that the MDH enzyme activity was increased, while fumarase activity was diminished. The MDH enzyme increase causes an effect similar to increasing PEPC activity. The oxaloacetate increase in the TCA cycle, therefore organic acids and resistance to salt stress will increase. In our experiment the salt stress, in the case of cv. Grand Brix, caused an increase in TCA cycle enzymes activities, It is 52% in CS, 78% in ICDH, 46% in fumarase, 55% in MDH, and 40% in PEPC (Table-3). The cv. Marmande RAF increased CS activity by 33% in the saline treatment. However, the other enzyme activities decreased by 64% in ICDH and 67% in fumarase and 28% in PEPC, while for the MDH no significant differences. (Table-3). Although there are few studies linking these enzymes with salt stress, one thing seems clear, an increased enzyme activity promotes organic acids accumulation which increases resistance to salinity. In addition, the TCA cycle enzyme reactions generate ATP molecules creating energy reserves (Ryan et al.

2001; Araújo et al. 2012; Nunes-Nesi et al. 2013). Considering the above, our results support the decision to consider the cv. Grand Brix as salinity tolerant genotype. In this aspect it could be that Marmande RAF does not have enough energy to their metabolic processes, and therefore, this cv. is more sensitive to stress. Complementary studies would be needed to support this idea.

With respect to organic acids, we can observe that the cv. Grand Brix showed higher organic acids concentrations that the cv. Marmande RAF in saline treatment (Table-4). This is vital to stress tolerance because of organic acid metabolism is crucial for several biochemical pathways, including energy production, amino-acid biosynthesis and at the whole plant level modulating plant adaptation to the environment (López-Bucio et al. 2000). Therefore, cv. Grand Brix presented better metabolic homeostasis that the cv. Marmande RAF when this was subjected to saline treatment.

Citrate is the first organic acid generated in the TCA cycle. This is generated in the first step of the cycle, the most important step of the cycle. This step is catalysed by CS and in our experiment the activity of this enzyme increases in both cv. in the saline treatment but increases more in cv. Grand Brix (52% in Grand Brix and 33% in Marmande RAF) (Table-3). This citrate increase could favour the ionic stress resistance in the plant. In fact, it has been observed in tobacco transgenic plants and *Arabidopsis thaliana*, a relationship between CS overexpression, citrate increase and resistance to Al^{+3} stress (Ryan et al. 2001).

Malate in the TCA cycle is generated from the conversion of fumarate to malate by fumarase enzyme, one of the key enzymes in the cycle. This is reflected by the increase of fumarase activity in the saline treatment in cv. Grand Brix (Table-3) produces an increase of malate (Table-4), while in cv. Marmande RAF just the opposite occurs (Table-3 and 4). However malate sometimes does not have that unique way of generation, the MDH can acts in the opposite direction to the cycle generating malate, MDH is one of the key enzymes cycle too. Richter et al. (2015) showed, in two corn hybrids, that the most salinity tolerant hybrid had higher malate concentration. In our experiment, the malate registered the highest concentration among the three organic acids assessed in both genotypes, with one difference. The malate concentration in cv. Grand Brix increased a 117% while the cv. Marmande RAF decreased by 11% with respect to the control (Table-4). This is important if we consider that malate plays a central role in plant nutrition in N_2 fixation, acquisition of P from infertile soils, and tolerance to Al^{3+} stress (Schulze et al. 2002).

Oxaloacetate is generated in the last step of the cycle by the conversion of malate to oxaloacetate by MDH enzyme. However, oxalacetate also may come from an anaplerotic pathway, this pathway is developed outside the mitochondria and is catalysed by the enzyme PEPC. These two enzymes presented more activity in the saline treatment in cv. Grand Brix, while in the saline treatment in Marmande RAF decreased or had no significant changes (Table-3). If we observed the increase in citrate (87%) and malate (117%) concentration in the cv. Grand Brix, we expected that oxaloacetate increase (only increased a 2% more than control) (Table-4). Oxaloacetate excess may be serving as a substrate for the

accumulation of other organic acids. Furthermore, MDH enzyme could be operating contrary to the cycle or forming amino acids from oxaloacetate. However we think that a possible explanation would be that oxaloacetate, could form sodium oxalate crystals and would be precipitated in the vacuoles, and therefore we did not observe an increase in its concentration. The ability of oxaloacetate to form calcium crystals and accumulate in vacuoles is known (Nakata 2003), the same thing could be happening with sodium. Thus, it could explain why the cv. Grand Brix presents an increased tolerance to salt stress despite accumulates more Na^+. Nevertheless, further studies are needed on this aspect.

6 Conclusion

This work leads to the conclusion that the influence of salt stress on TCA cycle enzymes will depend on the variety. We conclude that the increased activity of the TCA cycle enzymes helps saline stress resistance, as cv. Grand Brix (resistant genotype) has a higher enzyme activity. The genotypic comparison of the accumulation of organic acids reveals what we expected, more organic acids were accumulated in cv. Grand Brix (more resistant), while in cv. Marmande RAF (sensitive) decreased. Therefore an increase in organic acids concentration increases resistance to salt stress, as the organic acids can form complexes with Na^+, apart from improving other aspects such as nitrogen fixation or resistance to ionic stresses resulting from stress saline. The idea that oxaloacetate is precipitating Na^+ excess in the cv. Grand Brix could be an explanation of its greater tolerance to Na^+, however further studies are required.

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CAPÍTULO 3

Influencia del metabolismo de la prolina y glicina betaina en la tolerancia al estres salino en genotipos comerciales de tomate (*Solanum Lycopersicum* L.)

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Resumen

El tomate es uno de los cultivos con mayor importancia económica en el mundo y el estrés salino provoca una reducción en la cantidad y calidad de la producción del cultivo. El objetivo de este trabajo es verificar si la acumulación de prolina y glicina betaína (GB) y sus rutas metabolicas mejoran la tolerancia al estrés salino. Para este trabajo se utilizaron dos genotipos comerciales de *Solanum Lycopersicum* L., Grand Brix y Marmande RAF. Los parámetros analizados fueron parámetros de crecimiento, concentración de prolina y sus rutas metabolicas, GB y su síntesis por la betaína aldehído deshidrogenasa (BADH) y algunos aminoácidos relacionados. El estrés salino redujo la biomasa y la tasa de crecimiento relativa (TCR) en ambos genotipos, siendo este efecto mayor en Marmande RAF. Estos resultados, junto con la acumulación de prolina, indican que Grand Brix fue más tolerante al estrés salino. El aumento de la prolina en Grand Brix se produjo por la vía de la ornitina, dejando reprimida la vía del glutamato. Por otro lado, se encontró en ambos genotipos que BADH y GB disminuyen como mecanismo de tolerancia a la salinidad. Proponemos que, a diferencia de la prolina, la síntesis de GB puede producir H_2O_2 y por lo tanto la GB no actúa, en nuestro trabajo, como un soluto compatible, no mejorando la tolerancia a la salinidad.

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Influence of the proline metabolism and glycine betaine on tolerance to salt stress in tomato (*Solanum Lycopersicum* L.) comercial genotypes

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1 Abstract

Tomato is the crop with the greatest economic importance in the world and salinity stress causes a reduction in the quantity and quality of crop production. The objective of this work is to verify if the accumulation of proline and glycine betaine (GB) and their metabolisms improve tolerance to salt stress. Two commercial genotypes of *Solanum Lycopersicum* L., Grand Brix and Marmande RAF were used for this work. The analyzed parameters were growth parameters, proline concentration and its metabolism, GB and its above betaine aldehyde dehydrogenase (BADH) synthesis and some related amino acids. Saline stress reduced biomass and relative growth rate (RGR) in both genotypes, this effect being greater in Marmande RAF. These results, together with the proline accumulation indicate that Grand Brix is more tolerant to saline stress. The proline increase in Grand Brix came by the ornithine pathway, leaving the glutamate pathway repressed. On the other hand, it was found in both genotypes a BADH and GB decreases as a salinity tolerance mechanism. We propose that, unlike proline, GB synthesis can produce H_2O_2 thereby, GB not act, in our study, as a compatible solute and salt tolerance does not improve.

Keywords: Salt stress; *Solanum lycopersicum* L.; Proline; Proline metabolism; Glycine betaine; BADH

2 Introduction

Tomato is the crop with the greatest economic importance in the world. According to the FAO, in 2015 roughly 4,832,700 tonnes of tomato were produced only in Spain, cultivated on 58,134 Ha. A great part of this cultivation area is affected by salinity stress. In particular, salinity stress causes a reduction in the quantity and quality of crop production (Saito et al. 2008). Currently, the main challenge in world agriculture is to sustain the continuously growing global population, and this becomes more difficult due to climatic change, as this imposes further abiotic stress. In the coming years, several factors could exacerbate this situation, such as the continuous expansion of intensive agriculture and the use of poorer quality water. Therefore, it is of great importance to ascertain the impact of salt stress on tomato cultivation. Greatly limiting crop yield in semiarid and arid regions, salinity affects roughly 397 million Ha of soils in the world (Gong et al. 2013). This is particularly true in the Mediterranean area, where cultivation tends to take up small fields often with crops of high quality and commercial values, such as tomato (Lynch and Clair 2004).

Growth conditions under salinity stress trigger osmotic and ionic imbalances, alter the plant's metabolism and prompt oxidative stress through an increase in reactive oxygen species (ROS) production (Sumithra et al. 2006; Hossain and Dietz 2016). ROS are continuously generated during normal metabolic processes

in the mitochondria, peroxisome and cytoplasm. However, at a high level they are highly cytotoxic and can react with vital biomolecules, causing damages such as peroxidation, protein denaturation and DNA mutation (Saibi et al. 2015). The toxic effects of O_2^- and H_2O_2 can initiate a cascade of reactions that results in the generation of hydroxyl radicals and other harmful species such as lipid peroxides (Sumithra et al. 2006).

Salt stress generates a great Na accumulation. This accumulation causes osmotic stress and ionic imbalances in the plants, and thereby plant growth and its development decreased. Considering the importance of conserving the osmotic gradient, is very important the compatible osmolites role (Munns and Gilliham 2015). The solutes that accumulate during osmotic adjustment include amino acids (e.g., proline) and quaternary amines (e.g., glycine betaine). Proline and glycine betaine accumulations are prominent physiological responses of many higher plants to salinity stress. These compounds should, by definition, be non-toxic at high concentrations to cytoplasmic functions, allowing turgor maintenance and protection of macromolecular structures (Mansour and Ali 2017; Hannachi and Van Labeke 2018).

Proline often plays diverse roles under stress conditions, such as proteins stabilization, membranes, and subcellular structures, and the protection of cellular structures by scavenging ROS (Salinas et al. 2013). Oxidative stress can induce not only biosynthesis of antioxidant enzymes, also the proline synthesis, through the MAPK signal cascade, as does abscisic acid. However, induction of

proline biosynthesis under the effect of oxidative stress is poorly investigated although some researchers showed that proline can act as a ROS scavenger (Radyukina et al. 2011). Proline biosynthesis is upregulated by high radiation and osmotic stress whereas proline catabolism is activated in the dark and during stress relief (Kaur and Asthir 2015). Several authors have shown a strong correlation between the rise of proline levels and the capacity to survive salinity conditions; such as Jaarsma et al. (2013) that worked with 6 potato (Solanum tuberosum L.) cultivars subjected to salt stress, and showed that in the most sensitive varieties the proline accumulation was lower while in the most tolerant varieties proline accumulated. These authors also observed that this proline accumulation in the tolerant varieties, comes from a greater activity in the proline synthesis enzymes and a reduction in the Proline dehydrogenase (PDH) and not by a proteins degradation. Sarabi et al. (2017) also showed how proline accumulates in response to stress in salt tolerant varieties of melon (Cucumis melo L.). These authors showed that proline accumulation was greater in the most severe salinity treatments. In the sensitive varieties proline also was accumulated, although this accumulation was lower, besides their biomass was reduced in a more dramatic way. The proline increase in the tolerant varieties supports the idea that proline counteracts the osmotic stress caused by salt stress, providing these varieties a greater tolerance to this stress.

However, other authors reported the rise in proline levels in sensitive varieties to salt stress. Rokebul et al. (2017) showed in two alfalfa genotypes that the most sensitive genotype to salt stress also accumulated more proline, and also

presented more oxidative stress. Hannachi and Van Labeke (2018) showed in seedling of eggplant (*Solanum melongena L.*) as in the varieties most sensitive to salt stress, those that less germinated and accumulated more malondialdehyde (MDA), had a higher proline concentration. Therefore the results of these authors support the idea that proline is a stress indicator and does not increase tolerance to salt stress. The amino acid proline is probably the most widely distributed and discussed osmolyte accumulated by plants.

Proline and its metabolism are often regulated by other compounds with which they may have synergistic or antagonistic relationships, such as phytohormones (Per et al. 2017). Phytohormones usually interact synergistically with proline metabolism although not always. Per et al. (2017) showed some examples where Gibberellins (GAs) in mays, linseed and Oryza sativa L. plants, cytokinins (CKs) in tobacco plants or ethylene in mustard presented a positive relationship with proline accumulation. However, Per et al. (2017) also showed how the exogenous application of Indole-3-acetic acid (IAA) reduces proline accumulation in wheat under salt stress.

On the other hand, glycine betaine (GB) also plays different protective roles: the stabilization of enzymes and protein structures; the reduction of ROS levels under stress; the preservation of membrane stability under non-physiological conditions (Paradisone et al. 2015). Some authors reported the beneficial effect of GB at the whole plant level, reducing the osmotic imbalance caused by salt stress. Mahboob et al. (2017) showed 11 wheat genotypes with different tolerance to salt

stress, their results reflect 4 tolerant genotypes, of these 4 genotypes 3 had higher levels of GB under salt stress, and the last one showed a decrease in GB concentration. This study supports that genotypic variability is determinant in stress tolerance processes. Kaya et al. (2013) showed how the application of GB in maize plants subjected to salt stress improved their tolerance to stress. These authors also showed that the higher the applied dose, the greater the tolerance showed the plant.

However other authors showed a negative correlation between GB concentration and salt stress tolerance. Heuer (2003) observed that a foliar application of GB, reduced tomato plants growth, and in the treatments of plants subjected to salt stress plus GB application, growth was greatly reduced. Therefore GB was not helping to increase salt stress tolerance. Chen et al. (2007) showed a greater accumulation in barley genotypes that were more sensitive to salt stress.

Further studies are required given the current controversy about the role of proline and GB in salt stress tolerance. Probably the role of these osmolytes depends on the species and varieties studied. In this context, considering the compatible osmolyte importance, and its osmoprotector role in plant under salt stress, we investigate here the proline pathway, and GB and its synthesis enzyme in two tomato genotypes subjected to salinity stress. The main objective is to determine the importance of proline, GB and its associated enzymes in salt stress tolerance. Therefore this work consists of two hypotheses, first hypothesis: a more efficient proline metabolism and a proline accumulation promote tolerance

to salinity. Second hypothesis: a higher betaine aldehyde dehydrogenase (BADH) activity and a GB accumulation favour tolerance to salinity.

3 Experimental

3.1 Plant material

Seeds of *Solanum lycopersicum* L. Gran brix and Marmande RAF genotypes (Saliplant S.L., Spain) were germinated and grown for 30 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were placed on benches in an experimental greenhouse located in Southern Spain (Saliplant S.L., Motril, Granada). After 30 days, the seedlings were transferred to a growth chamber (Department of plant physiology, University of Granada) under the following controlled environmental conditions: Relative humidity 60-80%; Day/night temperatures 28/19 °C; 16/8 h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol $m^{-2}s^{-1}$ (measured at the top of the seedlings with a 190 SB quantum sensor, LI-CORInc., Lincoln, Nebraska, USA). Under these conditions, the plants were grown in hydroponic cultivation in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) of 3 L volume, 8 plants/tray. Throughout the experiment the plants were treated with a growth solution made up of 4 mM KNO_3, 3 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2 mM $MgSO_4{\cdot}7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4{\cdot}2H_2O$, 2 µM $MnCl_2{\cdot}4H_2O$, 1 µM $ZnSO_4{\cdot}7H_2O$, 0.25 µM $CuSO_4{\cdot}5H_2O$, 0.1 µM $Na_2MoO_4{\cdot}2H_2O$, 5 µM Fe-chelate (Sequestrene; 138 FeG100) and 50 µM H_3BO_3. This solution, with a pH of 5.5–6.0, was renewed every three days.

3.2 Experimental design and treatments

Treatment of salt stress started 38 days after germination and was maintained for 15 days. The control treatment received the nutrient solution, while the treatment salt stress received the nutrient solution plus 100 mM NaCl. The experimental design was a randomized complete block with two treatments, 8 plants per treatment and with 3 replications per treatment (n = 9).

3.3 Plant sampling and determination of the relative growth rate (RGR)

Plants of each treatment (53 days after germination) were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of leaves from each treatment were frozen at −30 °C for further work and biochemical assays and the other half of the plant material was lyophilised for 48h to obtain the dry weight (DW) and the subsequent analysis of the concentrations of nutrients. To determine the relative leaf growth rate, leaves from three plants per cultivar were sampled on day 38 after germination, immediately before starting the stress treatment (Ti). The leaves were lyophilised for 48h, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled 53 days after germination (15 days of treatments, Tf). RGR was calculated from the increase in leaf DW at the beginning and at the end of the saline-stress treatment, using the equation RGR = (ln DWf − ln DWi)/(Tf − Ti) where T is the time and the subscripts denote the final and initial sampling.

3.4 Proline Metabolism

Extraction of D-1-pyrroline-5-carboxylate synthetase (P5CS) was carried out according to Sumithra et al. (2006). Leaves were homogenised with extraction buffer containing 100 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 1 mM phenylmethylsulphonyl fluoride, and then centrifuged at 10,000g for 15 min. The supernatant was used for enzyme assays. P5CS activity was measured as described in Charest & Ton Phan (1990). The reaction mixture contained: 100 mM Tris-HCl (pH 7.2), 25 mM $MgCl_2$, 0.4 mM NADPH, 5 mM ATP and the enzyme extract. The reaction was initiated by addition of 75 mM sodium glutamate. The activity was measured as the rate of consumption of NADPH, monitored as a decreased in absorbance at 340 nm. For ornithine-d-aminotransferase (OAT) and proline dehydrogenase extraction, leaves were homogenised in 100 mM potassium phosphate buffer (pH 7.8). The homogenate was filtered and centrifuged at 12,000g for 20 min (4°C). OAT was assayed according to Charest & Ton Phan (1990) in 0.2 M Tris-KOH buffer (pH 8.0) containing 5 mM ornithine, 10 mM α-ketoglutarate and 0.25 mM NADH. The decrease in absorbance of NADH was monitored at 340 nm for 1 min after initiating the reaction with addition of enzyme extract. PDH activity was assayed as a reduction of NAD^+ at 340 nm (Charest & Ton Phan 1990). The reaction mixture contained 0.15 M Na_2CO_3-HCl buffer (pH 10.3) containing 2.67 mM L-proline and 10 mM NAD^+. Glutamate dehydrogenase (GDH) was assayed by measuring the decrease in absorbance due to consumption of NADH at 340 nm (Kanamori et al. 1972). The reaction mixture contained 0.2 M Tris-HCl buffer (pH 8.0), 1.5 M NH_4Cl, 0.5 M α-ketoglutaric acid, 3 mM NADH and 0.2 ml of enzyme

extract. The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine serum albumin as standard.

3.5 GB Concentration and BADH activity:

GB concentration was determined by the method of Grieve and Grattan (1983). GB was extracted from 38 mg of dry plant material in 1.5 ml of distilled water gently shaking for 24 h. Extract was filtered and added 2 ml of 2N H_2SO_4, the solution was incubated 16 h at 4°C and then centrifuged at 9,000g for 15 min at 0ºC. The pellet obtained by centrifugation was resuspended in 1,2-dichloroethane. After 2 h the GB content was measured by reading absorbance at 365 nm and quantified using a standard curve of GB.

BADH activity was determined by measuring the reduction of NAD^+ by BADH at 340 nm for 1 min (Kumar et al. 2004). The reaction mixture (1 ml) contained 50 mM HEPES-KOH buffer (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, 1 mM NAD^+, 1 mM betaine aldehyde and 100g protein.

3.6 Protein determination

The protein concentration of the extracts was determined according to the method of Bradford (1976) using bovine-serum albumin as the standard.

3.7 Determination of Na^+ and K^+ ions

The samples were mineralized by wet digestion according to Wolf (1982). To carry this out, 0.2 g of freeze-dried leaves were ground and mineralized with 98% H_2SO_4 and H_2O_2 to 30% at 300 °C. K^+ and Na^+ were analyzed by ICP-OES.

3.8 Soluble amino acids analysis

The soluble amino acids were extracted following the method of (Bieleski and Turner 1966) with some modifications. 0.1 g of fresh leaves were homogenised in 1ml of MCW (methanol: chloroform: water, 12:5:1). 50 µl of L-2 Aminobutyric acid was added as an internal standard. The mixture was centrifuged at 5000 rpm for 10 min. The resulting supernatant was added 700 µl of Milli-Q water and 1.2 ml of chloroform and incubated 24 h at 4 °C. Then, the aqueous phase was obtained, which was lyophilized and the resulting extract was diluted with 0.1 M HCl. Instrumental analysis of soluble amino acids was carried out using the precolumn AccQ Tag Ultra Derivatization Kit (Waters, Milford, MA, USA). Derivatization was performed according to the manufacturer's protocol. For derivatization, 60 µl of borate buffer was added to 10 µL of the sample, 10 µL 0.1 N NaOH and 20 µL reconstituted AccQ•Tag Ultra Reagent. LC fluorescence analysis was performed on the Waters Acquity® UPLC System equipped with the Acquity fluorescence detector. UPLC separation was performed on the AccQ Tag Ultra column (2.1 x 100 mm, 1.7 µm) from Waters. The flow rate was 0.7 mL min-1, and the column temperature was kept at 55ºC. The injection volume was 1 µL, and the detection was set at a 266-nm excitation wavelength and a 473-nm emission wavelength. The solvent system consisted of two eluents: 1:20 Dilution

of AccQ Tag Ultra eluent A concentrate and AccQ Tag Ultra eluent B. The profile was as follows: 0–0.54 min, 99.9% A and 0.1% B; 5.74 min, 90.9% A and 9.1% B; 7.74 min, 78.8% A and 21.2% B; 8.04 min, 40.4% A and 59.6% B; 8.05–8.64 min, 10% A and 90% B; 8.73–9.50 min, 99.9% A and 0.1% B.

3.9 Statistical analysis

All analyses were repeated in triplicate and the results were evaluated statistically using an analysis of variance ANOVA simple with a 95% confidence interval. A two-tailed ANOVA was applied to ascertain whether the saline treatment and the genotype significantly affected the results. The differences between the treatments means were compared using the test of the minor differences of Fisher (LSD) at a 95% probability level. Significance levels were expressed as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS not significant. The statistical software used was Statgraphics Centurion.

4 Results

4.1 Foliar biomass and RGR

Plant growth was determined using foliar biomass and RGR parameters. When the saline treatment is compared to its control and the genotypes were compared, the one more negatively influenced by salinity stress was Marmande RAF, showing a sharp reduction in both parameters. Nevertheless, Grand Brix also presented a reduction in salinity stress treatment, but this reduction was lower (Figure 1).

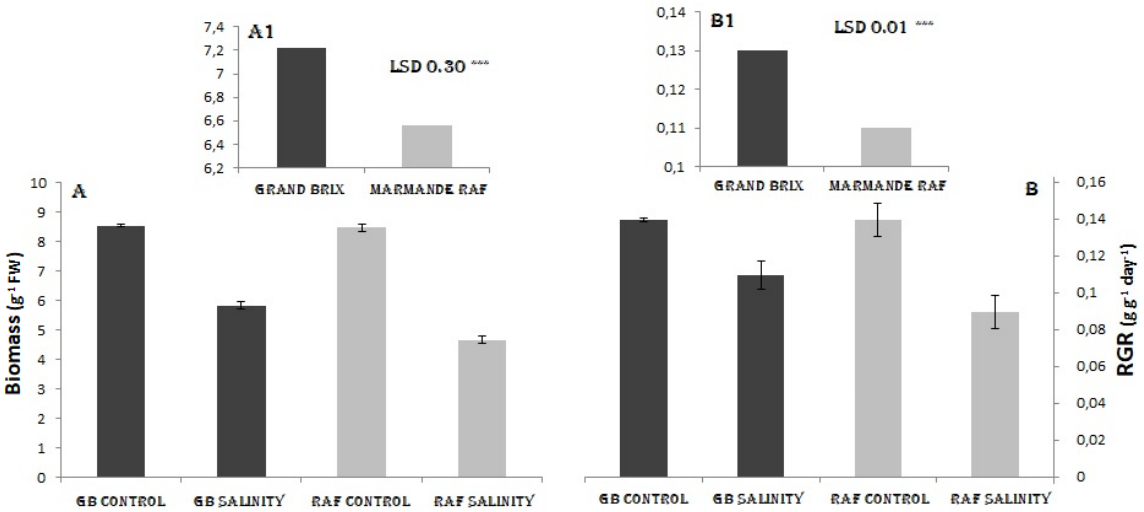

Figure 1: Effect of salt treatment on the foliar biomass (A) and RGR (B) in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF. The columns Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1 and B1 values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0.05). Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

4.2 Na and K concentration and Na/K ratio

Our results showed an accumulation of Na^+ ion in plants under the salinity treatment in comparison to control in both genotypes (Table 1). Regarding K^+ ion, the salinity treatment resulted in a decline in concentration in both genotypes (Table 1). As well as Na^+ ion, the Na/K ratio reached its highest value in the salinity treatment plants (Table 1). Finally, the comparison between these two genotypes revealed that the Grand Brix reached higher values for the three above-mentioned parameters (Table 1).

Table 1: Effect of salt treatment in the concentration of Na, K and Na/K ratio in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Na (mg g^{-1}DW)		K (mg g^{-1}DW)		Na/K (mg g^{-1}DW)	
	Grand Brix	Marmande	Grand Brix	Grand Brix	Grand Brix	Marmande
Control	3.29±0.28[b]†	2.70±0.01[b]	24.39±0.48[a]	22.33±0.17[a]	0.14±0.01[b]	0.12±0.00[b]
Salinity	18.78±0.07[a]	11.25±0.21[a]	16.34±0.11[b]	15.26±0.19[b]	1.15±0.00[a]	0.74±0.01[a]
P-value	***§	***	***	***	***	***
Grand Brix	11.03[a]‡		20.37[a]		0.65[a]	
Marmande	6.98[b]		18.80[b]		0.43[b]	
P-value	***		***		***	
$LSD_{0.05}$	1.28		0.59		0.07	

† Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05).
‡ Values are means (n = 18) and differences between means were compared using LSD test (P = 0.05).
§ Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

4.3 Proline Metabolism

Respecting proline metabolism there are two main synthesis pathways, GDH and OAT (Figure 2). Salt treatment was affected differently to genotypes for GDH and OAT activities (Table 3). These enzymatic activities raised significantly in Grand Brix grown under salinity treatment, maintaining without significant changes in Marmande RAF (Table 3). However, the comparative study of genotypes, for GDH and OAT, did not show significant differences when compared to each other (Table 3).

On the other hand, P5CS activity did not show significant changes in the salt treatment of any genotype. Nevertheless, when comparing the genotypes we observed that Grand Brix had a higher P5CS activity than Marmande RAF (Table 3). Proline metabolism and proline accumulation also depend on its degradation.

Our results showed a PDH activity decline caused by salinity treatment in both genotypes with respect to control (Table 3). Despite this, Marmande RAF presented greater PDH activity than Grand Brix (Table 3).

4.4 Proline and related amino acids

Grand Brix plants grown under salinity presented significantly greater proline concentration in the saline treatment, than Marmande RAF with respect to its control. Likewise, in the genotypes comparison, Grand brix also presented higher proline values (Table 2). Arginine and glutamate concentrations had similar behaviours in the genotypes, both showed a decrease in Grand Brix plants subjected to salt treatment compared to their control (Table 2). Regarding Marmande RAF both amino acids did not present significant differences in plants under salinity treatment compared to control. When the genotypes were compared for both amino acids, Grand Brix presented higher values (Table 2). Our results showed a decrease in glycine concentration in the case of saline treatments of both genotypes when compared with their controls. The genotype comparison showed higher values in the Marmande RAF genotype (Table 2).

Table 2: Effect of salt treatment in the proline, arginine, glycine and glutamate concentration in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Prolina (ug g^{-1} DW)		Arginine (ug g^{-1} DW)		Glycine (ug g^{-1} DW)		Glutamate (ug g^{-1} DW)	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	2,22±0,27[b]†	5,45±0,71[b]	10.18±0.39[a]	8.14±1.06[a]	0.30±0.04[a]	0.47±0.03[a]	8.27±0.09[a]	5.62±0.75[a]
Salinity	20,45±0,64[a]	22,03±1,65[a]	4.93±0.16[b]	5.44±0.51[a]	0.14±0.01[b]	0.23±0.03[b]	7.50±0.07[b]	6.71±0.69[a]
P-valor	***§	**	***	NS	*	**	**	NS

Grand Brix	13,74[a]‡	7.55[a]	0.22[b]	7.88[a]
Marmande	11,34[b]	6.79[b]	0.35[a]	6.17[b]
P-valor	*	*	**	**
$LSD_{0.05}$	2,22	1.44	0.07	1.18

Table 3: Effect of salt treatment in the P5CS, OAT, PDH, GDH activity in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	P5CS (Abs mg $prot^{-1}$ h^{-1})		OAT (Abs mg $prot^{-1}$ h^{-1})		PDH (Abs mg $prot^{-1}$ h^{-1})		GDH (Abs mg $prot^{-1}$ min^{-1})	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	0,29±0,05[a]†	0,16±0,03[a]	0,06±0,01[b]	0,15±0,02[a]	0,15±0,01[a]	0,27±0,02[a]	2,51±0,05[b]	2,77±0,04[a]
Salinity	0,31±0,02[a]	0,16±0,02[a]	0,22±0,09[a]	0,11±0,01[a]	0,07±0,01[b]	0,17±0,01[b]	3,04±0,07[a]	2,64±0,07[a]
P-valor	NS§	NS	*	NS	***	***	***	NS

Grand Brix	0,30[a]‡	0,14[a]	0,11[b]	2,78[a]
Marmande	0,16[b]	0,13[a]	0,22[a]	2,70[a]
P-valor	***	NS	***	NS
$LSD_{0.05}$	0,07	0,10	0,04	0,18

†Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).

‡ Values are means (n= 18) and differences between means were compared using LSD test (P= 0,05).

§Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0,05; **P< 0,01 and *** P< 0,001 relative to the control.

4.5 GB concentration and BADH activity

GB showed a decline in its concentration caused by the salinity treatment in both genotypes with respect to control (Table 4). BADH activity did not show significant changes by the salt treatment of any genotype (Table 4).

However, comparing both genotypes, we found differences for each genotype. Grand Brix showed a higher decrease in GB concentration than Marmande RAF, while Marmande RAF showed a higher decrease by BADH activity than Grand Brix (Table 4).

Table 4: Effect of salt treatment in the GB Concentration and BADH activity in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	GB ($mM\ g^{-1}DW$)		BADH ($Abs\ mg\ prot^{-1}\ min^{-1}$)	
	Grand Brix	Marmande	Grand Brix	Marmande
Control	$2,09\pm0,01^{a}$†	$3,78\pm0,05^{a}$	$0,55\pm0,08^{a}$	$0,31\pm0,09^{a}$
Salinity	$1,68\pm0,03^{b}$	$3,32\pm0,02^{b}$	$0,47\pm0,09^{a}$	$0,23\pm0,05^{a}$
P-value	***§	***	NS	NS
Grand Brix	$1,89^{b}$‡		$0,51^{a}$	
Marmande	$3,55^{a}$		$0,27^{b}$	
P-value	***		**	
$LSD_{0.05}$	0,16		0,16	

† Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05).
‡ Values are means (n = 18) and differences between means were compared using LSD test (P = 0.05).
§ Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

5 Discussion

5.1 Foliar biomass and RGR

Biomass and RGR are optimal indicators to evaluate plant stress and thus reflect plant growth (Gong et al. 2013). The growth reduction under salt stress conditions is well characterized in plants such as tomato, maize, and alfalfa (Li et al. 2010; Wang et al. 2011; Gong et al. 2014; Li et al. 2014). In our experiment, both parameters declined in the plants subjected to the saline treatment. The saline treatment reduced plant biomass by 32% in Grand Brix and by 52% in Marmande RAF (Figure 1A). As well as biomass, RGR was reduced in the salinity treatment by 19% in Grand Brix and by 39% in Marmande RAF (Figure 1B). Concerning the negative effect in relation to genotypes, Sánchez-Rodríguez et al. (2010) observed that the most tolerant tomato cultivar lowered its biomass and its RGR levels under water stress. Khaliq et al. (2015) concluded that the most sensitive maize cultivar also registred the lowest biomass under salinity stress. In our study, the greatest loss of biomass and RGR were found in Marmande RAF plants. In short, according to these results, we can define Marmande RAF as a more sensitive genotype of both regarding salinity stress.

5.2 Na and K concentration and Na/K ratio

The decline in biomass and RGR under salinity stress, are related to Na^+ accumulation and K^+ deficit, as these alter basic physiological processes such as photosynthesis (Li et al. 2010). In our experiment, the salinity treatment produced the greatest Na accumulation (an increase of 470% Grand Brix and an increase of 318% Marmande RAF; Table 1). Works such as Li et al. (2010) also reported

a greater Na accumulation in alfalfa plants grown under salinity. In addition, works as Gong et al. (2013) and Wang et al. (2011) in tomato or Li et al. (2014) in rice found a decline in K^+ concentration when plants were grown salt treatments. This agrees with our results in which the salt treatment in both genotypes registered the lowest K^+ concentrations (Table 1). Finally, our results a priori appear to indicate that Grand Brix should be more affected by the salinity treatment since this genotype accumulates more Na^+ and the Na^+/K^+ ratio is greater than in Marmande RAF; however, the growth data indicate that the more sensitive genotype was Marmande RAF (Table 1). This may be due to strategies such as Na^+ compartmentalization in vacuoles or the immobilization of this ion (Li et al. 2010), which could be reinforced in Grand Brix and therefore might be less affected by Na^+ accumulation than in Marmande RAF.

5.3 Proline Metabolism

It is well known that proline accumulates in plants during adaptation to various types of environmental stresses, several studies have observed proline accumulation in response to salinity and this accumulation help to reduce the salt injure effect in plants (Kumar et al. 2003; Jaarsma et al. 2013; Sarabi et al. 2017). In plants, Proline biosynthesis takes place via two pathways, namely, glutamate and ornithine pathways (Figure 2). Proline function is not restricted only as a compatible osmolyte, also is capable of detoxifying ROS by forming stable complexes. Thus, an increase in proline synthesis could increase resistance to these stress conditions in tomato plants (Rosales et al. 2007).

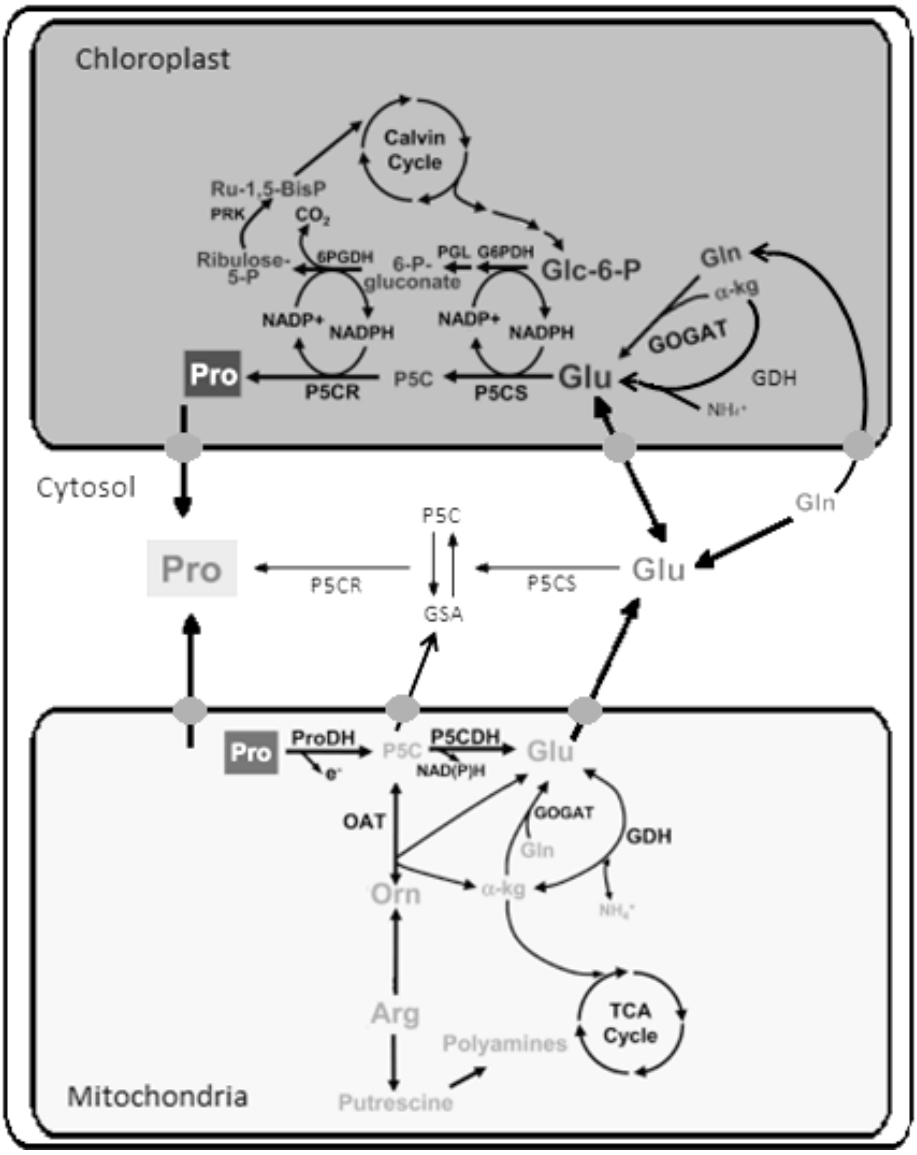

Figure 2. Schematic representation of proline biosynthesis and metabolism in plant cell.

In the glutamate pathway, proline biosynthesis begins with the phosphorylation and Glutamate reduction to an intermediate glutamic-5-semialdehyde (GSA) by the action of bifunctional enzyme P5CS, which is spontaneously cyclized into pyrroline-5-carboxylate (P5C). This intermediate P5C is finally reduced to Proline by the enzymatic catalysis of D1-pyrroline-5-carboxylate reductase (P5CR) (Figure 2) (Per et al. 2017). The main enzymes that accumulate glutamate are GDH and GOGAT (Figure 2). In our experiment Grand brix showed a 21% higher GDH activity, while Marmande RAF showed a 5% decrease in its activity (Table

3). This coincides with the results of Sumithra et al. (2006) that showed that in two Vigna radiata L. genotypes subjected to salt stress increased GDH activity with respect to control, increasing more in the tolerant genotype. GOGAT activity showed an increase by saline treatment in both genotypes, being higher in Grand Brix plants (Data not shown). The increase in GDH and GOGAT activities, contrary to what is expected, does not coincide with an increase in the concentration of glutamate (Table 3). However, in Grand Brix salt treatment we can observe a decrease in glycine concentration (Table 3), which is a precursor of glutamate (Figure 2). This could explain that the production of glutamate is taking place, but it is being spent at the same time in producing proline. This contributes to greater tolerance to salt stress of Grand Brix because it promotes proline synthesis. However, in general, proline accumulation in response to different types of stress is correlated with transcriptional activation of the gene-encoding P5CS, which is the key regulatory and rate-limiting enzyme in this biosynthetic pathway (Rosales et al. 2007; Saibi et al. 2015). Authors such as Jaarsma et al. (2013) in potato plants or Saibi et al. (2015) in Arabidopsis transgenic plants showed that in plants subjected to salt stress P5CS activity increase. Nevertheless, contrary to what we expected our P5CS activity increased 6% for Grand Brix and 2% for Marmande RAF, but both increases were not significant (Table 3). These results would be related to those shown by Ruiz et al. (2002) in greenbeans or Sánchez et al. (2002) in French beans plants, they showed a decrease in P5CS activity and an increase in proline concentration. These results indicate that P5CS activity is not defined as the limiting step in proline synthesis and accumulation under salt stress. Verslues and Sharma (2010) and Radyuina et al. (2011) showed the possibility that P5CS enzyme

genes could be activated in the presence of ROS, in particular H_2O_2. In previous experiments conducted by our research group with these same genotypes, it was found that H_2O_2 concentration was not significantly different between genotypes (De la Torre-Gonzalez et al. 2017). This could be the reason why our results show no significant changes in P5CS activity. Several authors show relationships between phytohormones and the regulation of proline metabolism or its accumulation (Radyuina et al. 2011; Per et al. 2017). Verslues and Sharma (2010) also showed that proline accumulation may be induced by abscisic acid (ABA) and salicylic acid (SA) by regulating P5CR expression. In previous studies with these same genotypes it was possible to verify how ABA and SA levels increased significantly in both genotypes, being this increase greater than in Grand Brix (De la Torre-Gonzalez et al. 2017). These data support that ABA and SA favours proline accumulation by P5CR and thus increases the tolerance to salt stress of Grand Brix.

Within the ornithine pathway, Ornithine can be transaminated to GSA by OAT activity and subsequently is converted to Proline via P5C. This P5C can be transformed into glutamate that will then be transported into the cytosol in the form of P5C (Figure 2) (Per et al. 2017). Authors such as Sánchez et al. (2001; 2002) and Ruiz et al. (2002) in greenbeans and in French beans showed that sometimes in plants subjected to stress the proline accumulation can come from the ornithine pathway. Our results coincide with those of these authors for Grand Brix genotype where OAT activity increased by 274%, while in Marmande RAF genotype this activity enzyme decreased by 25% (Table 3). Therefore a greater

activity of this enzyme would increase proline synthesis favouring tolerance to salt stress. Alvarez et al. (2003) showed that under salt stress glutamate is derived to proline synthesis. Proline synthesis would come from OAT pathway. Supporting these results we observed that Grand Brix presents a very significant reduction of the amino acid arginine (Table 2), which is the ornithine precursor (Figure 2).

Proline catabolism occurs in mitochondria and this process is catalyzed by PDH enzyme (Figure 2). Our results showed a 55% decrease in Grand Brix and a 39% decrease in Marmande RAF for PDH, this favour proline accumulation (Table 3). Jaarsma et al. (2013) showed a PDH activity decrease in sensitive potatoes (Solanum tuberosum L.) genotypes to salt stress. Ruiz et al. (2002) in greenbeans or Sánchez et al. (2002) in French beans plants, showed a decrease in PDH activity in plants under different stresses. Khavari-Nejad et al. (2013) described that PDH enzyme can generate toxic P5C levels, which can change homeostasis and redox state generating ROS. Therefore a decrease in PDH activity prevents the generation of ROS. In previous works of this research group, the same genotypes did not present a large ROS accumulation (De la Torre-Gonzalez et al. 2017). PDH is also regulated by phytohormones, thus the application of SA decreases PDH activity in lentil plants under salt stress (Per et al. 2017). In previous studies with these same genotypes it was possible to verify that SA level increased significantly in both genotypes, being this increase greater than in Grand Brix (De la Torre-Gonzalez et al. 2017). These data support that

SA favours proline accumulation by suppressing PDH activity and thus increases the tolerance to salt stress in Grand Brix.

Everything described above leads to a greater or lesser proline accumulation. Many papers support the proline accumulation in plants under different types of abiotic stresses (Kaur and Asthir 2015) such as salt stress (Jaarsma et al. 2013; Sarabi et al. 2017). Our data showed proline accumulation in both genotypes, however Grand Brix had a higher increase (821%) than Marmande RAF (304%) (Table 2). Proline has been related to ROS detoxification by authors such as Radyuina et al. (2011) who showed how oxidative stress was reduced by exogenous proline application in sage plants.

Proline is also regulated by phytohormones, Per et al. (2017) showed some examples where CKs reduce proline accumulation in wheat and tobacco plants subjected to salt stress, and GAs promote the proline accumulation in mays plants. Ethylene in mustard plants, ABA in Brassica rapa L. plants and even SA in lentil plants could favour proline accumulation in plants subjected to salt stress. The phytohormone data of the studied genotypes coincide with these authors since Grand Brix showed a concentration increase of these phytohormones. This probably increases tolerance to salt stress in Grand Brix, since proline reduces damage by oxidative and osmotic stress (De la Torre-Gonzalez et al. 2017).

5.4 GB Concentration and BADH activity

GB also plays different protective roles: the stabilization of structures, enzymes and proteins; the reduction ROS levels under stress; the preservation of membrane stability under non-physiological conditions (Paradisone et al. 2015). Authors such as, Mansour (2000), Ashraf and Foolad (2007) or Ahmad et al. (2016) report the beneficial effect of GB at the whole plant level, reducing the osmotic imbalance caused by salt stress. However, contrary to our expectations this study showed that Marmande RAF presented a major GB concentration, despite presenting a greater reduction in its synthesis enzyme BADH (Table 4). These data support the theory that GB is a stress indicator and does not contribute, at least to a large extent, to an increase in tolerance to salt stress. Nevertheless, authors like Mahboob et al. (2017) showed that a tolerance increase depends not only on the species but also on the different genotypes. These results agree with other authors which showed a negative correlation between GB concentration and stress salt tolerance. Heuer (2003) observed that applying GB to leaves, the tomato plants grew less, not only in salt treatments, but also in controls, and thereby GB was not helping to cope with salt stress. Chen et al. (2007) showed a GB accumulation in genotypes of barley sensitive to salt stress.

We think that GB could indirectly contribute to the alteration of coenzymes [ATP, NAD(P)H] turnover which is of primary importance to maintain to some extent the photosynthesis and respiration rates under stress conditions. In addition, GB could act as an inefficient compatible replacement solute, replacing the proline.

Besides, Chen and Murata (2002) showed that GB when is generated from choline, through choline monooxygenase enzyme (CMO), produces H_2O_2, which contributes to oxidative stress. This could explain why Marmande RAF despite having less BADH activity has a higher GB concentration (Table 4). In previous work using these same genotypes, it was observed that Marmande RAF showed a higher H_2O_2 concentration (De la Torre-Gonzalez et al. 2017)

6 Conclusion

In this work, we can conclude that the proline increases in plants grown under saline treatment contribute to improve tolerance to salt stress. This is more evident in the Grand Brix genotype since the increase in proline is greater. In our experiment, OAT enzyme shows a great increase in its activity, especially in Grand Brix, therefore this proline accumulation comes from the metabolic pathway controlled by OAT enzyme. PDH activity decreases in both genotypes, contributing to the proline accumulation and saline stress tolerance. On the other hand, GB acts as a stress indicator in these tomato genotypes. The enzyme that synthetizes GB (BADH) does not present a significant difference in the saline treatment. Because of this, we suggest that in these genotypes, GB can be synthesized by choline pathway. This synthesis generates H_2O_2 so it can be repressed to avoid oxidative stress, although more studies are needed to test this hypothesis. Also, this work showed that the proline metabolism is influenced by oxidative stress and phytohormones, enhancing or inhibiting both the synthesis and degradation enzymes, increasing saline stress tolerance due to the proline accumulation.

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CAPÍTULO 4

Estrés salino, ruta metabólica del nitrógeno y fotorrespiración, efecto de la tolerancia genotípica en plantas de tomate (*Solanum lycopersicum* L.).

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Resumen

El nitrógeno (N) es necesario para la síntesis de aminoácidos, proteínas, clorofila, ácidos nucleicos, lípidos y otros metabolitos con N en su estructura. En este sentido, el estrés salino produce una disminución en la calidad y cantidad de la producción de cultivos en todo el mundo debido a un desequilibrio osmótico e iónico que altera el metabolismo del N. El objetivo de este estudio es verificar si la variabilidad genotípica y una mejor regulación del metabolismo del N mejoran la tolerancia al estrés salino en las plantas de tomate. Para este estudio se usaron dos genotipos comerciales de Solanum Lycopersicum L., Grand Brix y Marmande RAF. Se analizaron las formas de N, su asimilación, el proceso de fotorrespiración, parámetros de eficiencia en el uso del N (NUE) y el perfil de aminoácidos. Una regulación más efectiva del metabolismo del N indica que Grand Brix es más tolerante al estrés salino. Una mayor actividad de las enzimas del ciclo GS/GOGAT podría promover una mejor asimilación del N en la planta, además de promover la generación de aminoácidos osmoprotectores como la prolina y mejorar la tolerancia al estrés salino.

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Salt stress, nitrogen pathway and photorespiration, genotypics tolerance effects in tomato plants (*Solanum lycopersicum* L.).

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Abstract

Nitrogen (N) is necessary for the synthesis of amino acids, proteins, chlorophyll, nucleic acids, lipids, and other metabolites with N in their structure. In this sense, saline stress produces a decrement in the quality and quantity of crop production around the world due to an osmotic and ionic imbalance that alters the N metabolism. The objective of this study is to verify if the genotypic variability and a better nitrogen metabolism regulation improve tolerance to salt stress in tomato plants. Two commercial genotypes of *Solanum Lycopersicum* L., Grand Brix and Marmande RAF were employed for this study. N forms, N metabolism, N use efficiency (NUE) parameters and amino acids profile were analysed. A more effective N metabolism regulation indicate that Grand Brix is more tolerant to saline stress. A greater GS/GOGAT cycle enzymes activity could promote a better N assimilation in the plant, besides it promotes the generation of osmoprotective amino acids such as proline and improve the salt stress tolerance.

Keywords: Salt stress; *Solanum lycopersicum* L.; Nitrogen metabolism; Amino acid; NUE; Photorespiration

1 Introduction

Saline stress produces a decrement in the quality and quantity of crop production around the world (Saito et al. 2008). In addition, nitrogen (N) is the most important mineral nutrient for plants. Given that N is the mineral element with the highest requirement by plants, N deficiency is a limiting factor for plant growth (Sánchez-Rodríguez et al. 2011). In saline environment, sodium (Na^+) and chloride (Cl^-) hinder the uptake, translocation and assimilation of plant nutrients such as ammonium (NH_4^+), potassium (K^+) or nitrate (NO_3^-) promoting the disruption of ion homeostasis (Ashraf et al. 2018).

N is necessary for the synthesis of amino acids (AAs), proteins, chlorophyll, nucleic acids, lipids, and other metabolites with N in their structure. After N compounds are uptaken by the root, NO_3^- can be reduced in the roots or shoots to form NH_4^+, this reduction is carried out by nitrate reductase (NR) and nitrite reductase (NiR) enzymes (Lea and Azevedo 2007). It is well known that NO_3^- reduction is the main control point in NO_3^- assimilation, because the complex regulation of NR is considered the limiting step in N assimilation (Sánchez-Rodríguez et al. 2011). NR and NiR activities are known to be repressed by NH_4^+ and there is evidence that these enzymes can be regulated by certain AAs or amides. Thus, Causin (1996) suggested that glutamine may act as a negative signal for NR. Regarding salinity, Debouba et al. (2007) observed in tomato leaves grown under salt stress a decrease in NR and NiR activities. They showed a decrease in the NO_3^- concentration and an increase in NH_4^+ concentration.

NH_4^+ in plants originates from direct uptake, NO_3^- reduction, nitrogenous compounds deamination, or from photorespiratory N cycle. NH_4^+ is assimilated in plants into AAs via glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle or via glutamate dehydrogenase (GDH) alternative NH_4^+ assimilation pathway (Ghanem et al. 2011). In addition, close related to N metabolism, photorespiration is important to maintain adequate N homeostasis and produce metabolites for other processes. Several enzymes are involved in photorespiration including glyoxylate oxidase (GO) and glyoxylate aminotransferase (GGAT) presented in peroxisomes (Shi-Wei et al. 2007). Authors such as Sarabhi et al. (2008) reported, in plants grown under salt stress, an increase in the activity of N cycle enzymes and photorespiration improves the N integration in the plant and prevents NH_4^+ toxic effect.

In leaves, glutamate and aspartate are the main products of N assimilation and therefore plants present a great content of these AAs, especially during the light period. Furthermore, many AAs are precursors for the biosynthesis of other N compounds such as nucleotides, phytohormones, or secondary metabolites. In addition, serine concentration might be higher under increased photorespiration conditions (Lea and Azevedo 2007). On the other hand, pools of all AAs are more induced during stress. For instance, it is known that proline concentration increases significantly in response to stress in some plants and proline is considered a compatible osmolyte (Hildebrandt et al. 2015). Protein degradation could be a compensatory mechanism under abiotic stress conditions to improve osmolarity. Thus, genotype difference in the AAs accumulation is considered as

an indicator to determine the saline stress tolerance in crop plants. Under saline stress, an increase in AAs concentrations may suggest that proteins are in a continuous turnover state (Surabhi et al. 2008). The interference in the ions uptake and the osmotic imbalance caused by salt stress, determine in part the N availability in the plant. This aspect is particularly relevant to estimate N use efficiency (NUE) and the environmental impact of N fertilization. NUE is defined as the biomass production per unit of N available in the soil and is divided into two fundamental processes: (1) N utilization efficiency (NUtE) i.e., the ability of plants to transfer this element to plant organs, and (2) N uptake efficiency (NUpE) i.e., the ability of plants to take up N from the soil (Abenavoli et al. 2016).

On the other hand, an adequate N supplementation could be an efficient method to improve the productivity in plant growth under saline stress. The relationship between N nutrition and salt stress had been studied because soil salinization is an emerging problem around the world (Ashraf et al. 2018). As NO_3^- is a key molecule for N metabolism, supporting the synthesis of N compounds in plants, the objective of this work is to investigate the effect of NaCl in NO_3^- reduction, NH_4^+ assimilation, nitrogen pathway, photorespiration and amino acids in tomato leaves. In addition, we want to verify if the genotypic variability improves tolerance to saline stress in tomato plants.

2 Material and methods

2.1 Plant material

Seeds of *Solanum lycopersicum* L. Gran brix and Marmande RAF genotypes were germinated and grown for 30 days in cell flats. The flats were placed on benches in an experimental greenhouse. After 30 days, the seedlings were transferred to a growth chamber under the following controlled environmental conditions: Relative humidity 60-80%; Day/night temperatures 28/19 °C; 16/8 h photoperiod. Under these conditions, the plants were grown in hydroponic cultivation in lightweight polypropylene trays of 3 L volume, 8 plants/tray. Throughout the experiment the plants were treated with a growth solution made up of 4 mM KNO_3, 3 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 6 mM KH_2PO_4, 1 mM $NaH_2PO_4 \cdot 2H_2O$, 2 µM $MnCl_2 \cdot 4H_2O$, 1 µM $ZnSO_4 \cdot 7H_2O$, 0.25 µM $CuSO_4 \cdot 5H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2H_2O$, 5 µM Fe-chelate (Sequestrene; 138 FeG100) and 50 µM H_3BO_3. This solution, with a pH of 5.5–6.0, was renewed every three days. Plant material was more widely described in previous works (De la Torre-González et al. 2017; De la Torre-González et al. 2018).

2.2 Experimental design and treatments

Treatment of salt stress started 38 days after germination and was maintained for 15 days. The control treatment received the nutrient solution, while the treatment salt stress received the nutrient solution plus 100 mM NaCl.

2.3 Plant sampling

Plants of each treatment (53 days after germination) were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). For the present work the analysis were done in the leaves, half of leaves from each treatment were frozen at −30 °C for further work and biochemical assays and the other half of the plant material was lyophilised for 48h to obtain the dry weight (DW) and the subsequent analysis of the concentrations of nutrients.

2.4 Analysis of N forms

NO_3^- was analyzed from an aqueous extraction of 0.1 g of DW in 10 ml of Millipore-filtered water. A 100-µl aliquot was taken for NO_3^- determination and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO_3^- concentration by spectrophotometry as performed by Cataldo et al. (1975). NH_4^+ was analyzed from an aqueous extraction and was determined by using the colorimetric method described by Krom (1980). Total reduced N concentration was analyzed from digested samples. A 1-ml aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 µM sodium phosphate, and 5.4% w/v sodium hydroxide), 15%/0.03% (w/v) sodium silicate/sodium nitroprusside, and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37 °C for 15 min, and organic N was measured by spectrophotometry according to the method of Baethgen and Alley (1989).

2.5 Enzyme extractions and assays

Leaves were ground in a mortar at 0 °C in 50 mM KH_2PO_4 buffer (pH 7.5) containing 2 mM EDTA, 2 mM dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 30,000g for 20 min. The resulting extract (cytosol and organelle fractions) was used to measure the enzyme activity of NR and GOGAT. The extraction medium was optimized for these enzyme activities so that they could be extracted together according to the same method (Groat and Vance 1981; Kaiser and Lewis 1984; Singh and Srivastava 1986).

The NR assay followed the methodology of Kaiser and Lewis (1984). The NO_2^- formed was colorimetrically determined at 540 nm after azocoupling with sulfanilamide and naphthylethylenediamine dihydrochloride according to the method of Hageman and Hucklesby (1971).

GOGAT activity was assayed spectrophotometrically at 30 °C by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986), always within 2 h of extraction. The decrease in absorbance was recorded for 5 min.

For the GO determination, fresh leaf tissue (0.25 g) was ground in a chilled mortar with PVPP and 1 ml of 50 mM Tris–HCl buffer (pH 7.8) with 0.01% Triton X-100 and 5 mM DTT. The homogenate was centrifuged at 30,000g for 20 min. The

supernatant was decanted and immediately used for the enzyme assay. GO was assayed as described by Feierabend and Beevers (1972) with modifications. A volume of assay mixture containing 50 mM Tris–HCl buffer (pH 7.8), 0.009% Triton X-100, 3.3 mM phenylhydrazine HCl (pH 6.8), 50 µl plant extract, and 5 mM glycolic acid (neutralized to pH 7 with KOH) was used to start the reaction. GO activity was determined by following the formation of glyoxylate phenylhydrazone at 324 nm for 2 min after an initial lag phase of 1 min.

For determination of GGAT and HR, leaves were ground in a chilled mortar in 100 mM Tris–HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at 20,000g for 10 min. The resulting extract was used to measure enzyme activity. The extraction medium was optimized for the enzyme activities such that they could be extracted together using the same method (Hoder et al. 1983).

GGAT activity was measured by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH. The reaction was assayed in a mixture containing 100 mM Tris–HCl (pH 7.3), 20 mM glutamate, 1 mM glyoxylate, 0.18 mM NADH, 0.11 mM pyridoxal-5-phosphate, 83 mM NH_4Cl, and 0.3 U GDH in a final volume of 0.6 ml (Igarashi et al. 2006).

HR assay was performed with 100 mM Tris–HCl (pH 7.3), 5 mM hydroxypyruvate, and 0.18 mM NADH. The activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm (Hoder et al. 1983).

GS was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis (1984). Leaves were ground in a mortar at 0 °C in 50 ml maleic acid-KOH buffer (pH 6.8) containing 100 mM sucrose, 2% (v/v) β-mercaptoethanol, and 20% (v/v) ethylene glycol. The homogenate was centrifuged at 30,000g for 20 min. The resulting extract was used to measure enzyme activity of GS. The reaction mixture used in the GS assay was composed of 100 mM KH_2PO_4 buffer (pH 7.5) with 4 mM EDTA, 1000 mM l-sodium glutamate, 450 mM $MgSO_4 \cdot 7H_2O$, 300 mM hydroxylamine, 100 mM ATP, and enzyme extract. Two controls were prepared, one without glutamine and the other without hydroxylamine. After incubation at 28 °C for 30 min, the formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride (Wallsgrove et al. 1979).

Aspartate aminotransferase (AAT) activity was assayed spectrophotometrically at 340 nm using the method published by Gonzalez and others (1995). AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8), 4 mM $MgCl_2$, 10 mM aspartic acid, and enzyme extract. The decrease in absorbance was recorded for 3 min.

2.6 Protein determination

The protein concentration of the extracts was determined according to the method of Bradford (1976) using bovine-serum albumin as the standard.

2.7 Soluble AAs analysis

The soluble AAs were extracted following the method of (Bieleski and Turner 1966) with some modifications. 0.1 g of fresh leaves were homogenised in 1ml of MCW (methanol: chloroform: water, 12:5:1). 50 µl of L-2 Aminobutyric acid was added as an internal standard. The mixture was centrifuged at 5000 rpm for 10 min. The resulting supernatant was added 700 µl of Milli-Q water and 1.2 ml of chloroform and incubated 24 h at 4 °C. Then, the aqueous phase was obtained, which was lyophilized and the resulting extract was diluted with 0.1 M HCl. Instrumental analysis of soluble AAs was carried out using the precolumn AccQ Tag Ultra Derivatization Kit (Waters, Milford, MA, USA). Derivatization was performed according to the manufacturer's protocol. For derivatization, 60 µl of borate buffer was added to 10 µL of the sample, 10 µL 0.1 N NaOH and 20 µL reconstituted AccQ•Tag Ultra Reagent. LC fluorescence analysis was performed on the Waters Acquity® UPLC System equipped with the Acquity fluorescence detector. UPLC separation was performed on the AccQ Tag Ultra column (2.1 x 100 mm, 1.7 µm) from Waters. The flow rate was 0.7 mL min-1, and the column temperature was kept at 55ºC. The injection volume was 1 µL, and the detection was set at a 266-nm excitation wavelength and a 473-nm emission wavelength. The solvent system consisted of two eluents: 1:20 Dilution of AccQ Tag Ultra eluent A concentrate and AccQ Tag Ultra eluent B. The profile was as follows: 0–0.54 min, 99.9% A and 0.1% B; 5.74 min, 90.9% A and 9.1% B; 7.74 min, 78.8% A and 21.2% B; 8.04 min, 40.4% A and 59.6% B; 8.05–8.64 min, 10% A and 90% B; 8.73–9.50 min, 99.9% A and 0.1% B.

2.8 Determination of NUtE and NUpE:

NUtE (Nitrogen-utilization efficiency) was calculated as plant tissue DW divided by TNC (total nitrogen content) (Siddiqi and Glass 1981). The results were expressed as g DW plant mg−1 N. NUpE (Nitrogen-uptake efficiency) was calculated following Elliot and Läuchli (1985). The results were expressed as mg N g−1 DW root.

2.9 Statistical analysis

All analyses were repeated in triplicate and the results were evaluated statistically using an analysis of variance ANOVA simple with a 95% confidence interval. A two-tailed ANOVA was applied to ascertain whether the saline treatment and the genotype significantly affected the results. The differences between the treatments means were compared using the test of the minor differences of Fisher (LSD) at a 95% probability level. Significance levels were expressed as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS not significant. The statistical software used was Statgraphics Centurion.

3 Results

3.1 N forms analysis

Saline treatment caused a decrease in the N forms analysed in both Grand Brix and Marmande RAF genotypes, with respect to their controls (Table 1). This decrease was significant for all parameters in both varieties, except for NH_4^+ which was not significant for Marmande RAF genotype. The reduction was

greater in Marmande RAF for total N and total reduced N, whereas NO_3^- reduction was similar in both genotypes and NH_4^+ reduction was greater in Grand Brix (Table 1). Nevertheless, comparing between the genotypes, Marmande RAF presented significantly higher values than Grand Brix genotype for these parameters (Table 1).

Table 1: Effect of saline treatment in the concentration of NO_3, NH_4, N tot red and N tot in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	NO_3 (mg g DW^{-1})		NH_4 (µg g DW^{-1})		Total reduced N (mg g DW^{-1})		Total N (mg g DW^{-1})	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	18.35±0.11[a]†	21.35±0.43[a]	8.00±0.30[a]	9.17±0.29[a]	46.02±0.82[a]	53.14±2.07[a]	72.38±0.95[a]	83.66±2.46[a]
Salinity	3.53±0.18[b]	4.35±0.14[b]	5.83±0.24[b]	8.44±0.34[a]	43.25±0.60[b]	43.25±0.96[b]	52.62±0.70[b]	56.04±1.30[b]
P-value	***§	***	***	NS	*	***	***	***

Grand Brix	10.94[b]‡	6.92[b]	44.64[b]	62.50[b]
Marmande	12.85[a]	8.81[a]	48.20[a]	69.85[a]
P-value	***	***	**	***
$LSD_{0.05}$	0.51	0.61	2.54	3.07

Table 2: Effect of saline treatment in the NR, GS, GOGAT and AAT activity in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	NR (µM NO_2 mg $prot^{-1}$ min^{-1})		GS (µM γ-GH $prot^{-1}$ min^{-1})		GOGAT (ΔAbs h^{-1} mg^{-1} prot)		AAT (ΔAbs h^{-1} mg^{-1} prot)	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	0.14±0.00[b]†	0.13±0.00[a]	13.17±0.26[b]	14.19±0.79[a]	0.11±0.01[b]	0.13±0.01[b]	0.08±0.01[b]	0.03±0.00[a]
Salinity	0.16±0.00[a]	0.13±0.00[a]	23.07±0.65[a]	12.53±0.20[b]	0.30±0.02[a]	0.24±0.01[a]	0.12±0.01[a]	0.04±0.01[a]
P-valor	***§	NS	***	*	***	***	**	NS

Grand Brix	0.15[a]‡	17.62[a]	0.21[a]	0.10[a]
Marmande	0.13[b]	13.36[b]	0.18[b]	0.03[b]
P-valor	***	***	*	***
$LSD_{0.05}$	0.00	1.10	0.03	0.01

†Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).

‡ Values are means (n= 18) and differences between means were compared using LSD test (P= 0,05).

§Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0,05; **P< 0,01 and *** P< 0,001 relative to the control.

3.2 N metabolism and photorespiration

Genotypes show very different results regarding N metabolism. Grand Brix plants grown under saline treatment showed a significant increase in NR, GS, GOGAT and AAT enzyme activities (Table 2). However, Marmande RAF presented different results, for these enzymes, in the saline treatment compared to its control. NR and AAT enzymes did not show significant changes in their activities, while GS activity showed a significant decrease in its activity in saline treatment. On the other hand, GOGAT activity registered a significant increase in its activity (Table 2). In addition, when the genotypes were compared, Grand brix showed greater activity for all enzymes (Table 2).

Regarding photorespiration enzymes, GO activity showed a significant decrease in both genotypes in the saline treatment compared to their controls. HR activity did not register significant differences in saline treatment in both genotypes. GGAT activity showed a significant increase in Grand Brix and a significant decrease in Marmande RAF when salinity was applied (Table 3). Comparing between genotypes, Marmande RAF presented significantly higher activities than Grand Brix genotype for all the enzymes mentioned above (Table 3).

Table 3: Effect of saline treatment in the GO, GGAT and HR activity in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	GO (ΔAbs min^{-1} mg^{-1} prot)		GGAT (ΔAbs h^{-1} mg^{-1} prot)		HR (ΔAbs min^{-1} mg^{-1} prot)	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	0.04 ± 0.00^{a}†	0.06 ± 0.00^{a}	0.18 ± 0.02^{b}	0.52 ± 0.04^{a}	0.02 ± 0.00^{a}	0.03 ± 0.00^{a}
Salinity	0.03 ± 0.00^{b}	0.05 ± 0.00^{b}	0.53 ± 0.02^{a}	0.34 ± 0.02^{b}	0.02 ± 0.00^{a}	0.03 ± 0.00^{a}
P-value	***§	***	***	**	NS	NS
Grand Brix	0.03^{b}‡		0.36^{b}		0.02^{b}	
Marmande	0.05^{a}		0.43^{a}		0.03^{a}	
P-value	***		*		**	
$LSD_{0.05}$	0.00		0.06		0.00	

† Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05).
‡ Values are means (n = 18) and differences between means were compared using LSD test (P = 0.05).
§ Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

3.3 AAs profile

Histidine and aspartate concentrations showed a similar tendency. Thus, Grand Brix presented a significant decrease for these 3 AAs, while Marmande RAF did not show significant differences in any of these 3 AAs in saline treatment. When we compared the genotypes in Grand Brix we observed a higher aspartate concentration, while Marmande RAF presented a higher histidine concentration (Table 4).

Serine registered in the treated plants a significant increase in Grand Brix, while Marmande RAF presented a significant decrease in its concentration with respect to its control (Table 4). When we compared genotypes, no significant differences were found for serine (Table 4).

Table 4: Amino acids concentration in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	Aspartate (ug g^{-1} DW)		Serine (ug g^{-1} DW)		Histidine (ug g^{-1} DW)		Total AAs	
	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande	Grand Brix	Marmande
Control	11.47±1.26[a]	3.79±0.33[a]	2.01±0.01[b]	3.78±0.29[a]	0.99±0.01[a]	1.10±0.09[a]	35.44±0.55[b]	26.18±1.96[b]
Salinity	4.62±0.03[b]	3.19±0.36[a]	4.33±0.12[a]	2.14±0.23[b]	0.73±0.01[b]	1.03±0.12[a]	42.69±1.04[a]	38.78±2.43[a]
P-value	***	NS	***	*	***	NS	**	*
Grand Brix	8.05[a]		3.17[a]		0.86[b]		39.07[a]	
Marmande	3.49[b]		2.96[a]		1.06[a]		32.48[b]	
P-value	***		NS		*		**	
$LSD_{0.05}$	1.56		0.45		0.17		3.84	

†Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).

‡ Values are means (n= 18) and differences between means were compared using LSD test (P= 0,05).

§Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0,05; **P< 0,01 and *** P< 0,001 relative to the control.

3.4 NUE parameters

NUtE registered a significant reduction in both genotypes, but this reduction was greater in Marmande RAF. On the other hand, NUpE showed a significant increase in both genotypes, being higher in Marmande RAF. When the genotypes were compared, Marmande RAF genotype presented a greater concentration of both parameters than Grand Brix genotype (Table 5).

Table 5: Effect of saline treatment in the concentration of NUtE and NUpE in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

	NUtE (g DW mg N^{-1})		NUpE (mg N g DW^{-1})	
	Grand Brix	Marmande	Grand Brix	Marmande
Control	0.04±0.00[a]†	0.07±0.00[a]	104.42±1.30[b]	82.22±2.48[b]
Salinity	0.03±0.00[b]	0.04±0.00[b]	131.57±1.96[a]	224.34±5.04[a]
P-value	***§	***	***	***
Grand Brix	0.04[b]‡		118.00[b]	
Marmande	0.05[a]		153.28[a]	
P-value	***		***	
$LSD_{0.05}$	0.00		6.20	

† Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P = 0.05).
‡ Values are means (n = 18) and differences between means were compared using LSD test (P = 0.05).
§ Levels of significance are represented by NS (non-significant) P> 0.05; * P< 0.05; ** P< 0.01 and *** P< 0.001 relative to the control.

4 Discussions

4.1 Growth parameters

Previous works from our research group concluded that the Marmande RAF genotype was more affected by salt stress than Grand Brix genotype. This conclusion was based on the results of parameters such as biomass, relative growth rate, Na^+ and K^+ concentration and Na^+/K^+ ratio (De la Torre-González et al. 2017; De la Torre-González et al. 2018).

4.2 NH_4^+ formation

NH_4^+ formation under salt stress conditions is well characterized in plants (Xu et al. 2012; Shao et al. 2015; Ashraf et al. 2018). NR and NiR are the first enzymes involved in the metabolic route of NO_3^- assimilation (Figure 1), in higher plants (Causin 1996). Debouba et al. (2007) observed a decrease in the NR activity in tomato leaves grown under salt stress. Likewise, some authors reported that, in plants grown under saline stress, NaCl can alter the ionic balance, causing a decrease in NO_3^- concentration and an increase in NH_4^+ concentration. (Xu et al. 2012; Shao et al. 2015; Ashraf et al. 2018). In our study, the saline treatment produced a NO_3^- decrease in both genotypes (-81% Grand Brix and -80% Marmande RAF Table 1). Works such as Debouba et al. (2006) also reported a lower NO_3^- concentration in tomato seedlings grown under salinity. It could be due to an uptake competition between Cl^- and NO_3^-. In our study, the NO_3^- decrease in both genotypes (Table 1) probably contributes to the biomass decrease presented by the genotypes grown under saline treatment (De la Torre-González *et al.* 2018). Some authors showed a decrease in NO_3^- associated with

a biomass reduction in plants grown under saline stress (Shao et al. 2015). On the other hand, in our study NR activity presented a slight increase in Grand Brix genotype and did not present changes in the Marmande RAF genotype (Table 2). This NR activity does not agree with NO_3^- and NH_4^+ results. Thereby, NH_4^+ must come from a different pathway to the NR and NiR pathway (Figure 1).

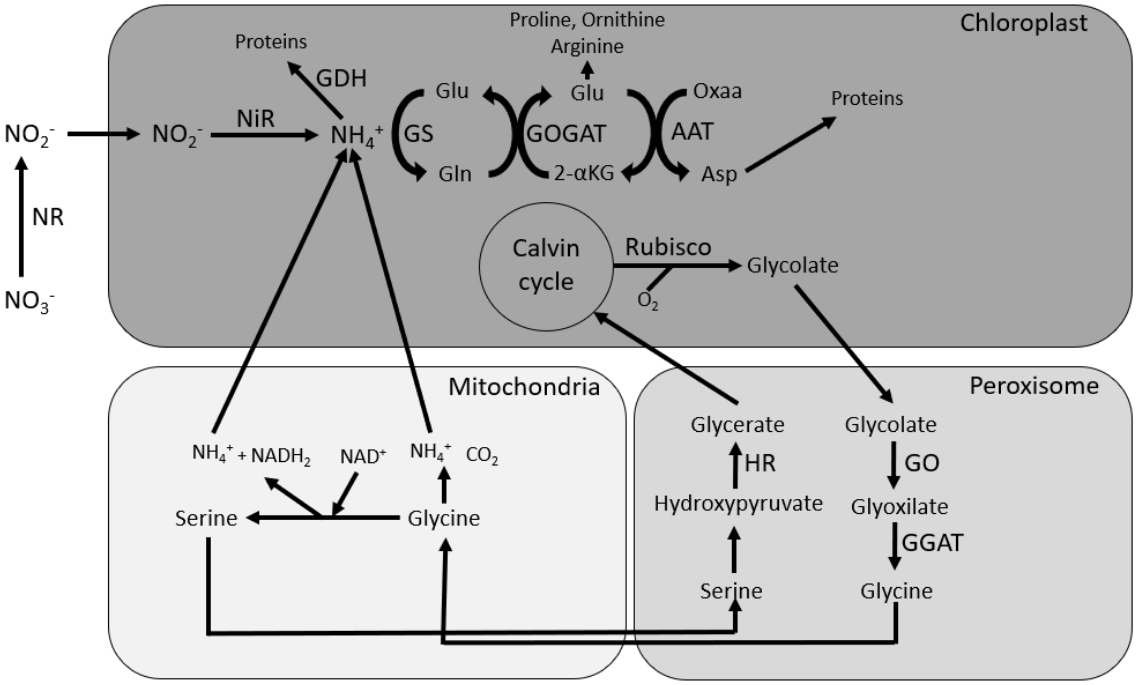

Figure 1. Schematic representation of nitrogen metabolism and photorespiration in plant cell.

Some authors report an increase in NH_4^+ concentration by photorespiration in plants under stress (Shi-Wei et al. 2007; Sánchez-Rodríguez et al. 2011). However, in our study, the general process of photorespiration is not enhanced (Table 3). Nevertheless, GGAT enzyme showed an increase in its activity in the genotype Grand Brix. An increase in this enzyme could indirectly generate NH_4^+ (Figure 1). In addition, authors as Debouba et al. (2007), found an increase in NH_4^+ concentration by an increased proteolysis, when tomato plants were grown

under saline treatment. The lower NH_4^+ concentration presented in Grand Brix, that does not present Marmande RAF (Table 1), might indicate that Grand Brix genotype maintains the activity of enzymes that incorporate N in the plant (Figure 1), this could contribute to a better tolerance to salt stress. Besides, a high NH_4^+ concentration can be toxic to the plant and reduce its growth (Surabhi et al. 2008).

4.3 NH_4^+ assimilation

The main pathway of NH_4^+ assimilation is the GS/GOGAT cycle (Figure 1). Sánchez-Rodríguez et al. (2011) showed that the most resistant tomato genotype presented a greater activity of GS, GOGAT and AAT enzymes, improving its water tolerance stress. Therefore, greater activity of the GS/GOGAT cycle would improve the tolerance to salt stress directly by the N incorporation. Thus, Surabhi et al. (2008) in two mulberry genotypes subjected to salt stress, concluded that a greater activity of these enzymes helps, on the one hand, to control NH_4^+ concentration in plants reducing its toxic effect, and on the other hand improves salt stress tolerance by counteracting the osmotic stress by osmoprotectors solutes accumulation such as proline (Proline result was showed in De la Torre-González et al. 2018). In addition, GOGAT enzyme generates glutamate (proline synthesis precursor; Glutamate result was showed in De la Torre-González et al. 2018). Therefore there is a connection between N and proline metabolism, this could indirectly improve the tolerance to salt stress by the proline synthesis precursor generation. In this sense, this research group did a previous work where a proline increase concentration in Grand Brix improved the salt stress tolerance in this genotype. (De la Torre-Gonzalez et al. 2018). Our results

showed an activity increase of these enzymes in Grand Brix (Table 2), this increase could improve saline stress tolerance and improve the N incorporation. Alternatively, GDH can incorporate NH_4^+ (Figure 1), the NH_4^+ turns into glutamate for synthesize proteins in response to high NH_4^+ levels under saline stress (Surabhi et al. 2008; Xu et al. 2012; De la Torre-González et al. 2018). In our study, this alternative pathway is enhanced in the Grand Brix genotype (Table 2) decreasing NH_4^+ concentration in this genotype (reducing its toxicity) and generating glutamate (De la Torre-González et al. 2018), which could increase tolerance to salt stress.

On the other hand, the lower reduction presented by Grand Brix in total N concentration (Table 1), supports the theory that Grand Brix maintains better N incorporation in the plant than Marmande RAF. This is reflected in a greater N concentration in the plant and contributes to a lower biomass reduction. In this sense, some authors have reported that a better N uptake and integration improve tolerance to salt stress (Lea and Azevedo 2007; Ashraf et al. 2018)

4.4 AAs profile

Histidine and aspartate often play a role in plants as an N reserve or as N transport to young leaves (Causin 1996; Hildebrandt et al. 2015; Galili et al. 2016). A decrease in these AAs concentration (Table 4) together with a greater GS/GOGAT cycle activity in the Grand Brix genotype (Table 2), could mean that the N is integrated more efficiently. Authors such as Zhonghua et al. (2011)

reported a decrease in these AAs when N nutrition is adequate in plants grown under salt stress.

Likewise, glycine and serine can be interconverted easily (Figure 1). However, glycine can be completely degraded forming CO_2 and NH_4^+ (Hildebrandt et al. 2015). Authors such as Mishra et al. (2016) reported, in rice genotypes with different tolerance degrees to saline stress, a glycine decrease while serine showed an increase in genotypes tolerant to salt stress. This is in agreement with our results and supports that Grand Brix presents a greater tolerance to salt stress. Glycine might be generating NH_4^+ through photorespiration and NH_4^+ can be integrated into the plant by the GS/GOGAT cycle or the GDH (Data of glycine and GDH showed in De la Torre-González et al. 2018) (Figure 1).

4.5 NUE

According to our results, the NUtE values obtained (Table 5), Grand Brix genotype presented a more effective N utilization. This is in agreement with the lower reduction in total N concentration described above (Table 1). However, NUpE values indicate that Marmande RAF has a better N uptake (Table 5). These values together with the biomass data (De la Torre-González et al. 2018) could indicate that although Marmande RAF has a better N uptake, this N might be integrated into the root and is not transported towards the leaves, this would explain the biomass decrease in Marmande RAF. On the contrary, although Grand Brix is less effective in the N uptake under salt stress, the N utilization was better in the leaves. This allowed increasing biomass production reducing the

NH_4^+ accumulation and generating more osmoprotector compounds. Abenavoli et al. (2016) studied NUE in different tomato genotypes obtaining similar results to our study i.e., the genotype that presented more NUtE also showed less NUpE. These authors conclude in their work that for the tomato genotypes NUtE could be more important in relation to the N metabolism. Considering these results, a greater NUtE could increase tolerance to salt stress. However, other authors, in tomato plants grown under water stress, obtained higher NUpE values in the most tolerant genotype (Sánchez-Rodríguez et al. 2011). Therefore, N uptake and N use could depend on genotype variability and stress type.

5 Conclusion

In this work, we can conclude that a better N metabolism regulation contributes to improving saline stress tolerance. This is more evident in the Grand Brix genotype, as this genotype presented a greater GS/GOGAT cycle activity integrating more efficiently the N and generating AAs osmoprotectors such as proline. This was reflected in the levels of total N and NUtE of Grand Brix, which were not as reduced as in Marmande RAF. In addition, the photorespiration might contribute to glycine generation, and this could generate a NH_4^+ source that can be used by the GS/GOGAT cycle or GDH enzyme. Therefore, the results obtained in this study could be used to generate genotypes more resistant to salt stress.

6 Author contribution statement

J.M.R. and B.B. conceived and designed research. A.T-G. and E.N-L. conducted experiments. A.T-G. and B.B. analysed data. A.T-G. wrote the manuscript. J.M.R. critically reviewed the manuscript. All authors read and approved the manuscript.

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DISCUSIÓN GENERAL

Como hemos comprobado a lo largo de esta Tesis Doctoral el estrés salino es un problema para el crecimiento y desarrollo de los cultivos a nivel mundial que está aumentando cada vez más (Mateo-Sagasta y Burke 2010; Abbas et al. 2013). Es por ello que necesitamos genotipos más tolerantes a este estrés para hacer frente a las condiciones de salinidad de muchos tierras de cultivo, sobre todo en las zonas costeras donde el problema será mayor en un futuro, y de esta manera poder abastecer la creciente demanda de alimentos de la población mundial.

En los capítulos 1, 2, 3 y 4 hemos descrito como el efecto toxico del estrés salino afecta a diferentes rutas y procesos metabólicos en los genotipos de tomate seleccionados. Además, en estos capítulos se describe como en el metabolismo de la planta podríamos encontrar procesos fisiológicos clave para mejorar la tolerancia al estrés salino, dejando puertas abiertas para la generación de nuevos genotipos más tolerantes a este tipo de estrés. Los procesos fisiológicos que determinan en nuestro trabajo una mayor tolerancia a la salinidad en el genotipo Grand Brix fueron una mayor concentración de ácidos orgánicos acompañado de una mayor actividad de las enzimas del ciclo TCA, una mayor síntesis de prolina y su acumulación, y una mayor actividad de las enzimas implicadas en la asimilación del N. Como resultado de la inducción de estos procesos el genotipo Grand Brix presentó una menor concentración de ROS (Imagen 1).

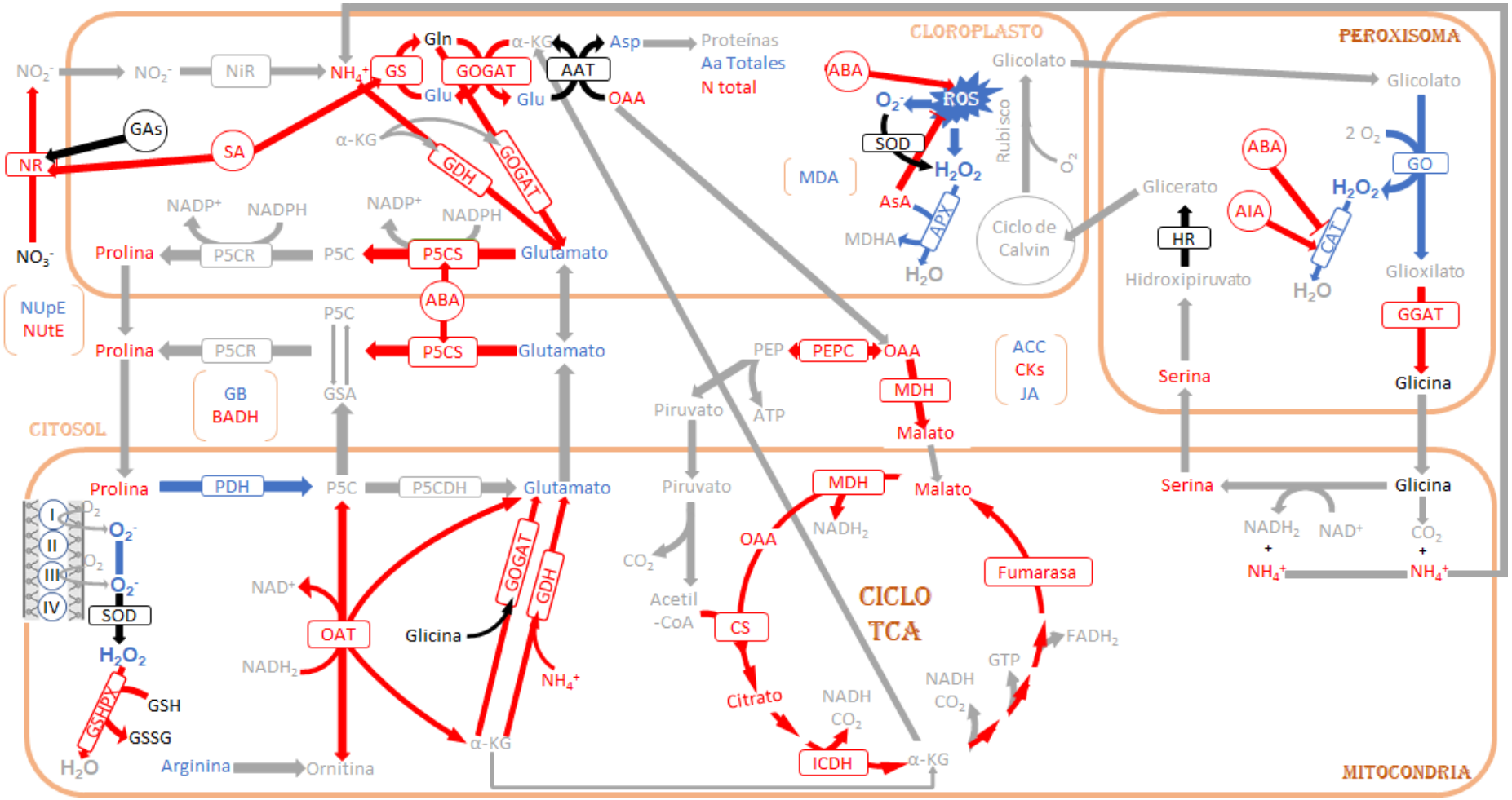

Figura 1: Representación esquemática de la interrelación de las rutas metabólicas y compuestos analizados en la presente tesis. En rojo – se representan procesos que se inducen de manera más significativa en Grand Brix que en Marmande RAF, en el tratamiento salino. En azul – se representan procesos que se inducen de manera más significativa en Marmande RAF que en Grand Brix, en el tratamiento salino. En negro – se representan procesos que se inducen de manera similar en ambos genotipos. En gris – se representa partes del metabolismo no estudiadas en la presente tesis

Además, de definir los procesos fisiológicos clave que se alteran frente a un estrés es muy importante conocer también la interrelación de las diferentes rutas metabólicas y compuestos analizados para intentar mostrar como una mejora de estos procesos fisiológicos podría ser clave para una mayor tolerancia al estrés salino en plantas, que es precisamente lo que intentaremos abordar en este capítulo de discusión general.

Tal como se describe en los capítulos 1, 2 y 3 el genotipo Grand Brix presento una mayor tolerancia al estrés salino que el genotipo Marmande RAF, esto se vio reflejado en la biomasa y la TCR. Ambos parámetros presentaron una menor disminución en el tratamiento salino en el genotipo Grand Brix. Sin embargo, la concentración de Na^+ fue mayor en este genotipo. En el capítulo 2 se describe como sería posible que el genotipo Grand Brix estuviera compartimentalizando este exceso de Na^+ en las vacuolas como oxalato-Na^+, evitando de esta forma su efecto citotóxico. Aunque ambos genotipos sobrevivieron al efecto toxico del estrés salino, estos presentaron algunas alteraciones en su metabolismo.

Los ROS se generan de manera constante en las plantas tanto en la membrana de las mitocondrias con la cadena de transporte de electrones como en los tilacoides con la fotosíntesis (Saibi et al. 2015) (Imagen 1). Sin embargo, bajo un estrés abiótico, como el estrés salino la producción de ROS aumenta por el efecto citotóxico del Na^+ y Cl^-. De hecho, la reducción de la capacidad fotosintética generada por el estrés salino es la principal causa de generación de ROS, que se acumulan en las hojas provocando un daño oxidativo en estructuras

celulares, compuestos y alterando el metabolismo (Acosta-Motos et al. 2017; Liang et al. 2018). En este sentido, Grand Brix mostró niveles más bajos de los principales ROS (O_2^- y H_2O_2) (Imagen 1) y de los indicadores de daño oxidativo como el MDA, lo que pudo favorecer que los procesos metabólicos y estructuras celulares estuvieran menos afectadas mejorando la tolerancia al estrés salino de forma directa e indirecta. Con respecto a los sistemas antioxidantes no hubo una clara diferencia entre genotipos. Por ejemplo, la principal enzima de detoxificación de ROS, la SOD, fue inducida de forma similar bajo estrés salino para ambos genotipos. Para el resto de enzimas y compuestos antioxidantes Grand Brix mostró una mayor actividad para la GSHPX y una mayor concentración de AsA (Imagen 1), mientras que el genotipo Marmande RAF lo hizo para enzimas como la APX o la CAT (Imagen 1). Atendiendo a estos resultados no pudimos observar una tendencia clara en los genotipos, sin embargo, la menor concentración de ROS y MDA si reflejan un menor estrés oxidativo en Grand Brix, lo cual como ya hemos comentado mejoraría la tolerancia a la salinidad.

Por otro lado, las fitohormonas influyen muchos procesos metabólicos, incluso en la generación y detoxificación de ROS (Imagen 1). Aunque en este sentido se necesitan más estudios para comprender en profundidad la influencia de las fitohormonas en los procesos metabólicos, pudimos observar en el genotipo Grand Brix un aumento significativo de algunas fitohormonas como el ABA, AIA, SA y una disminución de ACC (Imagen 1). Los cambios en la concentración de estas fitohormonas podrían estar generando una mayor tolerancia al estrés

salino en este genotipo. Estas fitohormonas regulan procesos como el desarrollo y crecimiento de las plantas, la senescencia o el cierre estomático, además de favorecer el aumento de la actividad de algunas enzimas como la NR o la GS (Imagen 1).

Otro proceso metabólico de gran importancia para las plantas es el ciclo TCA, interviene en la producción de energía y formación de precursores para la formación de Aa (López-Bucio et al. 2000). En condiciones de estrés salino los ácidos orgánicos generados en este ciclo mejoran los desbalances osmóticos provocados por los iones Na^+ y Cl^-, previenen el daño oxidativo (Hossain y Dietz 2016) y aumentan la resistencia del daño citotóxico de cationes como el Al^{3+} (Ryan et al. 2001). Actualmente existen bastantes estudios sobre el ciclo TCA, sin embargo, hay pocos estudios sobre su relación con el estrés salino. Por ello en la introducción general y en el capítulo 2 describimos como las plantas que presentan una mayor actividad enzimática del ciclo TCA, así como una mayor acumulación de ácidos orgánicos podrían contribuir a la mejora de la tolerancia del estrés salino, manteniendo la producción de energía y mejorando el estrés osmotico. En este sentido el efecto toxico del estrés salino fue menos perjudicial en el genotipo Grand Brix, el cual mostro un incremento de todas las principales actividades enzimáticas que generan los ácidos orgánicos malato, citrato y oxalacetato, además de presentar una mayor concentración de los propios ácidos orgánicos malato y citrato bajo estrés salino (Imagen 1). Por el contrario, Marmande RAF presento una disminución para la actividad de la mayoría de las enzimas del ciclo, lo que se tradujo en una disminución de la concentración de

ácidos orgánicos (imagen 1). Estos resultados, como hemos descrito anteriormente ayudarían a Grand Brix al mantenimiento energético y mejoraría el estrés osmotico, procesos que podrían ser clave en la tolerancia a la salinidad. Por otro lado, el ciclo TCA podría estar proporcionando α-KG para la asimilación de N (Imagen 1), lo cual podría estar sucediendo en el genotipo Grand Brix ya que como se describe en el capítulo 4 el ciclo GS/GOGAT parece estar inducido (Imagen 1). Por tanto, un aumento de la actividad del ciclo TCA sumado a una asimilación de N adecuada podrían ser factores clave para aumentar la tolerancia al estrés salino.

Las rutas metabólicas para la generación de prolina y GB han sido también muy estudiadas para la tolerancia ante estreses abióticos como el estrés salino. Esto es debido a que muchos autores creen que estos compuestos ayudan a la prevención del estrés osmotico en plantas, contrarrestando el efecto citotóxico de algunos iones como el Na^+ y el Cl^- en el caso del estrés salino (Sumithra et al. 2006; Hossain y Dietz 2016). Además, algunos autores describen como estos compuestos también podrían intervenir en la estabilización de proteínas membranas y estructuras celulares, además de prevenir el daño oxidativo generado por los ROS (Salinas et al. 2013). Por todo ello, en el capítulo 3 se describe el efecto de la prolina y la GB sobre la tolerancia a la salinidad.

En este caso ambos genotipos acumularon prolina bajo condiciones de estrés salino. Sin embargo, pensamos que la vía acumulación de este Aa fue diferente entre ambos genotipos. Pudimos observar (tal y como se describe en el capítulo

3) que la vía principal de síntesis de prolina (P5CS) (Imagen 1) no presentó un incremento significativo en ambos genotipos, lo que no se corresponde con la acumulación de prolina que si pudimos observar en los mismos. En este sentido, en el genotipo Grand Brix se observó el incremento de la enzima OAT (Imagen 1), la cual es una vía secundaria para la generación de prolina que no está muy estudiada, pero que podría verse favorecida en plantas bajo estrés abiótico como el estrés salino (Anwar et al. 2018). Esto unido a que la principal enzima de degradación de prolina PDH se encuentra menos inhibida en Marmande RAF pensamos que en el genotipo Grand Brix la acumulación de prolina viene proporcionada por una síntesis a través de esta vía secundaria, mientras que en el genotipo Marmande RAF la acumulación de prolina podría estar proporcionada por una degradación proteica a consecuencia del estrés salino (Imagen 1). Como podemos observar en la Imagen 1 la síntesis de prolina requiere glutamato como sustrato y este puede ser proporcionado a través de la enzima GDH, enzima que está relacionada con la asimilación de N. No obstante, aunque la actividad enzimática de la GDH incrementó en el genotipo Grand Brix bajo tratamiento salino, nosotros pensamos que el glutamato generado se está utilizando para la asimilación de N (Imagen 1), ya que esta ruta esta inducida en Grand Brix tal como describimos anteriormente. Esto está en concordancia también con los resultados de la concentración de glutamato y arginina (descritos en el capítulo 3), donde observamos una disminución de su concentración en el genotipo Grand Brix bajo salinidad (Imagen 1), que nos lleva a concluir que el glutamato está siendo utilizado como sustrato en la asimilación de N, procediendo por tanto la prolina principalmente en este genotipo de la síntesis a través de la enzima OAT (Imagen 1).

Por último, uno de los procesos metabólicos principales en las plantas es la asimilación del N, ya que genera compuestos nitrogenados, como los Aa, esenciales para el crecimiento y desarrollo de las plantas (Sánchez-Rodríguez et al. 2011). Actualmente, la relación entre la nutrición nitrogenada y el estrés salino está siendo muy estudiada ya que se considera un problema emergente a nivel mundial (Ashraf et al. 2018). Como hemos desarrollado en la introducción general y en el capítulo 4 el estrés salino afecta a la captación de nutrientes y en el caso del N, el Na^+ afecta a la absorción de NH_4^+ y el Cl^- a los NO_3^- generando alteraciones iónicas en las plantas (Ashraf et al. 2018). Por ello en el capítulo 4 se describe como el efecto toxico de la salinidad afectó a el proceso de asimilación del N. En líneas generales el efecto toxico del estrés salino provoco una disminución de las actividades enzimáticas del genotipo Marmande RAF, tanto en el ciclo GS/GOGAT (principal ruta de asimilación del N) como en la fotorrespiración, provocando una menor captación y acumulación de N y compuestos nitrogenados (Imagen 1). Sin embargo, la NR no presento diferencias significativas en este genotipo bajo estrés salino. Autores como Sarabhi et al. (2008) reflejan como un aumento de la actividad enzimática para la asimilación del N mejora la integración de este nutriente por parte de la planta e incluso previene un posible efecto toxico por una acumulación excesiva de NH_4^+. Por tanto, una mejor actividad enzimática para el proceso de asimilación de N mejoraría la tolerancia al estrés salino. En concordancia con estos autores, Grand Brix mostró un incremento de las actividades enzimáticas NR y las enzimas del ciclo GS/GOGAT lo que reflejan un aumento de la asimilación de N (Imagen 1), mejorando la tolerancia al estrés salino. El genotipo Marmande RAF presento una mayor acumulación de los Aas glutamato y aspartato (Imagen 1),

al igual que una mayor concentración de Aa totales. Sin embargo, el genotipo Grand Brix reflejó una mayor concentración de N total (Imagen 1). Estos datos nos llevan a pensar que Grand Brix podría presentar una mayor capacidad para la utilización del N asimilado por la planta, de hecho, así lo refleja los resultados del NUtE en Grand Brix, y que Marmande RAF esta sufriendo un proceso de degradación proteica probablemente a causa de un estrés osmotico y/o oxidativo generado por el estrés salino. Por último, destacar la ruta de asimilación de N (GS/GOGAT) la cual genera precursores/sustratos para otras rutas o procesos metabólicos como el glutamato para la síntesis de prolina, mejorando no solo la biosíntesis de proteínas sino la de compuestos osmoprotectores (Imagen 1).

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CONCLUSIONES/CONCLUSIONS

1. La reducción de los principales ROS (O_2^- y H_2O_2) en el genotipo Grand Brix mejoró la tolerancia al estrés salino reflejando un menor daño oxidativo y mayor producción de biomasa. Las principales vías de detoxificación de estos ROS fueron mediante la actividad GSHPX y el aumento en la concentración de AsA. Esto unido a un aumento de la concentración foliar de las fitohormonas ABA, AIA, SA y una disminucion de ACC podría explicar el aumento en el grado de tolerancia al estrés salino que observamos en este genotipo.

2. El ciclo de los acidos tricarboxilicos se define como proceso clave en la resistencia a la salinidad, puesto que el genotipo Grand Brix presentó una mayor actividad de las principales enzimas de este ciclo y una mayor acumulación de los ácidos orgánicos lo que mejoraría el equilibrio osmotico en las plantas sometidas al estrés salino. Por otro lado, destacar el ácido orgánico oxalato puede estar reduciendo en el genotipo Grand Brix el efecto fitotóxico del Na^+ mediante la compartimentalización en las vacuolas y/o apoplasto.

3. La vía secundaria para la síntesis de prolina a través de la enzima OAT podría ser una ruta determinante en la tolerancia a la salinidad. Esto unido a una menor inducción de la PDH en Grand Brix podría estar generando una mayor acumulación de prolina y por tanto una mayor tolerancia a la salinidad.

4. Una mayor actividad para las principales enzimas de asimilación de N, como la NR o el ciclo GS/GOGAT en plantas bajo estrés salino proporciona una constante tasa de generación de Aa y proteínas, además de precursores para otras rutas metabólicas esenciales para un correcto desarrollo y crecimiento de las plantas, mejorando de esta forma la tolerancia al estrés salino en el genotipo Grand Brix.

5. La variabilidad genotípica incluso en genotipos comerciales influye en el grado de tolerancia/resistencia al estrés salino, a través de la modificación de ciertos procesos fisiológicos como la detoxificación de ROS, el ciclo de los acidos tricarboxilicos, la sintesis de compuestos osmoprotectores y la asimilación de N.

Conclusions

1. The reduction of the main ROS (O_2^- and H_2O_2) in Grand Brix genotype improved tolerance to saline stress reflecting lower oxidative damage and increased biomass production. The main detoxification pathway of these ROS were through GSHPX activity and the AsA concentration increase. This together with an increase in the foliar phytohormones concentration ABA, AIA, SA and a decrease in ACC could explain the increase in the tolerance degree to saline stress that we observe in this genotype.

2. The tricarboxylic acids cycle is defined as a key process in salinity resistance, since the Grand Brix genotype showed a greater activity of the main enzymes of this cycle and a greater organic acids accumulation which would improve the balance osmotic in plants subjected to saline stress. On the other hand, highlighting the organic acid oxalate may be reducing in the Grand Brix genotype the phytotoxic effect of Na^+ by compartmentalization in vacuoles and/or apoplast.

3. The secondary pathway for proline synthesis through the OAT enzyme could be a determinant pathway in salinity tolerance. This together with a lower PDH induction in Grand Brix could be generating a greater proline accumulation and therefore a greater salinity tolerance.

4. Increased activity of main N assimilation enzymes, such as the GS/GOGAT cycle in plants under saline stress provides a constant Aa and protein generation rate, as well as precursors for other essential metabolic pathways for a correct plant development and growth, thereby improving tolerance to saline stress in Grand Brix genotype.

5. Genotypic variability even in commercial genotypes influences the tolerance/resistance degree to saline stress, through the modification of certain physiological processes such as ROS detoxification, tricarboxylic acids cycle, osmoprotective compounds synthesis and N assimilation.