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Abstract

Pepper culture is economically very important worldwide, although it is very sensitive to sub-optimal conditions of water and high salinity. However, the tolerance to these stresses can be improved by the grafting technique. Previous studies of the Valencian Institute for Agricultural Research and the Polytechnic University of Valencia have been conducted to select pepper accessions that showed tolerance to both stresses, after which a further selection of them was used as rootstocks to find physiological mechanisms of tolerance and to increase its agronomic profit. However, after all these studies, the available information in this regard is still scarce. Therefore, the objectives of this thesis were to: i) screen new tolerant pepper accessions under high salt concentrations and suboptimal water conditions, to increase the availability of tolerant genotypes to be used in future breeding programmes, with the final aim of obtaining new and improved tolerant rootstocks; ii) identify the short-term physiological mechanisms of water stress tolerance of a tolerant accession (A25) used as a rootstock; iii) identify the physiological mechanisms of short-term tolerance to salinity of a new tolerant hybrid rootstock (NIBER®); and iv) find the main molecular pathways of salinity tolerance of a tolerant accession (A25) compared to a sensitive one (A6) by a transcriptomic approach.

After conducting these studies, we firstly found a positive relationship between photosynthetic capacity and growth maintenance in plants that were tolerant to water or salt stress, both grafted or ungrafted; indeed, based mainly on this relationship, we selected accessions A34 and A31 as tolerant to salt and water stress, respectively. In addition, we were able to demonstrate that the main role of proline under salinity and water scarcity is not linked herein to the drop in osmotic potential; on the contrary, we identified different protective roles that, together with other antioxidant protective molecules such as phenols, contribute to the tolerance of pepper plants to these environmental stresses. Moreover, hydrogen peroxide, a reactive oxygen species, was found to play important roles in the antioxidant capacity of pepper, working as a signalling molecule under salinity stress. Furthermore, the drop in abscisic acid concentration and its signalling deregulation were also shown to maintain stomatal aperture and thus the growth of the scion when grafted onto tolerant rootstocks and ungrafted accessions under high salt concentration conditions. It was also demonstrated that a limitation of Na^+ transport to leaves, as well as a more efficient transport and accumulation of K^+ in roots and leaves, are essential to reach ion homeostasis and, thus, tolerance in pepper plants grafted onto tolerant rootstocks. Finally, the study of the molecular pathways of tolerance was a useful tool to confirm the physiological and agronomical behaviour of a pepper accession previously classified as tolerant, although new mechanisms were also found. The differentially expressed genes found were linked to hormonal signalling, plant growth and development, photoprotection, regulation of ion transporters and ROS detoxification.

Resumen

El pimiento es un cultivo muy importante a nivel mundial, pero es sensible a la falta de agua y a la salinidad. No obstante, se puede mejorar la tolerancia mediante la técnica del injerto. El Instituto Valenciano de Investigaciones Agrarias y la Universidad Politécnica de Valencia han realizado estudios previos para seleccionar accesiones de pimiento tolerantes a ambos estreses, utilizando después una selección de ellos como portainjertos para estudiar los mecanismos fisiológicos de tolerancia y aumentar la rentabilidad de su producción. Sin embargo, después de todos estos estudios, la información disponible es limitada. En este sentido, los objetivos que se han planteado en esta tesis doctoral fueron: i) seleccionar nuevas accesiones tolerantes de pimiento a la salinidad y escasez de agua, para aumentar la disponibilidad de genotipos tolerantes y usarlos en futuros programas de mejora, con el objetivo final de obtener nuevos portainjertos con una tolerancia mejorada; ii) identificar a corto plazo los mecanismos fisiológicos de tolerancia al estrés hídrico de una accesión tolerante (A25) usada como portainjerto; iii) identificar a corto plazo los mecanismos fisiológicos de tolerancia a la salinidad de un nuevo portainjerto híbrido tolerante (NIBER®); iv) encontrar los principales mecanismos moleculares de tolerancia a la salinidad de una accesión tolerante (A25) respecto a una sensible (A6) desde el punto de vista transcriptómico.

Una vez realizados estos ensayos, en primer lugar, pudimos relacionar positivamente la capacidad fotosintética y el mantenimiento del crecimiento en plantas tolerantes a estrés hídrico y salino, tanto sin injertar como injertadas; de hecho, basándonos principalmente en esta relación, seleccionamos las accesiones A34 y A31 como tolerantes a estrés salino e hídrico, respectivamente. Además, demostramos que el papel principal de la prolina en los estreses estudiados no está ligado a la bajada de potencial osmótico; sin embargo, se identificaron funciones protectoras de este aminoácido que, junto a otras moléculas antioxidantes como los fenoles, contribuyen en el pimiento a aumentar la tolerancia. Igualmente importante es el peróxido de hidrógeno, que se relacionó con la capacidad antioxidante en pimiento, funcionando como molécula señalizadora en estrés salino. Asimismo, la bajada de ácido abscísico y la modificación de la expresión de genes relacionados han sido también relevantes en condiciones de estrés salino para mantener la apertura estomática y, por consiguiente, el crecimiento en plantas sin injertar e injertadas sobre portainjertos tolerantes. Se demostró también que la limitación del transporte de Na^+ a hojas, así como el transporte y acumulación eficiente de K^+ en raíces y hojas, son esenciales para alcanzar la homeostasis iónica y por tanto la tolerancia en pimientos injertados sobre portainjertos tolerantes. Para finalizar, el estudio de las rutas moleculares fue una herramienta útil para confirmar el comportamiento fisiológico y agronómico de una accesión de pimiento previamente clasificada como tolerante a la salinidad, descubriendo además nuevos mecanismos no referenciados hasta el momento. Los genes diferencialmente expresados encontrados estaban relacionados con la señalización hormonal, el crecimiento y desarrollo de las plantas, la fotoprotección, la regulación de los transportadores de iones y la detoxificación de ROS.

Resum

El pimentó és un cultiu molt important mundialment, però és sensible a la falta d'aigua i la salinitat. No obstant això, es pot millorar la tolerància mitjançant la tècnica de l'empelt. L'Institut Valencià d'Investigacions Agràries i la Universitat Politècnica de València han fet estudis previs per a seleccionar accessions de pimentó tolerants a tots dos estressos i a continuació, una selecció d'entre elles es va utilitzar per a estudiar els mecanismes fisiològics de tolerància i augmentar la rendibilitat de la seua producció. No obstant això, després de tots aquests experiments, la informació encara és limitada. En aquest sentit, els objectius que s'han plantejat en aquesta tesi doctoral van ser: i) seleccionar noves accessions tolerants de pimentó a la salinitat i la falta d'aigua, per a augmentar la disponibilitat de genotips tolerants i usar-los en futurs programes de millora, amb l'objectiu final d'obtindre nous portaempelts amb una tolerància millorada; ii) identificar a curt termini els mecanismes fisiològics de tolerància a l'estrès hídric d'una accessió tolerant (A25) usada com portaempelt; iii) identificar a curt termini els mecanismes fisiològics de tolerància a la salinitat d'un nou portaempelt híbrid tolerant (NIBER®); iv) trobar els principals mecanismes moleculars de tolerància a la salinitat d'una accessió tolerant (A25) respecte a una sensible (A6) des d'un punt de vista de la transcriptòmica.

Després de realitzar aquests assajos, en primer lloc, vam poder relacionar positivament la capacitat fotosintètica i el manteniment del creixement en plantes tolerants a l'estrès hídric i salí, tant sense empeltar com empeltades; de fet, basant-nos principalment en aquesta relació, vam seleccionar les accessions A34 i A31 com tolerants a l'estrès salí i hídric, respectivament. A més a més, vam demostrar que el paper principal de la prolina en els estressos estudiats no està lligat a la baixada de potencial osmòtic; en canvi, es van identificar diferents funcions protectores d'aquest aminoàcid, que, junt a altres molècules antioxidants com els fenols, contribueixen en el pimentó a combatre'ls. Igualment important és el peròxid d'hidrogen, que es va relacionar amb la capacitat antioxidant del pimentó, funcionant com a molècula senyalitzadora a l'estrès salí. Així mateix, la baixada d'àcid abscísic i la modificació de l'expressió de gens relacionats de la seua senyalització han sigut també rellevants en condicions d'estrès salí per a mantindre l'obertura estomàtica i per tant el creixement en plantes sense empeltar i empeltades amb portaempelts tolerants. Es va demostrar també que la limitació del transport de Na^+ a les fulles, així com el transport i l'acumulació eficient de K^+ a les arrels i les fulles, són essencials per a aconseguir l'homeòstasi iònica i per tant la tolerància en pimentons empeltats damunt portaempelts tolerants. Per concloure, l'estudi de les rutes moleculars va ser un instrument útil per a confirmar el comportament fisiològic i agronòmic d'una accessió de pimentó prèviament classificada com a tolerant, descobrint a més nous mecanismes no trobats fins ara. Els gens diferencialment expressats trobats estaven relacionats amb la senyalització hormonal, el creixement i el desenvolupament de les plantes, la fotoprotecció, la regulació dels transportadors de ions i la detoxificació de ROS.

Index

Chapter 1

Introduction

1.1. General Aspects of *Capsicum* sp.

Capsicum sp. is a genus of the family Solanaceae that comprises more than 90 genera and 2500 species, and some play an important role in agriculture, like some species of the genus *Solanum* spp. (Ramchiary and Kole, 2019). Although it is distributed world-wide, it is thought to be native from the tropics and subtropics of America (Mongkolporn and Taylor, 2011). Data suggest that it was the first spice crop to be domesticated and cultivated around 6,000 thousand years ago (Ramchiary and Kole, 2019).

This genus, commonly named pepper, chile, chilli, ají or paprika, comprises around 31 species; five are called *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. and *C. pubescens* Ruiz & Pavón and have been domesticated (Bojórquez-Quintal *et al.*, 2014). The diversity of *Capsicum* sp. fruit is very high, with a wide variety of typologies, colours and pungency (**Figure 1.1.**). Depending on the region it is possible to find specific fruit types.

Of all these species, *C. annuum* is the most important crop both economically and due to the nutritional value of its fruit (Bojórquez-Quintal *et al.*, 2012), which have a high content of antioxidant compounds, fibre, carbohydrates and vitamin A (Navarro *et al.*, 2006; Condés Rodríguez, 2017; Gisbert-Mullor *et al.*, 2020).

Figure 1.1. Fruit diversity of the genus *Capsicum* sp.

1.2. Economic Importance

Pepper production has increased considerably in the last 24 years from 2.7 to 40.9 million tonnes, as its cultivated area, which has grown by almost 28% (1994-2018; FAOSTAT). Of all production, the fruit grown to be consumed fresh have increased the most. The continent that produces the most pepper to be consumed fresh and dry is Asia, with the 65% and 72% of the total production, respectively. In Europe, pepper production as fresh fruit is bigger, with 11% compared to 4% for dry fruit. The continent with smallest pepper production is Oceania (**Figure 1.2.**).

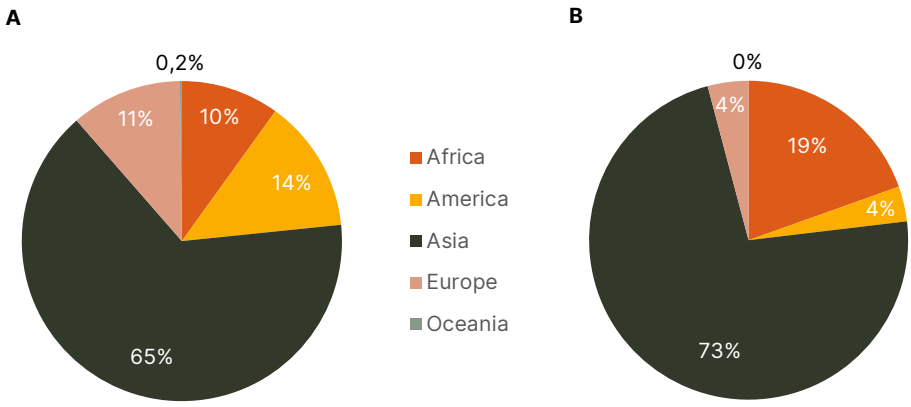

Figure 1.2. Percentage of production of pepper fruits consumed fresh (A) and dry (B) (2018; FAOSTAT).

Furthermore, fresh pepper was cultivated in 2018 in 128 countries worldwide, whereas pepper used as dried fruit was produced in 70 countries. Mainland China plays an important role in pepper production and is the first and second to produce fresh and dry fruit, respectively. In Spain, the majority of pepper production is for fresh fruit, and is the fifth most important country in the world (**Table 1.1.**).

Table 1.1. Production in thousands of tonnes of the world's top 10 most productive countries of fresh or dried pepper fruit (2018; FAOSTAT).

Fresh fruit		**Dry Fruit**	
Country	**Production (thousand tonnes)**	**Country**	**Production (thousands of tonnes)**
China, mainland	12,354.83	India	1,233.61
Mexico	1,957.92	China, mainland	248.24
Turkey	1,787.04	Thailand	158.15
Indonesia	1,268.16	Ethiopia	141.04
Spain	1,002.72	Pakistan	129.14
USA	846.15	Bangladesh	119.17
Nigeria	715.15	Myanmar	92.13
Egypt	524.28	Vietnam	83.76
Republic of Korea	330.98	Ghana	69.10
Italy	317.93	Ivory Coast	65.02

In Spain, the total cultivated surface of pepper has not increased in the last 10 years, but production and yield did by 17% and 23%, respectively (2007-2017; MAGRAMA). As a result, efficiency and productivity have notably improved. The region with the biggest area and most production assigned to pepper is Andalusia (South of Spain). In fact only this community sustains two thirds of the total pepper production (2017; MAGRAMA). The Valencian Community is the fifth most productive region in Spain, and contributes 4.7% to pepper production.

1.3. Pepper Production Challenges

Farming production has increased notably in recent for almost all crops in order to cover the world population's food demands. However, this has been linked with more pressure for ecosystems and the environment (FAO, 2018), which contributes to a new climate change scenario. With this situation, the risk of suffering biotic and/or abiotic stresses has increased, which reduces crop production and fruit quality. Depending on the crop and its location, plants are susceptible to specific types of stress. With pepper, drought, salt, extreme temperatures and different types of pathogens reduce fruit production and quality (Erickson and Markhart, 2002; De Pascale *et al.*, 2003; Condés Rodríguez, 2017; Aidoo *et al.*, 2018).

1.3.1. Biotic Stress

Biotic stress in plants is caused by specific living organisms, including viruses, bacteria, fungi, nematodes, insects, arachnids, or even other plants. These agents directly compete for the same nutrients as plants, diminish plant vigour and increase the risk of death (Singla and Krattinger, 2016). In fact pathogens reduce global crop production by 15% (Onaga and Wydra, 2016), as well as both pre- and post-harvest yields.

Several authors have demonstrated that pepper is very susceptible to **soil-borne pathogens**, especially *Phytophthora capsici*, *Verticillium dahlia* and *Meloidogyne* spp. (root-knot nematode) (Morra and Bilotto, 2006; Gisbert *et al.*, 2010). This type of disease is drastically enhanced due to long-term intensive agriculture, which implies enormous economic loss (Wang *et al.*, 2019), stunted growth, and yield and leaf modifications (**Figure 1.3.**).

Figure 1.3. Pepper plants affected (A) and unaffected (B) by the fungus *P. capsici*.

Pepper has been considered susceptible to more than 60 different **virus** species, depending on the region (Kenyon *et al.*, 2014). Among the symptoms of viruses on plants are mild leaf chlorosis or severe leaf curl, plant stunning, necrosis, dieback or even death (Ramchiary and Kole, 2019). The most important genera are *Potyvirus*, *Cucumovirus*, *Tospovirus*, *Tobamivus* and *Begomovirus*, which induce similar symptoms. Thus extra research is necessary to determine them (Ramchiary and Kole, 2019).

Insects, or arthropods in general, are also major constraints of pepper production. There is a wide variety of species that can attack pepper, including thrips (*Frankliniella occidentalis*), aphids (*Myzus persicae, Aphis gossypii*), whiteflies (*Bemisia tabaci, Trialurodes vaporariorum*), red spider mite (*Tetranychus urticae*), broad mite (*Polyphagotarsonemus latus*), beet armyworm (*Spodoptera exigua*) or fruit borer (*Heliothis armigera*) (**Figure 1.4.**) (Condés Rodríguez, 2017). The mechanisms of growth and survival in plants depend on specific species, but they generally cause growth and pepper production drop, and are vectors of different virus species (Amari *et al.*, 2008; Kenyon *et al.*, 2014).

Figure 1.4. Upper (A), underside (B) and detail of a nymph (C) of leaves infected by *T. vaporariorum*.

In general, increasing bacterial diversity in soil, soil solarisation, using resistant rootstocks, phytosanitary products or employing natural enemies are some of the strategies that are now available to reduce the incidence of all these pests and illnesses (Funderburk *et al.*, 2000; Morra and Bilotto, 2006; Lee *et al.*, 2007; Rekanovic *et al.*, 2007; Ros *et al.*, 2008; Wang *et al.*, 2019).

1.3.2. Abiotic Stress

Plants as sessile organisms live constantly in a changing environment that are often unfavourable and stressful for plants, and reduce growth and development. Of all stresses, abiotic stress is the major cause of declining yields (50% in major crops) (Tuteja, 2007). There are many different types of abiotic stresses responsible for crop decrease, such as drought, high salt concentrations or heavy metals, extreme temperatures or little availability of some nutrients. Under field conditions, they do not act independently, but combined, which increases the level of difficulty when they are being studied (Mittler, 2006).

Pepper normally requires tropical and semiarid climates for optimum production, which aggravates unfavourable water scarcity and quality conditions (Bojórquez-Quintal *et al.*, 2012). Besides, a wide variability of daily temperatures reduces marketable production, and the optimum temperature is 25°C (Pyshnaya *et al.*, 2016; Condés Rodríguez, 2017). Therefore, **water**, **salt** and **extreme temperature** stresses are the main problems for pepper production.

a. Salt Stress

It is estimated that around 20% of all irrigated land is affected by salt (45 million ha), which reduces the world crop production. In the Mediterranean Basin, it is considered the main cause of desertification, where only in Spain 3% of the total irrigated land is considered to be severely affected by salt stress (Machado and Serralheiro, 2017).

This stress is a multifaceted phenomenon, in which natural causes are normally responsible for primary salinity and human intervention of secondary salinity from overusing non-renewable natural sources and incorrect drainage systems (Vargas *et al.*, 2018). Accumulation of salt in soil is a problem not only for the environment and plants, but is also a socio-economic problem, with major crop losses and reduced quality water for human intake (Vargas *et al.*, 2018; Zaman *et al.*, 2018).

In this scenario, plants have developed a series of mechanisms to tolerate this condition, normally classified as **halophytes** or **glycophytes** depending on the ability to tolerate permanent high salt stress conditions or not (300-500 mM of NaCl), respectively. Most plants grown in agriculture are considered glycophytes, but not all species have the same degree of tolerance. By way of example, the majority of vegetable species are considered moderately sensitive (i.e. tomato, eggplant or potato), but can also be sensitive (i.e. carrot) and moderately tolerant (i.e. zucchini) (Ayers and Westcot, 1985). Many authors have classified pepper as moderately sensitive, sensitive

or highly susceptible (Ayers and Westcot, 1985; Bojórquez-Quintal *et al.*, 2014), which notably lowers yields and growth when plants are exposed to irrigation water with electrical conductivity above 4.4 dS/m (De Pascale *et al.*, 2003). The effects on pepper plants are diverse, including stunted growth and production, and the appearance of BER (Blossom End Root) on fruits during the period with the highest fruit growth rate (**Figure 1.5.**) (Rubio *et al.*, 2009; Pyshnaya *et al.*, 2016).

Figure 1.5. Marketable (A) and non-marketable pepper fruit affected by BER under salt stress conditions (B).

b. Water Stress

Population growth has increased water use, which combined with the global climate change situation, has diminished both water quality and availability (Odlare, 2014). Irrigated agriculture is a major water consumer and represents about 60% of total human water use. So in general terms, crops must face either less irrigation water (**water deficit**) or even total lack of water (**drought**) under water stress conditions (Pardo, 2010). By ensuring water availability and maximising its sustainable use, and by improving plant tolerance, production can be enhanced (Rouphael *et al.*, 2008a; Patanè *et al.*, 2011).

In areas like the Mediterranean Basin, where most horticultural species are grown, little irrigation decreases crop production (Patanè *et al.*, 2011). Indeed water stress decreases 39% of the marketable tomato plant yields (Patanè and Cosentino, 2010), 43% for mini-watermelon (Rouphael *et al.*, 2008a) or 29% for eggplant (Kirnak *et al.*, 2002). Bell pepper is considered one of the most susceptible horticultural plants to water stress given its large transpiring leaf area and high stomatal conductance (Delfine *et al.*, 2001; De Pascale *et al.*, 2003). Nonetheless, flowering and fruit development are the most critical development stages (Anjum *et al.*, 2012). Decreasing water availability in pepper by reducing or stopping irrigation has negative consequences for leaf area and biomass and also for marketable fruit production, as this stress also makes the presence of BER more noticeable in apical pepper fruit parts (Delfine *et al.*, 2001; De Pascale *et al.*, 2003; Condés Rodríguez, 2017; Sezen *et al.*, 2019).

c. Other Abiotic Stresses of Economic Importance in Pepper Cultivation

Although salt and water stress have long been studied, it is possible to find other relevant stresses that currently contribute to major crop losses worldwide which often act in synergy to acerbate negative effects (Prasad *et al.*, 2008; Pardo, 2010; Zandalinas *et al.*, 2018). In pepper cultivation, typical summer **high temperatures** frequently appear after anthesis and negatively influence the pollination of flowers and, consequently, limit reproductive development and yield (Pagamas and Nawata, 2008). Temperatures over 36/27°C (day/night) reduce seed production, induce abnormal seeds and reduce pepper fruit weight (Pagamas and Nawata, 2007). Furthermore, the physiological disease called BER, which is mentioned above, can be aggravated in water and salt stress, and normally needs to be combined with high temperatures and low relative humidity to appear in fruit (Condés Rodríguez, 2017).

Accumulation of **heavy metals** in soil is a relevant problem worldwide that negatively affects plants and human health. In pepper plants, the accumulation of different heavy metals, such as cadmium (Cd), copper (Cu), nickel (Ni) or lead (Pb), in tissues has been studied, and reduces fruit growth and production (Barut, 2019; Desoky *et al.*, 2019). Hence it is important to find solutions that diminish accumulation in both plants and soil, and consequently in the food chain.

Inefficient nutrition in pepper plants also disturbs normal growth, particularly when essential nutrients such as nitrogen (N), potassium (K) or phosphorus (P) are not added at sufficient concentrations (Medina-Lara *et al.*, 2008; Urrea-López *et al.*, 2014).

1.4. Tolerance to Salt Stress in Plants: Physiology and Genetics

Plants affected by soil or water salinization pass through two main types of phases that modulate stress responses. Immediately after roots have been exposed to a threshold sodium chloride (NaCl) concentration (normally 40 mM of NaCl) the **osmotic phase** starts, and is characterised by the osmotic effect of ions outside roots, that block water from entering root cells and cause plant dehydration (Munns and Tester, 2008). As a result, cell expansion and elongation diminish, as do plant growth and leaf expansion (Isayenkov and Maathuis, 2019). However, negative effects due to toxic accumulation of ions are not yet present. This first phase may last from a few hours to several days depending on the degree of plant tolerance and salt concentration (Munns, 2002). Furthermore, the physiological processes altered in this phase are not specific to salinity, but are linked with water stress as no toxic effect due to a high salt concentration has yet been found (Munns, 2002; Carillo *et al.*, 2011).

When concentrations reach toxic levels, the **ionic phase** starts, in which ions are toxic for plants and normally accumulate in old leaves (Munns and Tester, 2008). Consequently, photosynthesis is generally affected, along with the level of carbohydrates, enzymatic activity and growth (Chaves *et al.*, 2009). In this phase, the mechanisms that enhance extrusion and block influx of toxic ions (essentially Na^+ and Cl^-), compartmentalization and synthesis of organic compounds are essential for plants to tolerate salt stress (Munns and Tester, 2008).

Increased salt concentration not only affects all the aforementioned physiological processes, but also alters hormone signalling and synthesis, and produces reactive oxygen species (ROS) by oxidative stress.

1.4.1. Plant Growth and Development

One of the first effects in plants affected by salt stress is stunted plant growth. Osmotic and ionic stresses reduce crop biomass and production by lowering water uptake and through the injury of cells located in transpiring leaves. Nonetheless, the lowering growth rate is unequal in both phases. Osmotic stress generally has an instantaneous and more prominent effect on growth than ionic stress (Munns and Tester, 2008). Consequently, maintaining the plant growth rate, the root to shoot ratio, the leaf area or root morphology are signals of plant tolerance that have been widely confirmed by several authors in different species (Acosta-Motos *et al.*, 2017).

At the cellular level, osmotic stress disturbs many processes related to cell division, such as the regulation and progression of the cell cycle, the number of dividing cells, and even cell death in root tips and leaves (West *et al.*, 2004; Ogawa *et al.*, 2006). Cells exposed to salt stress pass firstly through a quiescent phase (QP) in which growth stops, followed by growth recovery at lower rates than under control conditions; the

duration of this QP and later recovery depend mainly on sensitivity to salt stress, salt concentration and plant organs (Julkowska and Testerink, 2015). Several types of cyclins (CYCB1;2, CycB1;1, CycA2;1, CDC2a), Forkhead-associated domains (FHA) or antioxidant enzymes are some regulators described in the bibliography that are transient or permanently affected (Burssens *et al.*, 2000; West *et al.*, 2004; Zhou *et al.*, 2007; Banu *et al.*, 2009).

Maintenance of cellular size is also compromised when salt blocks water from entering cells. A well studied cellular component that directly influences cell division, elongation and morphology under salt and osmotic stress is cell wall (Koiwa, 2009). Overexpression of aquaporins, expansins and microtubules contributes to cell wall expansion by turgor maintenance, cell wall relaxation and the guide patterning of cellulose microfibrils, respectively (Cabañero and Carvajal, 2007; Koiwa, 2009; Geilfus *et al.*, 2010; Julkowska and Testerink, 2015). Likewise, the composition of the carbohydrate and protein components of cell walls improves in salt-tolerant plants by the expression of a wide variety of genes; e.g. cellulose (CSLD5, CesA) (Zhu *et al.*, 2010; Li *et al.*, 2017), pectin (PME, PMEI) (Chen *et al.*, 2018) or lignin (COMT, CCOMT, PAL) (Zagorchev *et al.*, 2014; Le Gall *et al.*, 2015).

1.4.2. Ion Homeostasis

It is thought that the ions responsible for salt stress, normally Na^+ and Cl^-, enter roots passively by the symplast or apoplast via the epidermis and root cortex due to the ion gradient and osmotic pressure. The main mechanism by which they enter remains unclear today. After going into roots, they rapidly move to the xylem and reach photosynthetic tissues to cause extensive critical damage. The ability to efficiently stop such ions from entering inside cells, favour expulsion from the cytoplasm and compartmentalise into non-toxic organelles may improve tolerance to salt shock. Depending on the transported ions, different candidate genes have been discovered and analysed.

After plants have come into contact with NaCl, how Na^+ is sensed remains unclear, but it is known that it passively enters by the negative potential membrane of cells. As a result, symplastic uptake is theoretically assumed by non selective cation channels (NSCCs). High affinity K^+ transporters (HKTs), Arabidopsis K^+ transporter (AKT1), high-affinity K^+ uptake transporter (HAK) or even aquaporins have been considered possible candidates for Na^+ uptake in roots (**Fig. 1.6.**) (Assaha *et al.*, 2017).

Favouring **Na^+ extrusion** is a possible plant mechanism to reduce the Na^+ concentration in the cytoplasm, where it is toxic. Along this line, the SOS pathway (SALT OVERLY SENSITIVE) has been broadly studied. Component SOS1 of this pathway is a transmembrane Na^+/H^+ antiporter that was firstly proposed to require the interaction with complex SOS2/SOS3 to be active by phosphorylation, which improves the K^+/Na^+ ratio in the cytoplasm (**Fig. 1.6.**) (Shi *et al.*, 2000; Yue *et al.*, 2012). SOS1 antiporter activity regulation is, nonetheless, not as simple as was primarily proposed as other signals, such as phosphatidic acid or Mitogen-Activated Protein Kinase 6 (MPK6), have been

considered as regulators (Ji *et al.*, 2013). This pathway cannot be considered broadly expressed, rather in specific tissues and in the whole plant context as Na^+ expulsion could be a disadvantage for neighbour cells (Zhu, 2003). In this vein, it is expressed together with other key transporters like HKT1 in xylem loading in roots, Na^+ extrusion to the external medium, accumulation in xylem parenchyma cells and recirculation from photosynthetic tissues to phloem vessels (Pardo, 2010; Assaha *et al.*, 2017).

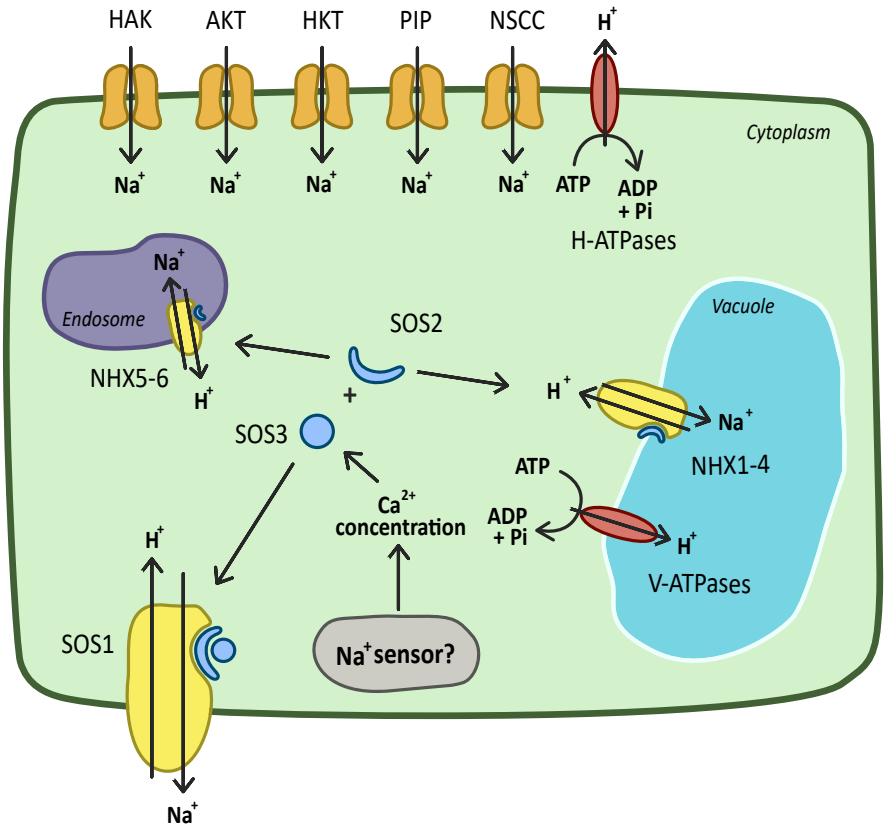

Figure 1.6. Transport of Na^+ in a plant cell under salt stress conditions. Yellow represents antiporters Na^+/H^+; orange denotes the passive transporters of Na^+; red depicts H^+ pumps.

It is also worth mentioning that **compartmentation in non-toxic organelles** is also key for tolerance. The Na^+/H^+ transporters in vacuoles (NHX1-4) and endosomes (NHX5-6) can regulate the concentration of cytoplasmic K^+ and Na^+ under control and saline conditions, respectively, and can control pH homeostasis and vesicular trafficking (**Figure 1.6.**) (Bassil *et al.*, 2012; Gálvez *et al.*, 2012). Many authors have demonstrated that overexpression of NHX genes improves salt tolerance in species like tomato, pepper or *Nicotiana tabacum* (Zhang *et al.*, 2008; Gálvez *et al.*, 2012; Bulle *et al.*, 2016). Regulation of vacuolar NHXs antiporters is mediated by SOS2 (Tuteja, 2007; Ji *et al.*, 2013).

In a hydrated form, Na^+ and and **potassium** (K^+) structurally and chemically share lots of similarities, which may lead to a cotransport of both cations by K^+ transporters when salt is present at high concentrations (Golldack *et al.*, 2003; Takahashi *et al.*, 2007). It is generally assumed that only K^+ is an essential nutrient that takes part in many physiological and biochemical processes in plants, which means that Na^+ entrance negatively affects plant development (Isayenkov and Maathuis, 2019). Maintaining the K^+/Na^+ ratio in roots and leaves is, thus, especially relevant to achieve tolerance and improve growth (Ghars *et al.*, 2008; Tiwari *et al.*, 2010).

Although Na^+ is the most studied ion, many species like *Vitis vinifera* or *Citrus* sp. show a better Na^+ exclusion strategy than **chloride** (Cl^-), with more detrimental effects on plants (Walker *et al.*, 2004; Hossain *et al.*, 2016). Nevertheless, in annual and vegetable species like pepper, Cl^- has a minor toxic effect due to its lesser contribution to reduce osmotic potential and photosynthesis (Penella *et al.*, 2015). Even if Cl^- is an essential micronutrient involved in many activities under control conditions, it may replace major macronutrient anions (NO_3^-, SO_4^-, Pi) and organic anions at high concentrations when it is transported, which thus disturbs anion homeostasis (Teakle and Tyerman, 2010). In this situation, mechanisms are necessary that regulate the entrance, extrusion and compartmentation of Cl^- with the coordinated activity of slow anion channels (SLAC), Cl^- channels (CLC), aluminium-activated malate transporters (ALMT) or nitrate transporters (NRT), among others (Wu and Li, 2019).

Finally, it is noteworthy that correct **calcium** (Ca^{2+}) concentration regulation in cells has been largely demonstrated to improve multiple stresses, including salt stress, by the regulation of multiple transporters in plants, such as SOS3 (or CBL4), PIP2 or NSCC activity (Ji *et al.*, 2013; Byrt *et al.*, 2017). Therefore, if Ca^{2+} is imbalanced after NaCl addition, plants may present increased sensitivity to salt stress as this cation is a very important secondary messenger implied in many activities (*see Section 1.5.2. for more information*).

1.4.3. Water Relations

After salt addition, soil water potential lowers in the osmotic stress phase and leads to the declined water uptake. Plants must consequently also decrease **water potential** (ψ_w) to improve water uptake through roots to avoid death. Apart from ψ_w,

hydraulic conductivity (Kh) affects the water movement rate and regulates water relations under salt stress because a decrease in roots can lower not only the delivery of salty water to shoots, but also the water potential (Negrão *et al.*, 2017). These two components are not the only parameters affected by salt stress, but also the **osmotic** (ψ_s) **and pressure** (ψ_P) **potential** or the relative water content (RWC) of different organs (Navarro *et al.*, 2003; Hegazi *et al.*, 2017). The incorrect adjustment of water levels in plants, thus, lowers the volume of water in the vacuole and the cytoplasm of cells, and stops cell expansion, elongation and division, stomatal opening or abscisic acid accumulation (Negrão *et al.*, 2017).

If plants need to maintain growth and development, they employ different mechanisms to lower the water potential and to consequently improve water uptake. These mechanisms are not normally specific for salt stress, but to all abiotic stresses, which implies a disequilibrium in water balance, like water stress or extreme temperatures (Rhodes, 2004). One of the most widely studied is the synthesis of small organic compounds, generally called **osmolytes, osmoprotectants or compatible solutes** that normally accumulate in the cytosol or cytoplasmic organelles (Rhodes, 2004). They include proline, glycine betaine and sugars (e.g. trehalose), which are the most widely studied ones in salt stress. They all have the ability to lower ψ_s and allow water uptake (Hasanuzzaman *et al.*, 2013). Studying the expression level of the main synthesis and degradation genes for these molecules has drawn the attention of different research groups. With proline, Δ-1-pyrroline-5-carboxylate synthase (P5CS), and Δ1-Pyrroline-5-carboxylate reductase (P5CR) are the most important biosynthetic genes, along with proline dehydrogenase (PDH) and Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH) degradative genes. Hence they are pivotal to maintain correct proline accumulation and to ensure salt stress tolerance (Huang *et al.*, 2013b; Singh *et al.*, 2014). The role of osmolytes includes not only improved water potential and water intake, but also decreased ROS accumulation or improved lipid peroxidation and membrane integrity (Banu *et al.*, 2009; Tiwari *et al.*, 2010).

Even if osmolyte accumulation is an advantage for plant tolerance to abiotic stresses, the energy costs that synthesis and accumulation imply may be detrimental for plant growth. Indeed they may not improve growth, but avoid wilting and ion toxicity, which would allow survival under salt stress for longer periods (Munns, 2005). This is why plants sometimes choose to invest in the accumulation of **non-compatible solutes** like ions in non-toxic organs rather than the synthesis of compatible solutes (Bojórquez-Quintal *et al.*, 2014; Theerawitaya *et al.*, 2020) to help turgor pressure of cells (Navarro *et al.*, 2003).

1.4.4. Reactive Oxygen Species (ROS) Scavenging

In addition to primary effects (osmotic and ionic stress), salt stress may cause secondary stresses like **oxidative stress** (Pang and Wang, 2008), which lead to ROS accumulation. This involves deleterious effects to proteins, lipids, carbohydrates and nucleic acids, as well as increased programmed cell death (Lin *et al.*, 2006). When salt is

present at high concentration, ROS generation grows and may even cause death. It has been largely demonstrated that, contrarily, a low ROS concentration, especially H_2O_2, can work as signalling molecules and improve tolerance to several abiotic stresses, including a high salt concentration (Dat *et al.*, 2000; Zhu *et al.*, 2016).

Studying ROS scavenging mechanisms has been considered by a broad range of studies to elucidate detailed information about how it takes place in plant cells and which molecules are responsible. Indeed catalase (CAT; EC 1.11.1.6), superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11) and peroxidase (POX; 1.11.1.7) have been the most frequently studied **antioxidant enzymes** under salt stress. Among all enzymatic scavengers, SOD is the most effective one, which constitutes the first line of defence against environmental stresses. It generates H_2O_2 from O_2^-. Then other enzymes like APX, CAT or POX detoxify H_2O_2 into other non-toxic molecules (Yang and Guo, 2018). For instance, pepper plants increase the expression of peroxidase CaPO2 and SOD activity under salt, improving its tolerance (Aktas *et al.*, 2012; Choi and Hwang, 2012) and tobacco plants improve the activity of APX and CAT enzymes (Badawi *et al.*, 2004; Al-Taweel *et al.*, 2007). In many cases, antioxidant enzymes simultaneously modulate other activities, as is the case of SOD and APX, which are responsible for cell wall formation during salt stress in *Arabidopsis thaliana* (Shafi *et al.*, 2015).

The production of proline and glycinebetaine, which decrease lipid peroxidation (Banu *et al.*, 2009); ascorbic acid (AsA), the main antioxidant in plants (Shalata and Neumann, 2001; Hernández *et al.*, 2017); or phenolic compounds, which function as hydrogen donators (Salah *et al.*, 2011; Pérez-Labrada *et al.*, 2019), are some of the main **non-enzymatic molecules** found in tolerant stressed plants. In pepper, AsA and proline have been associated with better tolerance to salt stress (Penella *et al.*, 2016).

1.4.5. Phytohormones

Phytohormone levels are another point of stress regulation that plays specific roles depending on the type and duration of stress, as well as the development stage and tissues. In any case, hormones must always be considered when we attempt to elucidate plants' mechanisms of tolerance.

In salt stress, **abscisic acid (ABA)** has long since been considered an essential hormone to regulate adaptive responses to salt stress. Its accumulation is triggered by the differential expression of key genes of biosynthetic and degradative pathways, like 9-cis-epoxycarotenoid dioxygenase 3 (NCED3), Arabidopsis aldehyde oxidase 3 (AAO3) and ABA-deficient 1 (ABA1) (Barrero *et al.*, 2006; Geilfus *et al.*, 2018). However, its activation and signalling under salt stress by genes like Sucrose non fermenting Kinase-1-Related protein kinase 2s (e.g. SnRK2.6), 2C protein phosphatases (PP2Cs) (e.g. ABI1, ABI2), SRM1, CAMTAs or DREBLP1 (Ohta *et al.*, 2003; Boudsocq *et al.*, 2004; Hong and Woo, 2005; Wang *et al.*, 2015; Krzywińska *et al.*, 2016; Büyük *et al.*, 2019) are even more important than ABA concentration for proper functioning. In line with

this, ABA controls stomatal closure and reduces transpiration, which are key to avoid dehydration in plant cells (Niu *et al.*, 2019) (*see Section 1.5.5. for more information*), but also modulates wax deposition, proline biosynthesis or ion homeostasis (Mills *et al.*, 2001; Ohta *et al.*, 2003; Sripinyowanich *et al.*, 2013; Farhoudi and Saeedipour, 2018). Even if they all help to salt tolerance, they may compromises plant growth and limit crop production (Farhoudi and Saeedipour, 2018).

Other hormones have also been associated with playing relevant roles by mediating salt stress responses. **Jasmonates (JA)** act as inductors of salt stress tolerance when conjugated as methyl jasmonate (MeJa) or isoleucine jasmonate (IleJa). Studying genes like JA carboxyl methyltransferase (JMT) or jasmonate amino acid synthetase 1 (JAR1) may be of interest (Ali and Baek, 2020). By external applications, these active forms improve growth and chlorophyll content in pepper (Sedigheh *et al.*, 2013) or growth and ROS scavenging in wheat (Qiu *et al.*, 2014). **Auxins**, on the other hand, normally decreases after salt addition because plants cannot face it (Sakhabutdinova *et al.*, 2003; Zörb *et al.*, 2013), which means that increasing the IAA concentration or auxin-related genes improves salt tolerance (Wang *et al.*, 2009; Guo *et al.*, 2018). Other hormones, like **gibberelins** or **brassinosteroids,** also decrease under salt stress conditions. Their expression generally involves growth and improves development, but in some cases signalling mechanisms have not yet been completely elucidated (Ryu and Cho, 2015).

1.4.6. Photosynthesis

One of the primary processes affected by salt or water stress is photosynthesis. When salt accumulates in roots after salinity addition, it induces osmotic stress, decreases water uptake and causes dehydration, as explained above. Consequently, plants limit water relations and carbon assimilation by stomata closure (defined as **stomatal limitations**). Stomata actively control CO_2, O_2 and H_2O exchanges with the medium, which means that stomata regulation improve photosynthesis and carbon fixation, which results in better growth and yield (Liu *et al.*, 2014). In both salt and water stresses, stomatal movement is closely connected to ABA accumulation, a critical point of stomatal closure regulation in several plants, such as carnation, tomato or citrus (Gómez-Cadenas *et al.*, 2002; Kwon *et al.*, 2019; Poór *et al.*, 2019). Albeit not very well documented, the genetic control of stomata plays a central role. Genes like guard cell slow anion channel-associated (SLACs) and SLAC homologues (SLAHs) have been proposed in tomato plants to be candidate genes (Liu *et al.*, 2014).

Apart from stomatal limitations, it is possible to also find in plants **non-stomatal limitations**, characterised by the toxic accumulation of Na^+ and Cl^- ions in photosynthetic tissues which, in turn, inhibit carbon assimilation (Stępień and Kłbus, 2006; Pan *et al.*, 2020). Such limitations in salt stress include the reduced activity of Calvin cycle enzymes, the disruption of chlorophyll biosynthesis, increased ROS creation in photosystems by electron transport chain constraints, and less efficient functioning and integrity of photosynthetic apparatus that leads to the inactivation of photosystems

and CO_2 fixation (Parida *et al.*, 2003; Stępień and Kłbus, 2006; Kalaji *et al.*, 2011; Hand *et al.*, 2017; Sasi *et al.*, 2018; Poór *et al.*, 2019; Pan *et al.*, 2020).

A large number of mechanisms are available in plants to reduce non-stomatal limitations and improve salt stress tolerance. The correct transport of imbalanced ions by different transporters like sodium hydrogen antiporter (NhaD)-type carriers, the Na^+/Pi symporter anion transporter (PHT), bile acid: sodium symporter family protein (BASS), the K^+/H^+ antiporter KEA family or McsS-Like (MSL2 and MSL3) are possible candidates for maintaining Na^+, K^+ and Cl^- homeostasis in the chloroplast (Aranda-Sicilia *et al.*, 2012; Wilson *et al.*, 2014; Zhao *et al.*, 2016; Pan *et al.*, 2020). Other mechanisms that contribute to improve non-stomatal limitations are the regulation of photosynthetic electron transport efficiency, improved PSI cyclic electron transport (Wu *et al.*, 2019) or the protection of thylakoid membranes, Rubisco and PSII extrinsic proteins by the action of compatible osmolytes like glycinebetaine (Ahanger *et al.*, 2014).

1.5. Tolerance to Water Stress in Plants: Physiology and Genetics

Water stress responses and mechanisms of tolerance are very similar to those described in salt stress conditions because in both cases it is possible to find osmotic stress that restricts water uptake. However with high salinity, water is found in soil, but plants cannot uptake it due to ion accumulation in root cells. This situation is often termed "**physiological drought**" (Lisar *et al.*, 2012). With water scarcity, the origin of the stress is not the marked accumulation of toxic ions, but a difficulty to obtain water because this molecule is scarce or nonexistent.

Plants optimise their physiology, genetics and metabolism to better optimise water uptake and use, and are generally classified into two different groups: stress avoidance and tolerance. In **water stress avoidance**, plants have developed a series of modifications, such as deeper root systems, modifications to their life cycle, reduced stomatal conductance, water accumulating in specialised cells or diminished leaf area. In **water stress tolerance,** plants have no special modifications to cope with this stress, but can change water relations, as well as physiological and biochemical processes like photosynthesis, membrane structure and stomatal aperture (Bray, 2001; Chaves *et al.*, 2003; Lisar *et al.*, 2012). The latter is the case of most agronomical species, including *Capsicum* sp.

1.5.1. Plant Growth and Development

It is a fact that after water deficit begins, plant growth and production decrease. Indeed, a reduction in the amount of water uptake by roots is one of the primary effects, followed by root damage (Zeiger and Taiz, 2010). Hence root growth immediately slows down and disturbs the root/shoot ratio (Manoj and Uday, 2007). Nonetheless, aerial tissues are even more affected as leaf area, plant height and aerial biomass (Gómez-del-Campo *et al.*, 2002; Ge *et al.*, 2012).

Cell walls are in charge of regulating the hydration level and cellular shape. Controlling the biosynthesis, transport and incorporation of cell wall polysaccharides, such as cellulose, pectins or hemicelluloses in roots by specific genes, has been demonstrated in wheat seedlings, whose degree of drought tolerance varies (Piro *et al.*, 2003). Balsamo *et al.* (2015) demonstrated by several *Arabidopsis thaliana* mutants that the composition of hemicellulose and pectins in leaves plays an important role in leaf biomechanical properties against cellular deformations in water stress treatment.

Cell wall expansion is even more relevant than its composition. Water stress tolerance can be achieved by modifying cell wall elasticity by either making them more elastic by turgor maintenance or decreasing elasticity to lower ψ_w with minimal leaf water loss (García *et al.*, 2007; Martínez *et al.*, 2007; Miranda-Apodaca *et al.*, 2018; Al-Yasi *et al.*, 2020). Controlling the expression of key genes (e.g. expansins) has been

reported to play key roles in cell wall expansion, cell shape and growth (Lü *et al.*, 2013; Liu *et al.*, 2019).

The cell cycle is also modified with osmotic stress in plants. After water deficit, tomato embryos decreased the accumulation of β-tubulin, DNA synthesis of phase S and the number of cells in the division phase, which were reactivated after rehydration (De Castro *et al.*, 2000). Via changes in microtubule cytoskeleton dynamics, *Brassica napus* plants can improve tolerance to water stress (Bagniewska-Zadworna, 2008).

1.5.2. Ion Homeostasis

Even if water stress does not involve the accumulation of toxic ions as in salt stress, it also disturbs plant mineral nutrition and unbalances ion homeostasis. Cell water deficit promotes ion accumulation and destabilises macromolecules, despite compatible osmolytes being able to prevent their interaction to protect cell components (Lisar *et al.*, 2012).

Independently of passive ion accumulation due to less water content, the active disequilibrium of some ions under suboptimal water conditions has been widely studied in osmotic stress. One of the most relevant ions is **calcium** (Ca^{2+}), a secondary messenger that implies many processes under osmotic stress like growth and development, ABA synthesis and signalling, ROS detoxification, stomatal movement or fruit quality (Suzuki *et al.*, 2003; Jiang *et al.*, 2013; Wang *et al.*, 2013; Naeem *et al.*, 2018). This ion also plays important roles in a variety of signal transduction pathways, including calcium-sensing proteins, calcium-dependent protein kinases (CDPK), cyclin-dependent-kinase (CDK), calcineurin B-like (CBL), calmodulin (CaM) or calmodulin-binding transcription factor (CAMTA), among others (Chung *et al.*, 2004; Li *et al.*, 2014b; Cui *et al.*, 2018). Expression of CaCDPK3 in pepper plants has, for example, been demonstrated to improve tolerance to drought (Chung *et al.*, 2004).

Water availability restrictions decrease nutrients, mainly **nitrogen (N)** which, in turn, affects growth (Lisar *et al.*, 2012). Disruption N-metabolism, by reducing key enzymes like nitrate reductase (NR, EC 1.6.6.1), glutamine synthetase (GS, EC 6.3.1.2) of glutamate dehydrogenase (GDH, EC 1.4.1.2), can disrupt photosynthesis and carbohydrate metabolism (Garg *et al.*, 1998; Xu and Zhou, 2006).

Water stress conditions have also been reported to unbalance **potassium** (K^+) levels, which leads to minor growth and osmotic adjustment in sensitive plants (Damon *et al.*, 2011). K^+ participates in broad functions in plants (reviewed in Ahanger *et al.*, 2014), but its role in the aperture of stomata by the regulation of K^+ channels (e.g. KAT1, KAT2 or GORK) is particularly important (Li and Assmann, 2009). According to Zahoor *et al.* (2017), increasing the K^+ concentration by external applications in photosynthetic tissues improves both photosynthesis, by higher stomatal conductance, and carbon assimilation which, in turn, spells improved tolerance to water stress.

1.5.3. Water Relations

The most remarkable event under water stress conditions is blocked water intake in plants because it causes dehydration and reduced growth. Most vegetative plants are unable to tolerate water contents below 60-30%, but some particular exceptions are found (Chen *et al.*, 2020). The ability to lower ψ_W under water scarcity conditions has been generally accepted as a tolerance mechanism, for both this stress and osmotic stress in general (*see Section 1.4.3. for more information*). However in extreme cases, a reduction in stomatal conductance (g_s), hydraulic conductivity (Kh) or ψ_W can avoid the cavitation of large vessels, which thus means a reduced xylem system (Saliendra *et al.*, 1995). It has been demonstrated that maintaining k by reducing the area of xylem vessels can contribute to tolerance in pepper plants (Guijarro-Real *et al.*, 2014).

By osmotic adjustment, the ψ_s can lower and, thus, permit water uptake. In salt stress, osmotic adjustment can be accomplished by either ion accumulation in non-toxic organelles or the synthesis and accumulation of neutral compounds, generally called **compatible osmolytes** (*see Section 1.4.3. for more information*). In water stress, only compatible osmolytes contribute to osmotic regulation. Proline and glycine betaine are among the most studied compatible osmolytes in horticultural species like pepper plants, and a positive correlation appears between the concentration and tolerance to water stress (Santos-Díaz and Ochoa-Alejo, 1994; Anjum *et al.*, 2012). It must be pointed out that looking for expression of their biosynthetic and degradative enzymes is an important point of studying water stress tolerance (Porcel *et al.*, 2004; Lv *et al.*, 2007).

By the overexpression of **aquaporins** in the plasma membrane and intracellular membranes, it is possible to maintain water relations. They mediate the transport of water and other small molecules in cells, and maintain water relations during water scarcity periods (Rao and Chaitanya, 2016). According to Sahitya *et al.* (2019), for example by the overexpression of PIP1-1 and PIP1-2 in tolerant pepper cultivar KCa-4884, it is possible to improve water stress tolerance, associated with improved hydraulic conductivity and photosynthesis.

1.5.4. Reactive Oxygen Species (ROS) Scavenging

The balance between ROS production and scavenging in plant cells is what distinguishes a tolerant and a sensitive plant, controlled by the ROS gene network (*see Section 1.4.4. for more information*). Prolonged and severe water deficit periods end in oxidative damage through the overproduction of ROS molecules, as with many other abiotic and biotic stresses. This, in turn, leads to a reduced photosynthesis machinery via altered stomatal regulation (Miller *et al.*, 2010).

The ability to scavenge them by enzymatic and non-enzymatic molecules is a determinant in suboptimal water conditions, as it is under salt stress conditions. Regarding **enzymatic antioxidants**, as already explained, most plants use SOD to scavenge O_2^-, one of the most detrimental ROS species, although other enzymes are also critical. According to Hu *et al.* (2010), SOD and APX activities increase with drought and heat

stress in a tolerant pepper cultivar. Choi and Hwang (2012) demonstrated the role of peroxidase CaPO2 in ROS scavenging with drought and salt stress tolerance in the Nockwang cultivar.

In **non-enzymatic compounds**, similar results have been found with both water and salt stress, which lead to modification of proline, glycinebetaine, sugars, AsA and phenolic compounds, which are the most studied ones. Improved tolerance occurs when they accumulate in tissues, as many studies have corroborated in pepper and other species (del Amor *et al.*, 2010; Shafiq *et al.*, 2014).

1.5.5. Phytohormones

On the signalling pathway, to sense water deficit many hormones are implicated either direct or indirectly. However, **ABA** has been widely proposed as the main hormone implicated in water scarcity and drought tolerance. Its specific roles can be elucidated by studying the regulation of either its biosynthetic and degradative key enzymes (NCED) or its signalling cascade (PYR, PYL, PP2C, SnRK2) (Pardo, 2010; Bhaskara *et al.*, 2012), which has been as well described in salt stress (*see Section 1.4.5. for more information*). If ABA performs crucial functions in salt stress tolerance, its role under suboptimal water conditions is even more important as plants must cope with a sudden lowering of water content with precise stomata control to avoid excess water by transpiration. Short-term stomata closure includes the action of the ABA signalling pathway through the action of SnRK2.6 (open stomata 1; OST1) which, in turn, controls the activity of ion channels like slow anion channel-associated 1 (SLAC1) and inward rectifying K^+ channel (KAT1 and KAT2) (Li and Assmann, 2009; Sreenivasulu *et al.*, 2012). If osmotic stress is maintained in the long term, high ABA accumulation levels can be detrimental for growth and development by impairing photosynthesis. Moreover, ROS accumulation and compatible osmolytes (Dubois and Inzé, 2020), as well as lignin and wax biosynthesis (Macková *et al.*, 2013; Li *et al.*, 2020), are long-term responses that depend on the ABA concentration and signalling.

Increased **ethylene** biosynthesis has also been found under different biotic and abiotic stresses, including water deficit. It is believed that important roles lead to stress acclimation. Different studies have concluded that ethylene limits stomata closure by inhibiting the ABA signalling pathway (Tanaka *et al.*, 2005; Chen *et al.*, 2013). Besides this function, ethylene response factors (ERFs) like TERF1 also play a role in plant defence against water stress by the enhanced expression of antioxidant enzymes like catalase or glutathione peroxidase in tobacco plants (Zhang *et al.*, 2016).

Jasmonates, along with **salicylic acid,** have been widely proposed as candidates to modulate root to shoot signalling after water stress. It has been demonstrated in tomato mutants that their synthesis is highly related to ABA biosynthesis (Muñoz-Espinoza *et al.*, 2015). They can mitigate negative effects by the modulation of photosynthesis and stomatal conductance, growth and development, ion transport or nitrate reductase activity (Li and Assmann, 2009; Ahanger *et al.*, 2014).

1.5.6. Photosynthesis

Photosynthetic machinery is very sensitive to a drop in the relative water content of plant cells, which negatively affects growth and crop production (Ge *et al.*, 2012; Sahitya *et al.*, 2019). As with salt stress (*see Section 1.4.6. for more information*), a lower photosynthesis rate is attributed to both stomatal and non-stomatal limitations (called metabolic impairment). Short-term photosynthetic limitations are related mostly to **stomatal conductance** (g_s) which, in turn, negatively arrests carbon assimilation and affects transpiration and gas exchange (Bray, 2001). By controlling ABA concentration and signalling, together with other hormones and ion transporters, the degree of stoma closure can be efficiently regulated, and water stress tolerance may improve. The role of the specific molecules that interconnect several pathways seems pivotal, as is OST1, which are very important in the connection between ABA signalling and the control of ion transporters, such as SLAC1 and QUAC1, in *Arabidopsis thaliana* guard cells (Mustilli *et al.*, 2002; Imes *et al.*, 2013).

Metabolic impairment is even more relevant than stomata limitations to improve photosynthesis in plants by the misregulation of the activity of key enzymes like sucrose phosphate synthase (SPS), nitrate reductase or Rubisco (Rao and Chaitanya, 2016). Rubisco has long since been considered the most critical point of regulation in the physiology of water-stressed plants in many species, such as subterranean clover, alfalfa or *Pistacea lentiscus* (Medrano *et al.*, 1997; Aranjuelo *et al.*, 2010; Galmés *et al.*, 2011). Its activity is down-regulated by low CO_2 and Rubisco concentrations and the increased concentration of tight-binding inhibitors, among others (Galmés *et al.*, 2011).

Limited photosynthesis by water stress leads to excessive light excitation and photooxidation, which reduce the maximum quantum yield of PSII (F_v/F_m) and enhance ROS accumulation. By lowering the electron transport chain rate, improving the PSI cyclic electron flow, avoiding chlorophyll degradation, scavenging ROS and synthesising xanthophylls or compatible osmolytes, plants can diminish negative effects and are more tolerant to water stress (Li *et al.*, 2006; Hu *et al.*, 2010; Upadhyaya *et al.*, 2012; Zivcak *et al.*, 2013, 2014). Improving the biosynthesis of some components of plants, such as wax and lignins, can act as important defence mechanisms against excessive non-stomatal transpiration, which may help water accumulation (Zhong *et al.*, 2020).

1.6. How to Improve Tolerance to Abiotic Stresses in Crop Plants: The Role of Grafting

One of the biggest challenges for agriculture and scientific research is developing new efficient strategies to improve the growth, development and commercial production of plants. Both biotic and abiotic stressful conditions have been exacerbated in recent decades due to a new climate change scenario, including several that were minor problems some time ago; for instance, high concentrations of heavy metals and salts in water and soil, variation in water regimes or extreme temperatures. Thanks to the effort of public and private research, now it is possible to find different solutions, although all cases involve limitations.

One possibility is the **application of different** natural or synthetic **molecules or organisms** that are beneficial in tolerant plants and are used to improve tolerance in sensitive ones. These applications are generally employed with leaves and internally, they move through the whole plant to reach the most vulnerable tissues. Among the most widely used molecules to ameliorate the negative effects of salt and water stress are **hormones** (e.g. ABA, IAA, salicylic acid) (Yildirim *et al.*, 2007; Kaya *et al.*, 2010; Li *et al.*, 2014a), **polyamines** (e.g. putrescine, spermidine) (Shu *et al.*, 2012; Khoshbakht *et al.*, 2018), **compatible osmolytes** (e.g. proline, glycinebetaine) (Mäkelä *et al.*, 1999; Kaya *et al.*, 2007), **nutrients** (e.g. Ca^{2+}, K^{+}) (Amjad *et al.*, 2014; Naeem *et al.*, 2018) and **antioxidant compounds** (e.g. AsA) (Dolatabadian *et al.*, 2008). With time the use of **biostimulants** is becoming important, defined by du Jardin (2015) as "any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content". Even if beneficial effects have been described, the wide variety of biostimulants and no accurate legal framework in force make their study, use and the determination of their specific functions difficult (Tanou *et al.*, 2017).

Enhancing plant tolerance by selecting specific loci of interest in plants by the **breeding** technique has been broadly used, which studies the intra- and interspecies variation of the desired characteristics, and are crucial for use in new tolerant cultivars. By **classical breeding**, important advances have been made in the last century (Ashraf, 2010) to either find tolerant cultivars to specific stresses or cross homozygous tolerant plants to obtain hybrids with enhanced characteristics. Nonetheless, due to the efforts and time invested in this technique, in recent decades important advances have been made in what is called **genetic breeding** (Hanin *et al.*, 2016). In this case, different genetic engineering techniques are used to obtain tolerant plants in order to introduce one or several genes associated with stress-responsive pathways (Lisar *et al.*, 2012). For example, pepper plants displayed increased tolerance to salt and water stress by the inoculation of genes TaNHX2 of wheat or a *chl*Cu/Zn SOD of tomato with *Agrobacterium tumefaciens* (Chatzidimitriadou *et al.*, 2009; Bulle *et al.*, 2016). Even if important advances have been made in this field, we are

still far from using them under real field conditions given the complexity of mechanisms of tolerance, the large quantity of genes involved and poor legal-social acceptation.

1.6.1. Grafting in Vegetables

An eco-friendly alternative technique that more attention has been paid with time is **grafting**. It was defined by Bie *et al.* (2017) as "the art of joining together two plant parts (a rootstock and a scion) by means of tissue regeneration, in which the resulting combination of plant parts achieves physical reunion and grows as a single plant". Even if it is a millenary technique in woody plants, interest in vegetables has increased since the beginning of the last century (Kubota, 2017). Nowadays, the use of grafting is generalised worldwide and is employed more and more on a commercial scale to improve production in many situations. Asia is the continent with the largest cultivated area of grafted vegetables as only South Korea and Japan produced 700 billion grafted seedlings in 2009. In European countries, Spain heads the list with the most grafted seedlings for crop production (Lee *et al.*, 2010).

Different grafting techniques are available on the market, and depend mainly on crops, although **tube grafting** is performed in most Cucurbitaceae and Solanaceae crops (**Figure 1.7.**). Although most commercial growers perform hand grafting, it is possible to find machines that automatically does this to improve efficiency and costs (Chang *et al.*, 2012). After grafting, the joining generally needs 7-14 days for the wound to heal and be acclimated to transplantation to the field.

Growers use rootstocks in plants for the main objectives of improving growth and commercial production when subjected to most biotic or abiotic constraints. The number of studies carried out in grafted plants to improve the tolerance, growth and production of plants is quite extensive, and many provide detailed information about the acquired physiological and molecular mechanisms of grafted plants compared to non-grafted or self-grafted plants (Rouphael *et al.*, 2008b; López-Marín *et al.*, 2013). Improved tolerance has been achieved in the stress that starts in roots, such as **salt stress** (Penella *et al.*, 2013, 2015, 2016, 2017a), **water deficit** (Penella *et al.*, 2014a, 2014b), **heavy metal toxicity** (Rouphael *et al.*, 2008b), **low phosphorus concentration** (Albacete *et al.*, 2015; Martínez-Andújar *et al.*, 2017) or **soil-borne diseases** (Burelle *et al.*, 2009). However, it is possible to improve stress that arrives directly to the scion, such as **extreme temperatures stress** (López-Marín *et al.*, 2013; Aidoo *et al.*, 2018) or pathogens like ***Bemisia tabacci*** (Žanić *et al.*, 2017, 2018).

In pepper, using tolerant rootstocks improves **commercial production,** as well as the number of fruits under salt and water stress (Penella *et al.*, 2013; López-Marín *et al.*, 2017). This practice can reduce unmarketable yield; indeed Penella *et al.* (2016) demonstrated, for example, that using rootstock A25 improved marketable yields up to the 40% compared to non-grafted plants, and reduced BER incidence in fruit from the 49% to 19%. Even if the physiological and molecular mechanisms of tolerance of grafted plants subjected to salt and water stresses have been well studied in plants like tomato or cucumber, information is still scarce in pepper.

Figure 1.7. Pepper grafted seedlings by the tube grafting technique before being transplanted in the field. The lower part is the rootstock and the upper part represents the variety. The grafting joining is usually held with a grafting clip.

Under salt stress, for example, the use of tolerant pepper rootstocks has demonstrated that, on the one hand, it limits the **transport of toxic ions** to the scion and, on the other hand, it favours retrieval transport from the phloem to the roots. In both scenarios, ionic stress is alleviated in shoots and protects photosynthetic tissues (Giuffrida *et al.*, 2013; Penella *et al.*, 2015, 2017a). Na^+ ions are expelled from the root cells of rootstocks or, on the contrary, accumulate in non-toxic organelles, by the expression of several genes (e.g. SOS1, HKT1;1 or NHX1), as Huang *et al.* (2013a) proposed for pumpkin-tolerant rootstocks. Cl^-, on the contrary, has been demonstrated to play a secondary role in contributing to ion toxicity in the high NaCl concentration scenario in grafted pepper plants and has, thus, been less studied (Penella *et al.*, 2015). Accumulation of both ions and compatible osmolytes in roots also favours the lowering of both ψ_w and ψ_s which, linked with vigorous root systems of tolerant rootstocks, improve water use efficiency (WUE) and osmotic adjustment under water or salt stresses conditions (Kumar *et al.*, 2017; Penella and Calatayud, 2018).

Long-distance transport of **ABA** from rootstocks to photosynthetic tissues has been proposed as a short-term mechanism of tolerance in relation to **photosynthesis** preservation, which decreases g_s and improves antitranspirant activity in several species when present at a high concentration (reviewed by Pérez-Alfocea *et al.*, 2011). In grafted cucumber plants, for example, enhanced ABA concentrations in roots and transport to leaves, as well as increased ABA-biosynthesis and signalling gene expressions in roots (NCED2, ABCG22, PP2C, SnRK2.1), seem to play important roles in preserving water status by stomata closure during the first 24 h of salt addition (Niu *et al.*, 2019) or 7 days after water deficit starts (Liu *et al.*, 2016b). Avoiding shoot toxicity and water flow maintenance through plants under salt and water stress, respectively, preserve long-term photosynthetic relations at high rates in tolerant pepper grafted plants (López-Marín *et al.*, 2017; Penella *et al.*, 2017a), which promotes the interchange of CO_2, O_2 and H_2O and, thus, avoids stomatal aperture limitations. Nevertheless, how short- or long-term ABA responses and gene expressions are linked with better tolerance are still to be elucidated in pepper species.

Grafting can also modify the signalling of other hormones in rootstock-scion interactions to improve tolerance to stress in many species. However, in peppers specific studies have not yet been conducted until the present. A well-studied vegetable is tomato, in which **cytokinins** (CK) have been demonstrated to play important roles in salt stress tolerance as less transport of CK from roots to shoots, and greater degradative enzyme cytokinin oxidase (CKX) activity, increase sensitivity in plants; on the contrary, tolerant grafted plants maintain CK at higher levels, which improves growth, and reduce leaf senescence (Albacete *et al.*, 2008, 2009). Hormones like **auxins** (e.g. IAA) and **ethylene** also play key roles in this interaction, and are positive and negative regulators of tolerance of grafted tomato plants to water scarcity, respectively (Cantero-Navarro *et al.*, 2016). In this vein, genes like ACC deaminase for ethylene or iaaM

and iaaH for auxins have been proposed as important genes to be taken into account to assess the regulation of such hormones (Ghanem *et al.*, 2011).

Non-stomatal limitations, such as **Rubisco** activity, improve in the scion when they are grafted onto tolerant rootstocks. Yang *et al.* (2015) demonstrated that bottle gourd rootstock, under salt stress conditions, improved Rubisco activity in watermelon plants by overexpressing key genes related to the regeneration of this enzyme (e.g. TPI, FBPA, SBPase, PRK), together with improved chlorophyll b content, which enhanced photosynthetic capacity by stomatal opening and restored PSII efficiency. Maintaining high Rubisco content levels and activity requirements, as well as **N metabolism** activation to transform nitrate into amino acids by the action of several enzymes (e.g. GOGAT, GS, GDH, NR, NiR), have been demonstrated in cucumber, tomato and pepper grafted onto tolerant rootstocks (Liu *et al.*, 2013; Sánchez-Rodríguez *et al.*, 2013; Penella *et al.*, 2014b).

Oxidative damage, caused by impaired photosynthesis and photooxidation in the scion, can be alleviated by using more robust rootstocks. Enhanced enzymatic antioxidant activities, such as CAT, APX, SOD or PRX, have been widely demonstrated to improve salt and water stresses tolerance in the grafted scion in many vegetable species, including pepper (Penella *et al.*, 2016; Zhang *et al.*, 2019). Accumulation of molecules such as polyamines, AsA or proline have been described to improve ROS scavenging in grafted tolerant plants (Penella *et al.*, 2016; Sánchez-Rodríguez *et al.*, 2016), which reduce the peroxidative damage of lipid membranes (Penella *et al.*, 2015). Despite physiological mechanisms having been widely described, the molecular mechanisms of ROS detoxification and its regulation in grafted plants still remain unclear. Liu *et al.* (2016a) have demonstrated in watermelon under control conditions that the codificant genes for glutathione S-transferases, ascorbate oxidase, peroxidase or some pentatricopeptide repeat (PPR) proteins, are up-regulated when plants are grafted onto tolerant rootstocks in relation to self-grafted plants, which are proposed as possible mechanisms to improve oxidative damage and, thus, tolerance when abiotic stresses start.

Therefore, the advantages of the grafting technique are generally very well-documented in the bibliography, which is why its use in vegetable species has grown in recent decades. It is also worth mentioning the main limiting factors that reduce the effectiveness of such technique. Of all those proposed in the bibliography, **compatibility** between scion and rootstock is one of the most important, which reduces crop production and even leads to plant death. It is possible to successfully join two plants from different species, or even from different genera, such as cucumber (*Cucumis sativus*) onto luffa (*Luffa cylindrica*) (Liu *et al.*, 2016b), tomato (*Solanum lycopersicum*) onto related wild species (*Solanum habrochaites*) (Venema *et al.*, 2008), cucumber or mini-watermelon (*Citrullus lanatus*) onto the hybrid *Cucurbita maxima* x *Cucurbita moschata* (Rouphael *et al.*, 2008b, 2008a) or eggplant (*Solanum melongera*) onto *Solanum incanum* (Gisbert *et al.*, 2011). Nonetheless, not all vegetables have interspecific

compatibility, as with pepper (*C. annuum*), for which it is possible to find a variable degree of compatibility even with different cultivars of the same species. On the contrary, using rootstocks from other species of the same genus, such as *C. chinense* or *C. baccatum*, or other related genera like *Solanum* sp. seriously diminish compatibility (Penella *et al.*, 2017b). This fact complicates finding tolerant rootstocks in *C. annuum* in relation to other vegetables, which is why it is so important to find new genetic resources of the same species by screening semi-cultivated and wild accessions that can be used as rootstocks, and in future breeding programmes (Penella *et al.*, 2013, 2014b). Additionally, using the double grafted plants technique has demonstrated improvements in other species to graft joining and better grafted plant development (Kawaguchi *et al.*, 2008; San Bautista *et al.*, 2011).

It is important to also evaluate **economic losses** linked with the fact that grafted plants need the double amount of germinated seeds and double seedling maintenance until the time grafting takes place. It is also necessary to resort to specialised people or machinery to successfully develop graft joining and to monitor environmental parameters, such as temperature and relative humidity, to succeed in this process (Lee *et al.*, 2010). In general terms, all these factors can double the price per plant. However, encouraging results demonstrate that grafting under biotic and abiotic stresses result in net benefits compared to non-grafted plants (Djidonou *et al.*, 2013; Rysin and Louws, 2015).

1.7. Thesis Objectives

Previous studies conducted in the Horticulture department of the Valencian Institute of Agriculture Research (IVIA) and the Vegetable Production Department of the Polytechnic University of Valencia (UPV) demonstrated the genetic diversity of a series of pepper accessions when faced with abiotic stresses following a physiological and agronomical approach (Penella *et al.*, 2013, 2014b). Some tolerant accessions were used as rootstocks and evaluated agronomically and physiologically under salt or water stress (Penella *et al.*, 2014a, 2015, 2016, 2017a). Even if all this information contributes to a better understanding of the mechanisms of tolerance of pepper rootstocks, some behaviours remain unclear and unknown when this doctoral thesis began. We decided to also address a new genetic approach as the information on the molecular pathways of tolerant pepper accessions that confer scions tolerance has not been explored in-depth in the literature to date. By taking all this together, the main objectives of this work are:

1. Screening new pepper accessions of the germplasm bank of the Institute of Conservation and Improvement of the Valencian Agrodiversity (COMAV) at high salt concentrations and under suboptimal water conditions to find new tolerant rootstocks and use them in breeding programmes (Chapter 2).
2. Identifying the short-term physiological tolerance mechanisms of an accession, previously classified as tolerant, under water stress conditions when used as a rootstock (Chapter 3).
3. Identifying the short-term physiological mechanisms of salinity tolerance of a new hybrid rootstock (NIBER®) that has been previously classified as tolerant to a high salt concentration based on agronomical approaches (Chapter 4).
4. Looking for the main molecular pathways of salt stress tolerance of a tolerant accession compared to a sensitive one by a transcriptomic approach (Chapter 5).

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Chapter 2

Physiological changes of pepper accessions in response to salinity and water stress

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2.1. Abstract

New sources of water stress and salinity tolerances are needed for crops grown in marginal lands. Pepper is considered one of the most important crops in the world. Many varieties belong to the genus *Capsicum* spp., and display wide variability in tolerance/sensitivity terms in response to drought and salinity stress. The objective was to screen seven salt/drought-tolerant pepper accessions to breed new cultivars that could overcome abiotic stresses, or be used as new crops in land with water and salinity stress. Fast and effective physiological traits were measured to achieve the objective. The present study showed wide variability of the seven pepper accessions in response to both stresses. Photosynthesis, stomatal conductance and transpiration reduced mainly under salinity due to stomatal and non-stomatal (Na^+ accumulation) constraints and, to a lesser extent, in the accessions grown under water stress. A positive relationship between CO_2 fixation and fresh weight generation was observed for both stresses. Decreases in Ψ_s and Ψ_W and increased proline were observed only when accessions were grown under salinity. However, these factors were not enough to alleviate salt effects and an inverse relation was noted between plant salt tolerance and proline accumulation. Under water stress, A31 was the least affected and A34 showed the best tolerance to salinity in terms of photosynthesis and biomass.

Additional keywords:
osmotic potential; photosynthesis; proline; salinity ions; water potential.

Abbreviations used:
A_N (maximum net CO_2 fixation rate); A_N/Ci (instantaneous carboxylation efficiency); C_i (substomatal CO_2 concentration); DW (dry weight); E (transpiration); ETc (evapotranspiration); FW (fresh weight); g_s (stomatal conductance to water vapour); Ψ_s (osmotic potential); Ψ_W (water potential).

2.2. Introduction

With the global scarcity of water resources and increased of salinity in water and soil, these abiotic stresses constitute major limiting factors in plant growth and, consequently, agriculture productivity is decreasing (Bray *et al.,* 2000).

Plant responses to water and salinity stresses are complex and involve adaptive changes and/or deleterious effects (De Oliveira *et al*., 2013). The outcomes of both stress types on plant performance are diverse, but have some points in common. The main effect when plants start becoming stressed is the reduced water content in their tissue, and therefore the closure of leaf stomatal complexes takes place. Consequently, transpiration (Bray *et al.,* 2000) and/or photosynthesis may decrease through reduced osmotic potential in the soil solution, which involves reduced water potential (Bojórquez-Quintal *et al*., 2014; Penella *et al*., 2014a, 2015, 2016). If salinity stress occurs, a specific ionic effect appears, mediated by the accumulation of toxic ions in cellular tissues (De Pascale *et al*., 2003) with imbalances between nutrients (Hasanuzzaman *et al*., 2013). All these factors have adverse effects on both plant growth and development at physiological and biochemical levels (Munns & James, 2003).

Plants have evolved mechanisms to overcome salinity and water deficit that allow them to perceive incoming stresses and to regulate their metabolic functions. In general, one of the important pathways to enhance water stress and salt tolerance is through osmotic adjustment (OA), in which leaf turgor remains necessary for stomatal opening and, thus, sustains photosynthesis and growth (Huang *et al.,* 2010; Nio *et al.,* 2011). Besides, various types of compatible solutes accumulate, such as sugars, proline, gycinebetaine or potassium, among others (Munns *et al.,* 1979; Morgan, 1992; Nio *et al.,* 2011), and can increase. These compounds can be added to the list of the non-enzymatic antioxidants that plants need to counteract the inhibitory metabolic effects of reactive oxygen species (ROS) provoked by stress (Gill & Tuteja, 2010; Penella *et al*., 2014a, 2016). They also play a role in both the stabilisation of enzymes and proteins and the protection of membrane integrity (Patade *et al.,* 2012). Salt tolerance may arise from the ability to tolerate osmotic stress, from mechanisms of salt exclusion or from intercellular ion compartmentalisation (Munns & Tester, 2008). These mechanisms are not normally exclusive, so plants can combine some of these strategies at the same time (Chaves *et al.,* 2003). Furthermore, tolerance levels may vary between species (Munns, 2002) and within cultivars of the same species (Chartzoulakis & Klapaki, 2000).

Understanding the tolerance mechanisms that occur at the whole plant level has implications for screening and distinguishes plants that are tolerant to salinity and water stress (Munns, 2002). In the climate change scenario, new sources of salt and water stress tolerance are needed for the crops grown in areas with salinity and scarcity water problems. This available genotypic variability in terms of tolerance to abiotic

stresses can provide plant species with a breeding opportunity to obtain better yields and production, and good fruit quality.

Pepper is a member of the family *Solanaceae* and is considered one of the most important crops in the Mediterranean area, where water shortage and salinity are major problems that limit productivity (Penella *et al.,* 2013, 2014b). Many crops belong to the genus *Capsicum* spp., and display wide genetic variability (Aktas *et al.,* 2006). Pepper has been classified from moderately sensitive to sensitive under salinity and water stress conditions (Tanji & Kielen, 2002; Penella *et al.,* 2015). In fact, some studies have reported reduced seedling growth with 50 mM concentration of NaCl (Chartzoulakis & Klapaki, 2000; De Pascale *et al.,* 2003). Sometimes pepper has been described as one of the most susceptible crops to water stress, mainly due to its large transpiring leaf surface and high stomatal conductance of water vapour (Alvino *et al.,* 1994; Delfine *et al.,* 2002). Consequently, pepper plants are particularly sensitive to water stress at flowering and fruit setting (Bosland & Votava, 2000). However, not all *Capsicum* genus cultivars have the same sensitivity to abiotic stresses (Penella *et al.,* 2013, 2014b; Aktas *et al.,* 2006). Therefore, the study and identification of the tolerance level and mechanisms of different pepper genotypes are immensely important to breed new cultivars that can overcome abiotic stresses, or be used as new crops in land with drought and salinity problems to help extend the cultivated property. For pepper, very rare information about genotype variability in terms of its behaviour under salinity and water stress is available.

Different physiological markers have been proposed as key traits to select salt and water stress tolerance. Our most recent works evaluated several pepper accessions. We selected some of them as a source of tolerance to salinity and water stress (Penella *et al.,* 2013, 2014b) using gas exchange as a useful technique to differentiate tolerance and susceptibility to these stresses.

In the present study, we tested new accessions of *Capsicum annuum* L. for them being the most economically important species from the *Capsicum* genus in the Mediterranean climate. Accessions selection was made according to previous results (Penella *et al.,* 2013; 2014b). To evaluate their behaviour under salinity and water stresses, we studied the physiological mechanisms that underlie tolerance strategies using efficient parameters to identify which pepper accessions are tolerant to salt and/or water stress to be use in marginal areas and/or in breeding programmes. Further, we describe the physiological parameters roles and discussing the possibility of using them as selection criteria for cast salt and water stress genotypes with tolerance. As predictive screening parameters to salinity and water stresses in these seven new pepper accessions, we measured photosynthesis (A_N), stomatal conductance (g_s), inner carbon (C_i), water potential (Ψ_W), osmotic potential (Ψ_S), proline content, ion concentrations and biomass and their relationships.

2.3. Material and methods

2.3.1. Plant material

The *C. annuum* accessions used herein were Numex X (A31), Numex sandia type 2 (A32), Numex conquistador type 2 (A33), BGV-11814 (A34), BGV-4349 (A35), SIURIYA 600 (A36) and KAPIYA UV (A37). A numerical code for each accession is indicated in brackets. All the accessions used in the present study belong to the COMAV Institute collection (Universitat Politècnica de València, Valencia, Spain). Seeds were germinated in moistened perlite at 28ºC under greenhouse conditions. Seedlings were transferred to 15 L pots that contained coconut coir fibre (Cocopeat, Projar Co., Spain) in a heated polyethylene greenhouse on 10 April 2016 in the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain). Plants were drip–irrigated with Hoagland's No. 2 nutrient solution containing (all in mM): 14 NO_3^-, 1.0 $H_2PO_4^-$, 2.0 SO_4^{2-}, 1.0 NH_4^+, 16.0 K^+, 4.0 Ca^{2+} and 2.0 Mg^{2+}. Micronutrients were also provided (all in μM): 15 Fe^{2+}, 10 Mn^{2+}, 5 Zn^{2+}, 30 B^{3+}, 0.75 Cu^{2+} and 0.6 Mo^{6+}) (Maynard & Hochmuth, 2007). The electrical conductivity (EC) of the nutrient solution was 1.4 dS/m and pH 6.1. The greenhouse conditions in this period varied between 16ºC and 25ºC and from 50% to 70% of relative humidity.

After 15 days in pots, plants were divided into three groups for the control, saline and water stress treatments. Salinity treatment began by adding NaCl (60 mM) to the irrigation solution to reach an EC of 6.8 dS/m. Drip irrigation was applied based on estimations of weekly crop evapotranspiration (ETc) (Allen *et al.,*1998), even though the nutrient saline solution was allowed to drain freely from pots and the control drainage was controlled from 10% to 20% depending on solar radiation. Water stress treatment began by reducing the volume of irrigation water to 60% of the control. The volume of each irrigation and the number of irrigations were scheduled to maintain drainage at between 10% and 20% (depending on solar radiation).

Eight plants per accession were used in each treatment. Physiological measurements were taken 1 month after the salinity and water deficit treatments began on fully expanded mature leaves (third or fourth leaf from the shoot apex) and completed in 1 day.

2.3.2. Biomass

All the plants were harvested immediately after physiological parameters were measured. Aerial parts and roots were separated and their fresh weight (FW) was recorded. They were dried at 70°C for 72 h in a laboratory oven and then weighed for the dry weight (DW) determinations. Salt and water tolerance efficiencies (Fischer & Wood, 1981) were calculated according to the formulae: (DWstress/DWcontrol)*100, where DWstress and DWcontrol are total dry weight (aerial and root) of each genotype under the stress (water or salinity) or control conditions.

2.3.3. Photosynthesis measurements

Maximum net CO_2 fixation rate (A_N, μmol $CO_2/m^2 \cdot s$), stomatal conductance to water vapour (g_s, mol $H_2O/m^2 \cdot s$) transpiration rate (E, mmol $H_2O/m^2 \cdot s$) and substomatal CO_2 concentration (C_i, μmol CO_2/mol (air)) were measured at the steady state under conditions of saturating light (1000 μmol/$m^2 \cdot s$), 400 ppm CO_2 and 23-25ºC leaf temperature cuvette with a LI-6400 (LI-COR, Nebraska, USA). Parameter A_N/C_i was calculated as instantaneous carboxylation efficiency. Gas exchange measurements were taken on the third or fourth leaf from the shoot apex from 9 am to 11 am (GMT). One measurement per plant was taken, and eight different plants were used (n=8) for each treatment and accession.

2.3.4. Water relations

The osmotic potential of leaf sap (Ψ_s in MPa) was measured by an osmometer (Digital osmometer Vapro 5520, Wescor, USA). Leaves were tightly wrapped in aluminium foil, frozen in liquid nitrogen and stored at -80ºC. After thawing, sap was collected by centrifuging at 8,000 rpm at 4ºC and placed in the osmometer (modified from Callister *et al.,* 2006). Osmolyte content (mmol/kg) was converted into MPa using the Van't Hoff equation (Penella *et al.,* 2014a). Leaf water potential (Ψ_w in MPa) was measured on the leaves sampled with a Schlolander pressure chamber (Wescor Model 600, PMS Instruments, Albany, USA). Two independent determinations were made on each replicate and plant, obtained from six plants per treatment and combination for Ψ_s and Ψ_w.

2.3.5. Proline determination

Proline content (mg/g DW) was determined as described by Bates *et al.* (1973). Leaf and root dried pepper tissue (0.02 g) was ground in 3% sulphosalicylic acid, the homogenate was centrifuged at 8,000 rpm for 5 min, filtered, and 0.60 mL of glacial acetic acid and 0.70 mL of ninhydrin reagent (2.5 g ninhydrin in 600 mL glacial acetic acid and 40 mL 6 N phosphoric acid) were added to an aliquot of the supernatant. The reaction mixture was boiled for 1 h at 100ºC, and readings were recorded at a wavelength of 520 nm in a spectrophotometer. Proline determination was made for n=4 for each treatment and accession.

2.3.6. Sodium and chloride ions analysis

The leaves and roots collected for n=4 samples of each treatment and accession were dried at 70ºC for 4 days. Dried samples (0.1-0.2 g) were burnt in a muffle furnace for 12 h at 550ºC. Ions were extracted with 2% nitric acid in an ultrasonic bath for 30 min at 4ºC. Na^+ concentration was measured by an atomic absorption spectrometer (A Analyst 200, Perkin Elmer).

The chloride concentration (Cl^-) in the dry plant material was extracted with 0.1 N HNO_3 in 10% (v/v) acetic acid and was determined by potentiometric titration with $AgNO_3$ in a chloride analyzer (Sherwood, MKII 926).

The results for both ions were expressed as [Na^+] or [Cl^-] salt stress/[Na^+] or [Cl^-] control for n=6 independent samples in leaves and roots.

2.3.7. Statistical analysis

The layouts of the experiments took a completely randomised design. Data of each accession for treatments (control, water and salinity stress) were subjected to one-way ANOVA`s. The mean comparisons were made using Fisher's least significance difference (LSD) test at $p<0.05$. The data of (DWstress/DWcontrol)*100 (Arcsin $X^{½}$ transformation) and [Na^+] or [Cl^-] salt stress/[Na^+] or [Cl^-] were subjected to a one-way ANOVA with genotype as the variability factor, and the means comparisons Fisher's least significance difference (LSD) test at $p<0.05$ was applied.

The data obtained in some measurement parameters were subjected to linear regression and analyses to identify the relationships between the physiological parameters.

2.4. Results

2.4.1. Biomass

The first step in this experiment was to detect the phenotypical variations regarding water and salinity tolerance in these seven pepper accessions. The pepper accessions grown under stress and control conditions showed significant differences in DW (**Fig. 2.1.A**). A31 and A32 were the accessions with the highest DW values under the control treatment. Both stresses significantly decreased parameter DW in all the accessions. Salinity generated the lowest DW biomass in all the accessions. Under drought, A31 showed the minor decrease and under salinity A34 stood out. The salt and drought tolerance indices were also determined to distinguish between sensitive or tolerant accessions (**Fig. 2.1.B**). Under water stress, A31 stood out with 72% and A36 obtained the lowest value with 46% inhibition, while the other accessions displayed similar values. Regarding salinity, A34 showed the higher percentage (53% compared to its control), and the rest exhibited values of around 24-39%.

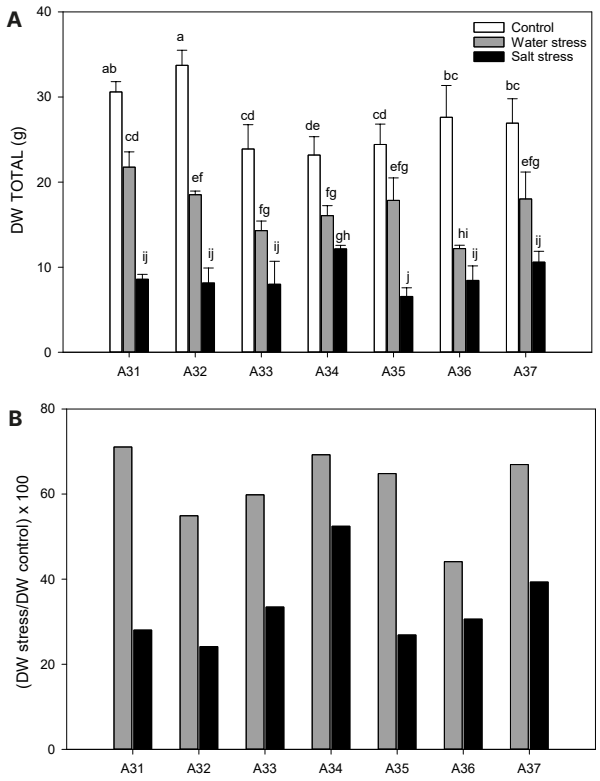

Figure 2.1. Total dry weight (A) and salt and water tolerance efficiencies (B) calculated as (DWstress/DWcontrol)*100 of pepper accessions after 1 month under water stress and under NaCl (60 mM) supplied to the nutrient solution. Different letters in (A) indicate significant differences at p<0.05 (LSD test) according to ANOVA, with treatments and accessions as the variability factors. In (B) for each histogram bar, the value is significantly different at p<0.05 (LSD test) to its control. Data are the mean for n=8 plants and SE.

2.4.2. Photosynthetic parameters

The leaf CO_2 assimilation rate (**Fig. 2.2.A**), stomatal conductance (**Fig. 2.2.B**) and transpiration (**Fig. 2.2.C**) were strongly reduced in pepper accessions exposed to salinity and in minor extends under water stress compared to controls. Under water stress A31 obtained the higher values of A_N, g_S and E without significant differences with its control; A36 gave the lowest values. The accession A34 showed the minor decrease of gas exchange parameters compared with the rest of pepper accessions under salinity but showed significant differences with its control.

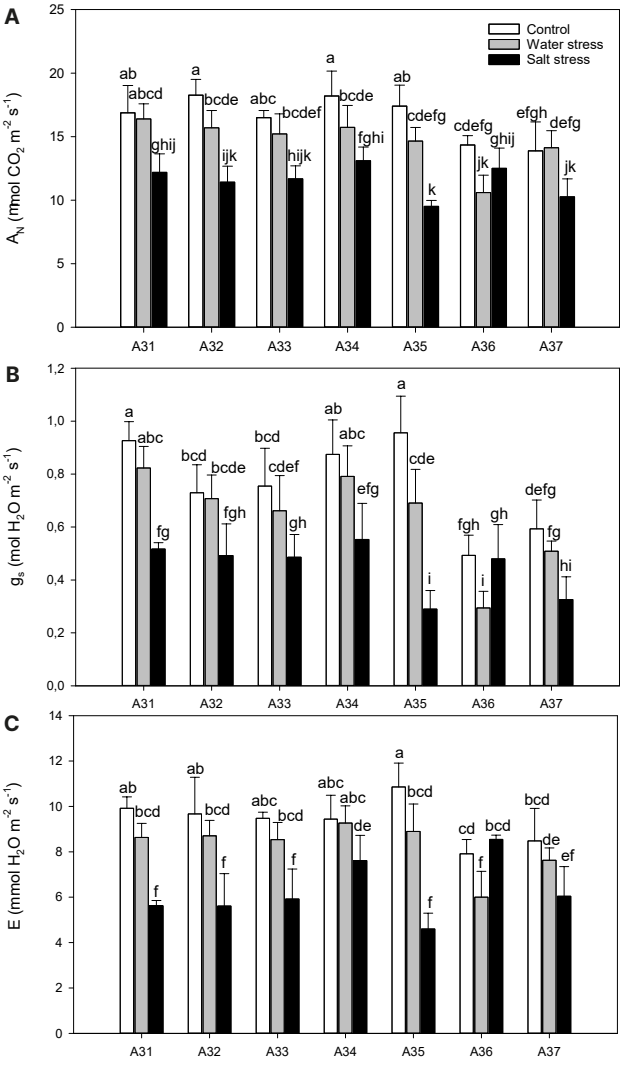

Figure 2.2. Leaf CO_2 assimilation (A_N) (A), stomatal conductance (g_S) (B) and transpiration (E) (C) in pepper accessions after 1 month under water and salt stress. Data are the mean values for n=8 plants ± SE. Different letters indicate significant differences at p<0.05 (LSD test).

2.4.3. Relation between photosynthesis and fresh weight

The data showed a positive relationship between A_N and FW (A_N= 0.0392FW + 8.914; r^2= 0.678; p<0.05) for all the values (**Fig. 2.3.A**). In the water stress group, A31 obtained the highest FW and photosynthesis values, and A36 gave the lowest ones. In the salinity group, A34 presented the highest biomass and the greatest increase in photosynthesis, while A35 displayed the worst behaviour.

2.4.4. Instantaneous carboxylation efficiency

Under control conditions, instantaneous carboxylation efficiency, expressed as A_N/C_i (**Fig. 2.3.B**), displayed the highest values, while the lowest values were obtained under the salinity treatment for all the studied pepper accessions. Under salinity, A37 was the accession with the greatest decrease in this parameter following A35 and A32. Under water stress, A36 gave the lowest A_N/C_i value with significant differences with the rest of the accessions under drought and control plants.

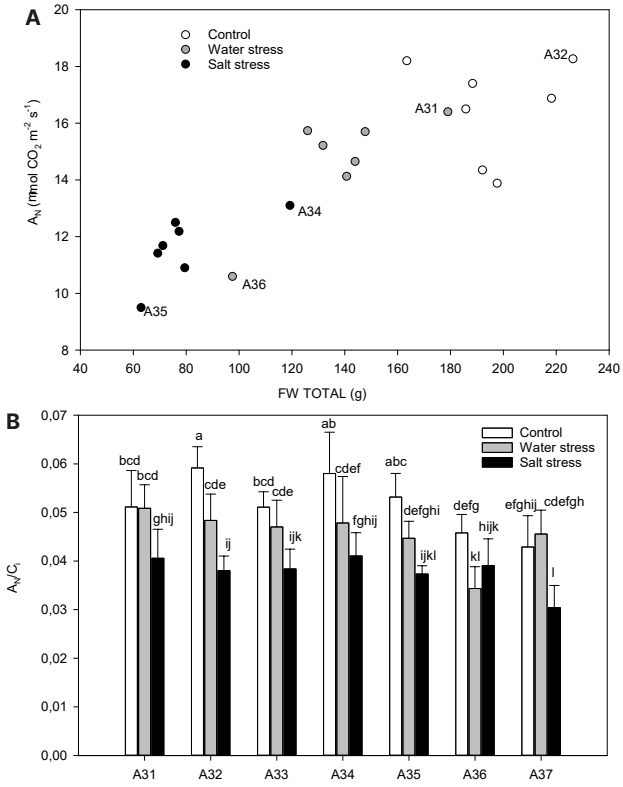

Figure 2.3. Relation between total fresh weight (FW total) and CO_2 fixation (μmol CO_2/m^2·s) under the control and the water and salinity stresses at the end of the experiment (A). Each point is the value mean for n=8 plants in each accession. Instantaneous carboxylation efficiency (A_N/C_i) for pepper accessions after 1 month under the control and water and salinity stresses (B). Data are the mean values for n=8 plants ± SE. Different letters indicate significant differences at p<0.05 (LSD test).

2.4.5. Water and osmotic potential

Leaf Ψ_W drastically lowered in response to salinity and led to large significant differences in A32, A33, A34 and A35 compared to their controls (p<0.05) (**Fig. 2.4.A**). During the drought period, Ψ_W remained higher for all the accessions than those exposed to salinity. It was noteworthy that A37 obtained significantly different Ψ_W values compared to its control, but not contrary to salt stress, where values were the lowest compared to the other accessions.

Leaf Ψ_s decreased in response to salt treatment, but not under water stress (**Fig. 2.4.B**). The salt-induced decrease in Ψ_s was more pronounced in A35, followed by A34 and A36, compared to the rest, and Ψ_s was not modified under water stress, except in A31 and A32 where Ψ_s values were biggest compared to their controls.

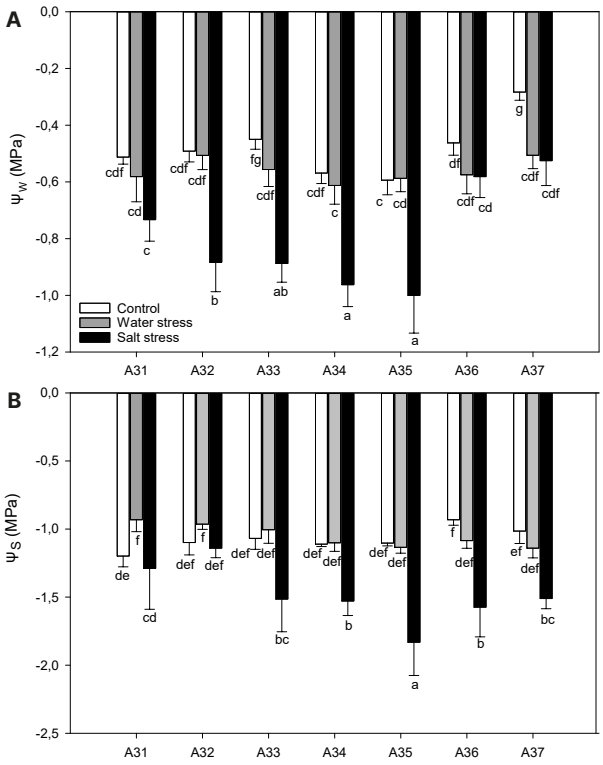

Figure 2.4. Water potential (Ψ_W, MPa) (A) and osmotic potential (Ψ_s, MPa) (B) in the leaves of pepper accessions under the control, water stress and salinity at the end of the experiment. Data are the mean values for n=6 ± SE for each treatment and accession. Different letters indicate significant differences at p<0.05 (LSD test).

2.4.6. Proline concentration in leaves and roots

Proline leaf accumulation occurred under salt stress in all the accessions (**Fig. 2.5.A**), but was not observed under water stress. The greatest increase was observed for A37, followed by A33 and A35.

For a given population, the proline concentration in roots was lower compared to leaves (**Fig. 2.5.B**). Erratic proline behaviour in this organ was observed, where accession A33 showed the most marked increase in proline content under salinity stress, whereas A35 and A36 displayed a decrease. Under water stress, accessions maintained similar values to their controls, except for A37 for which a decrease was noted (**Fig. 2.5.B**).

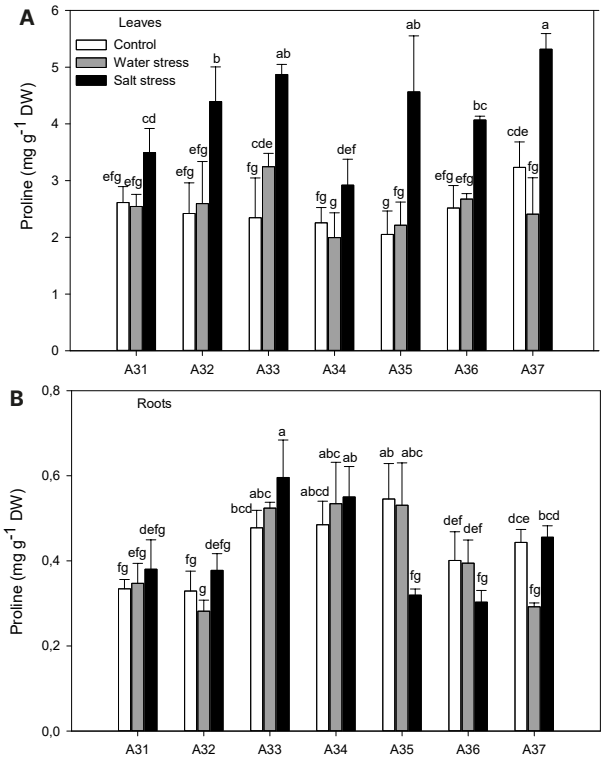

Figure 2.5. Changes in proline concentration (mg proline/g DW) in the leaves (A) and roots (B) of pepper accessions under the control and the water and salinity stresses. Data are the mean for n= 4 ± SE for each treatment and accession. Different letters indicate significant differences at $p<0.05$ (LSD test).

2.4.7. Sodium and chloride analysis

Increases in Na^+ and Cl^- were observed in all the pepper accessions under the salinity condition (**Fig. 2.6.**). Under water stress, the values were similar to the control (data not shown). In roots, Na^+ increased between 2-3.2-times were observed compared to its control values (**Fig. 2.6.A**). In leaves, Na^+ increased less compared with root levels in all the pepper accessions, except for A35 where the Na^+ leaf increase was 4.5-times than its control. Compared to the control, chloride accumulation (**Fig. 2.6.B**) was higher in leaves than in roots in some accessions (A34, A35 and A37), while the accession values for the rest were similar between roots and leaves.

For both ions and organs, an increase in each accession showed significant differences compared to its control.

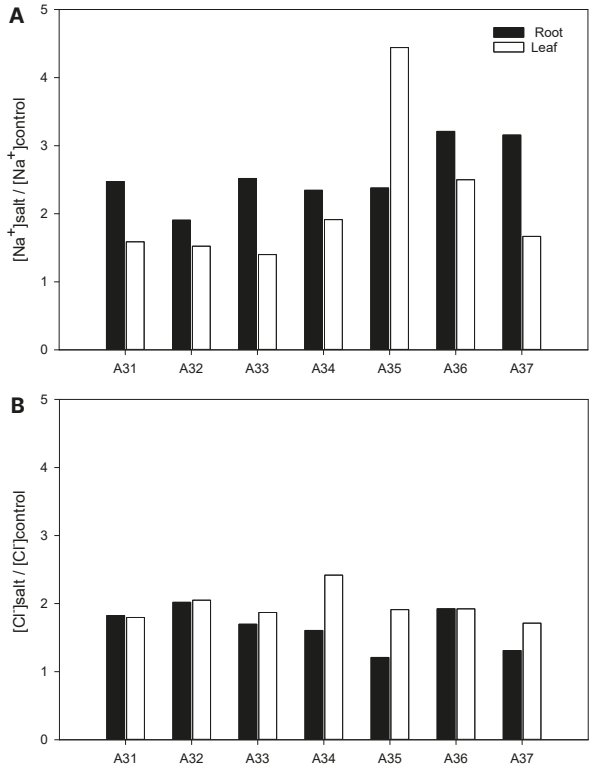

Figure 2.6. Ratio of the Na^+ (A) and Cl^- (B) concentration in the salt treatment and the control in roots and leaves for each pepper accession at the end of the experiment. For each histogram bar, the value is significantly different compared to its control after the LSD test at $p<0.05$. Data are the mean for n=6 independent samples in leaves and roots.

2.5. Discussion

The pepper accessions shown in this experiment exhibited physiological differences in response to drought and salinity stress. In particular, we obtained photosynthesis values connected to biomass, which indicated the ability to cope with these stresses. Therefore, these accessions would be suitable to be grown in semi-arid or salinity lands and/or to be used in breeding programmes as a source of tolerance.

It is well-known that water and salinity stress reduces plant growth and that there are differences among cultivars with peppers (Aktas *et al.,* 2006; Penella *et al.,* 2013, 2014b). According to our results, both stresses significantly suppressed the growth of pepper plants in dry weight terms, although their stress responses depended on the accession. It should be noted that among all accessions, A34 for salinity and A31 for water stress showed minor growth reduction; nevertheless, A31 under salinity experienced an important biomass reduction. However, the physiological, biochemical and genetics mechanisms involved in growth inhibition have not yet been well characterised (Misra *et al.,* 2002; Munns & Tester, 2008; Noreen *et al.,* 2010). Knowledge of plant certain capacities of cope with stress could be essential for characterising stress tolerance.

This differential growth of the seven pepper accessions under both stresses may have been due to a differential regulation of the distinct physiological attributes involved in growth processes. Previous studies have demonstrated a positive relationship between photosynthetic capacity and growth in the plants grown under both salinity (Praxedes *et al.,* 2010; Saleem *et al.,* 2011; Penella *et al.,* 2015; 2016) and water stress (Chaves *et al.,* 2002; Abbad *et al.,* 2004; Hassine *et al.,* 2008; Del Amor *et al.,* 2010). In spite of considerable reduction of carbon assimilation rate and biomass under both stresses for all accessions studied the genotype A31 under water stress showed the minor decrease for both physiological parameters showing not significant differences in A_N and a biomass reduction of 29% respect its control plants. Under salinity stress, among all accessions, the genotype A34 exhibited the higher photosynthesis rate and its biomass experienced the minor reduction respect the rest of accessions.

Reduced photosynthesis can be caused by stomatal closure and/or non-stomatal inhibition, and the latter is associated with damage in photosynthetic machinery (Flexas *et al.,* 2004). In our experiment, a high correlation between A_N and g_s was observed for all data (A_N = 13.304 g_s + 6.63, r^2= 0.8579; p<0.05). This apparent linearity indicates that g_s and A_N reduced in a coordinated relation. This finding agrees with the interpretations under water and salinity stress conditions made by several researchers (Cowan & Farquhar, 1977; Delfine *et al.,* 2002; Filippou *et al.,* 2014; Penella *et al.*, 2015). The equation of the relationship between both parameters (A_N *vs.* g_s) did not pass through the origin. This fact indicates that stomatal closure occurred earlier than CO_2 fixation (Delfine *et al.,* 2002), although A_N reduction was due mainly to stomata closure, the

partial inhibition of mesophyll conductance and/or photochemical efficiency cannot be ruled out.

Decrease in A_N/C_i indicates that both stresses affected the photosynthesis by metabolic limitations (Da Silva *et al.,* 2011; Penella *et al.,* 2015). The marked drop in A_N/C_i occurred more drastically under the salt-stressed pepper accessions. Excessive Na^+ and Cl^- accumulation is harmful and may disrupt the integrity of chloroplasts and decrease photosynthetic capacity (Munns & Tester, 2008; Chaves *et al.,* 2009; Rouphael *et al.,* 2012; Penella *et al.,* 2015). We observed how the accumulation of mainly Na^+, and of Cl^- to a lesser extent, occurred in the roots and leaves of all the pepper accessions. However, A35 stood out with the greatest Na^+ accumulation in its leaves, affecting directivity to photosynthetic apparatus, which could cause the lowest A_N, g_s, E and FW (and DW) values. Under water stress, A_N/C_i decreased to a lesser extent, which supports the notion that the major photosynthesis inhibition was mainly resulted from stomatal closure, and that A36 was the most affected genotype with the lowest growth and CO_2 fixation.

Under the osmotic stress provoked by water and salt stress in the root medium, plants lowered leaf Ψ_s in an attempt to maintain water uptake (decrease Ψ_w) with a positive turgor, which is indispensable for cell growth and maintaining photosynthetic performance (Yadollahi *et al.,* 2011; Penella *et al.,* 2015, 2016). In order to face water loss, plants accumulate many compatible (organic) metabolites to increase tolerance against tissue dehydration (Yoshiba *et al.,* 1997; Patakas *et al.,* 2002). Proline accumulation is believed to be one of the most important metabolites that are implied in osmotic adjustment. Moreover, several studies have attributed multiple roles to proline, such as signalling molecule that influences defence pathways, complex metabolic regulation and development processes, and protective compounds (see Szabados & Savouré, 2010). In our experiment, proline content increased considerably under the salinity conditions (from 29% for A34 to 64% for A37 compared with their controls), but not under water stress. When proline was taken as an osmolyte, the role it played to contribute to lower Ψ_s did not suffice under salinity (between 0.06 and 0.1MPa) to generate osmotic pressure (Smirnoff & Cumbes, 1989; Penella *et al.,* 2015). Therefore, the increase in proline under salt stress was unable to explain the observed decrease in Ψ_s, while the relationship between them was very weak (r^2= 0.027). Nevertheless, the largest proline amount observed in all the pepper accessions, except A34, was related with the greater salt sensitivity of these genotypes. These findings are consistent with the research reported for pepper into higher leaf proline in salt-sensitive genotypes (Penella *et al.,* 2015), or for other species, such as wheat (Colmer *et al.,* 2005), barley (Chen *et al.,* 2007), or rice (Lutts & Guerrier, 1995). Moreover, proline or other compatible solutes may protect plants by scavenging the oxygen-free radicals caused by salt stress (Huang *et al.,* 2010; Penella *et al.,* 2016), a role as signalling molecule, implicated in regulation and developmental processes, and should be considered a protective compound (see Szabados & Savouré, 2010). Under water stress, no changes were

observed in proline content respect to controls due to there were not differences in Ψ_W and Ψ_s was observed between treatments.

The adjustment of Ψ_s through inorganic salt-ion uptake is a strategy that implies a much lower energy cost for cells compared with the organic molecules synthesised in cells (Munns, 2002). Pepper accessions showed a better correlation between osmotic potential and Na^+ levels in leaves (Ψ_s = -0.361 [Na+] – 0.9288; r^2=0.666), but not for Cl^- accumulation (data not shown). The decreased Ψ_s in the pepper accession leaves subjected to salt stress was largely the result of strong Na^+ accumulation. This result was also observed by Chen *et al.* (2007) in barley genotypes, Abideen *et al.* (2014) in *Phragmite karka,* or Navarro *et al.* (2003) and Penella *et al.* (2015) in pepper plants.

Overall, all the analysed physiological parameters, photosynthesis and stomatal conductance, can be reliable indicators of biomass under water and salinity stress. Both salinity and water stress lead to reduce photosynthesis, stomatal conductance and growth. The present study evidences a wide variability of the seven pepper accessions in response to both stresses, and in both drought and salinity treatments. Under our conditions, growth inhibition occurred under water stress, provoked mainly by stomatal closure, where A31 was the less affected, and A36 was the most sensitive one and also correlated with minor CO_2 fixation and biomass. Under the salinity conditions applied in this experiment, our results showed that damage was greater compared with water stress. In this case, photosynthesis inhibition was due to the stomatal and non-stomatal effects caused by osmotic stress and toxic salt ion accumulation. Even with increased proline synthesis, the reduction in Ψ_s and Ψ_W was not enough to alleviate the salt effects. A34 was the most interesting accession due to its better tolerance to salinity with a major photosynthesis capacity, minor growth inhibition, but it had a lower proline concentration compared with A35, which suffered the worst adaptation. This genetic variation in response to both stresses can be exploited in pepper crops so they can be grown in marginal areas and/or in breeding programmes.

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Chapter 3

Pepper Rootstock and Scion Physiological Responses under Drought Stress

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3.1. Abstract

In vegetables, tolerance to drought can be improved by grafting commercial varieties onto drought tolerant rootstocks. Grafting has emerged as a tool that copes with drought stress. In previous results, the A25 pepper rootstock accession showed good tolerance to drought in fruit production terms compared with non-grafted plants and other rootstocks. The aim of this work was to study if short-term exposure to drought in grafted plants using A25 as a rootstock would show tolerance to drought now. To fulfil this objective, some physiological processes involved in roots (rootstock) and leaves (scion) of grafted pepper plants were analyzed. Pepper plants not grafted (A), selfgrafted (A/A), and grafted onto a tolerant pepper rootstock A25 (A/A25) were grown under severe water stress induced by PEG addition (-0.55 MPa) or under control conditions for 7 days in hydroponic pure solution. According to our results, water stress severity was alleviated by using the A25 rootstock in grafted plants (A/A25), which indicated that mechanisms stimulated by roots are essential to withstand stress. A/A25 had a bigger root biomass compared with plants A and A/A that resulted in better water absorption, water retention capacity and a sustained CO_2 assimilation rate. Consequently, plants A/A25 had a better carbon balance, supported by greater nitrate reductase activity located mainly in leaves. In the non-grafted and self-grafted plants, the photosynthesis rate lowered due to stomatal closure, which limited transpiration. Consequently, part of NO_3^- uptake was reduced in roots. This condition limited water uptake and CO_2 fixation in plants A and A/A under drought stress, and accelerated oxidative damage by producing reactive oxygen species (ROS) and H_2O_2; which were highest in their leaves, indicating great sensitivity to drought stress and induced membrane lipid peroxidation. However, drought deleterious effects were slightly marked in plants A compared to A/A. To conclude, the A25 rootstock protects the scion against oxidative stress, which is provoked by drought, and shows better C and N balances that enabled the biomass to be maintained under water stress for short-term exposure, with higher yields in the field.

Additional keywords:
drought, gas exchange, grafted,
oxidative stress, pepper,
rootstock, water relations.

3.2. Introduction

In agriculture, drought stress is one of the most limiting factors for growing crops, mainly due to a poor plant carbon balance, which is largely dependent on photosynthesis (Flexas *et al.*, 2009). This is associated with a significant drop in the leaf water potential and transpiration (Fahad *et al.*, 2017) which, in turn, affect nutrient absorption. These restrictions make plants more susceptible to photo damage by increasing reactive oxygen species (ROS) that may damage the cellular membrane and other vital molecules like DNA, lipids, and proteins (Fahad *et al.*, 2017). Metabolic alterations in plants by drought lead to significant yield reductions, which imply major economic loss and affect global food security. Bearing in mind that most climate change scenarios predict a more drought incidences, it is necessary to increase food production to satisfy the population's demand (Fahad *et al.*, 2017).

The selection of tolerant genotypes is a considerable challenge to improve productivity with limited water resources. Conventional plant breeding has had limited success at mitigating the effects of abiotic stress on plant productivity (Gilliham *et al.*, 2017; Lamaoui *et al.*, 2018). This can be ascribed to both the complexity of traits and lack of appropriate selection tools (Ashraf and Foolad, 2007; Schwarz *et al.*, 2010). In addition, it is very difficult to combine enhanced yields and superior product quality with tolerance to drought and other abiotic stresses (Finckh, 2008; Lammerts van Bueren *et al.*, 2011).

Genetic transformation could prove a powerful tool in plant breeding (Borsani *et al.*, 2003; Cuartero *et al.*, 2006; Martínez-Rodríguez *et al.*, 2008). However, lack of public acceptance of genetic engineering clearly indicates the need for alternative strategies to enhance abiotic stress tolerance (Munns, 2002; Estañ *et al.*, 2005).

One possible solution to cope with abiotic stress and reduce production losses involves using graft technology (Rivero *et al.*, 2003a; Colla *et al.*, 2010; Savvas *et al.*, 2010; Schwarz *et al.*, 2010; Sánchez-Rodríguez *et al.*, 2014). Some studies have demonstrated the efficiency of tolerant rootstocks in reducing the effects of drought on the scion by improving physiological performance and productivity through different approaches, like using a larger and vigorous root rootstock system capable of absorbing water and nutrients, and maintaining the root relative growth rate and leaf-relative water content more efficiently than non-grafted plants. This behavior has been observed in tomato (Sánchez-Rodríguez *et al.*, 2012; Yao *et al.*, 2016) and watermelon (Chouka and Jebari, 1999; Alan *et al.*, 2007; Rouphael *et al.*, 2008). Another alternative is active osmotic adjustment as it can contribute to improve the uptake of more water mediated by the accumulation of a range of osmotically active molecules, as reported in pepper (Anjum *et al.*, 2012; Penella *et al.*, 2014b) and tomato (Yao *et al.*, 2016) grafted plants. In addition, plants subjected to drought stress tend to overproduce ROS; the activation and/or modulation of an antioxidant defense system

plays an important role in conferring tolerance under drought and constitutes the first line of defense by reducing damage by lipid peroxidation in tomato grafted plants (Yao *et al.*, 2016). The ability to limit water loss can help to maintain the photosynthesis rate and improve NO_3^- assimilation in grafted plants under water deficit by allowing both plant growth and productivity (Rouphael *et al.*, 2008; Sánchez-Rodríguez *et al.*, 2013; Penella *et al.*, 2014b).

Recently, the number of reports on grafting as a mean to improve tolerance to drought has increased in mainly tomato, watermelon and cucumber (Kumar *et al.*, 2017). Nevertheless, the use of rootstocks that tolerate abiotic stresses is lacking in pepper plants because available commercial rootstocks provide limited profits (Lee *et al.*, 2010; Penella *et al.*, 2014a; Kyriacou *et al.*, 2017). Overall screenings to detect tolerant *Capsicum* plants are necessary to use them as rootstocks (Penella *et al.*, 2014a).

In our previous experiments, after wide screening of *Capsicum* accessions, a drought-tolerant genotype was selected to be used as a rootstock. It was tested in productivity terms (A25 code) and showed an increase in marketable fruit of 118% versus nongrafted plants (Penella *et al.*, 2017).

Thus present work aimed to (i) determine if shortterm drought stress exposure of seedling grafted plants onto A25 could express its tolerance to determine the highest productivity (Penella *et al.*, 2017); (ii) identify rootstock and scion physiological traits associated with drought tolerance to open up new strategies that improve crop performance under limited water supply conditions. Very few studies on simultaneous changes in the rootstock/scion are available, but none about the perception of scions and rootstock remodeling can be found (Li *et al.*, 2014; Liu *et al.*, 2016). To fulfill these objectives, we compared the behavior of photosynthetic, water relations, antioxidant mechanisms and oxidative index stress in non-grafted and self-grafted plants, and in those grafted onto a tolerant rootstock (A25), under drought stress and control conditions.

3.3. Materials and methods

3.3.1. Experimental Site and Greenhouse Conditions

The experiment was conducted in a Venlo-type glasshouse located in Moncada (Valencia, Spain; Latitude: 39.58951793357715, Longitude: -0.3955507278442383, 37 m above sea level) at the IVIA research institute.

During the experiments, plants were grown under natural light conditions with a maximum PAR of 1000 mmol m^{-2} s^{-1} (800–1,000 mmol m^{-2} s^{-1}), a mean temperature of 22°C (18–25°C) and a mean humidity of 60% (50–70%).

3.3.2. Plant Material and Management

Based on previous studies, a pepper accession of *Capsicum annuum* L. was used as a tolerant rootstock to water scarcity (code A25). Pepper cultivar "Adige" (code A) (Lamuyo type, Sakata Seeds, Japan) was used as a scion. Seeds were sown in March 2016 in 54-hole seed trays filled with enriched substrate for germination. Three months after sowing, plants were grafted by the tube-grafting method (Penella *et al.*, 2014b).

Three weeks after grafting (June 2016), seedlings were removed from substrate, and their roots were cleaned before being placed in 5 L polyethylene pots covered with aluminum sheets. Pots were filled with a nutrient solution containing (in mmol L^{-1}): 12.3 NO_3^-, 1.02 H_2PO_4, 2.45 SO_4^{2-}, 3.24 Cl^-, 0.6 NH_4^+, 5.05 K^+, 4.23 Ca^{2+}, 2.55 Mg^{2+}, 2.2 Na^+ and micronutrients (15.8 mM Fe^{2+}, 10.3 mM Mn^{2+}, 4.2 mM Zn^{2+}, 43.5 mM B^+, 2.14 mM Cu^{2+}), which were artificially aerated with an air pump. Electrical conductivity and pH were 2.14 dS m^{-1} and 6.7, respectively. Nutrient solution was added daily to compensate absorption. The water stress treatment was induced after a 7-day seedling acclimation in pots by adding 5% PEG 8000 (Sigma Co.) to the nutrient solution. The osmotic potential of the solutions, measured by a vapor osmometer (Digital osmometer, Wescor, Logan, UT, United States), was -0.55 MPa for 5% PEG and -0.05 MPa for the control solution (0% PEG).

The assay was based on three plant combinations: A (nongrafted plants of cultivar Adige), A/A (A grafted onto itself by showing the graft effect) and A/A25 (A grafted onto the A25 rootstock). The layout was completely randomized with four replications for each combination and six plants per replication.

All the physiological measurements were taken 1, 2, 4, and 7 days after treatment began (DAT). Measurements were taken in fully and expanded mature leaves (third to fourth leaf from the shoot apex), and also in lateral roots for some physiological measurements. The layout was randomized with 12 measurements (three plants per replication) per plant combination and treatment for the gas exchange measurement, and with four measurements (one plant per replication) in the other analysis of the physiological parameters. Biomass determinations were made only on 7 DAT using the

plants that were not involved in the physiological measurements. Eight plants per plant combination and treatment were analyzed (two plants per replication).

3.3.3. Gas Exchange Measurements

The CO_2 assimilation rate (A_N, mmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapor (g_s, mol H_2O m^{-2} s^{-1}), and substomatal CO_2 concentration (C_i, mmol CO_2 mol^{-1} air) were measured with a portable LI-COR 6400 infrared gas analyzer (Li-Cor Inc., United States). Measurements were taken under saturating light conditions (1,000 mmol quanta m^{-2} s^{-1}), with reference CO_2 (400 mmol CO_2 mol^{-1}) at 24°C (24°C ± 2) and 75% relative humidity (75% ± 10). The fully expanded (third to fourth leaf from the apex) and non-detached leaves were used for the measurements taken from 09:00 h to 11:00 h (UT C 01:00 h).

3.3.4. Biomass Determination

Root length and the fresh weight of roots and leaves were measured at the end of the experiment (7 DAT). Fresh roots and leaves were dried at 65°C for 72 h to determine dry weight.

3.3.5. Water Relations

Relative water content (RWC) was measured by weighing leaves before and after a 24 h rehydration with distilled water. Next they were dried at 65°C for 72 h and the measurement was repeated. RWC was determined by the equation RWC = (FW - DW)/(TW - DW) / 100, where FW, DW, and TW are fresh weight, dry weight, and turgid weight, respectively.

The leaf water potential at pre-dawn (Ψ_W) was measured with a Schlolander pressure chamber (Wescor Model 600, PMS Instruments, Albany, NY, United States) on detached fresh and mature leaves inside a greenhouse.

The osmotic potential of leaf sap (Ψ_s in MPa) was measured by an osmometer (Digital osmometer, Wescor, Logan, UT, United States). Leaves were detached, placed inside 1 mL tubes and quickly frozen at -20°C. After melting, sap was collected by centrifugation at 9,000 rpm for 1 min in 1.5 mL tubes to be used for the osmometer measurements. Osmolyte content (mmol kg^{-1}) was converted into MPa by the Van't Hoff equation.

3.3.6. Nitrate Reductase Activity

Nitrate reductase activity (Enzyme Code 1.7.1.1) was determined in vivo following the methods described by Hageman and Hucklesby (1971) and Jaworski (1971). Disks of 1 cm diameter from mature fresh leaves or 1 cm root pieces were collected. Samples (0.2 g) were suspended in plastic vials containing 10 mL of 100 mM potassium phosphate buffer (pH 7.5), 1% (v/v) n-propanol and 100 mM KNO_3. Plant samples were incubated in a water bath at 30°C for 60 min in the dark and placed in a boiling water

bath for 5 min to stop the enzymatic reaction. The nitrite released from plant material was determined colorimetrically at 540 nm (spectrophotometer PerkinElmer, Lambda 25) by adding 0.02% (w/v) N-naphthyl-ethylenediamine and 1% (w/v) sulfanilamide. A standard curve with KNO_2 was prepared to calculate the amount of NO_2 contained in the samples.

3.3.7. Determination of DPPH Radical-Scavenging Capacity

Determination of radical scavenging capacity (RSA) was carried out by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, proposed by Brand-Williams *et al.* (1995) with modifications. First 0.1 g of sample (leaves and roots) was frozen in liquid nitrogen and stored at -80°C. Samples were ground by a mortar with the addition of 80% (v/v) methanol. After 12 h at 4°C in a mixer, samples were centrifuged for 10 min at 10,000 x *g* and 4°C. A 10 µL volume of sample and 990 µL of 0.065 mM DPPH were taken and incubated for 30 min in the darkness at room temperature. Absorbance was measured at 515 nm. The percentage of inhibition of the DPPH radical was measured by the equation: [(DPPH absorption - sample absorption)/DPPH absorption] x 100.

3.3.8. Total Phenolic Content Analysis

Total phenolic content was determined according to Koç *et al.* (2010) with modifications. The fresh leaf and root samples (0.1 g) were frozen in liquid nitrogen and stored at -80°C. They were mixed with 1.5 mL of extraction solution [50% (v/v) methanol and 1% (v/v) HCl]. Samples were extracted in a boiling bath at 80°C for 15 min. Then 0.1 mL of root extract and 0.02 mL of leaf extract (diluted in 0.08 mL extraction solution) were mixed with 0.7 mL of Folin–Ciocalteu solution (Sigma-Aldrich®), diluted in the 1:10 proportion, and with 0.7 mL of 6% (w/v) Na_2CO_3. Samples were incubated at room temperature and in the darkness for 1 h before being subjected to absorbance measurement at 765 nm. Gallic acid was used as a standard.

3.3.9. Determination of Hydrogen Peroxide

H_2O_2 content was determined according to Sergiev *et al.* (1997) and Velikova *et al.* (2000) with slight modifications. First 0.25 g of FW (leaves and roots) was frozen in liquid nitrogen and conserved at -80°C. Samples were ground with a mortar and 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 x *g* at 4°C for 8 min. With the root samples, 1 mL of the supernatant was added to 0.5 mL of 100 mM potassium phosphate buffer (pH = 7) and 2 mL of 1 M KI. For another set of samples, 0.4 mL of leaves was diluted with 0.6 mL of 0.1% (w/v) TCA. Samples were incubated for 1 h at room temperature under dark conditions. Absorbance was measured at 390 nm. H_2O_2 content was given by a H_2O_2 standard curve.

3.3.10. Lipid Peroxidation Analysis

Lipid peroxidation was estimated through malondialdehyde (MDA) determinations by the thiobarbituric acid reaction, according to the protocol reported by Heath and Packer (1968), and modified in Dhindsa *et al*. (1981). First 0.1 g of sample (leaves and roots) was frozen in liquid nitrogen and kept at -80°C. Samples were ground with a mortar and 2 mL of 0.1% (w/v) TCA. Later the homogenate was centrifuged at 10,000 x *g* and 4°C for 5 min. Afterward, 2 mL of reaction buffer (TCA 20% C TBA 0.5%) were added and heated at 95°C for 30 min. The non-specific background absorbance reading at 600 nm was subtracted from the specific absorbance reading at 532 nm.

3.3.11. Statistical Analysis

The experiment was completely randomized, and every time measurements were separately subjected to a two-way ANOVA (Statgraphics Centurion for Windows, Statistical Graphics Corp.), where plant combinations and treatments were the factors of the analyses. After verifying the significance of the interaction for each variable (data not shown), a one-way ANOVA was performed by joining the plant combination and treatment. Means were compared by the Fisher's least significance difference (LSD) test at $P < 0.05$. There were no significant differences among replicates for each measured parameter.

3.4. Results

3.4.1. Gas Exchange Measurements

At 24 h after PEG addition, A_N dramatically dropped in the A plants, followed by the A/A plants compared to their control (**Fig. 3.1.A**), while plants A/A25 showed no significant differences between PEG treatment and the control. At the end of the experiment (7 DAT), all the plant combinations displayed significant differences with their control. The highest values went to the A/A25 control, followed by A/A25 PEG. The g_s values (**Fig. 3.1.B**) changed significantly from the beginning of the experiment in all the plant combinations, when low g_s values were recorded for plants A and A/A under PEG. Parameter C_i was higher in plants A and A/A under the PEG conditions on 4 DAT and 7 DAT (**Fig. 3.1.C**), but showed no significant differences for plants A/A25.

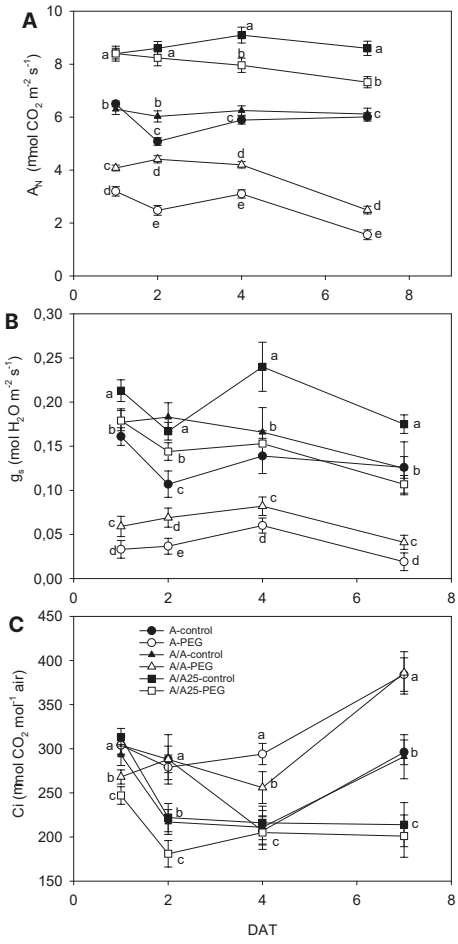

Figure 3.1. Net CO_2 assimilation rate (A_N; mmol CO_2 m^{-2} s^{-1}) (A); leaf stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) (B) and substomatal CO_2 concentration (C_i; mmol CO_2 mol^{-1} air) (C) in the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) at 0% PEG (control) or 5% PEG (water stress). Measurements were taken on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began). Data are the mean values for n = 12 ± SE. For each studied time, different letters indicate significant differences at $P < 0.05$ (LSD test).

3.4.2. Biomass Parameters

At the end of the experiment (7 DAT), root length (**Figure 3.2.A**), root DW (**Fig. 3.2.B**), and leaf DW (**Fig. 3.2.C**) decreased, with significant differences in the A and A/A plants exposed to stress compared with their control treatments. The A/A25 plants exposed to PEG underwent changes in root DW, with a significant decrease compared to the control plants, which was not the case for the other biomass parameters (root length and leaf DW). Under the control conditions, the biomass parameters were higher in the A/A25 plants for the root traits compared with plants A and A/A.

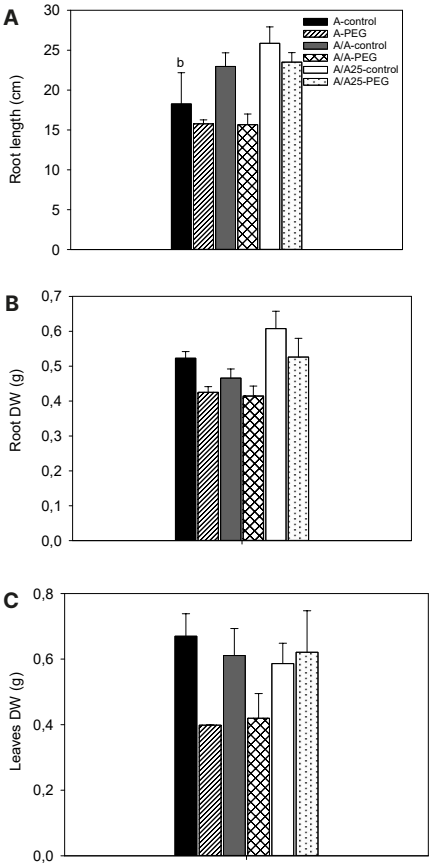

Figure 3.2. Root length (A), root dry weight (DW) (B), and leaf dry weight (C) in the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) at 0% PEG (control) or 5% PEG (water stress). Measurements were taken at the end of the experiment, 7 days after treatment with PEG began (7 DAT). Data are the mean values for n = 8 ± SE. For each studied time, different letters indicate significant differences at $P < 0.05$ (LSD test).

3.4.3. Hydric and Osmotic Relations

During the experiment, the pepper plants under the control conditions maintained a constant leaf RWC (**Fig. 3.3.**) above a value of 95%. The presence of PEG in the nutrient solution provoked a reduction in RWC from 2 DAT, which became more evident on 7 DAT for the A plants, followed by the A/A plants. The RWC in the A/A25 plants exposed to drought stress remained stable with similar values in the control plants during the experiment.

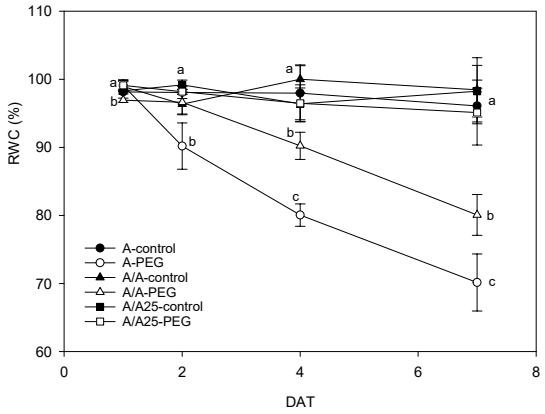

Figure 3.3. Effect of PEG addition at 0% (control) and 5% (water stress) on relative water content (RWC) on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began) in the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25). Data are the mean values for n = 4 ± SE. For each studied time, different letters indicate significant differences at P < 0.05 (LSD test).

The leaf water potential (Ψ_W) in the control plants did not show any significant difference for each measured time (**Fig. 3.4.**). The A plants under PEG showed changes in Ψ_W after 24 h after exposure to stress (1 DAT), which remained stable on 2 DAT and 4 DAT, when both had similar values. Then a sharp drop was observed on 7 DAT. In the A/A plants under the stress conditions, the drop in Ψ_W started on 2 DAT and reached a maximum decrease on 7 DAT. For the A/A25 plants under PEG, the Ψ_W values during the experiment were similar to the control plants.

The Ψ_s (**Fig. 3.5.**) lowered in relation to the exposure time to PEG. Within the first measured time frame (1 DAT), plants A and A/A displayed a drop in Ψ_s under the stress conditions, which was not found for plants A/A25. On 7 DAT, the maximal decrease was found for A, followed by plants A/A and A/A25.

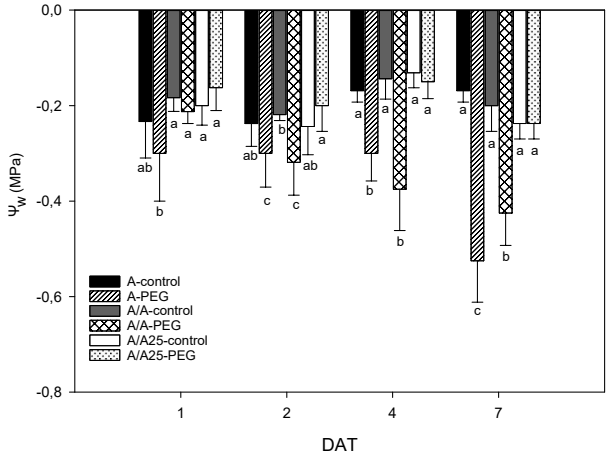

Figure 3.4. Leaf water potential (MPa) in the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) after addition of 0% PEG addition (control) and 5% PEG on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began). Data are the mean values for n = 4 ± SE. For each studied time, different letters indicate significant differences at P < 0.05 (LSD test).

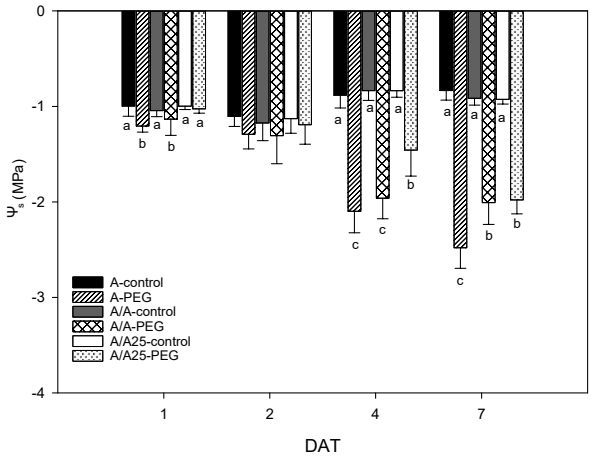

Figure 3.5. Leaf osmotic potential (MPa) in the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) after addition of 0% PEG (control) and 5% PEG on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began). Data are the mean values for n = 4 ± SE. For each studied time, different letters indicate significant differences at P < 0.05 (LSD test). The absence of letters on 2 DAT means no significant difference for both factors (plant and treatment).

3.4.4. Nitrate Reductase Activity

Nitrate reductase activity (NR) was higher in leaves (**Fig. 3.6.A**) than in roots (**Fig. 3.6.B**). In general terms, the opposite behavior was observed between both organs in activity terms during the experiment, with higher values in leaves that matched the lower values in roots for each time and plant combination. In leaves (**Fig. 3.6.A**), NR dramatically lowered 24 h after adding PEG to the A plants, but this decrease in the A/A plants was less marked compared with the A plants. On 2 DAT, the A/A plants underwent a sharp drop in NR activity under water stress, and the A/A25 plants underwent a reduction only on 7 DAT. In roots (**Fig. 3.6.B**), NR activity increased in all the pepper plant combinations under drought stress compared with the control plants from 1 DAT to 2 DAT. Afterward, activity lowered until 7 DAT, when the lowest values were obtained in the A/A25 plants, followed by A/A and finally A.

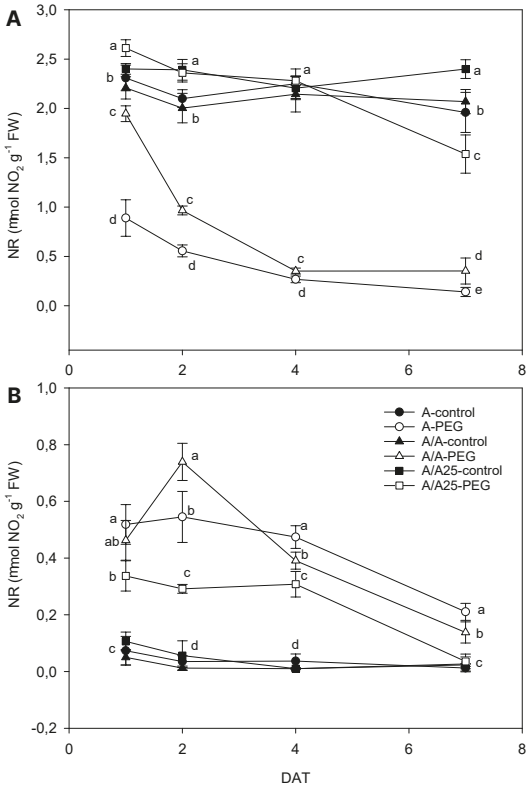

Figure 3.6. Nitrate reductase activity (NR) in the leaves (A) and roots (B) of the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) under the control conditions (0% PEG) and at 5% PEG on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began). Dates are the mean values for n = 4 ± SE. For each studied time, different letters indicate significant differences at P < 0.05 (LSD test).

3.4.5. DPPH-Radical Scavenging Activity

The leaves of the plants grown under drought stress displayed an increasing percentage of inhibition of the DPPH radical (**Fig. 3.7.A**). For the A plants under PEG, the increase was recorded earlier (1 DAT), whereas DPPH-RSA started from 4 DAT in A/A and A/A25. For the plants under drought stress on 7 DAT, maximum activity was found for the A plants followed by A/A, with minor activity for A/A25. DPPH-RSA was higher in leaves than in roots (**Fig. 3.7.**). In roots, major activity was exhibited on 1 DAT and 2 DAT in the plants exposed to PEG (**Fig. 3.7.B**). Afterward no significant differences were found in activity among treatments.

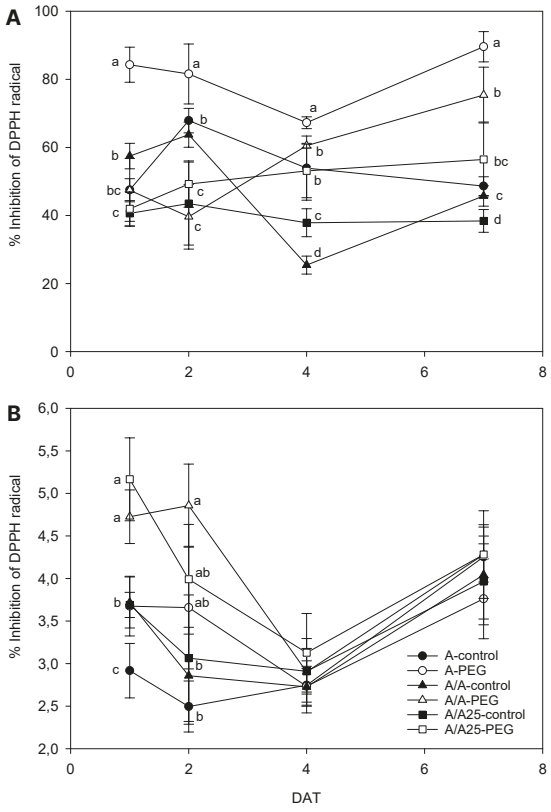

Figure 3.7. Percentage of inhibition of DPPH radical in the leaves (A) and roots (B) of the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) under the control conditions (0% PEG) and 5% PEG on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began). Data are the mean values for n = 4 ± SE. For each studied time, different letters indicate significant differences at P < 0.05 (LSD test). In (B), the absence of letters on 7 DAT means no significant difference for both factors (plant and treatment).

3.4.6. Total Phenolic Content

An increase in phenolic content was observed during the experiment in the leaves of the plants exposed to drought stress (**Fig. 3.8.A**). The most marked phenolic increase was found for plants A in the PEG treatment, with significant differences shown on 4 DAT and 7 DAT compared to the control plants. In roots (**Fig. 3.8.B**), phenolic contents were 10-fold lower than in leaves, and values were similar among treatments, except for the A/A plants under drought stress at 2 DAT, when a sharp drop was observed.

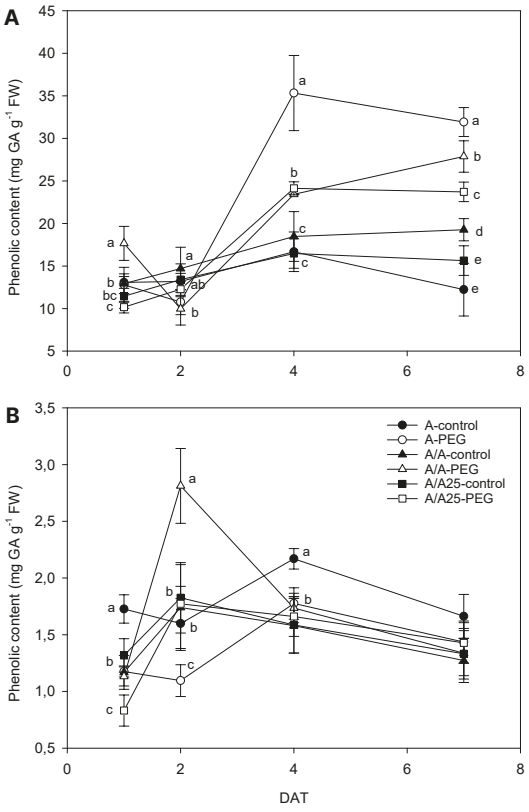

Figure 3.8. Changes in phenolic content in the leaves (A) and roots (B) of the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) under the control conditions (0% PEG) and 5% PEG on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began). Data are the mean values for n = 4 ± SE. For each studied time, different letters indicate significant differences at $P < 0.05$ (LSD test). In (B), the absence of letters on 7 DAT means no significant difference for both factors (plant and treatment).

3.4.7. H_2O_2 Concentration

The hydrogen peroxide level in leaves (**Fig. 3.9.A**) increased after exposing plants to drought stress from 4 DAT to 7 DAT, which was emphasized mainly in plants A and A/A with significant differences compared to the control plants. In roots (**Fig. 3.9.B**), the H_2O_2 concentration was approximately 10-fold lower than in leaves. The maximum concentrations for all the plant combinations were recorded on 2 DAT. At the end of the experiments, no significant differences were observed between plants and treatments.

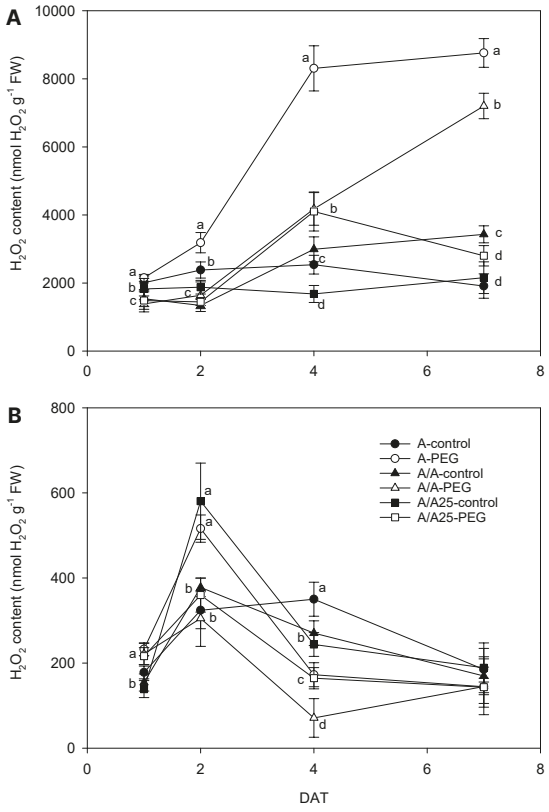

Figure 3.9. Hydrogen peroxide concentration in the leaves (A) and roots (B) of the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) under the control conditions (0% PEG) and 5% PEG on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began). Data are the mean values for n = 4 ± SE. For each studied time, different letters indicate significant differences at $P < 0.05$ (LSD test). In (B), the absence of letters on 7 DAT means no significant difference for both factors (plant and treatment).

3.4.8. Lipid Peroxidation

The MDA concentration in leaves increased with time from 2 DAT (**Fig. 3.10.A**). As a result, the highest MDA levels were found in the A plants, followed by the A/A plants under drought stress, and the A/A25 plants on 7 DAT. In roots (**Fig. 3.10.B**), lipid peroxidation increased and followed this trend in leaves during the experiment. On 7 DAT, the highest values were recorded for pepper plants A and A/A under drought stress.

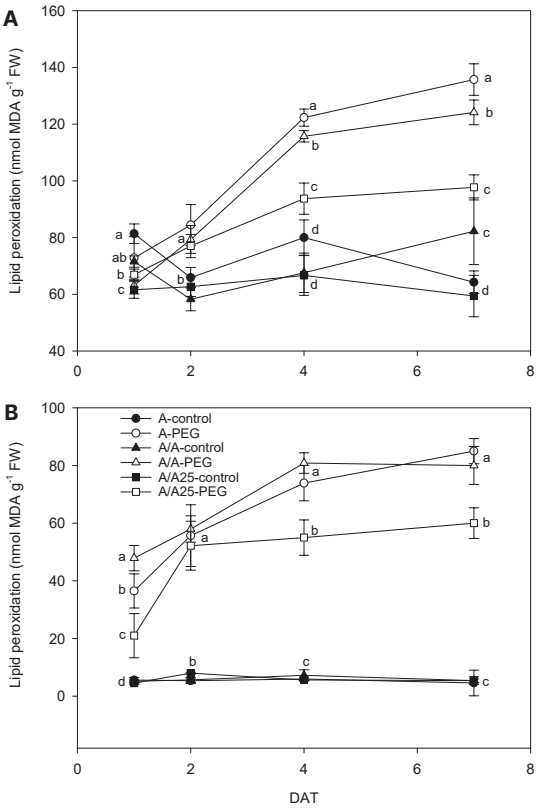

Figure 3.10. Malondialdehyde content (MDA) of the leaves (A) and roots (B) of the non-grafted pepper plants (cultivar Adige, A), self-grated plants (A/A), and plants grafted onto A25 (A/A25) under the control conditions (0% PEG) and 5% PEG on 1 DAT, 2 DAT, 4 DAT, and 7 DAT (days after treatment with PEG began). Data are the mean values for n = 4 ± SE. For each studied time, different letters indicate significant differences at P < 0.05 (LSD test).

3.5. Discussion

In this experiment, we observed numerous changes in the biochemical and physiological processes during the short drought stress treatment, which were provoked by the addition of 5% PEG. These processes were involved in the perception and transduction of stress in the scion depending on the employed rootstock. We found that pepper plants with the A25 rootstock (A/A25) displayed greater tolerance to drought stress in this short-term experiment, as indicated by the effects on the scion in terms of biomass conservation, photosynthesis and RWC maintenance, lower lipid peroxidation and greater NR activity in leaves compared with the non-grafted and self-grafted pepper plants. The fact that the scion (A) suffered less drought stress was related to the better tolerance of root parts (A25), which encompassed the greater vigor root system and the lower H_2O_2 concentration, lipid peroxidation and NR activity. These results indicated that scion performance is largely dependent on the tolerance of roots to drought stress, although the root to shoot communication involved in scion pepper stress responses is largely unknown.

In whole-plant terms, the effect of abiotic stresses is usually noticed as a reduction in photosynthesis and growth, and is associated with alterations in carbon and nitrogen metabolism (Loggini *et al.*, 1999; García-Mata and Lamattina, 2001). To support this idea, in our experiment drought stress negatively affected the aboveground mass production in the non-grafted and self-grafted pepper plants. The least biomass loss was found in the pepper plants of the Adige variety, grafted onto the A25 rootstock, which have been previously defined as tolerant, while Adige has been described as drought-sensitive (Penella *et al.*, 2017). Different drought stress effects were observed for the growth parameters in both roots and leaves; the growth inhibition of roots (DW) was lower (a reduction of 19% in A, 11% in A/A and 13% in A/A25 compared to their controls) versus leaves at a low osmotic potential of the solution (a reduction of 40% in A, 31% in A/A and a non-effect in A/A25). This result indicated that leaf growth was more sensitive to drought stress compared to roots, except for A/A25 where the effect of PEG was not detected in leaves. This differential response in the growth inhibition between roots and leaves under drought stress (in our results for plants A and A/A) has been observed by several authors (Westgate and Boyer, 1985; Sharp *et al.*, 1994; Hsiao and Xu, 2000). It resulted in a sudden reduction of Ψ_W in roots, which allowed water to enter. Then solutes had to enter to prevent dilution and maintain the osmotic forces needed for growth. On the contrary in leaves, osmotic adjustment occurs by slowly limiting their expansion (Hsiao and Xu, 2000). Beyond the biophysical aspect, there is compelling evidence that ABA accumulation under drought stress plays a pivotal role by inhibiting shoot growth, as in plants A and A/A, while a minor effect is observed in roots (Sharp *et al.*, 1994). Nevertheless, the behavior of plants A/A25 did not match

these results. Currently, we have no plausible explanation for this. All these results suggest that scion growth is largely dependent on rootstock tolerance to drought stress.

The growth reductions mediated by drought stress evidence a series of changes in various biochemical processes, such as photosynthesis (Urban *et al.*, 2017). Net CO_2 assimilation decreased suddenly 24 h after adding PEG to A, followed by plants A/A, while A/A25 maintained similar A_N values to the control up to 48 h (2 DAT); afterward A_N significantly lowered until the end of the experiment, but values were higher in A/A25 than in A and A/A under PEG, with sustained photosynthetic activity during drought (Rouphael *et al.*, 2008; Penella *et al.*, 2014b). These results agree with previous findings in which grafting onto a tolerant rootstock improved the photosynthesis performance of plants under drought stress (Schwarz *et al.*, 2010; Penella *et al.*, 2014b). In A/A25, the decrease in A_N on 4 DAT and 7 DAT was accompanied by a significant decrease in stomatal conductance, although the decline in g_s occurred earlier than the reduction in CO_2 fixation (1 DAT and 2 DAT). We could assume that stomata closure probably did not limit CO_2 acquisition by leaves under drought stress within the first two time frames (Delfine *et al.*, 2002). The decrease and the subsequent maintenance of the intercellular CO_2 concentration (C_i) in A/A25 under drought stress (compared to the A/A25 control plants) implied that stomatal limitations were responsible mainly for the reduction in A_N by drought stress (Delfine *et al.*, 2002; Rouphael *et al.*, 2008). In plants A and A/A under drought stress, the drastic decline of A_N was in line with the strong stomata closure from the beginning to the end when time measurements were taken (more marked in the A plants). However, C_i suddenly lowered on 1 DAT, 2 DAT, and 4 DAT, with a significant increase at the end of the experiment compared to the controls, which implies the existence of stomata and non-stomatal limitations related with changes in the cellular carbon metabolism, which can affect the growth mediated by reducing the biochemical capacity for carbon assimilation and utilization (Flexas *et al.*, 2004; Reddy *et al.*, 2004; He *et al.*, 2009). All this was true, except for plants A/A25, which showed no significant differences between plants under the control and PEG conditions (7 DAT).

Apart from the discussed changes in carbon assimilation, drought stress may affect several nitrogen metabolism stages (Feller and Vaseva, 2014; Penella *et al.*, 2014b) by inducing visible effects on biomass. One important step is the assimilation of nitrate into organic compounds. The activity of the first enzyme involved in this process, NR, was negatively influenced by drought; nitrogen assimilation is also coordinated with carbon assimilation as photosynthesis is required for NR activation (Kaiser and Huber, 2001). Nitrate reduction is sensitive to stomatal resistance and g_s decreases in plants under drought stress to preserve water loss when not only A_N drops, but NR also becomes less active (Kaiser and Huber, 2001; Yousfi *et al.*, 2012; Penella *et al.*, 2014b). In relation to this coordination between NR activity and A_N, we observed a quick decrease in NR of the A plant leaves 24 h after inducing drought stress, and after 48 h in the A/A plants, while the A/A25 plants preserved their activity until 4 DAT. At

the end of the experiment, the A plants, followed by A/A, did not show any NR activity and g_s dropped, while A/A25 maintained 64% enzyme activity and sustained g_s compared to the control. Some nonstomatal effects, such as reduced nitrate availability in plants, could inhibit NR gene transcription and decrease the stability of NR-mRNAs or post-translational factors, including inactivation through protein phosphorylation, and the induction of proteases could rapidly occur by decreasing NR activity as a result of drought stress (Ferrario *et al.*, 1995; Lillo *et al.*, 2004; Correia *et al.*, 2005). In most herbaceous plants, NO_3^- assimilation takes place predominantly in leaves (Scheurwater *et al.*, 2002). Accordingly, NR activity was greater in leaves than roots in all the plant combinations and treatments in our study. Nevertheless, the greatest root-NR activity was observed under drought stress, and maximum activities were displayed for the A plants, followed by the A/A plants, where lower leaf NR seemed to be partly compensated by an increase in root NR (Lexa and Cheeseman, 1997; Penella *et al.*, 2014b). Drought stress can decrease nitrate uptake by roots. Besides, the transfer of NO_3^- to leaves can be limited by stomata closure. Thus transpiration (data not shown) diminished as part of nitrate can reduce in roots. This happened in plants A and A/A as A/A25 had a higher root NR on 1DAT and 2DAT, be it to a lesser extent, because the higher g_s allowed NO_3^- transport to leaves for its reduction. This role of NR in leaves and roots under drought stress has been observed in grafted pepper (Penella *et al.*, 2014b), pea (Lexa and Cheeseman, 1997) or wheat plants (Yousfi *et al.*, 2012).

The decrease in Ψ_s during the PEG treatment could be a consequence of less water content in tissue and/or through the active osmotic adjustment involving the net accumulation of a range of osmotically active molecules in response to a drop in the Ψ_W in their environment. In pepper, short-term exposure with different osmotic potentials of the nutrient solution (Navarro *et al.*, 2003; Martínez-Ballesta *et al.*, 2004; Silva *et al.*, 2008) showed that the decrease in Ψ_W was not compensated by a reduction in Ψ_s and, as a result, the osmotic adjustment was negligible. According to our results, the decrease in the leaves of Ψ_W, Ψ_s and stomatal conductance in the PEG treatment showed that water uptake could not balance water loss and decreased RWC, along with there being fewer biochemical functions in A and A/A. This could indicate that osmotic adjustment was insignificant. Nevertheless, RWC was maintained in A/A25 with PEG addition, which could indicate an osmotic adjustment at the end of the experiment, when Ψ_W remained constant and Ψ_s decreased. Similar results have been obtained by Penella *et al.* (2014b). A deep root system and higher root biomass have shown as beneficial effects for acquiring water (Koevoets *et al.*, 2016), and could be one of the reasons for the unchanged RWC values in the A/A25 plants noted throughout the experiment. They suggest a typical conservative water strategy (Tardieu and Simonneau, 1998; García-Sánchez *et al.*, 2010; Sade *et al.*, 2012; Penella *et al.*, 2016). This would lead to more drought tolerance and would allow photosynthesis preservation, improved absorption, upward transfer and NO_3^- accumulation in leaves. Similar results have been shown in the susceptible tomato scion "Josefina" grafted onto drought stress-tolerant

"Zarina" rootstocks (Sánchez-Rodríguez *et al.*, 2013). However, the acclimation rate and drought stress duration are key factors that can influence depending on plant varieties and/or age.

This limited water uptake and CO_2 uptake in plants A and A/A under PEG conditions accelerated oxidative damage by producing ROS (Asada, 1999). Hydrogen peroxide is one type of ROS produced as a result of the dismutation of the superoxide radical, and a higher concentration damages both the cell and the whole plant to result in lipid peroxidation and membrane injury (Sairam and Srivastava, 2001). In the non-grafted tomato plants (Rivero *et al.*, 2003b), a major increase in H_2O_2 was observed under thermal shock stress compared with the grafted plants. In A, and to a lesser extent in the A/A pepper plants, drought stress caused excessive H_2O_2 accumulation in leaves, as well as high lipid peroxidation compared with the A/A25 plants and their controls. This result suggests that H_2O_2 in leaves is largely dependent on the adaptability of roots to drought stress. This effect has been observed in cucumber plants grafted onto fig leaf gourd (*Cucumis ficifolia*) or onto luffa (*Luffa cylindrical*), where a significantly low H_2O_2 concentration alleviated membrane lipid peroxidation at high temperature (Li *et al.*, 2014) compared with non-grafted plants. The authors attributed this reduction to increased CO_2 assimilation and ROS-scavenging activity. In roots, the maximum H_2O_2 concentration was observed on 2 DAT for the A-PEG and A/A25-control plants, but its concentration was 10-fold lower than in leaves. Afterward, levels lowered in this organ, whose concentration in leaves was "amplified." As a result of increasing H_2O_2, the MDA concentration was seen to be enhanced in both roots and leaves, with less lipid peroxidation damage in roots. A lower H_2O_2 concentration in A/A25 could be due to less ROS production or to the more efficient detoxification of this compound (Rivero *et al.*, 2003b). Antioxidant activity was evaluated by the effect of the extracted samples on the DPPH radical. Under the PEG conditions and at the end of the experiment, plants A/A25 showed the least radical scavenging activity, which indicates that the alleviation of oxidative stress occurred to a lesser extent in the A/A25 plants compared with the greatest activity in the A plants, followed by the A/A plants. In response to drought stress, plants can accumulate a wide range of antioxidants, including phenolic compounds (Keleş and Öncel, 2002). Phenolic compounds exhibit antioxidant activity by inactivating lipid free radicals or preventing the decomposition of hydroperoxides into free radicals (Pokorny, 2001). Our results showed that with PEG addition, the synthesis of phenols increased, but the most marked rise took place in the A plants, followed by A/A and A/A25. Even though the stimulation of antioxidant activity in plants A and A/A occurred simultaneously with higher phenol concentrations under drought conditions, an imbalance between ROS generation and scavenging systems might have occurred as the highest H_2O_2 and MDA levels was confirmed.

According to these results, grafting itself (A/A plants) has a slightly positive effect on the physiological parameters measured under drought stress compared with the A plants, probably due to enhanced endogenous hormone production as a result of the

grafting per se incision, which may influence the transport of hormones between roots and the scion that, in turn, could alter weak responses (Aloni *et al.*, 2008; Savvas *et al.*, 2011; Orsini *et al.*, 2013).

3.6. Conclusion

To conclude, our results suggest that plants A/A25 were more tolerant to drought stress given the response made in several physiological processes in the short term, which were maintained for 7 days under water stress, and even beyond this time given better fruit production (Penella *et al.*, 2017). Growth preservation in plants A/A25 after PEG addition was associated with maintained CO_2 assimilation and partly open stomata, which allowed water uptake and preserved RWC. The conservative water strategy involved minor oxidative stress as demonstrated by the lower H_2O_2 concentration and diminished membrane lipid peroxidation. These results could be attributed to the capacity to maintain shoot growth by the root system's conservative tolerance traits under drought stress. Consequently, the grafting of commercial cultivars onto drought-tolerant rootstock(s) such as A/A25 can be considered a valid strategy to improve drought stress tolerance. Nevertheless, other mechanisms, like the hormone signalling cascade (Cantero-Navarro *et al.*, 2016) or the mobility of genetic components (Haroldsen *et al.*, 2012), which were not contemplated herein, could also explain the improved drought tolerance of these grafted plants, and should be studied in future works.

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Chapter 4

Physiological characterization of a pepper hybrid rootstock designed to cope with salinity stress

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4.1. Abstract

In pepper crops, rootstocks that tolerate salt stress are not used because available commercial rootstocks offer limited profits. In this context, we obtained the hybrid NIBER®, a new salinity-tolerant rootstock that has been tested under real salinity field conditions for 3 years with 32%-80% higher yields than ungrafted pepper plants. This study aimed to set up the initial mechanisms involved in the salinity tolerance of grafted pepper plants using NIBER® as a rootstock to study root-shoot behavior, a basic requirement to help develop efficient rootstocks. Gas exchange, Na^+/K^+, total antioxidant capacity, nitrate reductase activity, ABA, proline, H_2O_2, phenols, MDA concentration and biomass were measured in ungrafted plants of cultivar Adige (A), self-grafted (A/A), grafted onto NIBER® (A/N) and reciprocal grafted plants (N/A), all exposed to 0 mM and 70 mM NaCl over a 10-day period. Salinity significantly and quickly decreased photosynthesis, stomatal conductance and nitrate reductase, but was lower in A/N plants compared to A, A/A and N/A. A/N plants showed decreases in the Na^+/K^+ ratio, ABA content and lipid peroxidation activity. This oxidative damage alleviation in A/N was probably due to an enhanced H_2O_2 level that activates antioxidant capacity to cope with salinity stress, and acts as a signal molecule rather than a damaging one by contributing a major increase in phenols and, to a lesser extent, in proline concentration. These traits led to a minor impact on biomass in A/N plants under salinity conditions. Only the plants with the NIBER® rootstock controlled the scion by modulating responses to salinity.

Additional keywords:
antioxidant capacity; graft; H_2O_2;
pepper; photosynthesis; rootstock

4.2. Introduction

New scenarios due to climatic change are affecting crop yield and quality. In this context, salinity is one of the most important environmental factors that limits plant growth, productivity, quality and the increasing demand for food crops (Ashraf, 2004; Srivastava and Kumar, 2015). More than 20% of cultivated land worldwide is affected by salt stress and this amount is increasing daily (Srivastava and Kumar, 2015). At the same time, the global population is expected to reach 9 billion by 2050. Thus increasing of agriculture productivity will be needed to meet food demands (Shelden and Roessner, 2013). To achieve the increased food production under salinity conditions, it is necessary to identify naturally occurring genetic variations within a crop species by screening varieties, wild genotypes and landraces that could provide salt tolerance (Roy *et al.*, 2011).

Pepper is an important crop that grows in most countries on our planet, and covers 1.93 million ha of crop-growing surface area (Penella and Calatayud, 2018). As a spice and fruit, the world's pepper production was 34 million tons in 2017 (Penella and Calatayud, 2018). Generally speaking, commercial pepper varieties need friable, well-drained, sandy loam soil with a pH of 6.5–7.5 for optimum production. Salt content in soil and irrigation water should be low. There are reports of a salinity resistance threshold of 1.5 dS m^{-1}, below which no effect on growth occurs, and a 14% drop in biomass production per additional 1 dS m^{-1} has been reported (Maas, 1973). Pepper and *Capsicum annuum* species in particular are highly susceptible to salt stress by showing blossom end rot (BER), lower yields and more unmarketable fruits (Penella *et al.*, 2015). Physiological changes have been analyzed in pepper under salt stress like membrane permeability and water channel activity alterations, ion imbalance, reduced total photosynthesis and stomatal conductance, and increasing reactive oxygen species production, which modify the carbon balance required to maintain both productivity and growth (Penella and Calatayud, 2018).

To minimize salinity damage in pepper crops, graft technology is an agronomic practice that can improve plant tolerance by using rootstocks capable of reducing the negative effect of external stress on the scion. In addition, grafted plants can avoid the problem associated with the "building or design" of tolerant varieties due to complexity of salinity traits and lack of practical selection tools; one example is genetic markers, which have made these tasks slow and inefficient (Flowers, 2004; Ashraf and Foolad, 2007; Schwarz *et al.*, 2010). Grafting can combine suitable commercial fruit quality characteristics and high production of a scion and tolerance traits to environmental factors from rootstock by working together like a single plant. Nevertheless, rootstocks that tolerate salt stress are not used in pepper plants because available commercial rootstocks offer limited profits (Lee *et al.*, 2010; Penella *et al.*, 2013; Kyriacou *et al.*, 2017).

There is a need to perform rigorous screenings to find *Capsicum* plants that tolerate salt stress so they can be used as pepper rootstocks. In this context, we screened physiological and phenotypically characterized accessions of pepper from gene banks before selecting those for their tolerance to salinity and then using them as rootstocks in grafted pepper plants (Penella *et al.*, 2013, 2015; López-Serrano *et al.*, 2017; Penella and Calatayud, 2018). The obtained results have allow to confirm that the tolerance to salinity of these grafted plants was expressed by maintaining scions presenting better physiological performance and, consequently, by increasing yields (Penella *et al.*, 2015, 2016, 2017). Afterward, a classic breeding program was applied to salinity-tolerant pepper accessions (*C. annuun* x *C. annuun*) have allowed obtain more uniform hybrids in terms of germination, growth and highest vigor to be used as rootstocks under salinity conditions. One of them, NIBER®, has been tested under real salinity field conditions for several years (Calatayud *et al.*, 2016) with higher yields (range of 32%-80%) than ungrafted plants and other commercial pepper rootstocks.

The aim of the present work was to evaluate the early physiological response of a tolerant rootstock under salt stress conditions using the hybrid NIBER®. To date, information about the initial mechanisms involved in the tolerance to of grafted pepper plants remains limited. The initial evaluation of root-shoot to physiological evolution is a basic requirement to help develop improved efficient rootstocks with the ability to cope with salinity and to ensure a better understanding of the response mechanisms of grafted pepper plants to imbalanced salinity.

To fulfill this objective, we compared the relative tolerance responses of ungrafted, self-grafted, grafted and reciprocal grafted pepper plants under both control and salinity conditions. Gas exchange, proline, phenols, hydrogen peroxide, radical scavenging capacity and nitrate reductase activity were measured in the leaves of all the pepper plants combinations. Na^+/K^+, Cl^- concentration and ABA levels were determined in both leaves and roots; in addition we analyzed the growth to acquire information to identify the mechanisms by which the NIBER® rootstock enhances tolerance to salinity.

4.3. Material and methods

4.3.1. Plant material

A new hybrid pepper salinity-tolerant rootstock, NIBER® *(Capsicum annuum x C. annuum)* (abbreviated herein as N), and the salt-sensitive pepper cultivar 'Adige' (abbreviated as A) (Lamuyo type, Sakata Seeds, Japan), were used as either a scion or rootstock. Four plant combinations were herein used: ungrafted A plants (A), self-grafted A plants (A/A), A grafted onto N (A/N) and N grafted onto A (N/A). The seeds of A and N were sown in 96 seedling trays filled with a peat-based substrate for germination early in March. After 2 months, the grafted plant combinations were performed by the tube-grafting method (Penella *et al.*, 2015). They were maintained in a chamber with relative humidity above 95% and air temperature around 28-29 °C for a 4-6 day period (Penella *et al.*, 2014). The grafted plants were then removed from the humidity chamber and placed in a greenhouse until transplanted. The ungrafted (A) plants were sown 2 weeks later to obtain plants with a similar biomass to that of the grafted plants upon transplantation (10-12 development leaves). The plants obtained by the above-mentioned procedure were utilized in greenhouse experiments at the end of May.

4.3.2. Hydroponic greenhouse experiment

The root systems of the plants were washed to clean the substrate and plants were placed in 5 L polyethylene pots, which were previously covered with aluminum sheets. Pots were filled with a standard nutrient solution for pepper (Sonneveld *et al.*, 1994) containing (in mmol L^{-1}): 12.3 NO_3^-, 1.02 H_2PO_4, 2.45 SO_4^{2-}, 3.24 Cl^-, 0.6 NH_4^+, 5.05 K^+, 4.23 Ca^{2+}, 2.55 Mg^{2+}, 2.2 Na^+ and micronutrients (15.8 µM Fe^{2+}, 10.3 µM Mn^{2+}, 4.2 µM Zn^{2+}, 43.5 µM B^+, 2.14 µM Cu^{2+}), which were artificially aerated with an air pump. The electrical conductivity (EC) and pH of this nutrient solution were 1.7 dS m^{-1} and 6.5, respectively. Nutrient solution was added daily to compensate for uptake. After of leaving seedling plants for 7 days to acclimatize to pots, the salinity treatment was initiated by adding NaCl (70 mM) to the nutrient solution to obtain an EC of 8.5 dS m^{-1} and a pH of 6.1.

While the experiment was underway, plants were grown in a Venlo-type greenhouse under natural light conditions (610-870 mmol m^{-2} s^{-1}). Temperature and relative humidity ranges were 21-25ºC and 52-72%, respectively.

The layout was a completely randomized design with four replications of six plants per combination (A, A/A, A/N and N/A).

All the physiological measurements were taken in days 1, 2, 4, 7 and 10 after the salt treatment (DAT) had started, except for ABA concentration and nitrate reductase activity, which were measured on 1DAT and 10DAT, and ion determination on 10DAT. Measurements were taken in fully and expanded mature leaves (3^{rd}-4^{th} leaf from the shoot apex) and in lateral roots for Na^+/K^+, Cl^- and ABA. They were taken in random

order in three plants per replication (12 measurements per plant combination and treatment) for the gas exchange measurement, and in four plants (1 plant per replication) in the other analysis of the physiological parameters.

4.3.3. Ion determination

Leaves and roots were dried in a laboratory oven at 70°C for 72 h before being burnt in a muffle furnace for 12 h at 550ºC. Ions were extracted with 2% nitric acid in an ultrasonic bath for 30 min at 40ºC. The Na^+ and K^+ concentrations were measured by ICP emission spectrometry (iCAP 6000, Thermo Scientific, Cambridge, UK). The chloride concentration (Cl^-) in the dry plant material was extracted with 0.1 N HNO_3 in 10% (v/v) acetic acid and was determined by potentiometric titration with $AgNO_3$ in a chloride analyzer (Sherwood, MKII 926, Cambridge, UK).

4.3.4. Gas exchange measurements

The CO_2 assimilation rate (A_N, µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) were determined on fully expanded leaves (3^{rd}-4^{th} leaf from the apex) in the steady state under saturating light conditions (1000 µmol m^{-2} s^{-1}) and with 400 ppm CO_2 by a LI-6400 infrared gas analyzer (LI-COR, Nebraska, USA) at 24ºC (24 ± 2ºC) and 65% relative humidity (65 ± 10%). The gas exchange measurements were taken from 9 am to 11 am (GMT).

4.3.5. Abscisic acid analysis

The thoroughly ground leaves and roots (about 0.1 mg fresh weight) on 1DAT and 10DAT were suspended in 80% methanol-1% acetic acid containing internal standards, and were mixed by shaking for 1 h at 4ºC. The extract was kept a 20ºC overnight and was then centrifuged. The supernatant was dried in a vacuum evaporator. The dry residue was dissolved in 1% acetic acid and passed through a reverse phase column (HLB Oasis 30 mg, Waters), as described in Seo *et al.* (2011). The final residues were dried and dissolved in 5% acetonitrile-1% acetic acid and hormones were separated by UHPLC with a reverse Accucore C18 column (2.6 µm, 100 mm long; Thermo Fisher Scientific) with a 2-55% acetonitrile gradient containing 0.05% acetic acid at 400 µL/min for 21 min.

Abscisic acid (ABA) was analyzed by a Q-Exactive mass spectrometer (Orbitrap detector, Thermo Fisher Scientific) by targeted Selected Ion Monitoring (tSIM; capillary temperature 300ºC, S-lens RF level 70, resolution 70.000) and electrospray ionization (spray voltage 3.0 kV, heater temperature 150ºC, sheath gas flow rate 40 µL/min, auxiliary gas flow rate 10 µL/min) in the negative mode.

The concentration of ABA in the extracts were determined using embedded calibration curves and the Xcalibur 4.0 and TraceFinder 4.1 SP1 programs. The internal standards for the quantification of all the different plant hormones were deuterium-labeled hormones.

4.3.6. Nitrate reductase activity

Nitrate reductase activity (NR) in leaves (Enzyme Code 1.7.1.1) was determined *in vivo* on 1DAT and 10DAT following the methods described by Hageman and Hucklesby (1971) and Jaworski (1971). Discs (1 cm in diameter) were collected from mature fresh leaves. Samples (0.2 g of fresh weight) were suspended in plastic vial containing 10 mL of 100 mM potassium phosphate buffer (pH 7.5), 1% (v/v) n-propanol and 100 mM KNO_3. Plant samples were incubated in a water bath at 30°C for 60 min in the dark and placed in a boiling water bath for 5 min to stop the enzymatic reaction. The nitrite released from the plant material was determined colorimetrically at 540 nm (spectrophotometer PerkinElmer, Lambda 25) by adding 0.02% (w/v) N-Naphthyl-ethylenediamine and 1% (w/v) sulfanilamide. A standard curve with KNO_2 was prepared to calculate the amount of NO_2 contained in the samples. NR activity was expressed as a percentage of NR in the salt treatment *versus* the control on 1DAT and 10DAT.

4.3.7. Proline determination

Proline content was determined as described by Bates *et al.* (1973). Dry leaves (20 mg) were ground in 3% sulfosalicylic acid, the homogenate was filtered, and glacial acetic acid and ninhydrin reagent were added to an aliquot of the filtrate. The reaction mixture was boiled at 100ºC for 1 h, and readings were taken in a spectrophotometer at a wavelength of 520 nm.

4.3.8. Total phenolic content

Total phenolic content was determined according to Koç *et al.* (2010) with modifications. Fresh leaf samples (0.1 g) were frozen in liquid nitrogen and stored at -80°C. They were mixed with 1.5 mL of extraction solution (50% (v/v) methanol and 1% (v/v) HCl). Samples were extracted in a boiling bath at 80°C for 15 min. Next 0.02 mL of the leaf extracts (diluted in 0.08 mL extraction solution) were mixed with 0.7 mL of Folin–Ciocalteu solution (Sigma-Aldrich®), and diluted at the proportion of 1:10, and 0.7 mL of 6% (w/v) Na_2CO_3. Samples were incubated at room temperature and in the dark for 1 h before being subjected to absorbance measurements at 765 nm. Gallic acid was used as a standard.

4.3.9. Hydrogen peroxide determination

H_2O_2 content was determined according to Sergiev *et al.* (1997) and Velikova *et al.* (2000) with slight modifications (López-Serrano *et al.*, 2019). First 0.25 g of fresh leaves was frozen in liquid nitrogen and kept at -80°C. Samples were ground in a mortar and 2 mL of 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 g and 4°C for 8 min. Then 0.4 mL of the supernatant was diluted with 0.6 mL of 0.1% (w/v) TCA. Finally, 0.5 mL of 100 mM potassium phosphate buffer (pH= 7) and 2 mL of 1 M of KI were added. Samples were incubated for 1 h at room temperature and in the dark and absorbance was measured at 390 nm. H_2O_2 content was given by a H_2O_2 standard curve.

4.3.10. Lipid peroxidation determination

Lipid peroxidation was estimated by malondialdehyde (MDA) determinations using a thiobarbituric acid reaction, according to the protocol reported by Heath and Packer (1968) and modified in Dhindsa *et al.* (1981). First 0.1 g of fresh leaves was frozen in liquid nitrogen and kept at -80°C. Samples were ground in a mortar and 2 mL of 0.1% (w/v) TCA. Later the homogenate was centrifuged at 10,000 g and 4°C for 5 min. Afterward, 2 mL of reaction buffer (TCA 20% + TBA 0.5%) were added and heated at 95°C for 30 min. The non specific background absorbance reading at 600 nm was subtracted from the specific absorbance reading at 532 nm.

4.3.11. DPPH radical-scavenging capacity

Radical scavenging capacity (RSA) was determined by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, proposed by Brand-Williams *et al.* (1995) with modifications. Namely, 0.1 g of fresh leaves was frozen in liquid nitrogen and stored at -80°C. Samples were ground in a mortar with the addition of 80% (v/v) methanol. After 12 h at 4°C in a mixer, samples were centrifuged for 10 min at 10,000 g and 4°C. A 10-µL volume of sample and 990 µL of 0.065 mM DPPH were taken and incubated for 30 min in the dark at room temperature. Absorbance was measured at 515 nm. The percentage of the inhibition of the DPPH radical was measured by this equation: [(DPPH absorption – Sample absorption)/ DPPH absorption] x 100 (López-Serrano *et al.*, 2019).

4.3.12. Biomass measurements

Roots and stems length and total dry weight of biomass (roots+leaves+stems) were measured at the end of the experiment (10DAT). The plants were dried at 65º C for 72 h to determine dry weight.

4.3.13. Statistical analysis

The results were subjected to a two-way ANOVA analysis (Statgraphics Centurion for Windows, Statistical Graphics Corp.) with treatment and plant combinations used as factors of the analyses. Each time of measurement (DAT) was separately analyzed. In all the parameters where the interaction was significant, the plant combinations and treatment were analyzed together by a one-way ANOVA. In the case of biomass parameters (root length, stem length and total dry biomass), interaction was not significant, but the genotype was, so a one-way ANOVA was performed separating both treatments. In the case on nitrate reductase, since the values were referenced by the percentage of salt with respect to control, one-way ANOVA considering only the plant combinations was carried out. Means were compared by the Fisher's least significance difference (LSD) test at $P < 0.05$. No significant differences were found among the four replicates for each measured parameter.

4.4. Results

4.4.1. Ions determination

The Na^+/K^+ ratio at the end of experiment (10DAT) was higher in roots than in leaves for all the plant combinations and treatments (**Fig. 4.1.**). In leaves, Na^+/K^+ significantly decreased ($P<0.05$) in the A/N plants under salt applications (**Fig. 4.1.A**). Under the control conditions of leaves, A/N showed a decrease with significant differences with N/A (**Fig. 4.1.A**). In the root compartment (**Fig. 4.1.B**), the Na^+/K^+ values increased in all the plant combinations under salt treatment. Na^+/K^+ were significantly higher in the ungrafted (A) and N/A plants, and the lower values were measured in A/N with significant differences. In the control treatment, all grafting combinations had significantly lower Na^+/K^+ values compared to the ungrafted plants (**Fig. 4.1.B**).

The Cl^- concentration in both leaves and roots (**Fig. 4.1.C-D**) increased with NaCl addition in all the plant combinations, although the Cl^- concentration was higher in roots. The highest Cl^- levels in leaves were obtained for N/A plants (**Fig. 4.1.C**), whereas no significant differences were found among all the plant combinations in roots (**Fig. 4.1.D**). In the control treatment, no significant differences appeared among the plant combinations in both leaves and roots (**Fig. 4.1.C-D**).

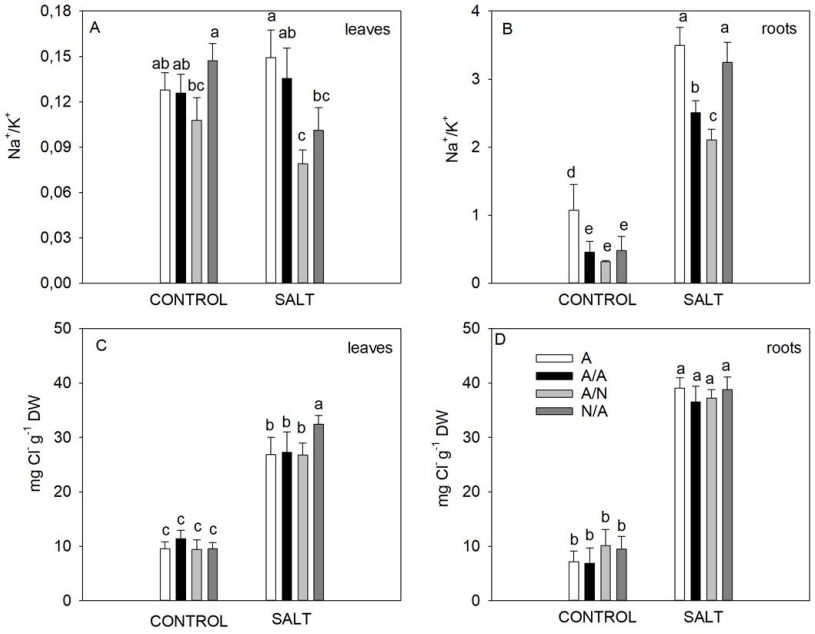

Figure 4.1. The Na^+/K^+ ratio (A, B) and Cl^- concentration (C, D) in the leaves and roots of ungrafted pepper plants (cultivar Adige, A), self-grafted (A/A), A grafted onto N (A/N) and N grafted onto A (N/A) after addition of NaCl at 0 mM (Control) and 70 mM (Salt) for 10-day exposures. Data are the mean values for n=4. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

4.4.2. Photosynthesis rate and stomatal conductance

Figs. 4.2. and **4.3.** showed the changes along the experiment for A_N (**Fig. 4.2.A-D**) and g_s (**Fig. 4.3.A-D**) under control and salt treatment. The photosynthetic rate (**Fig. 4.2.**) significantly dropped in all the plants ($P < 0.05$) in response to salt stress and reached null values at the end of experiment, except for A/N (**Fig. 4.2.C**) where the A_N values were higher with significant differences between salt and the control conditions from 4DAT to 10DAT.

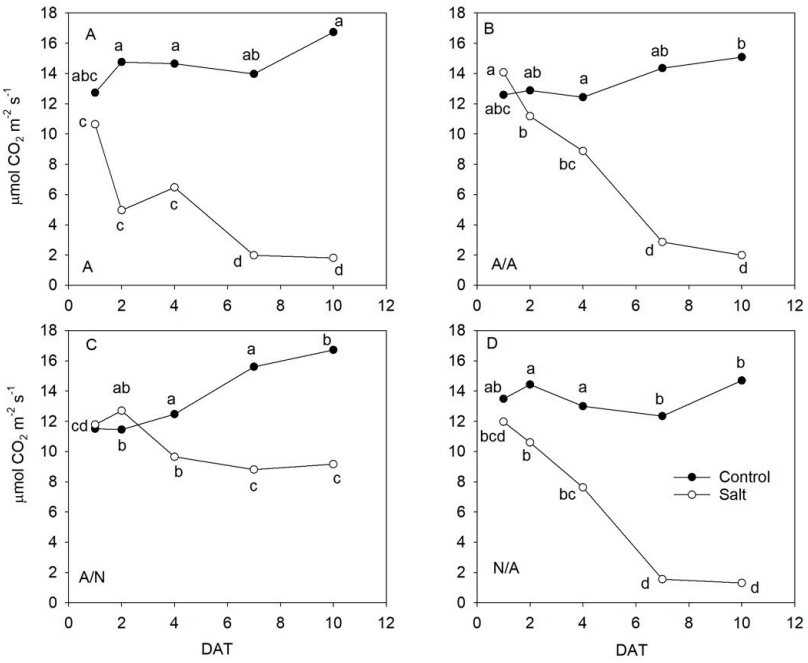

Figure 4.2. The net CO_2 assimilation rate (A_N; µmol CO_2 m^{-2} s^{-1}) of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=12. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

A drop in g_s under salt treatment was observed in all the plants with almost total stomatal closure (**Fig. 4.3.**). A minor drop in g_s was noted for A/N plants under salt stress showing the highest values of g_s respect the other salt-plant combinations with a significant difference at the end of experiment (**Fig. 4.3.C**).

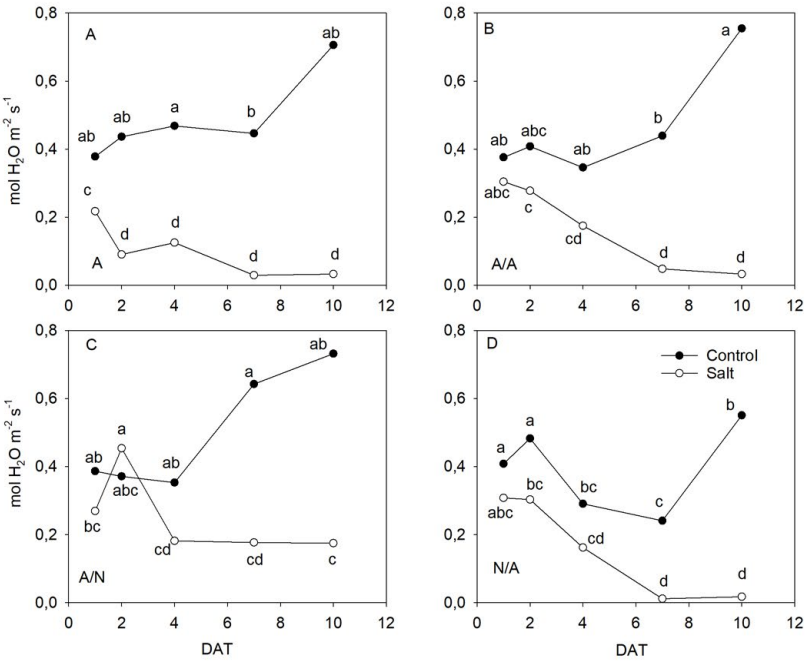

Figure 4.3. Leaf stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) of the ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=12. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

4.4.3. ABA analysis

After 1DAT and 10DAT, the average ABA concentration values (**Fig. 4.4.**) were higher in leaves than in roots. In leaves, significantly higher ABA concentrations were found under salinity conditions compared to the control ones in all the plant combinations (**Fig. 4.4.A, C**). Under the salinity conditions, the minimum ABA values in leaves went to A/N plants (**Fig. 4.4.A, C**). In roots (**Fig. 4.4.B, D**), the ABA levels were different depending on both plant combinations and salt time exposure. On 1DAT, the maximum values were found for A and A/N plants (**Fig. 4.4.B**). However on 10DAT, the highest ABA values were analyzed in A and A/A plants (**Fig. 4.4.D**).

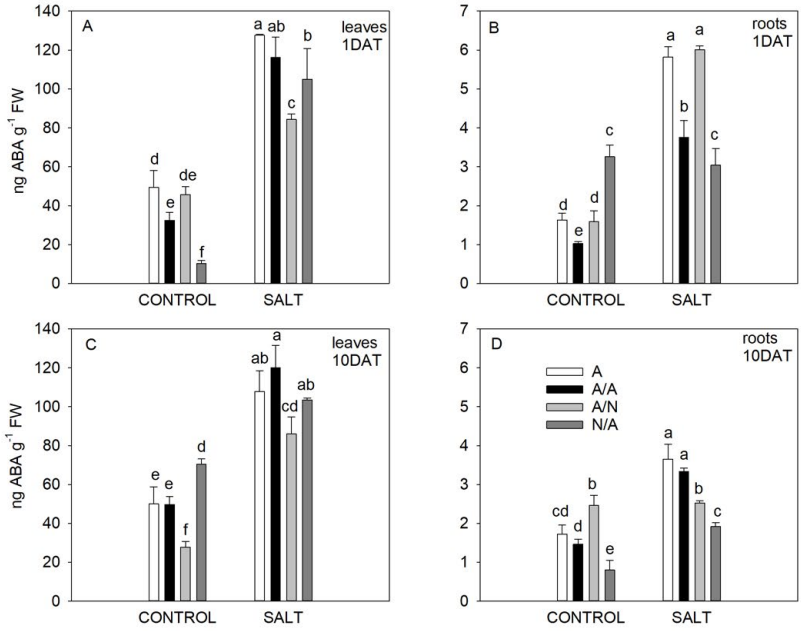

Figure 4.4. ABA contents (ng ABA g^{-1} DW) in the leaves (A, C) and roots (B, D) of the ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each plant combination and time, different letters indicate significant differences at $P < 0.05$ (LSD test).

4.4.4. Percentage of nitrate reductase activity in the salt treatment vs. the control

On 1DAT and 10DAT, the effect of salt addition induced reduction in the percentage of NR activity in leaves compared to their respective control in all plants combinations (**Fig. 4.5.**). Nevertheless, in A/N the reduction was lower compared to other plants combinations with significant differences observed between them both 1DAT and 10DAT (**Fig. 4.5.**).

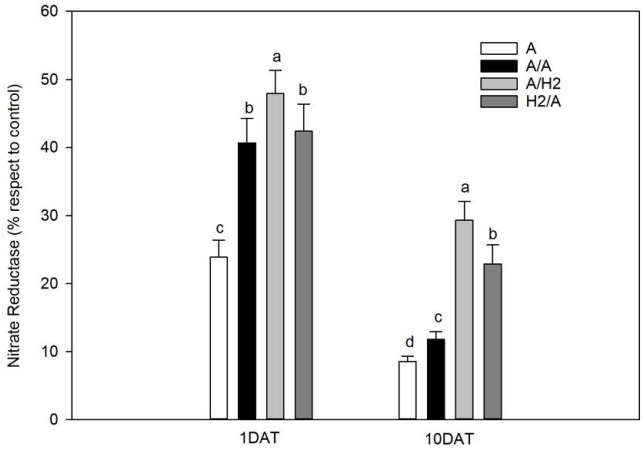

Figure 4.5. Nitrate reductase activity expressed as a percentage compared to its control (% vs. control) in the leaves of the ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each plant combination and time, different letters indicate significant differences at $P < 0.05$ (LSD test).

4.4.5. Proline analysis

The proline concentration in leaves (**Fig. 4.6.**) was always higher under salinity compared to the control condition from 1DAT to 10DAT. The maximum proline values appeared on 7DAT in all the plant combinations. Afterward the drop in concentration became more marked in A/N (**Fig. 4.6.C**) and N/A (**Fig. 4.6.D**) until 10DAT, but with higher values compared to A and A/A.

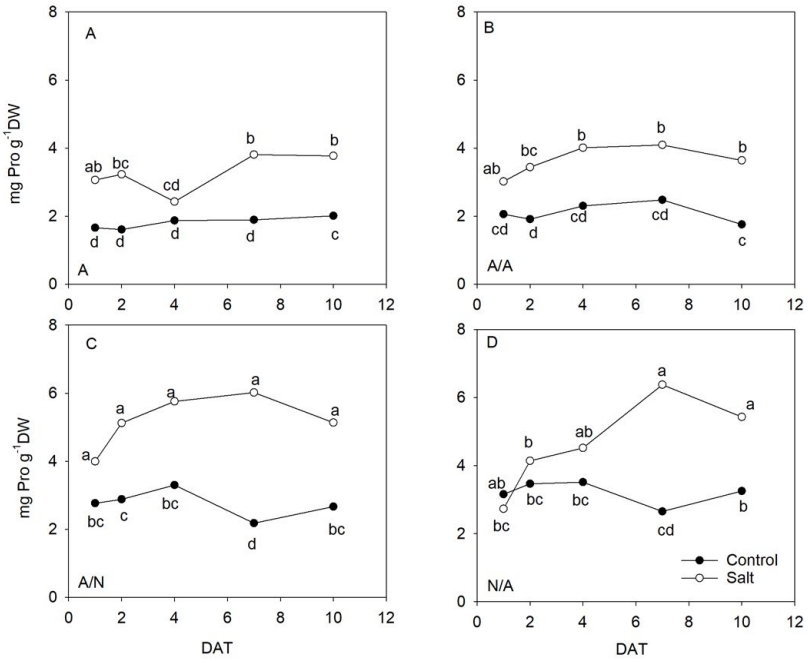

Figure 4.6. Proline concentration (mg Pro g-1 DW) in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

4.4.6. Total phenols analysis

The phenol concentrations in leaves under the salinity conditions (**Fig. 4.7.**) were higher with significant differences for all the plant combinations compared to the controls on each day after the start of treatment application. From 1DAT to 7DAT, A/N (**Fig. 4.7.C**) was the plant combination with the highest phenol concentrations compared to other plant types. The lowest phenol levels were found for N/A plants (**Fig. 4.7.D**).

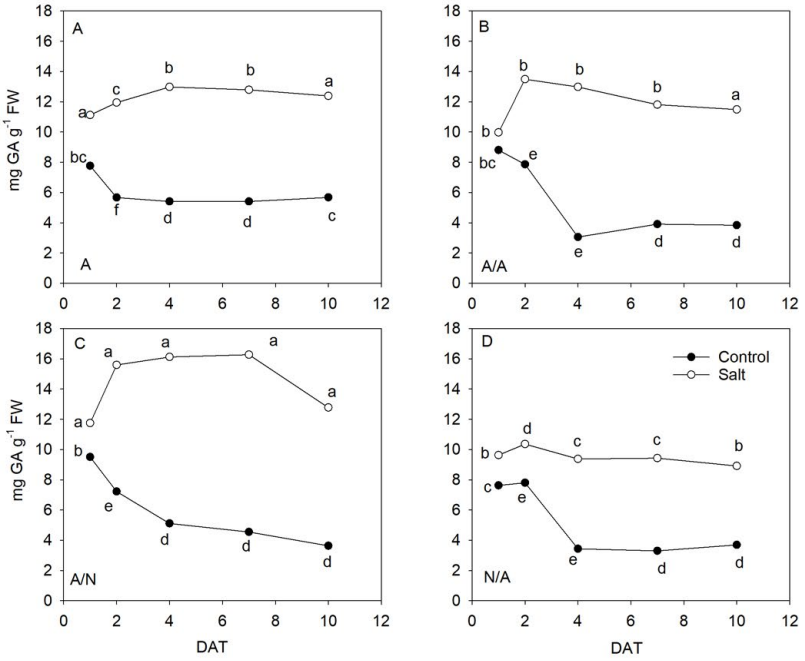

Figure 4.7. Changes in phenolic content (mg GA g^{-1} FW) in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

4.4.7. H_2O_2 determination

The hydrogen peroxide concentration in the A plant leaves (**Fig. 4.8.**) increased after salt exposure (**Fig. 4.8.A**) on 1DAT. In the other plant combinations, the increase in H_2O_2 was observed on 2DAT (except for A/N plants). A/N plants (**Fig. 4.8.C**) showed the highest H_2O_2 concentration, which increased from 2DAT to 7DAT, after which time the H_2O_2 levels remained constant until 10DAT.

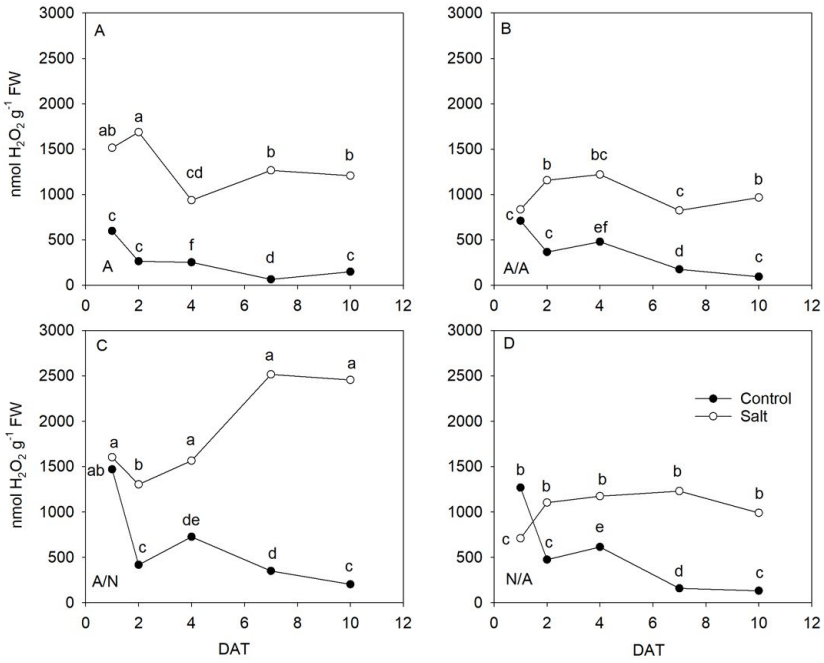

Figure 4.8. Hydrogen peroxide concentration (nmol H_2O_2 g^{-1} FW) in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at P < 0.05 (LSD test).

4.4.8. Lipid peroxidation

The MDA concentration in leaves (**Fig. 4.9.**) was higher in all the plant combinations under the salinity conditions and increased during the exposure time. At the end of experiment (10DAT), A/N plants (**Fig. 4.9.C**) displayed the smallest differences between control and salt stress compared to the other plants, followed for A/A (**Fig. 4.9.A**), N/A (**Fig. 4.9.D**) and A (**Fig. 4.9.A**), with the highest lipid peroxidation levels for salt treatment.

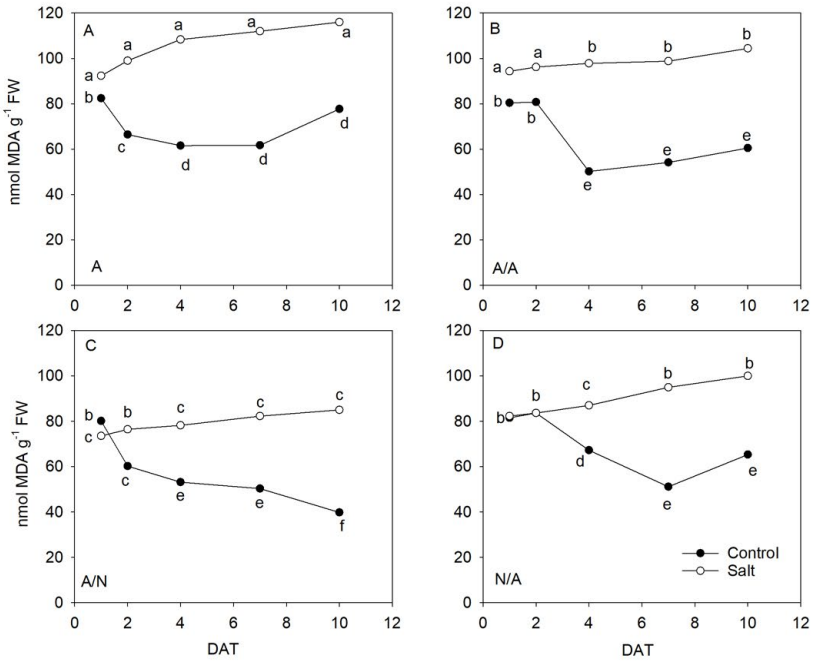

Figure 4.9. Malondialdehyde (MDA) content (nmol MDA g^{-1}FW) in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

4.4.9. DPPH-Radical Scavenging Activity

The leaves of the plants grown under salt stress obtained an increased percentage of inhibition radical DPPH compared to their control plants (**Fig. 4.10.**). Maximum activity was found for A/N (**Fig. 4.10.C**) plants on 7DAT and 10DAT under the salinity conditions.

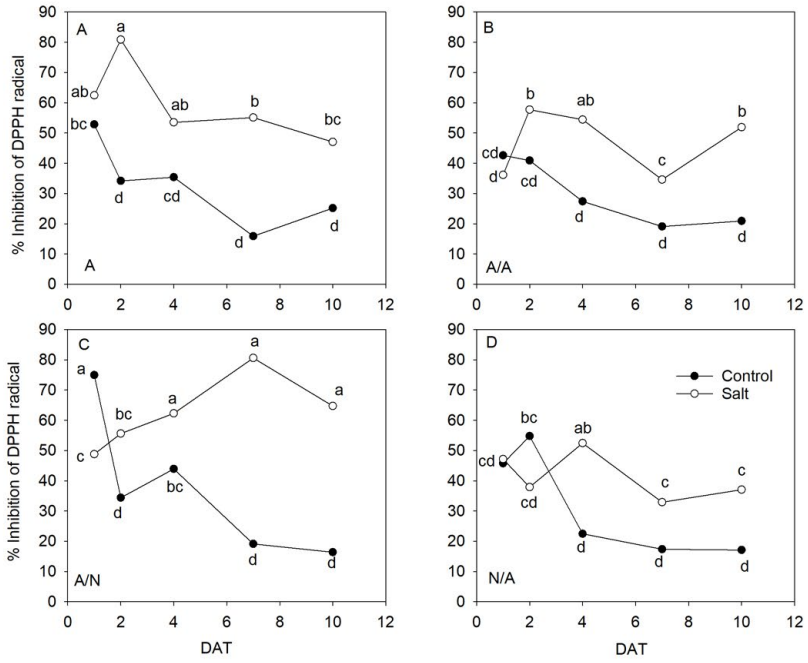

Figure 4.10. Percentage of inhibition of DPPH radical in the leaves of ungrafted pepper plants (cultivar Adige, A) (A), self-grated plants (A/A) (B), A grafted onto N (A/N) (C) and N grafted onto A (N/A) (D) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 1DAT, 2DAT, 4DAT, 7DAT and 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each study time, different letters indicate significant differences at $P < 0.05$ (LSD test).

4.4.10. Biomass measurements

At the end of the experiment (10DAT), the root length (**Fig. 4.11.A**), the shoot length (**Fig. 4.11.B**) and the total dry weight (**Fig. 4.11.C**) were significantly higher in A/N under salinity conditions compared to all other plant combinations. Under control condition, the highest root length was measured in A/N and N/A, however for the other biomass parameters significant differences were not observed between plant combinations.

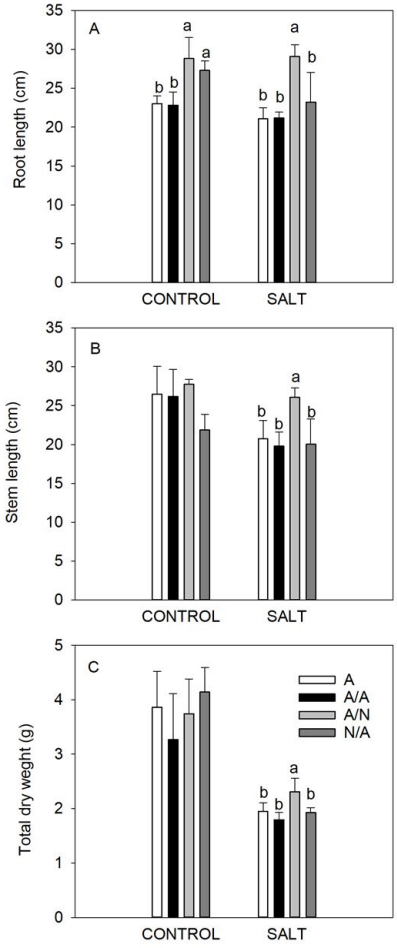

Figure 4.11. Root length (A), stem length (B) and total dry weigh (roots + stem + leaves) (C) of the ungrafted pepper plants (cultivar Adige, A), self-grated plants (A/A), A grafted onto N (A/N) and N grafted onto A (N/A) after addition of NaCl at 0 mM (Control) and 70 mM (Salt). Measurements were taken on 10DAT (days after treatment with NaCl began). Data are the mean values for n=4. For each plant combination and treatment, different letters indicate significant differences at $P < 0.05$ (LSD test). Not significant differences for stem length and total dry weight under control conditions are denoted with the absence of the letters above the bars.

4.5. Discussion

Vegetable grafting is an effective technique in increasing salt tolerance (Colla *et al.*, 2010). Some rootstocks, mainly hybrids for tomato, melon and cucumber, have demonstrated tolerance to salinity (Colla *et al.*, 2006; Savvas *et al.*, 2011; Huang *et al.*, 2013). To date, grafting onto pepper rootstocks has not been a feasible solution to cope with salinity given the unsatisfactory performance of available rootstocks (Kyriacou *et al.*, 2017; Penella and Calatayud, 2018). In previous field studies conducted under water salinity conditions, the hybrid NIBER®, obtained for that purpose, has been demonstrated as an effective rootstock in overcoming salinity and improved production compared to ungrafted or other commercial rootstocks (Calatayud *et al.*, 2016). The high yield obtained under the salinity conditions has been reported in other grafted vegetables, such as melon, watermelon or cucumber, grafted onto the hybrid *Curbita maxima* x *C. moschata* (Romero *et al,* 1997; Alan *et al.*, 2007; Colla *et al.*, 2012), or tomato grafted onto *S. lycopersicum* x *S. habrochaites* (Savvas *et al.*, 2009). These findings demonstrate that grafting directly and positively affects plant production.

For many crops, a significant factor that contributes to salinity tolerance is the ability to manage concentrations of toxic ions inside plants (Munns and Tester, 2008). Of all the different strategies, the capacity of salt ions exclusion and/or retention in roots, better maintenance of potassium homeostasis, or compartmentation of salt ions in the vacuole are available (Fernández-García *et al.*, 2004; Colla *et al.*, 2010). Moreover in grafted plants, the graft itself can act as a barrier to limit salt ions from the rootstock to the scion (Edelstein *et al.*, 2011). In this study, the Cl^- concentration under salinity did not show any significant differences among the plant combinations in roots, and only N/A-leaves exhibited the highest Cl^- levels on 10DAT. This result suggests that the graft effect itself does not act as a selective barrier to limit Cl^- movement from root to leaves by showing a uniform Cl^- concentration-distribution between root and leaves. Similar results were obtained by Edelstein *et al.* (2011) in melon grafted onto pumpkin. Further Cl^- accumulation exceeded that of Na^+ in all the plant combinations. This agrees with the results obtained by Navarro *et al.* (2002) in 'Orlando', and also with Chartzoulakis and Klapaki (2000) in 'Sonar' pepper varieties or in grafted pepper plants (Penella *et al.* 2015). A higher external Cl^- concentration could be linked to a major passive uptake root component, these might occur when the membrane potential is less negative than Cl^- equilibrium potential allowing for a passive influx and a very slightly active Cl^- uptake system (Altman and Mendel, 1973; Skerrett and Tyerman, 1994). However, for many vegetables like cucumber, melon, watermelon, tomato, eggplant and pepper, Na^+ ion is the primary cause of ion-specific damage (Tester and Davenport, 2003; Varlagas *et al.*, 2010, Penella *et al.*, 2015). Na^+ is largely a result of its capacity to compete with K^+ for essential binding sites for cellular function; moreover, regulation of ion homeostasis and selectivity of Na^+/K^+ discrimination are closely linked to a lower Na^+ concentration

and its relation to salt tolerance (Volkmar *et al.*, 1998; Munns and Tester, 2008). The significant depletion of the Na^+/K^+ ratio occurred only in the plants grafted onto N (A/N plants) in both leaves and roots to reduce the Na^+ load due to a higher K^+ concentration and/or lower Na^+ uptake compared to another plant combinations. Furthermore, the Na^+/K^+ ratios in roots undergoing the salinity treatment were higher compared with leaves (average of all plant combinations Na^+/K^+ was 24 fold higher in roots than leaves), regardless of the plant combination. The lowest Na^+ concentration in leaves to favor K^+ levels could be due to Na^+ retention and accumulation in roots (Edelstein *et al.*, 2011). Grafted plants had a higher K^+ content, which is apparently related to higher salt tolerance showing lower inhibited extent of stem and root and plant growth under salinity conditions (Zhu *et al.*, 2008; Huang *et al.*, 2009; Colla *et al.*, 2010, Nawaz *et al.* 2016) as just occurred in A/N plants in contrast to the other plant combination. In fact in the tomato-grafted plants, salt tolerance was associated with K^+, but not with Na^+ concentration (Albacete *et al.*, 2009).

The lowest foliar Na^+/K^+ ratio in the grafted A/N plants could possibly diminish the phytotoxic effect of salinity on photosynthesis (Ruiz *et al,*. 2005), facilitating the maintainance of growth (Rouphael *et al.*, 2012). The net CO_2 assimilation rate dropped in all the plant combinations under the salinity conditions, and this decrease was accompanied by a significant reduction in stomatal conductance. A/N plants showed a higher A_N than the self-grafted (A/A), non grafted (A) and reciprocal self-grafted (N/A) plants and the correlation analysis suggest that total DW was positively related with A_N (R^2 = 0.886 at 10DAT) indicating that plant growth was directly linked to photosynthesis. These results agree with previous findings which revealed that tolerant rootstocks can improve photosynthesis performance and growth under the salt treatment (Moya *et al.*, 2002; Massai *et al.*, 2004; He *et al.*, 2009, Rouphael *et al.*, 2012). However, g_s significantly decreased and more markedly compared to A_N, with values close to zero and reduced plants' ability to supply CO_2 to the photosynthetic apparatus (Piñero *et al.*, 2014). These results coincide with another finding which showed that g_s was very sensitive to salt stress (Jiang *et al.*, 2006; He *et al.*, 2009); although the least stomatal closure at the end of experiment was observed in A/N plants under the salinity conditions. A decrease in g_s has been observed in melon-, cucumber-, pepper- and tomato-grafted plants in response to salinity when tolerant rootstocks were also used (He *et al.*, 2009; Rouphael *et al.*, 2012; Penella *et al.*, 2015).

According to our results, salinity induced ABA accumulation, which could cause stomatal closure (Zhu, 2001; Finkelstein *et al.*, 2002), regardless of the root genotype (Holbrook *et al.*, 2002). In our experiment, the ABA concentration in leaves affected g_s and a linear correlation was found for both parameters on 1DAT and 10DAT (R^2= 0.84 and 0.90, respectively). This observation falls in line with the results for sweet pepper under salinity stress observed by Piñero *et al.* (2014). The A/N plant leaves showed a lower ABA concentration with higher stomatal opening, but the reciprocal grafted N/A plants exhibited a similar ABA concentration to plants A/A and A. This situation

indicates that the ABA levels in leaves were dependent on rootstock. There is evidence to show that a reduction in g_s is associated with an increased in ABA in roots prior to a detectable increase in leaf ABA (Davies and Zhang, 1991). The relation between both parameters was consistent on 1DAT (R^2= 0.72), but not on 10DAT (R^2= 0.40), according to our results. It is possible that the control of stomata conductance for a longer time (10DAT) was exerted by leaf metabolic activity (leaf water status, change in ion transport or transpiration stream) rather than by the ABA produced by roots, and/or ABA could be synthesized in leaves (Munns and Cramer, 1996; Holbrook *et al.*, 2002; Manzi *et al.*, 2017).

Nitrate reductase is sensitive to g_s and becomes less active when the stomata are closed (Kaiser and Huber, 2001; Yousfi *et al.*, 2012) limiting the assimilation of nitrate into organic compounds inducing visible effects on biomass (López-Serrano *et al.*, 2019). In this study, we observed diminished NR activity *versus* its control on 1DAT and 10DAT in all the plant combinations. However, only A/N plants maintained 50% (1DAT) and 30% (10DAT) enzyme activity, and both sustained the highest g_s and growth. This trend has been observed in pepper-, tomato- and cucumber-grafted plants in earlier studies (Liu *et al.*, 2013; Penella *et al.*, 2015, Ruiz *et al.*, 2005).

The low photosynthesis rate increased ROS formation in a very early response stage (Formentin *et al.*, 2018). The accumulation of an excessive ROS level may react with proteins, DNA and lipids, which could lead to redox imbalance and oxidative stress to cause metabolic dysfunction (Gill and Tuteja, 2010; Hossain *et al.*, 2015). To prevent ROS oxidative damage, plants up-regulate antioxidant enzymes and molecules to strike a balance between the formation rate and ROS removal (Munns and Tester, 2008). Salt-induced ROS are predominantly represented by H_2O_2 (Pang and Wang, 2008). Although H_2O_2 has been described to play a signaling role to plant processes related with abiotic stress acclimation in the last decade: antioxidative defense, up-/down-regulation of ABA, promotion of gibberellic acid biosynthesis or improvement of the K^+/Na^+ ratio in seedlings (Kim *et al.*, 2008; Shu *et al.*, 2016; Formentin *et al.*, 2018; Niu *et al.*, 2018). Furthermore, H_2O_2 has been considered a second messenger as it mediates adaptive responses to abiotic stress (Neill *et al.*, 2002; Yu *et al.*, 2003; Liu *et al.*, 2010; Baxter *et al.*, 2014; Hossain *et al.*, 2015). H_2O_2 accumulation has also been found to precede signaling activation, or has even been found to be the consequence of signaling (Hossain *et al.*, 2015). Under our salinity conditions, all the plant combinations increased the H_2O_2 concentration to show significant differences with its control. Particularly in A/N plants, H_2O_2 levels were the highest (with significant differences) compared to other plant combinations. The increased H_2O_2 in A/N plants has been associated with higher total antioxidant capacity and lower lipid peroxidation, with a less marked effect on the photosynthetic system (Zandalinas *et al.*, 2016). In tomato, an enhanced H_2O_2 level has been found to modulate the expression of stress and to up the defense genes related with antioxidant capacity (Zhou *et al.*, 2014). A, A/A and N/A plants showed minor antioxidant capacity (and significant differences with A/N

plants) and a major MDA concentration, which tends to show greater lipid peroxidation in salt-sensitive than salt-tolerant cultivars under salt stress (Zhu *et al.*, 2008; Penella *et al.*, 2015) thereby inhibiting biomass production (MDA concentration-total DW, R^2= 0.70 at 10DAT). These results indicate that H_2O_2 could be positively used by A/N plants to activate antioxidant capacity to help fight against salt stress (Hossain *et al.*, 2015) by acting as a signal molecule rather than a damaged plant system (Bose *et al.*, 2014; Rejeb *et al.*, 2015; Formentin *et al.*, 2018). Other molecules like proline and phenols could work well for salinity protection (Parida and Das, 2005; Ashraf and Foolad, 2007; Szabados and Savouré, 2010). Some studies suggest that proline may play a role as an enzyme-stabilizing agent under NaCl stress (Demir and Kocaçaliskan, 2001), reduce peroxidative damage to lipid membranes due to salt-dependent oxidative stress (Huang *et al.*, 2009), and play an important role as a compatible osmolyte (Szabados and Savouré, 2010). A_N-enhanced proline biosynthesis has been described to help prevent photosynthetic apparatus damage (Ashraf *et al.*, 2008). The increase in proline (2-fold times) herein observed was detected in the plant combination in which N was used as both a scion and rootstock compared to plants A or A/A. These results could indicate that N is implicated in more proline transport from roots to leaves for A/N plants by contributing to proline accumulation in leaves and/or could stimulate proline synthesis in N/A plant leaves (An *et al.*, 2013). Although proline metabolism has long since been studied in several crops, very little is known about the signaling pathways, biosynthesis, degradation and transport that regulate stress-induced accumulation, and this knowledge is vital to develop plants for stress tolerance (Kishor *et al.*, 2005; Szabados and Savouré, 2010).

Another metabolic process to be associated with tolerance responses to salinity stress in plants involves phenolic compounds (Parida and Das, 2005). Increasing phenolic content has been correlated with salt stress tolerance in watermelon plants grafted onto squash (Evrenosoğlu *et al.*, 2010) or in tomato-grafted plants (Ali and Ismail, 2014). Phenol compounds help avoid ROS formation, display antioxidant action and protect the photosynthetic apparatus (Harborne and Williams, 2000). According to our results, a significant increase in total phenols was detected in A/N plants under salinity treatment compared to the other plant combinations, which coincides with antioxidant capacity stimulation, minor lipid peroxidation formation and higher photosynthetic rates.

Grafting is an integrative reciprocal process in which both the scion and rootstock can influence salt tolerance (Etehadnia *et al.*, 2008). The importance of root characteristics in regulating salinity has been documented mainly in terms of the role in the control of toxic ions, water uptake, biomass and molecules signaling from root to leaves that modulate plant responses to salinity (Albacete *et al.*, 2009; He *et al.*, 2009; Colla *et al.*, 2010; Niu *et al.*, 2018; Penella and Calatayud, 2018). In contrast, other authors (Santa-Cruz *et al.*, 2002; Chen *et al.*, 2003; Zhu *et al.*, 2008) have suggested that salt tolerance in grafted plants is attributed to the scion genotype. This might be due to either differences in the salt tolerance of both the rootstock and scion used in the ex-

periments or the applied salinity dose (Huang *et al.*, 2013). In this study, the reciprocal graft (N/A) was done to examine whether plant tolerance to salinity can be helped by the rootstock or scion. In response to salinity N/A plants showed dramatically reduced photosynthesis and biomass, similarly to that obtained in A plants and A/A, which was associated with other physiological factors like greater stomatal resistance and higher ABA leaf concentration, minor phenol levels and lower antioxidant activities with major lipid peroxidation. These results suggest that A roots are less able to adapt to changes under salinity. Similar results have been found in cucumber grafted onto luffa under drought stress (Liu *et al.*, 2013) or in cucumber grafted onto pumpkin under salinity (Huang *et al.*, 2013), where reciprocal grafted plants showed no salinity tolerance. However, different tolerant mechanisms to cope with salinity can be used in grafted plants (Colla *et al.*, 2010).

This work has led to a better understanding of the response mechanisms of grafted plants to imbalanced salinity. We demonstrate that the new pepper rootstock, NIBER®, could influence scion behavior by preserving its plant physiology performance and growth. The time-course analysis showed that the reduction in ABA leaf content in the plants grafted on to NIBER® under salinity allowed to keep stomata open, strike an appropriate photosynthesis balance and lead to NR activation. The increases in endogenous H_2O_2 in these plants acted as a signaling molecule by activating the defense mechanism (increase in total antioxidant capacity, proline and phenols), which tips the balance to ROS scavenging. The least damage caused to the metabolism in the plants grafted onto NIBER® was strengthened to maintain ion homeostasis in relation to the ability to lower the Na^+/K^+ ratio, all which mitigating the reduction of the biomass imposed by salt stress. This ability is a cost-effective trait of salt tolerance in plants.

4.6. References

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Chapter 5

Uncovering Salt Tolerance Mechanisms in Pepper Plants: a Physiological and Transcriptomic Approach

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Under Review

5.1. Abstract

Background:
Pepper is one of the most cultivated crops worldwide, but is sensitive to salinity. This sensitivity to salinity is dependent on varieties and our knowledge about how they can face such stress is limited. Thus, a physiological and transcriptomic analysis was carried out to study mechanisms of tolerance. Tolerant and sensitive accessions, respectively called A25 and A6, were grown for 14 days under control conditions and irrigated with 70 mM of NaCl. Biomass, photosynthetic parameters, ion concentration and differentially expressed genes were analysed.

Results:
Transcriptomic changes between the accessions under both control and stress conditions could explain the physiological behaviour of A25 by improved growth (e.g. expansins), starch metabolism (e.g. BAM1), ion homeostasis (e.g. CBL9, HAI3, BASS1), photosynthetic protection (e.g. FIB1A, TIL, JAR1) and antioxidant activity (e.g. PSDS3, SnRK2.10). In addition, misregulation of ABA and other stress signalling genes would appear crucial to explain the different sensitivity to NaCl in both accessions.

Conclusions:
After analysing the physiological behaviour and transcriptomic results, we have concluded that A25 accession utilizes different strategies to cope better salt stress, being ABA-signalling a pivotal point of regulation. It has also been established a network of genes that could cooperate in the defence response to salinity in pepper plants.

Additional keywords:
Abscisic Acid; Growth;
Ion homeostasis; Photosynthesis,
Salt stress; Tolerance; Pepper.

We sincerely thank Javier Forment and Lorena Latorre-García from IBMCP (Valencia, Spain) for helping with the performance and analysis of the transcriptomic study

Abbreviations:

7DAT, 7 days after treatment;
14DAT, 14 days after treatment;
ABA, abscisic acid;
A_N, CO_2 fixation rate;
AKT1, Arabidopsis K^+ transporter 1;
BAM1, beta-amylase 1;
BAM5, beta-amylase 5;
BASS1, sodium bile acid symporter family;
CAMTA5, calmodulin-binding transcription activator 5;
CBL9, calcineurin B-like protein 9;
CCB3, Cofactor assembly, complex C (B6F);
CcdA, Cytochrome c biogenesis protein family;
CDC2, Cell division control 2;
CEL5, cellulase 5;
CER1, eceriferum 1;
C_i, substomatal CO_2 concentration;
CSD1, copper/zinc superoxide dismutase 1;
CSLE1, cellulose synthase-like E1;
CYP38, cyclophilin 38;
DEG, differentially expressed gene;
DW, dry weight;
E, transpiration rate;
EC, electrical conductivity;
EXLB1, expansin-like B1;
EXPA4, expansin A4;
EXPA13, expansin A13;
FIB1A, Fibrillin 1A;
FW, fresh weight;
g_s, stomatal conductance to water vapour;
HAB1, hypersensitive to ABA1;
HAI3, highly ABA-induced PP2C protein 3;
J8, Chaperone DnaJ-domain superfamily protein;
JA, jasmonate;
JAR1, jasmonate resistant 1;
KASI, 3-ketoacyl-acyl carrier protein synthase I;
LACS2, long-chain acyl-CoA synthetase 2;
MIOX1, myo-inositol oxygenase 1;
MIOX5, myo-inositol oxygenase 5;
NDHG, NADH, ubiquinone/ plastoquinone oxidoreductase, chain 6;
OCT4, organic cation/ carnitine transporter 4;
PHT1;4, phosphate transporter 1;4;
PME1, Pectin methylesterase 1;
PMEI13, plant invertase/ pectin methylesterase inhibitor superfamily protein;
PORA, protochlorophyllide oxidoreductase A;
PP2C, protein phosphatase 2C;
PPD1, photosystem II reaction centre PsbP family protein;
PRX66, peroxidase 66;
PRX71, peroxidase 71;
PSII, photosystem II;
PSAE-2, photosystem I subunit E-2;
PSAG, photosystem I subunit G;
PSAO, photosystem I subunit O;
PSBP-1, photosystem II subunit P-1;
RH, relative humidity;
ROS, reactive oxygen species;
SnrK2.5, SNF1-related protein kinase 2.5;
SnRK2.10, SNF1-related protein kinase 2.10;
SPDS3, spermidine synthase 3;
TIL, temperature-induced lipocalin;
TROL, thylakoid rhodanese-like protein;
TT4, chalcone and stilbene synthase family protein;
TTA1, Class I heat shock protein, putative/Titania 1;
TUB8, tubulin beta 8;
WS6D, o-acyltransferase (WSD1-like) family protein;
XK-1, Xylulose kinase-1.

5.2. Background

Pepper (*Capsicum annuum* L.) is one of the most important cultivated horticultural species worldwide. Production has increased over the last 20 years from 17 to 36 million tons, and the cultivated area has expanded by about 35% [1]. However, several stresses still significantly affect peppers, which decrease yields and fruit quality. The most important stress is biotic, but peppers are also affected by some abiotic stresses. One of the most relevant ones is salt stress as pepper plants are considered moderately sensitive, sensitive or highly susceptible [2, 3]. The source of a high salt concentration that affects plants may be either soil or irrigation water [4]. Some studies, such as [5], have observed that dry weight and marketable yield decreased by 46% and 25%, respectively, when pepper plants were exposed to an electrical conductivity of water of 4.4 dS m^{-1}.

The root is the first organ affected by the accumulation of toxic ions like Na^+ and Cl^- [3], which firstly exert osmotic stress and later ionic stress when ions exceed the toxic threshold [6]. These ions also move rapidly to photosynthetic organs and cause several negative effects. Indeed salt accumulation in plant tissues provokes changes in the physiological metabolism, such as nutritional imbalances, and generates reactive oxygen species (ROS), among other physiological disorders [7, 8] that lead to reduce biomass and crop production. However, some species are able to deal with these negative effects and can be tolerant to salt stress. To reach this condition, a complex network of genes related to salt tolerance is necessary [9], which modify physiological and biochemical plant responses.

In agricultural species, growers have always tended to select genotypes with increased commercial production, commonly linked to improved tolerance to specific stresses. As a result, it is now possible to find a wide diversity of accessions that differs in terms of grades of tolerance to stresses. In the case of pepper, several authors have demonstrated that the severity of negative effects depends on the variety [10–13].

This intraspecies variation may be a source of information to find factors like genes, proteins or metabolites related to tolerance, which can be used in, for example, conventional breeding programmes or genetic engineering technologies [9], or to be employed as tolerant rootstocks in grafted plants [14, 15].

Several articles can be found about transcriptomic studies that deal with understanding the genetic mechanisms responsible for the tolerance of pepper plants to different stresses, such as heat stress, chilling or leaf curl virus [16–19]. On the other side, some authors have studied specific genes related to salinity tolerance in pepper [20–22], but the genetic programmes that are differentially expressed in tolerant and sensitive pepper plants when salt stress is present have not yet been studied in-depth.

Consequently, this study compared two pepper accessions previously classified by us as tolerant (A25) and sensitive (A6) to salt stress after analysing a series of physio-

logical and agronomical parameters [11, 23]. This study included the measurements of plant biomass, photosynthesis and ions uptake to evaluate physiological traits, as well as a transcriptomic assay, by microarrays, to elucidate the genetic programmes that were expressed and are responsible for tolerance to salt stress. This analysis could reveal the underlying mechanisms in pepper to cope with salinity stress and open up new strategies to improve crop performance under salinity conditions.

5.3. Results

5.3.1. Biomass

In order to evaluate whether plants maintained the same growth rate after NaCl treatment, dry biomass was measured in both roots and aerial organs at 14DAT. Under the control conditions, both accessions obtained higher values compared to the salt stress conditions (**Fig. 5.1.**). Nevertheless, growth under the control conditions differed between accessions as A25 obtained higher values. The tolerant A25 accession better maintained both aerial and root dry weight under salt stress conditions compared to A6 accession at the end of the experiment (14DAT).

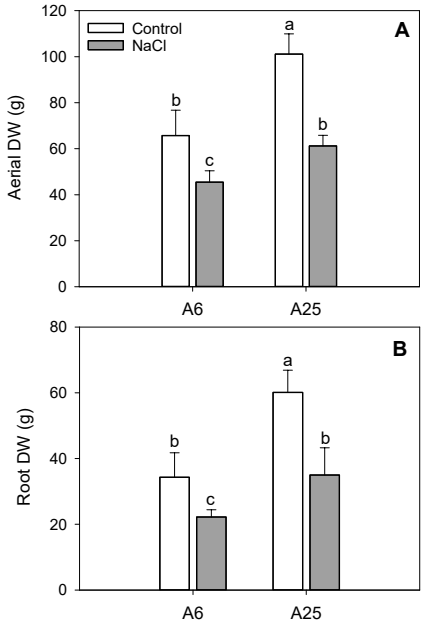

Fig. 5.1. Dry weight of the aerial part (A) and the root zone (B), in the accessions A6 and A25, under control and salt stress (70 mM NaCl) conditions. Measurements were taken at the end of the experiment (14DAT). Data are the mean of 6 replicates and the error bars belong to the standard deviation. Different letters indicate significant differences at $P<0.05$ (LSD test).

5.3.2. Gas Exchange Measurements

As photosynthesis is one of the first processes affected after salt stress addition, it is very important to evaluate its different parameters and how they progress with exposure time. In this experiment, A_N, g_s, C_i and E were analysed at 7DAT and 14DAT (**Fig. 5.2.**). At the first measurement time (7DAT), A25 showed no significant differences in A_N and C_i (**Fig. 5.2.A, C**) between the control and salt stress conditions. Conversely, g_s and E decreased in the stressed plants (**Fig. 5.2.B, D**) but, compared to A6, the photosynthetic parameters in A25 were better maintained as A6 obtained the lowest values of them all.

At the end of the experiment (14DAT) under the control conditions, a better response was observed in stomatal conductance and transpiration with the tolerant accession A25 (**Fig. 5.2.B, D**), unlike A_N and C_i, which remained unchanged (**Fig. 5.2.A, C**). Under the salt stress conditions, the situation differed due to the drop in the photosynthetic parameters, whose values significantly fell in the two studied accessions to similar values, with no significant differences noted between them.

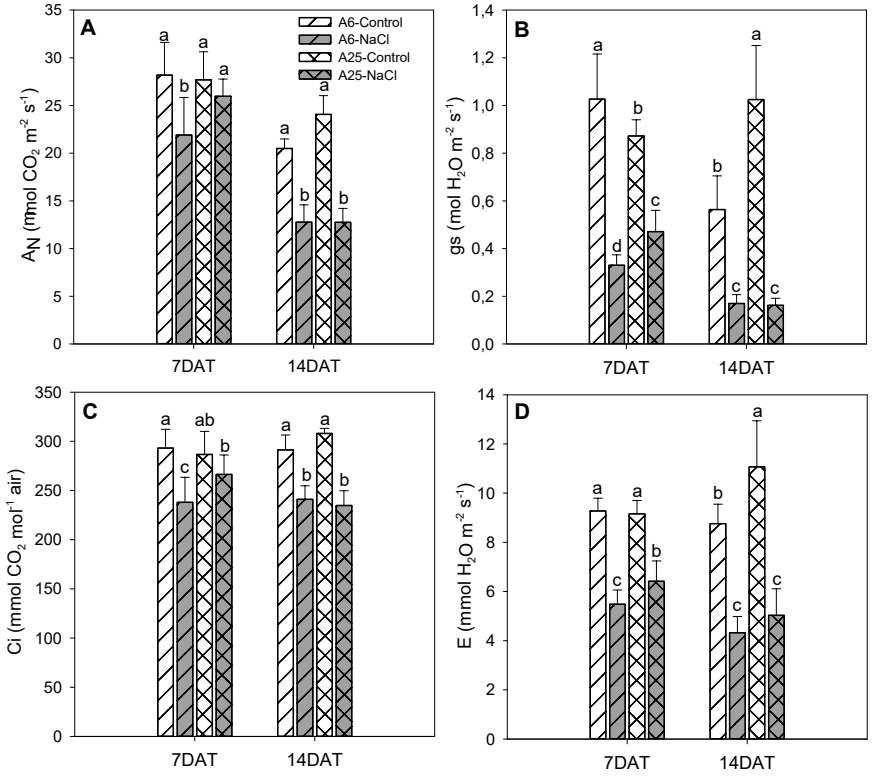

Fig. 5.2. CO_2 fixation rate (A_N, µmol CO_2 m^{-2} s^{-1}) (A), stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}) (B), substomatal CO_2 concentration (C_i, µmol CO_2 mol^{-1} air) (C) and transpiration rate (E, mmol H_2O m^{-2} s^{-1}) (D) under control and salt stress (70 mM NaCl) conditions. Measurements were taken after 7 days (7DAT) and 14 days (14DAT) of the experiment. Data are the mean of 5 replicates and the error bars belong to the standard deviation. For each studied time, different letters indicate significant differences at $P<0.05$ (LSD test).

5.3.3. Ion Determination

Exposure to high NaCl concentrations disrupts ion homeostasis in plant cells. Thus the evaluation of the ion concentration in different tissues after exposure to stress was crucial for this experiment. For this purpose, Na^+, K^+ and Cl^- concentrations were measured at the end of the experiment (14DAT) in leaves (**Fig. 5.3.A, C, E**) and roots (**Fig. 5.3.B, D, F**). With Na^+ (**Fig. 5.3.A, B**), the concentration rose when plants were subjected to salt stress. It is worth mentioning that the levels in roots were higher than leaves for both accessions and treatments, especially in A25, which showed more accumulation compared to A6.

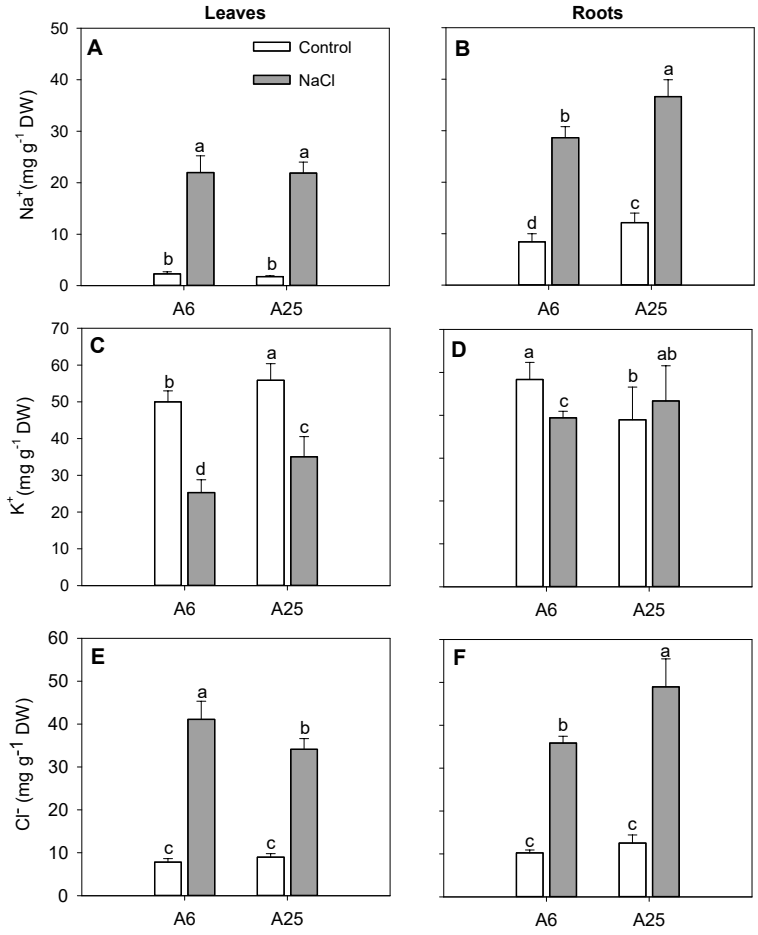

Fig. 5.3. Na^+ (A, B), K^+ (C, D) and Cl^- concentration (E, F) in leaves (A, C, E) and roots (B, D, F) in the accessions A6 and A25 under control and salt stress (70 mM) conditions. Measurements were taken at the end of the experiment (14DAT). Data are the mean of 6 replicates and the error bars belong to the standard deviation. Different letters indicate significant differences at $P<0.05$ (LSD test).

Regarding the K^+ concentration (**Fig. 5.3.C, D**), the main accumulation took place in roots where the concentration was higher than in leaves. Nonetheless, this concentration dropped in the salt stress treatment, except in the roots of the A25 accession, where it maintained. In addition, K^+ levels were higher in A25 under salt stress in both leaves and roots.

Lastly, a higher Cl^- concentration was detected under the salt conditions in all the studied organs and accessions compared to the control (**Fig. 5.3.E, F**). However, the concentration in the A25 accession under salt stress rose in roots and lowered in leaves compared to A6. Under the control conditions, no significant differences were found in any of the studied organs.

5.3.4. Transcriptomic Expression Results

A microarray experiment analysis was performed to know the transcriptomic changes that could explain the sodium chloride resistance of the A25 accession.

Under the control conditions when the A25 accession was compared to A6, 196 and 315 genes were up- and down-regulated, respectively (**Fig. 5.4.A, B**). Of all these genes, it is important to highlight the up-regulated genes related to cell wall biosynthesis and expansion (PMEI13, TUB8, EXPA13, XK-1, PME1, CEL5, CSLE1), wax and fatty acid biosynthesis (KASI, LACS2), cell division (CDC2), vitamin transport (BASS1), ABA-signalling (SnRK2.10, TINY2 and ERD4) and photosynthesis (PSBP-1). The genes related to the formation of cellular barriers, such as lignins (PRX71, PRX66) and waxes (WS6D, CER1), were down-regulated in A25 compared to A6. The down-regulation of the genes involved in stress protection (CAMTA5, JAR1, CBL9) and photosynthesis (NDHG, CCB3) (**Table 5.1., 5.2.**; **Additional file 1**) was noteworthy.

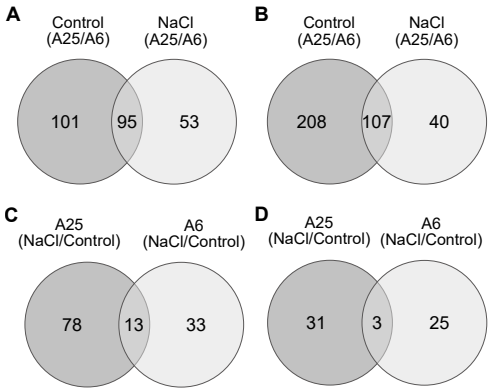

Fig. 5.4. Overlap of the up-regulated (A, C) and down-regulated (B, D) DEGs between the accessions in the different comparisons at 14DAT. A and B represent Venn diagram analysis of DEGs in A25 respect to A6 in control and salt stress conditions. C and D represent Venn diagram analysis of DEG of each accession when salt stress is compared to control conditions.

Table 5.1. Summary of the specific differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to control conditions. It is represented both the up (FC>1) and down-regulated genes (FC<1), as well as the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	**Short name**	**FC**	***P*-value**	***C. annuum* code**	***A. thaliana* code**
DNA-directed RNA polymerase subunit beta (Protein of unknown function. DUF642)	-	3.1	8.80E-03	CA01g20890	AT3G08030
Pectin lyase-like superfamily protein	-	2.3	6.00E-03	CA00g70080	AT3G07820
Expansin A13	EXPA13	2.3	0.05	CA04g04060	AT3G03220
Rubisco methyltransferase family protein	-	1.9	9.18E-03	CA08g02430	AT1G24610
Plant invertase/pectin methylesterase inhibitor superfamily protein	PMEI13	1.9	0.04	CA03g15820	AT5G62360
cation/hydrogen exchanger 14	CHX14	1.4	0.02	CA06g25650	AT1G06970
Tubulin beta 8	TUB8	1.4	0.02	CA06g25000	AT5G23860
Peroxidase superfamily protein	-	0.7	0.03	CA00g44710	AT2G37130
GDSL-like Lipase/Acylhydrolase superfamily protein	-	0.7	0.04	CA10g03820	AT5G45960
Eceriferum 1	CER1	0.7	0.02	CA01g27070	AT1G02205
Photosystem I assembly protein	YCF3	0.6	0.01	CA00g81520	ATCG00360
Calcineurin B-like protein 9	CBL9	0.6	0.04	CA01g33680	AT5G47100
O-acyltransferase (WSD1-like) family protein	WSD6	0.6	0.02	CA00g64820	AT3G49210
Cytochrome P450. family 86. subfamily A. polypeptide 8	CYP86A8	0.6	0.04	CA08g07320	AT2G45970
Beta-amylase 5	BAM5	0.6	0.02	CA07g12430	AT4G15210
Rubisco methyltransferase family protein	LSMT-L	0.5	0.01	CA11g04070	AT1G14030
Cellulose synthase family protein	CEV1	0.5	0.01	CA01g20250	AT5G05170
Pectin lyase-like superfamily protein	-	0.5	0.03	CA09g01850	AT3G53190
Jasmonate resistant 1	JAR1	0.4	1.45E-03	CA08g08190	AT2G46370
Calmodulin-binding transcription activator 5	CAMTA5	0.3	1.46E-03	CA01g14110	AT4G16150

Table 5.2. Summary of the common differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to control and salt stress conditions. It is represented both the up (FC>1) and down-regulated genes (FC<1), as well as the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	**Short name**	**Control**		**NaCl**		***C. annuum* code**	***A. thaliana* code**
		FC	***P*-value**	**FC**	***P*-value**		
Cell division control 2	CDC2	13.6	9.57E-05	6.9	1.15E-03	CA12g18420	AT3G48750
Xylulose kinase-1	XK-1	11.5	6.96E-06	11.0	1.48E-05	CA12g08890	AT2G21370
Sodium Bile acid symporter family	BASS1	7.1	7.34E-03	22.0	2.28E-04	CA09g06260	AT1G78560
SNF1-related protein kinase 2.10	SnRK2.10	3.6	3.20E-04	4.0	1.78E-04	CA08g14400	AT1G60940
Pectin methylesterase 1	PME1	2.5	3.37E-05	2.7	2.15E-05	CA03g36990	AT1G53840
Early-responsive to dehydration stress protein (ERD4)	ERD4	2.4	5.47E-04	2.0	4.87E-03	CA08g02700	AT1G30360
Photosystem II subunit P-1	PSBP-1	2.1	1.34E-04	2.3	4.80E-05	CA07g07930	AT1G06680
3-ketoacyl-acyl carrier protein synthase I	KASI	2.0	0.02	4.2	1.49E-04	CA01g00840	AT5G46290
3-ketoacyl-acyl carrier protein synthase I	KASI	2.0	0.02	4.2	1.49E-04	CA01g00830	AT5G46290
Cellulase 5	CEL5	1.7	3.86E-03	2.0	4.59E-04	CA11g09950	AT1G22880
Integrase-type DNA-binding superfamily protein	TINY2	1.7	3.86E-03	2.0	4.59E-04	CA08g04820	AT5G11590
Long-chain acyl-CoA synthetase 2	LACS2	1.6	3.86E-03	1.9	6.64E-04	CA08g18140	AT1G49430
Cellulose synthase like E1	CSLE1	1.5	0.02	1.5	0.02	CA05g16620	AT1G55850
ERD (early-responsive to dehydration stress) family protein	-	0.7	0.03	0.7	0.03	CA06g26780	AT4G02900
Peroxidase 71	PRX71	0.6	4.03E-04	0.6	2.18E-04	CA12g06550	AT5G64120
Peroxidase 71	PRX71	0.5	1.49E-03	0.7	0.04	CA12g06580	AT5G64120
Eceriferum 1	CER1	0.5	0.03	0.5	0.05	CA01g19130	AT1G02205
Peroxidase 66	PRX66	0.4	1.25E-04	0.6	0.01	CA03g16810	AT5G51890
Xyloglucan endotransglucosylase/hydrolase 7	XTH7	0.3	1.13E-05	0.4	3.30E-05	CA02g24640	AT4G37800
NADH:ubiquinone/plastoquinone oxidoreductase, chain 6	NDHG	0.3	1.05E-03	0.4	9.44E-03	CA08g09370	ATCG01080
Cofactor assembly, complex C (B6F)	CCB3	0.1	6.96E-06	0.1	2.04E-05	CA02g03840	AT5G36120

The response of each accession to salt stress (NaCl/Control) was very different as only 13 up- and 3 down-regulated genes were commonly expressed (e.g. CSD1, MIOX1) (**Fig. 5.4.C, D**). In relation to A25 accession 78 and 31 genes were specifically expressed (**Fig. 5.4.C, D**). The genes related to defence against stress (JAR1, CAMTA5, CBL9, HAB1), the cell wall (MIOX5, EXLB1), polyamine biosynthesis (SPDS3), photoprotection (FIB1A) and starch degradation (BAM1) were up-regulated (**Table 5.3.**; **Additional file 2**). Conversely, the photosynthesis-related genes (PSAG, PSAO, PORA, CYP38) and a phosphatase PP2C related to ABA signalling (HAI3) were significantly repressed. For the A6 accession, 33 and 25 specific up- and down-regulated genes were respectively found (**Fig. 5.4.C, D**), in which the genes related to cell expansion (EXPA4), photosynthesis (PSBP-1, TROL, PSAE-2) and starch degradation (BAM5) were down-regulated (**Table 5.4.**; **Additional file 2**). As a result of all this, the observed transcriptomic response to salt stress was more robust in our tolerant accession.

We also analysed the A25 transcriptome compared to A6 under salt stress conditions. The comparison revealed 95 up- and 107 down-regulated genes, which were also differentially expressed under the control conditions (**Fig. 5.4.A, B**). However, A25 specifically showed for the stress conditions that 53 genes were up- and 40 genes were down-regulated. (**Fig. 5.4.A, B**). The genes related to chaperones (J8, TTA1), photosynthesis (CcdA), ion homeostasis (OCT4, PHT1;4; TIL), cell expansion (EXPA4), flavonoid biosynthesis (TT4) and ABA signalling (SnrK2.5) were up-regulated, while the genes involved in photosynthesis (PPD1, PORA) and wax biosynthesis (CER1) were down-regulated (**Table 5.5.**; **Additional file 1**).

Table 5.3. Summary of the common differentially expressed genes after 14DAT in the comparison NaCl/Control in leaves in the accession A25. It is represented both the up (FC>1) and down-regulated genes (FC<1), as well as the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05).

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Expansin-like B1	EXLB1	6.1	4.10E-02	CA01g06350	AT4G17030
Calmodulin-binding transcription activator 5	CAMTA5	4.3	7.05E-03	CA01g14110	AT4G16150
Beta-amylase 1	BAM1	2.7	0.05	CA03g02770	AT3G23920
Calcineurin B-like protein 9	CBL9	2.7	2.25E-03	CA01g33680	AT5G47100
Hypersensitive to ABA1	HAB1	2.3	0.02	CA08g03850	AT1G72770
Jasmonate resistant 1	JAR1	2.1	0.01	CA08g08190	AT2G46370
Myo-inositol oxygenase 5	MIOX5	2.0	0.04	CA12g20180	AT5G56640
Fibrillin 1A	FIB1A	1.7	0.04	CA02g18750	AT4G04020
Spermidine synthase 3	SPDS3	1.6	0.05	CA03g19440	AT5G53120
Highly ABA-induced PP2C protein 3	HAI3	0.7	0.04	CA06g24830	AT2G29380
Cyclophilin 38	CYP38	0.5	0.05	CA02g29500	AT3G01480
Photosystem I subunit G	PSAG	0.5	0.02	CA07g20940	AT1G55670
Photosystem I subunit O	PSAO	0.4	0.04	CA06g22830	AT1G08380
Protochlorophyllide oxidoreductase A	PORA	0.1	0.04	CA10g00480	AT5G54190

Table 5.4. Summary of the common differentially expressed genes after 14DAT in the comparison NaCl/Control in leaves in the accession A6. It is represented both the up (FC>1) and down-regulated genes (FC<1), as well as the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05).

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Cellulose synthase-like D3	CSLD3	3.9	6.06E-03	CA01g07920	AT3G03050
Expansin A4	EXPA4	0.7	0.04	CA02g18410	AT2G39700
Beta-amylase 5	BAM5	0.7	0.03	CA07g12420	AT4G15210
Photosystem II subunit P-1	PSBP-1	0.7	0.04	CA07g07930	AT1G06680
Thylakoid rhodanese-like protein	TROL	0.5	0.04	CA08g08250	AT4G01050
Photosystem I subunit E-2	PSAE-2	0.5	0.04	CA06g28140	AT2G20260
Beta-amylase 5	BAM5	0.5	6.63E-03	CA07g12430	AT4G15210

Table 5.5. Summary of the specific differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to salt stress conditions. It is represented both the up (FC>1) and down-regulated genes (FC<1), as well as the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05).

Full name	**Short name**	**FC**	***P*-value**	***C. annuum* code**	***A. thaliana* code**
Temperature-induced lipocalin	TIL	3.39	1.01E-03	CA07g02210	AT5G58070
Chaperone DnaJ-domain superfamily protein	J8	2.7	3.92E-03	CA00g87730	AT1G80920
Chalcone and stilbene synthase family protein	TT4	2.4	0.03	CA05g17040	AT5G13930
Organic cation/carnitine transporter4	OCT4	2.18	5.81E-03	CA07g18590	AT3G20660
Temperature-induced lipocalin	TIL	1.87	0.03	CA09g18430	AT5G58070
Class I heat shock protein, putative (DUF1423)/ Titania 1	TTA1	1.7	7.81E-03	CA04g04530	AT1G14740
SNF1-related protein kinase 2.5	SnRK2.5	1.5	0.04	CA12g16870	AT5G63650
Expansin A4	EXPA4	1.42	0.02	CA02g18410	AT2G39700
Cytochrome c biogenesis protein family	CcdA	1.42	0.04	CA07g18200	AT5G54290
Phosphate transporter 1;4	PHT1;4	1.4	0.04	CA03g05830	AT2G38940
Photosystem II reaction center PsbP family protein	PPD1	0.69	0.04	CA01g31620	AT4G15510
Eceriferum 1	CER1	0.69	0.01	CA00g87940	AT1G02205
Protochlorophyllide oxidoreductase A	PORA	0.15	0.02	CA10g00480	AT5G54190

5.4. Discussion

In this work, we analysed gene expression in two pepper accessions, one tolerant (A25) and another sensitive (A6), which displayed different growth and behaviour under salt stress conditions [11, 23]. Several sections below explain the main processes affected by this complex gene regulation network in response to salinity stress.

5.4.1. Hormonal Signalling

Hormone signalling and biosynthesis have been considered an essential point of the regulation of plant tolerance or susceptibility [24]. Accordingly, our results uncover many of the genes involved in jasmonates (JAs) and abscisic acid (ABA) synthesis, degradation or signalling that could explain the behaviour of the analysed accessions.

Jasmonates are key elements in the regulation of a wide range of processes when different abiotic stresses are present [25–27]. However, they need to be conjugated with a series of compounds to be active [28]. We found that gene jasmonate resistant 1 (JAR1) was up-regulated when salt stress and control were compared in the A25 accession, but it was absent in A6. This gene catalyses the formation of an active jasmonyl-isoleucine (JA-Ile) conjugate. Several authors have demonstrated by external applications that JAs improve the activity of different antioxidant enzymes, growth and development, photosynthetic activity and Na^+ homeostasis [25, 29, 30].

Hormone ABA is well-known to play a central role in tolerance to different abiotic stresses as it performs a wide variety of functions in plant growth and development, it regulates plant water balance by stomata opening, and it plays a crucial role in osmotic stress tolerance [31]. Increasing ABA concentration and signalling are wide responses of the tolerance described by several authors, which favours stomata closure and, thus, avoids excess transpiration. However, this fact also compromises plant growth as it diminishes photosynthetic activity [24, 32]. In our experiment, we found several DEGs in A25 described as regulators of ABA, or are regulated by ABA signalling (HAB1, ERD4, CAMTA5, Tiny2, CBL9, Snrk2.5, Snrk2.10, HAI3) and, thus, play a central role in controlling tolerance.

Of all the ABA-related genes found in A25, one of the most relevant ones was the up-regulated gene hypersensitive to ABA1 (HAB1). HAB1 encodes a functional type 2C protein phosphatase (PP2C) and has been reported as a positive or negative regulator of ABA signalling, depending on the splice variant [33, 34]. Overexpression of this gene has been reported, in fact, that leads to a minor or major ABA sensitivity, modifying stomata opening and gene expression [34, 35].

A family of transcription factors, which has been reported to be regulated by ABA and plays an important role in stress tolerance, is the Calmodulin-binding transcription activators family (CAMTA) [36]. It has been demonstrated that the CAMTA family can bind to the promoters of different members of the dehydration-responsive-

element-binding (DREB) transcription factors family and modulate the stress response [37]. In our case, we found the up-regulation of CAMTA5 genes and DREB member TINY2 in the A25 accession, which may indicate that both genes enhanced the response to salt stress by improving growth, development, the expression of stress-responsive genes or ABA-mediated stomatal closure [38–41].

5.4.2. Biomass and Cell Growth

Salt stress negatively affects cell growth and plant biomass. However, greater biomass conservation is considered a sign of tolerance [42–45]. In this study, and at 14DAT, a better maintenance of root and aerial biomass was found in A25 compared to the A6 accession under the salt stress conditions. Biomass preservation is usually associated with the differential expression of a wide variety of genes related to cell growth and division, some of which were identified in this experiment. One of these genes is an ABA-related gene called ERD4 (early-responsive to dehydration 4), which was up-regulated in the A25/A6 comparison under both the control and salt stress conditions. This gen has been described in the bibliography as being overexpressed in tolerant transgenic *A. thaliana* plants when salt is added [46].

Proper progress through the cell division cycle is also very important for correct growth. We found an up-regulated gene in both treatments in the A25/A6 comparison, called cell division control 2 (CDC2), which regulates the G1/S and G2/M transitions in mitosis [47]. It has been demonstrated that abiotic stresses, such as drought, can negatively affect CDC2 activity [48]. As the expression in the A25 accession was 6.91-fold higher in salt stress, cell division rhythm improved.

Cell growth is also determined by cell expansion, which may be restricted by salt stress [49]. An important family of genes related to this function is expansins, which are responsible for the non-enzymatically loosening and extension of plant cell walls [50]. The analysis of the expression of these genes is crucial to determine how stress affects plant growth. An increased expression of the expansin genes was found in the A25 accession under salt stress compared to the control or the A6 accession. This finding suggests that A25 improved cell wall expansion and turgor, which may lead to better growth and development, as other authors have already demonstrated [51, 52].

5.4.3. Starch Degradation

Abiotic stresses may also affect starch content as it may be remobilised to release energy, sugars, carbon and derived metabolites when photosynthesis is limited [53]. Soluble sugars may interact with hormones, genes and proteins, especially those related to photosynthetic metabolism, by regulating diverse pathways [54]. Consequently, growth and development are highly dependent on plant efficiency by metabolising starch. In this study, β-amylase 1 (BAM1) was found to be up-regulated only under the salt stress conditions in the A25 accession. Under osmotic and salt stress, several authors have demonstrated that this chloroplastic gene is responsible for transitory

starch degradation in guard and mesophyll cells of mature leaves [55, 56], which suggests similar roles in A25.

5.4.4. Ion Homeostasis

When plants come into contact with salt, it is crucial to maintain ion homeostasis to avoid toxic accumulation. Plants cope with this situation by different mechanisms that can contribute to salt tolerance, some of which are very well documented in the bibliography [8, 57].

One of the most important and abundant cations in plants cells is K^+, which decreases under salt stress conditions because of replacement with Na^+. So maintaining the K^+/Na^+ ratio plays a very important role in salt stress tolerance and is considered a biomarker [8, 15]. The enhanced K^+ homeostasis in the A25 accession in both organs would seem to indicate that A25 possessed some mechanism to keep K^+ inside cells; one possible candidate that could explain it is AKT1, a passive transporter that specifically introduces K^+ into root and mesophyll cells [58, 59]. Thus we detected the up-regulation of the negative regulator of ABA signalling CBL9 (calcineurin B-like protein 9) and the down-regulation of positive regulator HAI3 (Highly ABA-Induced 3) in the A25 accession under salt stress conditions [60]. These genes play opposite roles in the regulation of AKT1 as CBL9 is a positive regulator [61], and HAI3 could be a repressor as this gene presents a high homology to HAI2 [62, 63].

The accumulation of Cl^- ions and especially Na^+ in pepper plant tissues, performs diverse physiological functions [14, 15]. When Na^+ reaches toxic levels, plants may lower the influx into cells and improve efflux and compartmentalisation in other organelles where ions are not toxic [6]. In our experiment, we found that Na^+ was accumulated in the roots of both accessions after 14DAT. As this accumulation was especially pronounced in A25, and root biomass had improved compared to the A6 seedlings, this effect could be associated with compartmentalisation in vacuoles or other organelles, as other authors have already demonstrated [3, 64]. Despite the negative effect on plant growth deriving from its toxic effect, accumulation of ions under salinity can help to maintain the turgor pressure of plants [14, 65]. The adjustment of the osmotic potential through inorganic ion uptake implies a much lower energy cost than that conferred by the organic molecules synthesised in cells [66].

With leaves, Na^+ was equally accumulated in both accessions, but biomass improved only in A25, a cue that Na^+ management was diverse in both pepper accessions. In line with this, we noticed that ion transport in leaves was closely linked to the protection of chloroplasts in A25 as we found some related genes. One of these genes was BASS1 (bile acid/sodium symporter 1), which was up-regulated in the control and salt stress treatments in the A25/A6 comparison. This gene, which encodes a symporter of Na^+ and pantoate, a precursor of Vitamin B5, played a double role in A25: on the one hand, it conferred protection from Na^+ toxicity in chloroplasts to conserve photosynthesis responses; on the other hand, the pantothenate cycle was promoted [67, 68]. We also

found the gene TIL (temperature-induced lipocalin), which was up-regulated in A25 compared to A6 under salinity stress, which can avoid excess Na^+ and Cl^- accumulation in chloroplasts by protecting chlorophyll b degradation in this way [69].

An up-regulated gene found in the A25/A6 comparison under the salt stress conditions was OCT4 (organic cation/carnitine transporter 4), which lowered the concentration of toxic Na^+ in the cytoplasm by accumulating in vacuoles. This family of genes is responsible for the symport of Na^+ and organic molecules like carnitine [70, 71]. So it would play an important role in osmotic balance through ionic homeostasis in our tolerant accession.

5.4.5. Photoprotection

When plants come into contact with salt stress, one of the primary affected processes is photosynthesis. The photosynthetic parameters herein analysed reflected that only A25 maintained them at 7DAT compared to the control conditions, although both accessions were equally affected by the end of the experiment. These reasons suggest that A25 kept the plant's photosynthetic capacity levels high for longer times [72]. In addition, some genes which possessed functions to protect photosynthesis were differentially expressed in both accessions. In line with this, we found the up-regulation of ABA-related gene fibrillin 1A (FIB1A) in the A25 NaCl/control, which suggests that fibrillin was accumulated in chloroplasts and, consequently, improved protection and efficiency of PSII [73]. Together with FIB1A, other previously explained genes contributed to photoprotection, such as TIL, BASS1 or JAR1.

5.4.6. ROS Scavenging

When photosynthesis is disturbed by salt stress, a series of secondary effects is detected, such as oxidative stress, which may lead reactive oxygen species (ROS) to toxic levels [74]. The ability to reduce the quantity of all these molecules by efficient ROS-scavenging mechanisms is vital for acquiring tolerance. In this study, we found up-regulated antioxidant mechanisms in both accessions, some of which were specific of A25. Of them, one of the most relevant ones was the up-regulation of gene SPDS3 (spermidine synthase 3), which catalyses the formation of spermidine, a polyamine that improves multiple processes in plants, such as ROS scavenging, the K^+/Na^+ ratio and PSII efficiency by protecting thylakoid membranes and chlorophyll content [75–77]. To support the role of polyamines in salt stress and ROS homeostasis, we also found another up-regulated gene in A25, called sucrose non-fermenting 1-related protein kinase 2-10 (SnRK2.10), which regulates the gene expression, protein level and/or enzymatic activity of several ROS-related enzymes, and is also involved in H_2O_2 accumulation and ascorbate cycle regulation in *A. thaliana* [78].

5.4.7. Conclusions

After analysing the physiological parameters and DEGs of both accessions, we conclude that different tolerance strategies simultaneously took place in the A25 accession after

exposure to salt stress, with ABA-signalling being a pivotal point of regulation, and an important network was established between different genes to reveal the complex response induced by salinity. These results provide valuable results about salt stress mechanisms of an important crop like pepper. It is noteworthy that we also found several genes that probably contributed to tolerance, but their functions have not yet been discovered. Therefore, an in-depth study into all these genes could be interesting to conduct future research.

5.5. Methods

5.5.1. Plant Material

Based on previous studies [11, 23], two accessions of *C. annuum* were selected depending on their grade of tolerance to salt stress: code A6 was sensitive and code A25 was tolerant.

Seeds were sown in 104-hole seed trays filled with enriched substrate for germination. When plants had 6-8 real leaves, they were placed in 5-litre polyethylene pots covered with aluminium sheet (roots were previously cleaned of substrate). Pots were filled with a nutrient solution containing (in mmol L^{-1}) 12.3 NO_3^-, 1.02 H_2PO_4, 2.45 SO_4^{2-}, 3,24 Cl^-, 5.05 K^+, 4.23 Ca^{2+}, 2.55 Mg^{2+} and micronutrients (15.8 µM Fe^{2+}, 10.3 µM Mn^{2+}, 4.2 µM Zn^{2+}, 43.5 µM B^+ and 1.4 µM Cu^{2+}), which was artificially aerated. The electrical conductivity (EC) and pH of this nutrient solution were 1.7 dS m^{-1} and 6.5, respectively. The nutrient solution was added daily to compensate for absorption. After 14 days of plant acclimation, salt stress was induced by the addition of NaCl 70 mM by replacing the plant pot solution to obtain an EC of 8.5 dS m^{-1} and a pH of 6.1. The layout design was completely randomised with 10 plants per accession and treatment.

During the culture and experiment, plants were grown in a greenhouse at the Polytechnic University of Valencia (UPV, Valencia, Spain) under natural light conditions (800-1,000 µmol m^{-2} s^{-1}), with a temperature range of 18-25°C and 50-70% relative humidity (RH).

All the parameters were measured 14 days after stress induction, except in the photosynthetic parameters, where measurements were taken after 7 days (7DAT) and 14 days (14DAT) of treatment.

5.5.2. Biomass Determination

Six replications per accession and treatment were harvested at the end of the experiment (14DAT) for the biomass parameters. Aerial organs and roots were separated and weighed (FW). Immediately afterwards, they were dried by placing them in an oven at 65°C for 72 h. After this time, everything was weighed again to determine dry weight (DW).

5.5.3. Gas Exchange Measurements

CO_2 fixation rate (A_N, µmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}), substomatal CO_2 concentration (C_i, µmol CO_2 mol^{-1} air) and transpiration rate (E, mmol H_2O m^{-2} s^{-1}) were measured with a portable LI-COR 6400 (Li-Cor Inc.) infrared gas analyser at 7DAT and 14DAT. Measurements were taken under saturating light conditions (1,000 µmol quanta m^{-2} s^{-1}), reference CO_2 of 400 µmol CO_2 mol^{-1}, on fully expanded leaves (3rd-4th leaf from the apex) at a cuvette temperature of 24°C and

75% RH. Measurements were taken from 09:00 h to 12:00 h (UT+01:00). The layout was randomised with five replications per accession and treatment.

5.5.4. Ion Determination

Six replications per accession and treatment of leaves and roots were collected and dried at 65°C for 72 h at the end of the experiment (14DAT). Dried samples were ground with a mortar and used for the ionic analysis.

With Na^+ and K^+, samples (0.2 g for leaves, 0.1 g for roots) were burnt in a muffle furnace for 12 h at 550°C. Ions were extracted with 2% nitric acid in an ultrasonic bath for 30 min at 40°C. Na^+ and K^+ concentrations were measured by an ICP emission spectrometry (iCAP 6000, Thermo Scientific. Cambridge, England, UK).

The chloride concentration (Cl^-) in the dry plant material (0.125 g) was extracted with 0.1 N HNO_3 in 10% (v/v) acetic acid and was determined by potentiometric titration with $AgNO_3$ in a chloride analyser (Sherwood, MKII 926).

5.5.5. Extraction and Quality Measurement of Total RNA

Three replications of leaves per treatment and accession were frozen in liquid nitrogen immediately after harvest and conserved at -80°C at 14DAT. At the time of RNA extraction, samples were ground to a fine powder with a mortar and liquid nitrogen. Total RNA was extracted using the MACHEREY-NAGEL NucleoSpin® RNA kit. Approximately 0.1 g was weighed, and RNA was obtained following the protocol "RNA purification from cultured cells and tissue" by the producer; DNase treatment was used to remove DNA from samples and was acquired from the same producer. Total RNA was eluted in 50 µL of RNAse-free water and was immediately aliquoted and conserved at -80°C. The total RNA samples with 260/280 and 260/230 ratios > 2 (measured by a NanoDrop ND1000) and RNA integrity (RIN) value > 7.0 (measured by the Agilent 5067-1511 Bioanalyzer 2100 System) were used for microarray hybridisation.

5.5.6. Microarray Hybridisation

The RNA extracted from the leaf samples was prepared for microarray hybridisation at the Genomic Service of the IBMCP Institute (Instituto de Biología Molecular y Celular de Plantas) in Valencia (Spain) by Agilent technologies. cDNA synthesis and labelling on Agilent Tomato microarrays were carried out using the Agilent One Colour RNA Spike-in Kit and the Agilent Low Input Quick Amp Labeling Kit. Microarray hybridisation and washing were next performed with the Agilent Gene Expression Hybridization kit and Gene Expression Wash Buffers. Agilent microarray 4*44k (Agilent G2519F) was selected for hybridisation (reference AMADID 22270 *Tomato*). Microarray scanning was done with a GenePix 4000B (Axon Molecular Devices, Sunnyvale, USA) and data were extracted by the Agilent Feature Extraction software, version 9.5.1.

5.5.7. Microarray Data Analysis

The obtained spot intensity values were analysed on the Babelomics 5 platform [79]. Firstly, raw data were normalised, which consisted in a background correction, rescaling all the microarrays to a unique final distribution and reshaping data to a suitable distribution. At this point, data were transformed from tomato probes to pepper and *Arabidopsis thaliana* genes by the Bioinformatics service at the IBMCP Institute in Valencia (Spain) to then take the average among all the probes of the same pepper gene. Raw data were then separated into categories (accession and treatment) and analysed by a class comparison test. All the differentially expressed genes (DEGs) of the class comparison, both up- and down-regulated, were described as their orthologue of *A. thaliana* by the database of Araport 11.

5.5.8. Statistical Analysis

The experiment layout was a completely randomised design. The data from the biomass, gas exchange measurements and nutritional analyses were subjected to a two-way ANOVA (Statgraphics Centurion XVI for Windows, Statistical Graphics Corp.), where both accession and treatment were considered to be the factors of the analysis. With the photosynthesis parameters, 7DAT and 14DAT were analysed independently. As the interaction between both factors was significant, a one-way ANOVA was performed by joining both factors of the two-way ANOVA. Ulterior comparisons were made using Fisher's least significance difference (LSD) test at $P<0.05$ with the same software.

All the statistical analyses of the microarrays were done using the Babelomics platform. Different treatments of the same accession (Salt/Control) and distinct accessions of the same treatment (A25/A6) were compared by a Limma test to compare genes, and a Benjamini and Hochberg test was run to reduce the false discovery rate. The adjusted *P*-value was selected at 0.05.

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5.7. Supplementary Information

Additional file 1. Table S1-S6. Total differentially expressed genes when A25 and A6 accessions are compared, in both control and salt stress conditions.

Table S1. Specific differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to control conditions. It is represented only up-regulated genes (FC>1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
C2H2 and C2HC zinc fingers superfamily protein	TT1	5.9	6.94E-03	CA12g20170	AT1G34790
ChaC-like family protein	-	4.6	8.73E-04	CA12g18980	AT5G26220
DNA-directed RNA polymerase subunit beta (Protein of unknown function. DUF642)	-	3.1	8.80E-03	CA01g20890	AT3G08030
Pathogenesis-related thaumatin superfamily protein	-	2.8	0.01	CA03g32740	AT1G73620
Phosphate-responsive 1 family protein	EXO	2.7	5.22E-03	CA00g36520	AT4G08950
Mob1/phocein family protein	MOB1-like	2.6	0.02	CA01g08350	AT5G45550
Major facilitator superfamily protein	-	2.6	0.03	CA03g02690	AT2G33280
Basic pathogenesis-related protein 1	PRB1	2.4	3.44E-03	CA01g31060	AT2G14580
Ribosomal protein L12/ ATP-dependent Clp protease adaptor protein ClpS family protein	-	2.3	7.10E-04	CA09g00270	AT3G06040
Pectin lyase-like superfamily protein	-	2.3	6.00E-03	CA00g70080	AT3G07820
Expansin A13	EXPA13	2.3	0.05	CA04g04060	AT3G03220
Peptidemethionine sulfoxide reductase 1	PMSR1	2.1	5.28E-03	CA03g25350	AT5G61640
UDP-N-acetylglucosamine (UAA) transporter family	-	2.1	0.03	CA01g19370	AT1G12600
Pre-mRNA-processing protein 40A	PRP40A	2.0	0.04	CA00g84420	AT1G44910
Sieve element occlusion amino-terminus protein	SEOR1	2.0	0.05	CA11g08750	AT3G01680
Formate dehydrogenase	FDH	2.0	0.03	CA02g29530	AT5G14780
Serine racemase	SR	2.0	0.01	CA01g34140	AT4G11640
Rubisco methyltransferase family protein	-	1.9	9.18E-03	CA08g02430	AT1G24610
Plant invertase/pectin methylesterase inhibitor superfamily protein	PMEI13	1.9	0.04	CA03g15820	AT5G62360
Alpha-galactosidase 1	AGAL1	1.8	4.88E-03	CA05g06220	AT5G08380

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Heavy metal transport/detoxification superfamily protein	-	1.8	0.01	CA03g22080	AT5G50740
Chloroplast RNA-binding protein 33	CP33	1.8	0.04	CA00g33110	AT3G52380
NADH dehydrogenase 3	NAD3	1.8	0.03	CA07g05040	ATMG00990
UDP-Glycosyltransferase superfamily protein	-	1.8	0.02	CA05g17400	AT4G14090
Ubiquitin-conjugating enzyme19	UBC19	1.8	0.05	CA11g10540	AT3G20060
Lsd one like 1	LOL1	1.8	0.04	CA00g77830	AT1G32540
Glycosyl hydrolase family protein	-	1.8	4.31E-03	CA11g15500	AT5G20950
C2H2-like zinc finger protein	-	1.8	0.02	CA11g19180	AT3G45260
PTEN 2	PEN2	1.7	0.03	CA02g14740	AT3G19420
WEB family protein (DUF827)	-	1.7	0.05	CA06g04300	AT2G40480
Zinc finger (C3HC4-type RING finger) family protein	-	1.7	0.02	CA06g09820	AT3G54780
Glyoxal oxidase-related protein	-	1.7	0.04	CA07g00290	AT3G57620
Peptide transporter 1	PTR1	1.7	0.02	CA00g00870	AT3G54140
ELM2 domain protein	-	1.7	0.02	CA06g18500	AT1G26580
Phloem protein 2-B10	PP2-B10	1.7	0.04	CA05g19940	AT2G02360
ChaC-like family protein	-	1.7	1.27E-03	CA01g30410	AT5G26220
BUB1-related (BUB1: budding uninhibited by benzymidazol 1)	BUBR1	1.7	0.03	CA04g23550	AT2G33560
RNA polymerase Rpb7 N-terminal domain-containing protein	-	1.7	0.03	CA07g01190	AT1G06790
Yeast autophagy 18 B-like protein	ATG18B	1.7	0.01	CA07g01370	AT4G30510
Homocysteine methyltransferase 2	HMT2	1.7	7.66E-03	CA03g07080	AT3G63250
Acyl carrier protein 4	ACP4	1.7	0.02	CA03g31150	AT4G25050
FASCICLIN-like arabinogalactan 1	FLA1	1.7	0.04	CA07g20370	AT5G55730
Uroporphyrinogen decarboxylase	HEME1	1.7	0.03	CA10g01670	AT3G14930
Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	MEE67	1.6	0.03	CA11g16140	AT3G10110
Hydroxyproline-rich glycoprotein family protein	-	1.6	3.21E-03	CA00g67160	AT2G18910
Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	-	1.6	0.03	CA02g24530	AT2G22760
Protein kinase superfamily protein	-	1.6	3.58E-03	CA02g18990	AT5G38260
NB-ARC domain-containing disease resistance protein	RPP13	1.6	9.47E-03	CA09g07300	AT3G46530

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Glutathione S-transferase THETA 1	GSTT1	1.6	2.93E-03	CA01g02960	AT5G41210
RNA methyltransferase family protein	-	1.6	0.02	CA07g21530	AT5G64150
Histone superfamily protein	HTB1	1.6	0.02	CA03g10400	AT1G07790
C3H4 type zinc finger protein	-	1.6	0.02	CA11g19800	AT4G32600
Concanavalin A-like lectin protein kinase family protein	-	1.6	0.02	CA03g00560	AT5G10530
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	-	1.6	0.02	CA01g23950	AT1G26850
Early nodulin-like protein 17	ENODL17	1.6	0.03	CA02g27870	AT5G15350
Tetratricopeptide repeat (TPR)-like superfamily protein	EMB3101	1.6	0.05	CA07g09730	AT1G05600
Subtilase family protein	ARA12	1.6	0.05	CA01g03820	AT5G67360
Nucleoside diphosphate kinase 2	NDPK2	1.5	2.94E-03	CA06g20240	AT5G63310
ASF1 like histone chaperone	SGA2	1.5	0.03	CA11g00080	AT1G66740
Cyclopropyl isomerase	CPI1	1.5	0.02	CA12g20380	AT5G50375
Plant invertase/pectin methylesterase inhibitor superfamily protein	-	1.5	0.01	CA01g33230	AT5G62350
Tetratricopeptide repeat (TPR)-like superfamily protein	-	1.5	0.05	CA04g20670	AT3G15200
Transducin family protein / WD-40 repeat family protein	MSI2	1.5	0.05	CA08g19320	AT2G16780
Hypothetical protein	-	1.5	0.02	CA02g25480	AT3G50370
Mitochondrial glycoprotein family protein	-	1.5	0.04	CA03g32230	AT1G15870
Calcium dependent protein kinase 1	CPK1	1.5	0.02	CA12g07790	AT5G04870
MATE efflux family protein	-	1.5	0.02	CA07g00340	AT5G52450
Ribosomal protein L18e/L15 superfamily protein	-	1.5	0.05	CA06g19960	AT1G70600
Translation protein SH3-like family protein	-	1.5	0.04	CA10g21320	AT1G57860
Serine carboxypeptidase-like 42	scpl42	1.5	0.01	CA04g13000	AT5G42240
NAD(P)-linked oxidoreductase superfamily protein	ATB2	1.5	5.87E-03	CA03g04090	AT1G60710
CLK4-associating serine/ arginine-rich protein	-	1.5	0.05	CA02g11010	AT4G36980
Proliferating cell nuclear antigen 2	PCNA2	1.5	0.02	CA06g25030	AT2G29570
RING/U-box superfamily protein	-	1.4	0.01	CA03g37160	AT3G14250
RNA-dependent RNA polymerase family protein	-	1.4	0.05	CA00g76620	AT2G19930
Cation/hydrogen exchanger 14	CHX14	1.4	0.02	CA06g25650	AT1G06970

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
NAD(P)-linked oxidoreductase superfamily protein	-	1.4	0.03	CA01g31480	AT4G33670
Alpha/beta-Hydrolases superfamily protein	-	1.4	0.01	CA02g11330	AT5G38520
Major facilitator superfamily protein	-	1.4	0.04	CA03g20620	AT5G17010
MATE efflux family protein	-	1.4	0.03	CA10g01710	AT3G23550
NB-ARC domain-containing disease resistance protein	-	1.4	2.07E-03	CA06g00580	AT3G46730
Tubulin beta 8	TUB8	1.4	0.02	CA06g25000	AT5G23860
DNA topoisomerase, type IA, core	-	1.4	0.03	CA00g58410	AT2G32000
F-box/associated interaction domain protein	-	1.4	0.03	CA04g18470	AT1G43005
F-box family protein	-	1.4	0.02	CA00g67750	AT3G06240
Alpha/beta-Hydrolases superfamily protein	-	1.3	0.05	CA03g36870	AT3G14360
GDSL-like Lipase/Acylhydrolase superfamily protein	-	1.3	0.01	CA02g12860	AT5G45670
Zinc ion binding / nucleic acid binding protein	-	1.3	0.03	CA04g12090	AT2G01050
SNF7 family protein	VPS2.1	1.3	9.91E-03	CA09g17000	AT2G06530
Early nodulin-like protein 9	ENODL9	1.3	0.03	CA07g19480	AT3G20570
Long-chain fatty alcohol dehydrogenase family protein	-	1.3	0.02	CA03g04850	AT4G28570
Alkaline-phosphatase-like family protein	-	1.3	0.04	CA00g81180	AT2G22530
NB-ARC domain-containing disease resistance protein	-	1.3	0.04	CA01g33820	AT4G27220
Cysteine-rich RECEPTOR-like kinase	CRK8	1.3	0.04	CA00g15340	AT4G23160
NAD(P)-binding Rossmann-fold superfamily protein	-	1.3	0.05	CA01g02470	AT4G11410
Damaged DNA binding 2	DDB2	1.3	0.01	CA00g57630	AT5G58760
Methionine aminopeptidase 1D	MAP1D	1.3	0.04	CA02g25570	AT4G37040
TolB protein-like protein	-	1.2	0.05	CA06g01340	AT4G01870
Glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	-	1.2	0.02	CA00g36190	AT2G33160
NC domain-containing protein-like protein	-	1.2	0.02	CA02g19070	AT3G02700
SET domain-containing protein	CLF	1.2	0.04	CA00g89910	AT2G23380

Table S2. Specific differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to control conditions. It is represented only down-regulated genes (FC<1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
ADP-ribosylation factor family protein	TTN5	0.8	0.05	CA07g08910	AT2G18390
NB-ARC domain-containing disease resistance protein	RPP13	0.8	0.03	CA12g20470	AT3G46530
BTB/POZ domain with WD40/YVTN repeat-like protein	-	0.8	0.04	CA00g75010	AT4G30940
Glutamine-tRNA ligase, putative / glutaminyl-tRNA synthetase. putative / GlnRS	OVA9	0.8	0.05	CA11g00840	AT1G25350
Uncoupling protein 2	UCP2	0.8	0.02	CA09g10550	AT5G58970
ACT domain-containing protein	ACR9	0.8	0.03	CA05g18730	AT2G39570
Receptor kinase 3	RK3	0.8	0.05	CA03g00160	AT4G21380
SKP1 interacting partner 4	SKIP4	0.8	0.04	CA08g07070	AT3G61350
C2H2-like zinc finger protein	-	0.8	0.04	CA10g15040	AT3G45260
ARM repeat superfamily protein	LFR	0.8	0.05	CA03g06500	AT3G22990
Phosphofructokinase family protein	-	0.8	0.02	CA12g18280	AT1G76550
Tubby like protein 2	TLP2	0.8	0.04	CA04g07310	AT2G18280
BPS1-like protein	-	0.8	0.04	CA08g14490	AT1G22030
ARID/BRIGHT DNA-binding domain-containing protein	-	0.8	0.03	CA12g15060	AT3G43240
Disease resistance protein (TIR-NBS-LRR class)	-	0.8	0.05	CA12g19890	AT5G17680
O-fucosyltransferase family protein	-	0.8	0.04	CA00g52440	AT1G20550
Cytochrome P450. family 72, subfamily A. polypeptide 15	CYP72A15	0.8	0.02	CA01g32190	AT3G14690
Translocase inner membrane subunit 17-2	TIM17-2	0.8	0.05	CA05g05170	AT2G37410
Ferritin 2	FER2	0.8	0.05	CA05g15360	AT3G11050
Tobamovirus multiplication protein	Cand3	0.8	0.03	CA02g11960	AT3G59090
Transmembrane protein	-	0.7	0.05	CA01g21710	AT5G55570
Plastid transcriptionally active 16	PTAC16	0.7	0.04	CA06g04680	AT3G46780
UDP-glucosyl transferase 71D1	UGT71D1	0.7	0.05	CA05g18350	AT2G29730
F-box protein 2	FBX2	0.7	0.05	CA11g14780	AT5G21040
Methyltransferase	-	0.7	0.04	CA08g12050	AT5G01710
Receptor kinase 3	RK3	0.7	8.59E-03	CA01g08140	AT4G21380

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Terpene synthase 21	TPS21	0.7	8.59E-03	CA09g15240	AT5G23960
Translation initiation factor SUI1 family protein	-	0.7	0.05	CA07g19530	AT1G54290
Endoplasmic reticulum oxidoreductins 1	ERO1	0.7	0.02	CA00g72080	AT1G72280
Heme binding protein	-	0.7	0.05	CA01g22320	AT3G62370
Granulin repeat cysteine protease family protein	RD21B	0.7	0.04	CA12g16580	AT5G43060
Protein phosphatase 2C family protein	-	0.7	0.02	CA04g12930	AT1G43900
Ca(2)-dependent nuclease family protein	AtCaN2	0.7	0.04	CA00g93270	AT2G40410
RNA processing FACTOR	RPF1	0.7	8.59E-03	CA00g82510	AT1G12700
F-box family protein	-	0.7	0.03	CA03g32370	AT5G07610
Kinase superfamily with octicosapeptide/ Phox/Bem1p domain-containing protein	-	0.7	0.03	CA07g00400	AT5G57610
Nuclear factor Y. subunit A1	NF-YA1	0.7	0.03	CA06g10300	AT5G12840
DNAse I-like superfamily protein	-	0.7	0.03	CA02g28970	AT3G63240
Class II aminoacyl-tRNA and biotin synthetases superfamily protein	NS1	0.7	0.02	CA03g23760	AT4G17300
Zinc finger protein 2	ZFP2	0.7	0.03	CA00g69610	AT5G57520
Tetratricopeptide repeat (TPR)-like superfamily protein	-	0.7	0.03	CA04g09580	AT4G21065
ARM repeat superfamily protein	RST1	0.7	0.04	CA02g29940	AT3G27670
Aluminium activated malate transporter family protein	-	0.7	0.03	CA00g64590	AT4G00910
Transducin/WD40 repeat-like superfamily protein	EMB2757	0.7	0.03	CA00g84410	AT4G29860
UDP-Glycosyltransferase superfamily protein	UGT84A2	0.7	0.04	CA09g18160	AT3G21560
PLAC8 family protein	-	0.7	0.01	CA10g14920	AT2G37110
Leucine-rich repeat (LRR) family protein	-	0.7	0.05	CA10g18520	AT5G21090
S-locus lectin protein kinase family protein	-	0.7	0.02	CA02g17410	AT1G11410
Peroxidase superfamily protein	-	0.7	0.03	CA00g44710	AT2G37130
ENTH/VHS/GAT family protein	-	0.7	0.02	CA08g06090	AT4G32760
Glucose-1-phosphate adenylyltransferase family protein	CYT1	0.7	0.03	CA03g13750	AT2G39770
GDSL-like Lipase/Acylhydrolase superfamily protein	-	0.7	0.04	CA10g03820	AT5G45960

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
TLD-domain containing nucleolar protein	-	0.7	0.04	CA12g12170	AT4G39870
Zinc finger (C3HC4-type RING finger) family protein	-	0.7	0.04	CA00g95920	AT3G45560
CAAX protease self-immunity protein	-	0.7	0.04	CA00g50240	AT2G35260
Phototropic-responsive NPH3 family protein	-	0.7	0.04	CA07g12540	AT3G22104
Copia-like polyprotein/retrotransposon	-	0.7	0.02	CA01g15740	AT1G21280
Polynucleotidyl transferase. ribonuclease H-like superfamily protein	-	0.7	0.02	CA00g25010	AT5G42965
F-box/LRR protein	-	0.7	0.02	CA05g00740	AT5G63520
Transmembrane protein	-	0.7	0.02	CA01g01230	AT5G60000
Splicing endonuclease 1	SEN1	0.7	0.03	CA06g23700	AT3G45590
S-adenosylmethionine synthetase 2	SAM-2	0.7	0.01	CA00g62160	AT4G01850
Nuclear factor Y, subunit A5	NF-YA5	0.7	0.02	CA12g14730	AT1G54160
Sucrose transporter 4	SUT4	0.7	0.03	CA04g17270	AT1G09960
Putative AT-hook DNA-binding family protein	-	0.7	0.02	CA12g15570	AT5G49700
Pyruvate orthophosphate dikinase	PPDK	0.7	0.04	CA01g23570	AT4G15530
Ubiquitin-specific protease 15	UBP15	0.7	0.04	CA07g16320	AT1G17110
Ubiquitin-like superfamily protein	-	0.7	0.03	CA03g02960	AT2G43210
Receptor like protein 7	RLP7	0.7	0.02	CA09g12200	AT1G47890
Phosphoenolpyruvate carboxykinase 1	PCK1	0.7	0.01	CA04g17220	AT4G37870
Alpha/beta-Hydrolases superfamily protein	-	0.7	0.02	CA02g05520	AT5G16120
Eceriferum 1	CER1	0.7	0.02	CA01g27070	AT1G02205
UDP-glycosyltransferase 73B4	UGT73B4	0.7	0.04	CA09g18040	AT2G15490
AP2/B3-like transcriptional factor family protein	VRN1	0.7	0.03	CA04g19550	AT3G18990
Fibrillin 1A	FIB1A	0.7	0.04	CA02g18750	AT4G04020
RAB geranylgeranyl transferase beta subunit 1	RGTB1	0.7	0.02	CA03g31010	AT5G12210
C2H2-like zinc finger protein	DOT5	0.7	0.03	CA00g88960	AT1G13290
Sensitivity to red light reduced protein (SRR1)	SRR1	0.7	0.03	CA00g43180	AT5G59560
Ankyrin repeat family protein	-	0.6	0.04	CA01g32270	AT1G03670
P-loop containing nucleoside triphosphate hydrolases superfamily protein	-	0.6	0.03	CA06g10000	AT5G58370

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
CCCH-type zinc finger protein with ARM repeat domain-containing protein	OXS2	0.6	4.63E-03	CA03g36600	AT2G41900
RHOMBOID-like protein 3	RBL3	0.6	0.02	CA03g16240	AT5G07250
Senescence regulator (Protein of unknown function. DUF584)	-	0.6	0.02	CA02g18230	AT1G11700
Homeobox 7	HB-7	0.6	0.04	CA01g00300	AT2G46680
NagB/RpiA/CoA transferase-like superfamily protein	-	0.6	0.03	CA12g08460	AT3G07300
Hypothetical protein (DUF810)	-	0.6	0.04	CA03g04710	AT2G33420
Phytochromobilin:ferredoxin oxidoreductase, chloroplast / phytochromobilin synthase (HY2)	HY2	0.6	0.04	CA01g13000	AT3G09150
Photosystem I assembly protein	YCF3	0.6	0.01	CA00g81520	ATCG00360
Cytochrome P450, family 72, subfamily A, polypeptide 15	CYP72A15	0.6	0.02	CA00g98060	AT3G14690
Seven in absentia of Arabidopsis 2	SINAT2	0.6	0.01	CA01g11670	AT3G58040
Eukaryotic aspartyl protease family protein	-	0.6	0.01	CA00g75560	AT3G20015
Concanavalin A-like lectin protein kinase family protein	-	0.6	0.01	CA04g04250	AT5G06740
Calcineurin B-like protein 9	CBL9	0.6	0.04	CA01g33680	AT5G47100
Nuclear factor kappa-B-binding protein	-	0.6	0.01	CA02g31340	AT5G13950
Cytochrome P450, family 71, subfamily B, polypeptide 35	CYP71B35	0.6	0.03	CA12g05210	AT3G26310
O-acyltransferase (WSD1-like) family protein	WSD6	0.6	0.02	CA00g64820	AT3G49210
Terpene synthase 21	TPS21	0.6	0.02	CA12g05180	AT5G23960
Cytochrome P450, family 86, subfamily A, polypeptide 8	CYP86A8	0.6	0.04	CA08g07320	AT2G45970
Beta-amylase 5	BAM5	0.6	0.02	CA07g12430	AT4G15210
General regulatory factor 12	GRF12	0.6	0.04	CA10g13730	AT1G26480
Inositol monophosphatase family protein	FBP	0.6	7.00E-03	CA12g11990	AT1G43670
Calcium-binding EF-hand family protein	-	0.6	0.04	CA03g18530	AT4G38810
Transcription factor-like protein	-	0.6	0.03	CA12g00150	AT3G14880
Germin-like protein 5	GLP5	0.6	0.04	CA08g13340	AT1G09560
Protein-tyrosine phosphatase-like, PTPLA	PAS2	0.6	8.77E-03	CA05g02070	AT5G10480
Phloem protein 2-B10	PP2-B10	0.6	2.27E-04	CA12g20010	AT2G02360

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Leucine-rich repeat protein kinase family protein	-	0.6	0.03	CA09g17210	AT1G72460
Terpene synthase 21	TPS21	0.6	0.04	CA03g18030	AT5G23960
Endoplasmic reticulum-type calcium-transporting ATPase 3	ECA3	0.6	0.04	CA10g17960	AT1G10130
Sugar transporter protein 7	STP7	0.6	8.73E-04	CA03g12450	AT4G02050
Protein kinase superfamily protein	NCRK	0.6	0.04	CA11g18840	AT2G28250
F-box and associated interaction domains-containing protein	-	0.6	0.04	CA02g11640	AT1G11620
Glycosyl hydrolase family 35 protein	-	0.6	0.02	CA10g07090	AT2G16730
IBR domain-containing protein	ARI8	0.6	0.03	CA08g00300	AT1G65430
Elongator complex protein	-	0.6	0.04	CA02g23490	AT2G18410
Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	-	0.6	0.01	CA01g01610	AT2G46150
Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein	-	0.6	0.03	CA11g00740	AT1G68390
Cytochrome P450, family 94, subfamily B, polypeptide 2	CYP94B2	0.6	0.02	CA02g30770	AT3G01900
Lysophosphatidyl acyltransferase 5	LPAT5	0.6	0.01	CA08g18340	AT3G18850
RING/U-box superfamily protein	-	0.6	0.01	CA02g00170	AT4G30370
Homeodomain-like transcriptional regulator	-	0.6	0.01	CA05g15200	AT5G58900
P-loop containing nucleoside triphosphate hydrolases superfamily protein	KINESIN-13A	0.6	4.39E-03	CA05g18020	AT3G16630
Zinc transporter 5 precursor	ZIP5	0.6	0.03	CA10g19490	AT1G05300
D-isomer specific 2-hydroxyacid dehydrogenase family protein	-	0.6	0.04	CA03g34810	AT1G79870
Cytochrome P450, family 82, subfamily C, polypeptide 4	CYP82C4	0.6	0.01	CA10g18220	AT4G31940
Trehalose phosphatase/synthase 11	TPS11	0.6	0.02	CA07g00130	AT2G18700
MAP kinase 20	MPK20	0.6	6.83E-03	CA07g15380	AT2G42880
NAD(P)-binding Rossmann-fold superfamily protein	-	0.6	5.22E-03	CA12g22560	AT3G46170
Mitochondrial substrate carrier family protein	BAC2	0.6	0.02	CA06g16670	AT1G79900
Kinase family with leucine-rich repeat domain-containing protein	-	0.6	7.69E-05	CA04g03150	AT1G35710

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Calcium-dependent lipid-binding (CaLB domain) family protein	C2	0.6	7.69E-05	CA12g11530	AT3G17980
Vamp/synaptobrevin-associated protein 27-2	VAP27-2	0.6	7.02E-03	CA08g11200	AT1G08820
Alpha/beta-Hydrolases superfamily protein	-	0.6	0.04	CA05g18360	AT4G02340
Histone superfamily protein	-	0.6	0.04	CA04g15730	AT5G10980
ZIM-like 1	ZML1	0.6	5.92E-03	CA04g15250	AT3G21175
Mitochondrial F0-ATPase subunit 9	ATP9	0.6	0.04	CA01g30670	ATMG01080
Mitochondrial ribosomal protein S14	RPS14	0.6	0.02	CA10g05440	AT2G34520
SET domain group 37	SDG37	0.6	0.02	CA06g12160	AT2G17900
Nitrate transporter 1.7	NRT1.7	0.6	0.03	CA11g05750	AT1G69870
Transducin/WD40 repeat-like superfamily protein	-	0.6	0.02	CA03g03170	AT5G56190
F-box family protein	-	0.5	8.51E-03	CA11g20210	AT3G06240
Transmembrane protein	-	0.5	5.07E-04	CA04g04190	AT1G65720
Metallopeptidase M24 family protein	-	0.5	0.04	CA08g13170	AT1G09300
Ribosomal protein L31e family protein	-	0.5	0.02	CA00g85720	AT2G19740
Non-specific phospholipase C2	NPC2	0.5	0.02	CA01g12500	AT2G26870
Trichome birefringence-like 38	TBL38	0.5	0.01	CA02g11220	AT1G29050
Dynein light chain type 1 family protein	-	0.5	5.87E-03	CA09g15090	AT5G20110
Yellow stripe like 2	YSL2	0.5	0.02	CA03g20380	AT5G24380
Leucine-rich repeat receptor-like protein kinase family protein	-	0.5	0.02	CA05g13330	AT4G08850
Transmembrane proteins 14C	-	0.5	0.05	CA10g20280	AT3G57280
BAK1-interacting receptor-like kinase 1	BIR1	0.5	0.03	CA00g75620	AT5G48380
Major facilitator superfamily protein	-	0.5	0.01	CA01g27680	AT5G19640
Rubisco methyltransferase family protein	LSMT-L	0.5	0.01	CA11g04070	AT1G14030
BTB and TAZ domain protein 1	BT1	0.5	0.03	CA06g19860	AT5G63160
Transducin/WD40 repeat-like superfamily protein	-	0.5	0.03	CA02g13530	AT5G45760
Homeodomain-like superfamily protein	RVE8	0.5	0.03	CA10g14780	AT3G09600
TatD related DNase	-	0.5	0.02	CA06g02600	AT3G52390
Transcription elongation factor	-	0.5	0.02	CA00g00620	AT5G47920
Cytochrome C assembly protein	CCB203	0.5	7.24E-03	CA02g26520	ATMG00960
DUF4228 domain protein	-	0.5	9.85E-03	CA02g25370	AT5G67620

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
RecA DNA recombination family protein	RECA2	0.5	8.59E-03	CA05g20060	AT2G19490
Cellulose synthase family protein	CEV1	0.5	0.01	CA01g20250	AT5G05170
Neurogenic locus notch-like protein	-	0.5	0.03	CA08g10440	AT4G14746
ARM repeat superfamily protein	-	0.5	0.01	CA02g24200	AT3G03440
Tetratricopeptide repeat (TPR)-like superfamily protein	-	0.5	0.01	CA07g04400	AT1G80130
Alpha/beta-Hydrolases superfamily protein	-	0.5	0.02	CA04g06250	AT1G66900
Hercules receptor kinase 2	HERK2	0.5	0.04	CA06g10660	AT1G30570
Terpenoid cyclases/Protein prenyltransferases superfamily protein	-	0.5	0.01	CA08g16380	AT3G25810
F-box family protein	MEE66	0.5	0.03	CA01g31560	AT2G02240
Tudor/PWWP/MBT superfamily protein	-	0.5	8.70E-03	CA06g21350	AT3G03140
Aluminium induced protein with YGL and LRDR motifs	AILP1	0.5	0.02	CA01g24020	AT5G19140
CLEC16A-like protein	-	0.5	0.04	CA07g06430	AT3G28430
Beta-galactosidase 8	BGAL8	0.5	0.03	CA02g16380	AT2G28470
Rubber elongation factor protein (REF)	-	0.5	0.02	CA03g08410	AT3G05500
Pectin lyase-like superfamily protein	-	0.5	0.03	CA09g01850	AT3G53190
AAA-type ATPase family protein	-	0.5	6.70E-03	CA06g20340	AT1G02890
NAD(P)-binding Rossmann-fold superfamily protein	-	0.5	0.01	CA12g16290	AT3G55310
CRINKLY4 related 3	CCR3	0.5	0.01	CA00g47510	AT3G55950
SHI-related sequence 5	SRS5	0.5	0.03	CA04g06890	AT1G75520
DNA repair protein RadA-like protein	-	0.4	6.44E-03	CA03g35970	AT5G50340
DNAJ heat shock family protein	-	0.4	2.23E-03	CA08g06460	AT5G25530
O-methyltransferase family protein	-	0.4	0.02	CA06g05520	AT4G35160
DEAD/DEAH box RNA helicase family protein	FANCM	0.4	0.01	CA04g09250	AT1G35530
Quinone reductase family protein	-	0.4	7.34E-03	CA06g25220	AT5G58800
ARM repeat protein interacting with ABF2	ARIA	0.4	0.03	CA09g08790	AT5G19330
Mitovirus RNA-dependent RNA polymerase	-	0.4	0.04	CA11g07030	AT2G07749
Seed maturation protein	-	0.4	0.02	CA03g07450	AT3G22490
Transducin/WD40 repeat-like superfamily protein	AtATG18a	0.4	2.10E-03	CA01g30560	AT3G62770

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Jasmonate resistant 1	JAR1	0.4	1.45E-03	CA08g08190	AT2G46370
NPK1-related protein kinase 3	NP3	0.4	0.02	CA01g24590	AT3G06030
Receptor-like protein kinase 4	RLK4	0.4	0.02	CA08g07410	AT4G00340
Mental retardation GTPase activating protein	-	0.4	0.02	CA02g23130	AT5G16030
NAC domain containing protein 1	NAC1	0.4	0.04	CA07g21470	AT1G56010
Pseudouridine synthase family protein	-	0.4	0.02	CA03g21840	AT5G51140
Peroxisomal adenine nucleotide carrier 1	PNC1	0.4	0.01	CA00g43860	AT3G05290
NAD(P)-binding Rossmann-fold superfamily protein	-	0.4	9.89E-03	CA12g22690	AT3G46170
Sulfite exporter TauE/SafE family protein	-	0.4	3.78E-03	CA09g01690	AT2G36630
Protein phosphatase 2C family protein	-	0.4	6.70E-03	CA05g20050	AT3G02750
Cytochrome P450 superfamily protein	-	0.4	0.04	CA12g16400	AT1G66540
Hypothetical protein (DUF1639)	-	0.3	9.61E-04	CA00g58480	AT1G55340
Histidine kinase-. DNA gyrase B-, and HSP90-like ATPase family protein	NOV	0.3	0.02	CA03g00360	AT4G13750
Calmodulin-binding transcription activator 5	CAMTA5	0.3	1.46E-03	CA01g14110	AT4G16150
Cytochrome P450 superfamily protein	-	0.2	5.08E-04	CA06g19760	AT5G08250
CCT motif family protein	-	0.2	0.01	CA03g19650	AT5G53420
P-loop containing nucleoside triphosphate hydrolases superfamily protein	-	0.2	5.51E-03	CA04g14480	AT1G01910
Homeodomain-like superfamily protein	-	0.2	6.15E-04	CA01g08210	AT5G45580
LOB domain-containing protein 42	LBD42	0.2	0.02	CA11g01060	AT1G68510
Undecaprenyl pyrophosphate synthetase family protein	cPT4	0.1	6.57E-05	CA10g14100	AT5G58770

Table S3. Specific differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to salt stress conditions. It is represented only up-regulated genes (FC>1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Hypothetical protein	-	6.5	0.03	CA03g15370	AT4G26450
Alpha/beta-Hydrolases superfamily protein	-	4.5	2.29E-03	CA09g00860	AT2G36290
Tetratricopeptide repeat (TPR)-like superfamily protein	-	4.0	0.05	CA05g17010	AT1G04770
Sterol-4alpha-methyl oxidase 1-1	SMO1-1	3.6	0.02	CA01g11080	AT4G12110
Temperature-induced lipocalin	TIL	3.4	1.01E-03	CA07g02210	AT5G58070
Myotubularin-like phosphatases II superfamily	MTM1	2.7	0.05	CA12g05660	AT3G10550
Chaperone DnaJ-domain superfamily protein	J8	2.7	3.92E-03	CA00g87730	AT1G80920
Formyltransferase	pde194	2.6	0.04	CA02g24050	AT1G66520
Basic chitinase	HCHIB	2.5	0.01	CA03g31390	AT3G12500
Peptidase S41 family protein	-	2.4	9.44E-03	CA06g09990	AT3G57680
Chalcone and stilbene synthase family protein	TT4	2.4	0.03	CA05g17040	AT5G13930
Proteolysis 6	PRT6	2.2	0.04	CA10g14530	AT5G02310
Catalytic LigB subunit of aromatic ring-opening dioxygenase family	LigB	2.2	0.03	CA08g00170	AT4G15093
Organic cation/carnitine transporter4	OCT4	2.2	5.81E-03	CA07g18590	AT3G20660
P-loop containing nucleoside triphosphate hydrolases superfamily protein	-	2.2	0.02	CA03g22090	AT5G35970
WUSCHEL related homeobox 4	WOX4	2.1	9.28E-04	CA04g18420	AT1G46480
Non-structural protein	-	2.0	0.02	CA09g11810	AT1G03180
Serine/arginine-rich splicing factor-like protein, putative	SC35	1.9	0.01	CA04g08840	AT5G64200
DA1-related protein 4	DAR4	1.9	4.37E-03	CA02g02530	AT5G17890
Sulfotransferase 12	SOT12	1.9	0.02	CA11g03250	AT2G03760
Auxin efflux carrier family protein	PIN3	1.9	0.03	CA05g08660	AT1G70940
Ubiquitin-like superfamily protein	-	1.9	0.03	CA12g01310	AT4G05230
Temperature-induced lipocalin	TIL	1.9	0.03	CA09g18430	AT5G58070
3'-5' exonuclease domain-containing protein / K homology domain-containing protein / KH domain-containing protein	-	1.8	7.99E-03	CA03g36100	AT2G25910

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
DNA/RNA polymerases superfamily protein	ORF158	1.8	4.65E-03	CA00g76850	ATMG00860
Class I heat shock protein, putative (DUF1423)	TTA1	1.7	7.81E-03	CA04g04530	AT1G14740
General transcription factor group E6	GTE6	1.7	0.04	CA05g10620	AT3G52280
Magnesium transporter, putative (DUF803)	-	1.6	0.01	CA11g12750	AT1G34470
Phosphoribosyltransferase family protein	-	1.6	0.03	CA07g15420	AT2G42910
Nudix hydrolase homolog 17	NUDT17	1.6	0.02	CA12g02260	AT2G01670
Inorganic pyrophosphatase 1	PS2	1.6	0.02	CA03g36140	AT1G73010
Serine hydroxymethyltransferase 3	SHM3	1.5	0.02	CA12g18510	AT4G32520
SNF1-related protein kinase 2.5	SNRK2.5	1.5	0.04	CA12g16870	AT5G63650
HCO3- transporter family	-	1.5	0.04	CA01g22650	AT3G62270
HCO3- transporter family	-	1.5	0.04	CA01g22660	AT3G62270
Ubiquitin-specific protease 12	UBP12	1.5	0.05	CA05g19150	AT5G06600
Ferritin 4	FER4	1.4	0.01	CA05g09460	AT2G40300
Profilin 1	PRF1	1.4	0.04	CA11g20130	AT2G19760
Aspartic proteinase A1	APA1	1.4	0.04	CA07g11500	AT1G11910
THUMP domain-containing protein	-	1.4	0.03	CA11g10530	AT5G12410
Expansin A4	EXPA4	1.4	0.02	CA02g18410	AT2G39700
Cytochrome c biogenesis protein family	CcdA	1.4	0.04	CA07g18200	AT5G54290
NHL domain-containing protein	-	1.4	0.03	CA11g07390	AT1G23890
SGNH hydrolase-type esterase superfamily protein	-	1.4	0.03	CA06g19700	AT5G62930
Transmembrane protein	-	1.4	0.03	CA06g27190	AT1G67020
Phosphate transporter 1;4	PHT1;4	1.4	0.04	CA03g05830	AT2G38940
BCL-2-associated athanogene 6	BAG6	1.4	0.04	CA08g07920	AT2G46240
Tetratricopeptide repeat (TPR)-like superfamily protein	TPR4	1.4	0.04	CA03g04290	AT1G04530
Nucleoporin interacting component (Nup93/Nic96-like) family protein	-	1.4	0.05	CA05g04820	AT2G41620
Trichome birefringence-LIKE 36	TBL36	1.3	0.04	CA12g06670	AT3G54260
GATA transcription factor 1	GATA1	1.3	0.02	CA03g02360	AT3G24050
Zinc induced facilitator-like 1	ZIFL1	1.3	0.04	CA10g22410	AT5G13750
Zinc finger C-x8-C-x5-C-x3-H type family protein	-	1.3	0.05	CA08g14890	AT1G10320

Table S4. Specific differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to salt stress conditions. It is represented only down-regulated genes (FC<1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
ABC transporter family protein	ABCF1	0.8	0.03	CA11g17940	AT5G60790
NAD(P)-binding Rossmann-fold superfamily protein	-	0.8	0.02	CA08g11450	AT5G06060
Arogenate dehydratase 2	ADT2	0.7	0.01	CA11g14180	AT3G07630
Alpha/beta-Hydrolases superfamily protein	-	0.7	0.03	CA08g05220	AT5G11650
Squalene epoxidase 3	SQE3	0.7	0.04	CA02g07240	AT4G37760
Cyclin-D1-binding protein	-	0.7	0.03	CA11g00110	AT1G22970
Subtilase family protein	-	0.7	0.02	CA10g14810	AT3G14240
Photosystem II reaction center PsbP family protein	PPD1	0.7	0.04	CA01g31620	AT4G15510
Eceriferum 1	CER1	0.7	0.01	CA00g87940	AT1G02205
Protein kinase superfamily protein	-	0.7	0.02	CA11g07190	AT1G24030
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	CCoAOMT1	0.7	8.97E-03	CA02g14470	AT4G34050
Protein kinase superfamily protein	CDL1	0.7	0.01	CA10g11900	AT5G02800
Alternative oxidase 1A	AOX1A	0.6	0.02	CA09g09850	AT3G22370
Glycosyl hydrolase superfamily protein	-	0.6	0.02	CA11g17020	AT2G27500
O-Glycosyl hydrolases family 17 protein	-	0.6	0.01	CA10g21610	AT1G64760
Hypothetical protein (DUF1997)	-	0.6	2.29E-03	CA02g01000	AT5G39530
SPFH/Band 7/PHB domain-containing membrane-associated protein family	-	0.6	0.01	CA03g20940	AT4G27585
Multidrug resistance-associated protein 14	ABCC10	0.6	0.05	CA03g19050	AT3G59140
Transmembrane amino acid transporter family protein	-	0.6	0.01	CA04g10860	AT5G15240
Pentatricopeptide (PPR) repeat-containing protein	-	0.6	0.01	CA12g07360	AT5G04810
Dimethylallyl. adenosine tRNA methylthiotransferase	-	0.5	8.05E-03	CA04g19040	AT4G33380
Hypothetical protein	-	0.5	8.05E-03	CA06g09500	AT1G64870
RING-box 1	RBX1	0.5	5.71E-03	CA09g17140	AT5G20570
BSD domain-containing protein	-	0.5	3.19E-03	CA04g17350	AT1G10720

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Auxin efflux carrier family protein	PILS1	0.5	0.04	CA04g23480	AT1G20925
NHL domain-containing protein	-	0.5	0.02	CA11g07380	AT1G70280
Allantoinase	ALN	0.5	2.36E-03	CA02g15930	AT4G04955
4-phosphopantetheine adenylyltransferase	COAD	0.5	9.25E-04	CA06g04390	AT2G18250
4-phosphopantetheine adenylyltransferase	COAD	0.5	9.25E-04	CA00g94400	AT2G18250
2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	-	0.5	9.25E-04	CA12g07770	AT4G23340
Major facilitator superfamily protein	-	0.5	3.52E-02	CA02g06500	AT4G36790
RNA-binding KH domain-containing protein	-	0.5	2.36E-03	CA06g26010	AT3G13230
Auxin efflux carrier family protein	PLS3	0.4	0.01	CA04g23470	AT1G76520
Gamma-glutamyl hydrolase 1	GGH1	0.4	0.04	CA07g16860	AT1G78660
Protein kinase family protein	THE1	0.4	0.02	CA10g00440	AT5G54380
Zinc-binding alcohol dehydrogenase family protein	-	0.3	2.18E-04	CA12g18000	AT5G42250
Alpha/beta-Hydrolases superfamily protein	-	0.3	6.24E-03	CA11g14470	AT3G09690
Alpha/beta-Hydrolases superfamily protein	-	0.2	3.65E-04	CA05g18260	AT4G02340
GATA transcription factor 17	GATA17	0.1	0.02	CA12g21330	AT3G16870
Protochlorophyllide oxidoreductase A	PORA	0.1	0.02	CA10g00480	AT5G54190

Table S5. Common differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to control and salt stress conditions. It is represented only up-regulated genes (FC>1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Oxidoreductase family protein	-	17.8	8.49E-07	2.5	7.68E-03	CA01g05600	AT4G17370
Cell division control 2	CDC2	13.6	9.57E-05	6.9	1.15E-03	CA12g18420	AT3G48750
Xylulose kinase-1	XK-1	11.5	6.96E-06	11.0	1.48E-05	CA12g08890	AT2G21370
SNF2 domain-containing protein / helicase domain-containing protein	PIE1	10.4	4.48E-06	23.3	6.59E-07	CA04g21650	AT3G12810
NAD(P)-binding Rossmann-fold superfamily protein	-	10.1	1.76E-03	38.6	4.84E-05	CA05g12020	AT5G06060
Polynucleotidyl transferase. ribonuclease H-like superfamily protein	-	7.4	4.20E-04	8.6	2.49E-04	CA07g17410	AT3G15140
Sodium Bile acid symporter family	BASS1	7.1	7.34E-03	22.0	2.28E-04	CA09g06260	AT1G78560
SKU5 similar 5	SKS5	6.7	2.71E-07	6.3	3.31E-07	CA12g17970	AT1G76160
Cytochrome P450, family 706, subfamily A, polypeptide 4	CYP706A4	5.0	5.71E-05	3.6	4.34E-04	CA00g86940	AT4G12300
Hypothetical protein	-	4.9	2.35E-04	5.8	1.17E-04	CA00g88030	AT5G43680
tRNA modification GTPase	-	4.4	8.28E-03	4.3	0.01	CA04g15120	AT1G78010
NAD(P)-binding Rossmann-fold superfamily protein	SDR2	3.6	0.02	4.8	6.19E-03	CA06g14350	AT3G51680
SNF1-related protein kinase 2.10	SnRK2.10	3.6	3.20E-04	4.0	1.78E-04	CA08g14400	AT1G60940
Adenine phosphoribosyltransferase-like protein, putative (DUF2358)	-	3.5	6.94E-03	4.6	1.96E-03	CA08g10760	AT3G04890
Hydroxyproline-rich glycoprotein family protein	EXT-like	3.5	1.58E-06	3.6	1.64E-06	CA07g19920	AT4G26750
Hypothetical protein (DUF674)	-	3.1	2.12E-05	3.2	2.55E-05	CA06g18830	AT3G09110

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Ternary complex factor MIP1 leucine-zipper protein (Protein of unknown function, DUF547)	-	3.0	3.56E-04	1.8	0.05	CA02g25660	AT4G37080
5′-3′ exonuclease family protein	-	3.0	1.05E-03	3.4	6.27E-04	CA03g33080	AT1G18090
Glutaredoxin family protein	-	3.0	6.26E-03	5.1	2.74E-04	CA10g20020	AT3G57070
Signal peptide peptidase-like 1	SPPL1	2.9	7.65E-04	2.8	1.15E-03	CA03g17890	AT4G33410
CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein	-	2.5	2.10E-03	2.0	0.02	CA01g31110	AT3G19690
Disease resistance protein (CC-NBS-LRR class) family	-	2.5	2.10E-03	2.0	0.02	CA00g93700	AT5G35450
Pectin methylesterase 1	PME1	2.5	3.37E-05	2.7	2.15E-05	CA03g36990	AT1G53840
Protein kinase superfamily protein	-	2.4	0.01	2.4	0.02	CA12g16510	AT1G53050
Early-responsive to dehydration stress protein (ERD4)	ERD4	2.4	5.47E-04	2.0	4.87E-03	CA08g02700	AT1G30360
Syntaxin of plants 61	SYP61	2.4	3.42E-05	2.3	5.84E-05	CA12g17160	AT1G28490
Ribosomal protein L7/L12 domain-containing protein	-	2.4	6.57E-05	2.3	8.88E-05	CA06g01410	AT1G70190
AMP-dependent synthetase and ligase family protein	AAE11	2.4	4.38E-04	1.9	6.24E-03	CA02g20380	AT1G66120
Alba DNA/RNA-binding protein	-	2.3	6.98E-03	2.0	0.03	CA03g01910	AT3G07030
ACT domain repeat 6	ACR6	2.3	0.02	2.3	0.02	CA08g06080	AT3G01990
Importin alpha isoform 1	IMPA-1	2.3	0.02	2.3	0.02	CA04g15350	AT3G06720
Minichromosome instability 12 (mis12)-like protein	MIS12	2.2	8.89E-03	2.0	0.02	CA03g29160	AT5G35520
Disease resistance protein (CC-NBS-LRR class) family	-	2.2	3.31E-05	2.2	4.80E-05	CA00g73250	AT5G35450
Protein phosphatase 2C family protein	APD6	2.1	1.01E-03	2.2	7.62E-04	CA10g07130	AT4G38520
Homoserine kinase	HSK	2.1	2.75E-03	1.8	0.01	CA00g97170	AT2G17265
Photosystem II subunit P-1	PSBP-1	2.1	1.34E-04	2.3	4.80E-05	CA07g07930	AT1G06680

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Alpha-L-fucosidase 1	FUC1	2.0	3.10E-04	1.8	1.15E-03	CA00g64130	AT2G28100
3-ketoacyl-acyl carrier protein synthase I	KASI	2.0	0.02	4.2	1.49E-04	CA01g00840	AT5G46290
3-ketoacyl-acyl carrier protein synthase I	KASI	2.0	0.02	4.2	1.49E-04	CA01g00830	AT5G46290
Ubiquitin-conjugating enzyme 10	UBC10	2.0	0.02	1.9	0.04	CA03g19120	AT5G53300
Hyaluronan / mRNA binding family	-	2.0	3.29E-05	1.9	8.76E-05	CA01g33750	AT4G16830
Adenine nucleotide alpha hydrolases-like superfamily protein	-	2.0	0.02	2.0	0.03	CA09g05970	AT3G53990
NAD(P)-linked oxidoreductase superfamily protein	PLR1	2.0	0.01	2.0	0.01	CA03g20520	AT5G53580
RING/FYVE/PHD zinc finger superfamily protein	-	2.0	2.75E-03	1.7	0.02	CA03g30050	AT3G47550
Tetratricopeptide repeat (TPR)-like superfamily protein	-	2.0	0.02	2.1	0.01	CA12g15360	AT2G40720
Flocculation protein (DUF1296)	-	1.9	0.02	2.2	9.43E-03	CA05g09200	AT3G07660
HAL2-like protein	HL	1.9	7.65E-04	1.9	1.15E-03	CA02g16270	AT5G54390
Hypothetical protein	-	1.9	2.09E-04	2.0	1.23E-04	CA12g00990	AT1G12020
S-locus lectin protein kinase family protein	B120	1.9	1.25E-04	1.4	0.02	CA09g03210	AT4G21390
Importin alpha isoform 1	IMPA-1	1.9	3.31E-03	1.9	4.64E-03	CA06g00340	AT3G06720
Monofunctional riboflavin biosynthesis protein RIBA 3	RIBA3	1.9	4.13E-03	1.7	0.01	CA11g13410	AT5G59750
Phototropic-responsive NPH3 family protein	-	1.8	6.70E-03	1.9	5.81E-03	CA03g34080	AT3G15570
Ribonuclease H2 subunit C-like protein	-	1.8	2.23E-03	1.6	0.01	CA00g50550	AT2G39440
Plant invertase/pectin methylesterase inhibitor superfamily	VGDH1	1.8	3.01E-03	1.8	3.20E-03	CA06g27340	AT2G47030
Isopropylmalate dehydrogenase 2	IMD2	1.8	6.57E-05	1.6	3.53E-04	CA11g00650	AT1G80560
With no lysine (K) kinase 6	WNK6	1.8	0.02	2.0	0.01	CA00g64570	AT3G18750
Cytochrome P450, family 706, subfamily A, polypeptide 6	CYP706A6	1.8	3.01E-04	1.6	9.28E-04	CA00g86950	AT4G12320
Nucleotide-diphospho-sugar transferases superfamily protein	PGSIP8	1.8	2.43E-04	1.9	6.27E-05	CA06g12360	AT4G16600

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Cellulase 5	CEL5	1.7	3.86E-03	2.0	4.59E-04	CA11g09950	AT1G22880
Integrase-type DNA-binding superfamily protein	TINY2	1.7	3.86E-03	2.0	4.59E-04	CA08g04820	AT5G11590
CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein	-	1.7	1.05E-03	1.6	2.46E-03	CA02g07880	AT5G66590
Long-chain acyl-CoA synthetase 2	LACS2	1.6	3.86E-03	1.9	6.64E-04	CA08g18140	AT1G49430
Protein kinase superfamily protein	-	1.6	2.51E-03	1.4	0.04	CA09g14790	AT2G07180
DUF1685 family protein	-	1.5	1.05E-03	1.5	2.96E-03	CA02g13520	AT2G42760
Late embryogenesis abundant protein	LEA27	1.5	0.01	1.7	4.69E-03	CA08g07780	AT2G46140
Plant transposase (Ptta/En/Spm family)	-	1.5	7.34E-03	1.5	8.05E-03	CA10g05500	AT3G30200
Annexin 1	ANNAT1	1.5	0.01	1.5	0.03	CA00g85100	AT1G35720
Pectin lyase-like superfamily protein	-	1.5	7.28E-03	1.5	0.0076476	CA07g21080	AT5G19730
Zn-dependent exopeptidases superfamily protein	-	1.5	0.05	1.6	0.04	CA11g01260	AT5G20660
Zn-dependent exopeptidases superfamily protein	-	1.5	0.05	1.6	0.04	CA11g01250	AT5G20660
General regulatory factor 11	GRF11	1.5	2.75E-03	1.7	7.32E-04	CA04g15570	AT1G34760
Hypothetical protein	-	1.5	0.01	1.7	3.19E-03	CA01g22430	AT1G26750
Hypothetical protein	-	1.5	2.19E-04	1.6	1.28E-04	CA00g43890	AT1G54920
FAD-binding Berberine family protein	-	1.5	0.03	1.6	0.02	CA03g20190	AT1G30760
RHOMBOID-like 1	RBL1	1.5	2.91E-03	1.6	1.03E-03	CA06g04920	AT2G29050
Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	-	1.5	0.04	1.7	0.01	CA00g60460	AT2G01080
Cellulose synthase like E1	CSLE1	1.5	0.02	1.5	0.02	CA05g16620	AT1G55850
Transducin/WD40 repeat-like superfamily protein	-	1.5	0.04	1.6	0.02	CA09g01910	AT5G03450

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Subtilase family protein	ARA12	1.4	5.02E-03	1.4	7.99E-03	CA01g33340	AT5G67360
Transferring glycosyl group transferase	-	1.4	0.02	1.6	2.74E-03	CA03g30800	AT3G23760
Pentatricopeptide repeat (PPR-like) superfamily protein	-	1.4	0.02	1.5	6.60E-03	CA11g01190	AT3G21470
2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	DMR6	1.4	0.02	1.5	6.60E-03	CA05g08790	AT5G24530
Hypothetical protein	-	1.4	0.02	1.5	6.60E-03	CA03g07990	AT2G47485
APO RNA-binding protein (DUF794)	APO1	1.4	0.02	1.5	5.24E-03	CA01g03450	AT1G64810
Extra-large G-protein 1	XLG1	1.4	6.20E-03	1.4	0.01	CA02g26040	AT2G23460
Ribosomal protein S3Ae	-	1.4	0.04	1.4	0.0273687	CA03g07130	AT4G34670
P-loop containing nucleoside triphosphate hydrolases superfamily protein	-	1.4	0.02	1.4	0.02	CA02g03140	AT4G04180
2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	DMR6	1.4	0.02	1.5	8.78E-03	CA07g07070	AT5G24530
Dual specificity protein phosphatase (DsPTP1) family protein	SEX4	1.4	0.01	1.5	2.88E-03	CA12g05430	AT3G52180
Male gametophyte defective 1	MGP1	1.4	1.92E-03	1.2	0.05	CA00g54070	AT2G21870
Major facilitator superfamily protein	-	1.3	0.03	1.3	0.03	CA00g85190	AT3G45710
TolB protein-like protein	-	1.3	0.05	1.3	0.05	CA06g00090	AT4G01870
Y-family DNA polymerase H	POLH	1.3	0.04	1.3	0.04	CA01g16510	AT5G44740
Shortage in chiasmata 1	SHOC1	1.3	0.03	1.4	8.05E-03	CA03g17760	AT5G52290
Pathogenesis-related thaumatin superfamily protein	-	1.2	0.05	1.4	4.79E-03	CA08g19030	AT4G38670

Table S6. Common differentially expressed genes after 14DAT in the comparison A25/A6 in leaves subjected to control and salt stress conditions. It is represented only down-regulated genes (FC<1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Golgin family A protein	-	0.8	0.02	0.8	0.02	CA05g01550	AT4G22320
B-box type zinc finger protein with CCT domain-containing protein	-	0.8	0.01	0.8	8.97E-03	CA03g08890	AT2G47890
Mutator transposase MUDRA protein	-	0.8	0.02	0.8	0.01	CA03g17550	AT5G15685
Receptor like protein 33	RLP33	0.8	0.03	0.8	0.03	CA00g91270	AT3G05660
Uncharacterized protein family (UPF0016)	-	0.8	0.01	0.7	4.79E-03	CA10g17910	AT1G25520
DNAJ heat shock N-terminal domain-containing protein	OWL1	0.8	0.02	0.8	0.03	CA01g05000	AT2G35720
Glycosyl hydrolases family 32 protein	-	0.8	0.02	0.8	0.03	CA03g15480	AT1G62660
ATPase family associated with various cellular activities (AAA)	EMB1968	0.7	0.01	0.7	5.81E-03	CA00g68930	AT1G21690
Calcium ion-binding protein	-	0.7	0.04	0.6	3.19E-03	CA02g14070	AT4G12700
Global transcription factor group A2	GTA2	0.7	0.03	0.6	2.29E-03	CA04g10530	AT4G08350
Glucose-6-phosphate dehydrogenase 2	G6PD2	0.7	0.03	0.7	0.02	CA00g82040	AT5G13110
ERD (early-responsive to dehydration stress) family protein	-	0.7	0.03	0.7	0.03	CA06g26780	AT4G02900
K-box region and MADS-box transcription factor family protein	AP3	0.7	0.04	0.7	0.03	CA04g21160	AT3G54340
UDP-glucosyl transferase 76E2	UGT76E2	0.7	7.59E-03	0.6	1.35E-03	CA10g13310	AT5G59590
2-aminoethanethiol dioxygenase. putative (DUF1637)	-	0.7	7.28E-03	0.7	0.02	CA03g29170	AT1G18490
2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	-	0.7	0.01	0.7	0.04	CA03g05480	AT1G06620

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Remorin family protein	-	0.6	0.02	0.6	9.43E-03	CA03g17720	AT3G48940
Yellow stripe like 1	YSL1	0.6	4.63E-03	0.6	2.36E-03	CA01g00350	AT4G24120
Condensin-2 complex subunit H2-like protein	HEB2	0.6	9.47E-03	0.7	0.04	CA12g21380	AT3G16730
NAC domain containing protein 76	NAC076	0.6	3.96E-04	0.6	4.84E-05	CA06g25420	AT4G36160
P-loop containing nucleoside triphosphate hydrolases superfamily protein	-	0.6	0.03	0.6	0.01	CA04g02500	AT1G65810
P-loop containing nucleoside triphosphate hydrolases superfamily protein	-	0.6	0.02	0.7	0.04	CA03g09750	AT1G03030
Actin cytoskeleton-regulatory complex pan-like protein	-	0.6	5.65E-03	0.7	0.03	CA07g19110	AT1G50660
Chaperonin 60 beta	CPN60B	0.6	2.07E-03	0.5	6.26E-05	CA03g34990	AT1G55490
Cadherin EGF LAG seven-pass G-type receptor, putative (DUF3527)	-	0.6	0.04	0.4	3.19E-03	CA03g04560	AT2G33360
Heat shock transcription factor A6B	HSFA6B	0.6	6.89E-03	0.7	0.04	CA06g08710	AT3G22830
GATA transcription factor 26	GATA26	0.6	0.01	0.5	2.29E-03	CA06g12750	AT4G17570
Trypsin family protein	-	0.6	0.02	0.6	0.03	CA02g09960	AT2G35155
O-acyltransferase (WSD1-like) family protein	FOP1	0.6	4.63E-03	0.6	2.76E-03	CA00g64780	AT5G53390
C2H2 and C2HC zinc fingers superfamily protein	ZAT11	0.6	8.55E-03	0.5	3.33E-03	CA11g13540	AT2G37430
Peroxidase 71	PRX71	0.6	4.03E-04	0.6	2.18E-04	CA12g06550	AT5G64120
Alpha/beta-Hydrolases superfamily protein	-	0.6	8.73E-04	0.6	1.96E-03	CA02g23610	AT5G38220
Peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein	-	0.6	2.69E-04	0.6	9.69E-04	CA07g18040	AT1G52870
Trigalactosyldiacylglycerol 1	ABCI14	0.6	3.97E-04	0.6	1.25E-03	CA00g77030	AT1G19800
Leucine-rich receptor-like protein kinase family protein	HAE	0.6	0.02	0.6	0.02	CA12g11720	AT4G28490
CA-responsive protein	-	0.6	1.82E-03	0.6	1.68E-03	CA03g36280	AT1G17665

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
HXXXD-type acyl-transferase family protein	-	0.6	0.01	0.6	0.04	CA05g05020	AT3G26040
Transmembrane protein	-	0.6	1.19E-03	0.6	3.31E-03	CA01g05330	AT5G18250
RING/U-box superfamily protein	XERICO	0.6	1.01E-03	0.6	1.02E-03	CA09g13850	AT2G04240
NSP-interacting kinase 3	NIK3	0.6	2.52E-03	0.5	2.50E-03	CA11g08110	AT1G60800
Squamosa promoter binding protein-like 3	SPL3	0.6	0.03	0.5	0.01	CA02g15200	AT2G33810
Isopropyl malate isomerase large subunit 1	IIL1	0.6	4.59E-03	0.5	4.69E-03	CA03g04320	AT4G13430
Ribosomal protein L1p/L10e family	-	0.6	9.47E-03	0.5	1.97E-03	CA05g06180	AT3G58660
Single hybrid motif superfamily protein	-	0.5	1.03E-03	0.5	4.21E-04	CA06g17040	AT1G52670
DNAse I-like superfamily protein	-	0.5	1.49E-03	0.7	0.02	CA08g00190	AT1G43760
ALG6. ALG8 glycosyltransferase family	-	0.5	1.16E-04	0.5	6.05E-05	CA02g23360	AT5G38460
Peroxidase 71	PRX71	0.5	1.49E-03	0.7	0.04	CA12g06580	AT5G64120
E3 ubiquitin-protein ligase, putative (DUF177)	-	0.5	1.14E-04	0.5	3.30E-05	CA02g03790	AT3G19800
Lactate/malate dehydrogenase family protein	-	0.5	2.07E-04	0.6	4.65E-03	CA12g04980	AT5G58330
IQ-domain 18	IQD18	0.5	8.55E-03	0.3	5.45E-05	CA10g05830	AT1G01110
Alpha/beta-Hydrolases superfamily protein	-	0.5	3.66E-05	0.5	5.45E-05	CA10g13180	AT5G36210
Haloacid dehalogenase (HAD) superfamily protein	-	0.5	6.64E-04	0.6	9.03E-03	CA03g21900	AT3G58830
Serine-rich protein-like protein	-	0.5	1.03E-03	0.5	4.19E-04	CA09g13860	AT5G55980
Alpha/beta-Hydrolases superfamily protein	-	0.5	0.03	0.1	1.82E-05	CA05g18250	AT4G02340
Structural maintenance of chromosomes flexible hinge domain protein	-	0.5	3.38E-04	0.6	3.60E-03	CA12g07740	AT3G11760
Eceriferum 1	CER1	0.5	0.03	0.5	0.05	CA01g19130	AT1G02205
UDP-glucosyl transferase 85A2	UGT85A2	0.5	6.12E-06	0.5	2.55E-05	CA07g06270	AT1G22360
UDP-Glycosyltransferase superfamily protein	UGT85A1	0.5	6.12E-06	0.5	2.55E-05	CA04g15390	AT1G22400

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Senescence-associated family protein. putative (DUF581)	-	0.4	2.35E-04	0.5	1.02E-03	CA04g15090	AT1G78020
Pentapeptide repeat-containing protein	-	0.4	8.59E-06	0.4	3.01E-06	CA01g04750	AT1G12250
Putative lysine decarboxylase family protein	-	0.4	4.12E-05	0.5	1.43E-04	CA07g20100	AT1G50575
Ferric reduction oxidase 6	FRO6	0.4	4.68E-06	0.6	8.76E-05	CA08g13530	AT5G49730
Peroxidase 66	PRX66	0.4	1.25E-04	0.6	0.01	CA03g16810	AT5G51890
RS2-interacting KH protein	RIK	0.4	4.05E-05	0.5	7.36E-04	CA02g01240	AT3G29390
Chaperone DnaJ-domain superfamily protein	DJC82	0.4	1.84E-03	0.4	9.69E-04	CA08g12000	AT3G05345
Hypothetical protein	-	0.4	6.64E-04	0.4	6.74E-04	CA09g03150	AT2G40955
Protein kinase superfamily protein	IBS1	0.4	2.46E-06	0.5	4.80E-05	CA03g29720	AT1G18670
Indole-3-butyric acid response 1	IBR1	0.4	2.35E-04	0.5	2.73E-03	CA05g01470	AT4G05530
Leucine-rich repeat protein kinase family protein	-	0.4	0.01	0.3	8.69E-03	CA04g03240	AT3G47570
Ankyrin repeat family protein	-	0.4	3.42E-05	0.4	4.96E-05	CA08g01500	AT2G01680
Terpenoid cyclases/Protein prenyltransferases superfamily protein	GA1	0.4	7.91E-04	0.5	0.02	CA01g32990	AT4G02780
Xyloglucan endotransglucosylase/hydrolase 7	XTH7	0.3	1.13E-05	0.4	3.30E-05	CA02g24640	AT4G37800
RNA-binding (RRM/RBD/RNP motifs) family protein	-	0.3	3.49E-04	0.3	4.15E-04	CA08g06390	AT2G27790
Carboxyl terminus of HSC70-interacting protein	CHIP	0.3	5.15E-04	0.5	8.59E-03	CA06g26430	AT3G07370
Uridine-ribohydrolase 1	URH1	0.3	1.55E-04	0.3	2.00E-04	CA09g00730	AT2G36310
Nuclear factor kappa-B-binding protein	-	0.3	2.99E-04	0.6	0.04	CA02g31330	AT5G13950
Chaperone (DUF2930)	HCF208	0.3	5.04E-04	0.4	5.48E-03	CA03g16400	AT5G52110
Trichome birefringence-like 19	TBL19	0.3	2.75E-03	0.4	0.02	CA02g08060	AT5G15900

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
LRR and NB-ARC domains-containing disease resistance protein	-	0.3	0.02	0.3	0.03	CA11g19950	AT3G14460
Leucine-rich repeat protein kinase family protein	-	0.3	1.77E-05	0.4	1.84E-04	CA08g02880	AT4G34220
RHO guanyl-nucleotide exchange factor 12	ROPGEF12	0.3	9.64E-05	0.3	4.80E-05	CA03g34780	AT1G79860
Tetratricopeptide repeat (TPR)-like superfamily protein	-	0.3	3.13E-05	0.3	1.82E-05	CA00g87070	AT4G16835
Acyl-CoA N-acyltransferases (NAT) superfamily protein	-	0.3	7.41E-07	0.3	3.31E-07	CA11g07200	AT1G24040
Phosphatidic acid phosphohydrolase 2	PAH2	0.3	7.46E-07	0.4	7.48E-06	CA00g67090	AT5G42870
Maternal effect embryo arrest 12	MEE12	0.3	2.47E-05	0.3	8.15E-05	CA11g15340	AT2G02955
Diacylglycerol kinase family protein	LCBK2	0.3	2.76E-06	0.3	1.64E-06	CA08g07600	AT2G46090
Plasma membrane intrinsic protein 1;5	PIP1;5	0.3	4.03E-04	0.4	4.37E-03	CA03g12700	AT4G23400
NADH:ubiquinone/plastoquinone oxidoreductase, chain 6	NDHG	0.3	1.05E-03	0.4	9.44E-03	CA08g09370	ATCG01080
Phospholipase C 2	PLC2	0.3	3.42E-05	0.5	4.57E-03	CA10g08530	AT3G08510
PIF1 helicase	-	0.3	5.78E-04	0.3	9.28E-04	CA02g21240	AT5G28780
Microtubule-associated protein 65-2	MAP65-2	0.2	2.10E-03	0.2	9.63E-04	CA07g19910	AT4G26760
UBX domain-containing protein	-	0.2	6.23E-06	0.3	2.45E-05	CA08g04530	AT4G00752
Zinc finger protein 4	ZFP4	0.2	6.42E-04	0.2	1.97E-04	CA05g07400	AT1G66140
1-aminocyclopropane-1-carboxylate synthase 4	ACS4	0.2	9.53E-06	0.3	2.55E-05	CA07g05340	AT2G22810
RNA-directed DNA polymerase (reverse transcriptase)-related family protein	-	0.2	2.26E-06	0.3	5.70E-06	CA10g03700	AT1G43730
ABC1 family protein	-	0.2	9.24E-04	0.2	1.45E-03	CA01g09700	AT5G24810
Transmembrane protein	-	0.2	2.76E-06	0.3	4.84E-05	CA12g03760	AT4G04190
Copine (Calcium-dependent phospholipid-binding protein) family	RGLG3	0.2	1.16E-04	0.3	3.46E-03	CA03g09720	AT5G63970

Full name	Short name	FC Control	*P*-value Control	FC NaCl	*P*-value NaCl	*C. annuum* code	*A. thaliana* code
Hypothetical protein	-	0.2	4.49E-06	0.3	4.80E-05	CA06g17050	AT1G80180
Galactose mutarotase-like superfamily protein	-	0.2	2.46E-06	0.1	1.64E-06	CA03g09520	AT3G47800
Zinc ion binding protein	-	0.2	0.04	0.09	0.01	CA09g12230	AT4G13970
DNA-directed RNA polymerase family protein	RPOC2	0.1	6.23E-06	0.2	5.45E-05	CA10g08960	ATCG00170
UDP-glucosyltransferase 73B2	UGT73B2	0.1	8.10E-07	0.2	3.01E-06	CA05g07050	AT4G34135
O-methyltransferase family protein	-	0.1	2.35E-04	0.1	2.18E-04	CA02g04210	AT4G35160
Amino-terminal glutamine amidohydrolase	-	0.09	7.41E-07	0.1	1.64E-06	CA05g01260	AT2G41760
Pentatricopeptide repeat (PPR) superfamily protein	-	0.05	8.49E-07	0.08	3.01E-06	CA03g32750	AT3G18020
Cofactor assembly, complex C (B6F)	CCB3	0.05	6.96E-06	0.06	2.04E-05	CA02g03840	AT5G36120

Additional file 2: Table S7-S12. Total differentially expressed genes when NaCl and control conditions are compared, in both A25 and A6 accessions.

Table S7. Specific differentially expressed genes after 14DAT in the comparison NaCl/Control in leaves of A25 accession. It is represented only up-regulated genes (FC>1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when $P<0.05$). Genes without abbreviation are represented with "-".

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Undecaprenyl pyrophosphate synthetase family protein	cPT4	9.1	1.02E-03	CA10g14100	AT5G58770
Expansin-like B1	EXLB1	6.1	0.04	CA01g06350	AT4G17030
Purine permease 11	PUP11	5.6	0.02	CA01g06760	AT1G44750
Cell wall / vacuolar inhibitor of fructosidase 1	C/VIF1	5.1	0.01	CA12g21170	AT1G47960
Calmodulin-binding transcription activator 5	CAMTA5	4.3	7.05E-03	CA01g14110	AT4G16150
Transketolase family protein	MAB1	4.1	0.02	CA11g14500	AT5G50850
Haloacid dehalogenase-like hydrolase (HAD) superfamily protein	-	3.8	0.04	CA03g07690	AT2G32150
Homeodomain-like superfamily protein	-	3.7	0.01	CA01g08210	AT5G45580
RmlC-like cupins superfamily protein	-	3.6	0.03	CA01g21540	AT5G39150
RmlC-like cupins superfamily protein	-	3.6	0.03	CA03g05750	AT5G39180
Cytochrome P450 superfamily protein	-	3.1	0.01	CA06g19760	AT5G08250
Nucleotide-diphospho-sugar transferases superfamily protein	IRX9-L	3.0	0.01	CA11g20390	AT1G27600
Histone deacetylase 3	HDA3	2.8	0.04	CA11g17720	AT3G44750
WRKY DNA-binding protein 23	WRKY23	2.8	0.01	CA01g22410	AT2G47260
Quinone reductase family protein	-	2.8	9.74E-03	CA06g25220	AT5G58800
Beta-amylase 1	BAM1	2.7	0.05	CA03g02770	AT3G23920
Calcineurin B-like protein 9	CBL9	2.7	2.25E-03	CA01g33680	AT5G47100
Major facilitator superfamily protein	-	2.6	6.19E-03	CA01g27680	AT5G19640
Rubisco methyltransferase family protein	LSMT-L	2.6	6.19E-03	CA11g04070	AT1G14030
Nuclear factor Y, subunit A1	NF-YA1	2.5	0.04	CA01g20270	AT5G12840
F-box/RNI-like superfamily protein	-	2.5	0.02	CA07g19460	AT3G58860
Uncharacterized protein family (UPF0114)	-	2.5	0.02	CA01g09140	AT4G19390
Lactoylglutathione lyase / glyoxalase I family protein	GLYI7	2.3	0.04	CA03g31680	AT1G80160
Serine/arginine-rich splicing factor-like protein, putative	SC35	2.3	0.01	CA04g08840	AT5G64200

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Hypersensitive to ABA1	HAB1	2.3	0.02	CA08g03850	AT1G72770
ARM repeat superfamily protein	-	2.2	0.01	CA06g12170	AT1G77600
Fumarylacetoacetase	-	2.2	0.02	CA12g01110	AT1G12050
Hypothetical protein	-	2.2	0.01	CA06g17050	AT1G80180
Jasmonate resistant 1	JAR1	2.1	0.01	CA08g08190	AT2G46370
RING/U-box superfamily protein	SGR9	2.1	0.03	CA09g03170	AT5G02750
Myo-inositol oxygenase 5	MIOX5	2.0	0.04	CA12g20180	AT5G56640
RNA-binding (RRM/RBD/RNP motifs) family protein	SR34	2.0	0.02	CA00g67000	AT1G02840
HXXXD-type acyl-transferase family protein	-	2.0	0.01	CA00g56990	AT5G39090
DA1-related protein 4	DAR4	1.9	0.01	CA02g02530	AT5G17890
Calcium-binding EF-hand family protein	-	1.9	0.02	CA03g18530	AT4G38810
AP2/B3-like transcriptional factor family protein	VRN1	1.9	9.74E-03	CA04g19550	AT3G18990
GDSL-like Lipase/Acylhydrolase superfamily protein	-	1.9	8.06E-03	CA10g03820	AT5G45960
Transmembrane amino acid transporter family protein	-	1.8	0.04	CA12g04200	AT5G15240
Endoplasmic reticulum oxidoreductins 1	ERO1	1.8	2.17E-03	CA00g72080	AT1G72280
Oxoglutarate/iron-dependent oxygenase	-	1.8	0.01	CA02g08750	AT3G28490
Transcription factor-like protein	ZW2	1.8	0.02	CA02g19080	AT1G58330
Transducin/WD40 repeat-like superfamily protein	-	1.8	0.02	CA04g14930	AT1G78070
Homeodomain-like transcriptional regulator	-	1.8	0.03	CA05g15200	AT5G58900
Homeobox 12	HB-12	1.8	0.02	CA08g08650	AT3G61890
Vamp/synaptobrevin-associated protein 27-2	VAP27-2	1.8	0.02	CA08g11200	AT1G08820
P-loop containing nucleoside triphosphate hydrolases superfamily protein	KINESIN-13A	1.8	0.01	CA05g18020	AT3G16630
Sec23/Sec24 protein transport family protein	-	1.8	0.03	CA04g00360	AT5G43670
Leucine-rich repeat protein kinase family protein	-	1.7	0.04	CA02g13990	AT5G45840
Transmembrane protein	-	1.7	9.74E-03	CA06g27190	AT1G67020
Phosphate transporter traffic facilitator1	PHF1	1.7	0.05	CA12g05520	AT3G52190

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Maternal effect embryo arrest 22	MEE22	1.7	0.04	CA02g03510	AT2G34780
Fibrillin 1A	FIB1A	1.7	0.04	CA02g18750	AT4G04020
D-isomer specific 2-hydroxyacid dehydrogenase family protein	HPR3	1.7	0.05	CA01g30750	AT1G12550
Disease resistance protein (TIR-NBS-LRR class) family	-	1.6	0.02	CA00g95750	AT4G11170
Alpha/beta-Hydrolases superfamily protein	-	1.6	0.03	CA09g00850	AT2G36290
Diphthamide synthesis DPH2 family protein	-	1.6	0.05	CA06g13640	AT5G62030
Receptor like protein 7	RLP7	1.6	0.02	CA09g12200	AT1G47890
Ubiquitin-specific protease 15	UBP15	1.6	0.04	CA07g16320	AT1G17110
E3 ubiquitin-protein ligase	-	1.6	0.01	CA01g17200	AT1G33490
BCL-2-associated athanogene 6	BAG6	1.6	0.01	CA08g07920	AT2G46240
Spermidine synthase 3	SPDS3	1.6	0.05	CA03g19440	AT5G53120
Splicing endonuclease 1	SEN1	1.6	0.04	CA06g23700	AT3G45590
Receptor like protein 6	RLP6	1.6	0.01	CA12g22850	AT1G45616
Uridine kinase-like 4	UKL4	1.5	0.02	CA10g01110	AT4G26510
Transmembrane protein	-	1.5	0.02	CA04g04190	AT1G65720
2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	-	1.5	0.03	CA09g00310	AT1G06620
2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	-	1.5	0.03	CA09g00320	AT1G06620
Aldehyde dehydrogenase 7B4	ALDH7B4	1.5	0.04	CA03g35250	AT1G54100
RING/U-box superfamily protein	-	1.5	0.04	CA10g21240	AT3G58030
Phosphatidic acid phosphohydrolase 2	PAH2	1.4	0.02	CA00g67090	AT5G42870
Kinase superfamily with octicosapeptide/Phox/Bem1p domain-containing protein	-	1.4	0.04	CA07g00400	AT5G57610
Nuclear factor Y, subunit A1	NF-YA1	1.4	0.05	CA06g10300	AT5G12840
O-methyltransferase 1	OMT1	1.4	0.04	CA03g21170	AT5G54160
Pumilio 1	PUM1	1.4	0.05	CA06g24760	AT2G29200
Kinase family with leucine-rich repeat domain-containing protein	-	1.3	0.03	CA04g03150	AT1G35710
Calcium-dependent lipid-binding (CaLB domain) family protein	C2	1.3	0.03	CA12g11530	AT3G17980
Octicosapeptide/Phox/Bem1p family protein	-	1.3	0.04	CA01g31360	AT5G64430
Cysteine-rich RECEPTOR-like kinase	CRK8	1.3	0.04	CA09g02690	AT4G23160

Table S8. Specific differentially expressed genes after 14DAT in the comparison NaCl/Control in leaves of A25 accession. It is represented only down-regulated genes (FC<1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Methionine aminopeptidase 1D	MAP1D	0.8	0.05	CA02g25570	AT4G37040
Pentapeptide repeat-containing protein	-	0.7	0.04	CA01g04750	AT1G12250
Peptide transporter 3	PTR3	0.7	0.02	CA03g09480	AT5G46050
Adenylate kinase family protein	-	0.7	0.05	CA09g18490	AT5G35170
Highly ABA-induced PP2C protein 3	HAI3	0.7	0.04	CA06g24830	AT2G29380
Leucine-rich repeat protein kinase family protein	-	0.7	0.04	CA02g09120	AT5G14210
Subtilase family protein	-	0.7	0.02	CA10g14810	AT3G14240
Plant invertase/pectin methylesterase inhibitor superfamily protein	-	0.7	0.03	CA03g15860	AT5G62360
SAUR-like auxin-responsive protein family	SAUR66	0.7	0.05	CA12g01640	AT1G29500
Serine/threonine protein kinase 2	S6K2	0.6	0.02	CA10g10590	AT3G08720
Amidase family protein	-	0.6	0.03	CA11g15360	AT4G34880
ACT domain repeat 4	ACR4	0.6	0.02	CA00g55790	AT1G69040
Cyclophilin 38	CYP38	0.5	0.05	CA02g29500	AT3G01480
Early nodulin-like protein 17	ENODL17	0.5	0.01	CA02g27870	AT5G15350
Glutamyl-tRNA (Gln) amidotransferase subunit A (DUF620)	-	0.5	0.02	CA08g17110	AT3G19540
Phloem protein 2-B10	PP2-B10	0.5	0.03	CA05g19940	AT2G02360
Photosystem I subunit G	PSAG	0.5	0.02	CA07g20940	AT1G55670
Plant invertase/pectin methylesterase inhibitor superfamily protein	-	0.5	0.05	CA03g15820	AT5G62360
Glyoxal oxidase-related protein	-	0.5	0.02	CA07g00290	AT3G57620
Photosystem I subunit O	PSAO	0.4	0.04	CA06g22830	AT1G08380
Ribosomal protein L12/ ATP-dependent Clp protease adaptor protein ClpS family protein	-	0.4	5.53E-03	CA09g00270	AT3G06040
lsd one like 1	LOL1	0.4	9.24E-03	CA00g77830	AT1G32540
Pre-mRNA-processing protein 40A	PRP40A	0.4	0.02	CA00g84420	AT1G44910
ChaC-like family protein	-	0.3	0.03	CA12g18980	AT5G26220
Ankyrin repeat family protein	-	0.3	9.24E-03	CA03g14190	AT2G31820
Proteinase inhibitor I4, serpin (DUF716)	-	0.3	0.04	CA01g11840	AT1G55240

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Germin 3	GER3	0.3	0.02	CA07g08820	AT5G20630
Germin 3	GER3	0.3	0.01	CA03g07830	AT5G20630
Trehalose-6-phosphate synthase	TPS1	0.2	0.02	CA02g12830	AT1G78580
Oxidoreductase family protein	-	0.2	3.98E-04	CA01g05600	AT4G17370
Protochlorophyllide oxidoreductase A	PORA	0.1	0.04	CA10g00480	AT5G54190

Table S9. Specific differentially expressed genes after 14DAT in the comparison NaCl/Control in leaves of A6 accession. It is represented only up-regulated genes (FC>1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
High affinity nitrate transporter 2.7	NRT2.7	7.9	0.04	CA02g00260	AT5G14570
Alpha/beta-Hydrolases superfamily protein	-	5.0	2.39E-03	CA05g18250	AT4G02340
Auxin efflux carrier family protein	-	4.6	2.39E-03	CA04g23470	AT1G76520
Cellulose synthase-like D3	CSLD3	3.9	6.06E-03	CA01g07920	AT3G03050
Tyrosine transaminase family protein	TAT7	3.5	5.13E-03	CA12g18900	AT5G53970
Alpha/beta-Hydrolases superfamily protein	-	3.3	0.03	CA11g14470	AT3G09690
Alpha/beta-Hydrolases superfamily protein	-	3.3	0.01	CA05g18260	AT4G02340
BUB1-related (BUB1: budding uninhibited by benzymidazol 1)	BUBR1	2.5	6.08E-03	CA04g23550	AT2G33560
Zinc-binding alcohol dehydrogenase family protein	-	2.5	6.14E-03	CA12g18000	AT5G42250
RING-box 1	RBX1	2.3	6.08E-03	CA09g17140	AT5G20570
Formin homolog 6	FH6	2.2	0.04	CA02g25170	AT5G67470
NSP-interacting kinase 3	NIK3	2.0	0.03	CA05g08950	AT1G60800
bZIP transcription factor family protein	TGA7	2.0	0.04	CA12g13050	AT1G77920
4-phosphopantetheine adenylyltransferase	COAD	1.8	0.01	CA00g94400	AT2G18250
4-phosphopantetheine adenylyltransferase	COAD	1.8	0.01	CA06g04390	AT2G18250
2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	-	1.8	0.01	CA12g07770	AT4G23340
Transmembrane amino acid transporter family protein	-	1.8	0.02	CA04g10860	AT5G15240
P-loop nucleoside triphosphate hydrolases superfamily protein with CH (Calponin Homology) domain-containing protein	-	1.8	0.04	CA01g02620	AT1G63640
Allantoinase	ALN	1.8	0.03	CA02g15930	AT4G04955
Serine carboxypeptidase-like 42	scpl42	1.8	6.63E-03	CA04g13000	AT5G42240
Alpha-galactosidase 1	AGAL1	1.7	0.03	CA05g06220	AT5G08380
Cytochrome P450. family 76. subfamily G. polypeptide 1	CYP76G1	1.6	0.03	CA10g16230	AT3G52970

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
Microsomal glutathione s-transferase	-	1.6	0.03	CA02g18880	AT1G65820
Multidrug resistance-associated protein 6	ABCC8	1.6	6.28E-03	CA00g70570	AT3G21250
Uncharacterized protein family (UPF0497)	-	1.6	0.04	CA10g12480	AT2G28370
Protein kinase superfamily protein	-	1.5	0.04	CA02g18990	AT5G38260
Pentatricopeptide repeat (PPR) superfamily protein	-	1.5	0.02	CA00g71780	AT3G08820
Alpha/beta-Hydrolases superfamily protein	-	1.5	0.01	CA08g05220	AT5G11650
Dentin sialophosphoprotein. putative (DUF1296)	-	1.5	0.05	CA12g15230	AT3G13990
Histone deacetylase-like protein	-	1.5	0.02	CA10g08190	AT2G14825
Cyclin-D1-binding protein	-	1.4	0.05	CA11g00110	AT1G22970
Cysteine-rich RECEPTOR-like kinase	CRK8	1.4	0.04	CA00g15340	AT4G23160
Allantoate amidohydrolase	AAH	1.3	0.03	CA02g09690	AT4G20070

Table S10. Specific differentially expressed genes after 14DAT in the comparison NaCl/Control in leaves of A6 accession. It is represented only down-regulated genes (FC<1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC	*P*-value	*C. annuum* code	*A. thaliana* code
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	-	0.7	0.04	CA02g30290	AT3G01660
Expansin A4	EXPA4	0.7	0.04	CA02g18410	AT2G39700
Beta-amylase 5	BAM5	0.7	0.03	CA07g12420	AT4G15210
Lipoyltransferase 2	LIP2	0.7	0.03	CA01g05290	AT1G04640
Photosystem II subunit P-1	PSBP-1	0.7	0.04	CA07g07930	AT1G06680
SOUL heme-binding family protein	HBP5	0.7	0.01	CA09g15500	AT5G20140
Transmembrane protein	-	0.7	0.03	CA01g21710	AT5G55570
Ribosomal protein L22	RPL22	0.7	0.04	CA11g09310	ATCG00810
Ribosomal protein L22	RPL22	0.7	0.04	CA08g01730	ATCG00810
Phloem protein 2-B10	PP2-B10	0.6	3.69E-03	CA12g20010	AT2G02360
Heme binding protein	-	0.6	0.01	CA01g22320	AT3G62370
Enoyl-CoA hydratase/isomerase D	ECHID	0.6	0.03	CA10g00450	AT1G60550
Pyridoxamine 5'-phosphate oxidase family protein	-	0.5	0.03	CA02g29200	AT2G04690
Inositol 1,3,4-trisphosphate 5/6-kinase family protein	ITPK1	0.5	0.01	CA03g22040	AT5G16760
RecA DNA recombination family protein	RECA2	0.5	0.04	CA05g20060	AT2G19490
NB-ARC domain-containing disease resistance protein	RPP13	0.5	0.01	CA05g06820	AT3G46530
Thylakoid rhodanese-like protein	TROL	0.5	0.04	CA08g08250	AT4G01050
Photosystem I subunit E-2	PSAE-2	0.5	0.04	CA06g28140	AT2G20260
Beta-amylase 5	BAM5	0.5	6.63E-03	CA07g12430	AT4G15210
3-ketoacyl-acyl carrier protein synthase I	KASI	0.5	0.05	CA01g00840	AT5G46290
3-ketoacyl-acyl carrier protein synthase I	KASI	0.5	0.05	CA01g00830	AT5G46290
Transducin/WD40 repeat-like superfamily protein	AtATG18a	0.4	0.01	CA01g30560	AT3G62770
SNF2 domain-containing protein / helicase domain-containing protein	PIE1	0.4	0.04	CA04g21650	AT3G12810
Receptor-interacting protein	-	0.4	0.05	CA10g05770	AT4G21445
Transcription elongation factor	-	0.4	6.63E-03	CA00g00620	AT5G47920

Table S11. Common differentially expressed genes after 14DAT in the comparison NaCl/Control in leaves of A25 and A6 accessions. It is represented only up-regulated genes (FC>1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC A25	*P*-value A25	FC A6	*P*-Value A6	*C. annuum* code	*A. thaliana* code
Beta vacuolar processing enzyme	BETA-VPE	8.5	3.98E-04	2.6	0.04	CA01g04690	AT1G62710
Amino acid permease 6	AAP6	3.2	8.06E-03	2.4	0.04	CA12g15790	AT5G49630
Myo-inositol oxygenase 1	MIOX1	3.0	0.01	2.4	0.04	CA06g16540	AT1G14520
Rhamnogalacturonate lyase family protein	-	2.3	1.02E-03	1.9	6.06E-03	CA04g16980	AT1G09890
Peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein	-	2.3	0.02	2.0	0.05	CA10g03410	AT4G03410
Protein PIN-LIKES 1	PILS1	2.1	0.04	3.6	5.00E-03	CA04g23480	AT1G20925
Cytochrome P450. family 72, subfamily A. polypeptide 7	CYP72A7	1.9	0.05	2.0	0.04	CA07g07090	AT3G14610
FTSH protease 6	FTSH6	1.9	1.13E-03	1.7	5.13E-03	CA02g19050	AT5G15250
STRESS RESPONSE SUPPRESSOR 1	STRS1	1.8	0.02	2.1	9.17E-03	CA01g08040	AT1G31970
Pentatricopeptide repeat-containing protein At1g09220, mitochondrial	-	1.6	0.01	1.6	0.02	CA06g00230	AT1G09220
Copper/zinc superoxide dismutase 1	CSD1	1.6	0.02	2.1	2.64E-03	CA01g25550	AT1G08830
IQ-domain 13	IQD13	1.6	0.03	1.6	0.03	CA06g08130	AT3G59690
tolB protein-related	-	1.4	0.02	1.4	0.04	CA06g00090	AT4G01870

Table S12. Common differentially expressed genes after 14DAT in the comparison NaCl/Control in leaves of A25 and A6 accessions. It is represented only down-regulated genes (FC<1). It is represented the fold change (FC) and the adjusted *P*-value obtained for each gene (significant differences were considered when *P*<0.05). Genes without abbreviation are represented with "-".

Full name	Short name	FC A25	*P*-value A25	FC A6	*P*-value A6	*C. annuum* code	*A. thaliana* code
1-aminocyclopropane-1-carboxylate synthase 4	ACS4	0.6	0.04	0.5	0.03	CA07g05340	AT2G22810
Poly (ADP-ribose) polymerase 3	PARP3	0.5	0.02	0.6	0.04	CA05g04960	AT5G22470
Hypothetical protein	-	0.3	0.02	0.3	0.05	CA03g24300	AT5G50335

Chapter 6

General Discussion

Classical genetic crop breeding has been developed considerably in the last century to provide solutions to the farming challenges and troubles of the new scenario of climate change, although such solutions are not always efficient. Vegetable grafting has been promoted and consolidated in the last decades as an alternative environmentally-friendly technique to face these issues (Colla *et al.*, 2006; Rouphael *et al.*, 2008). Indeed, the advantages of using tolerant rootstocks have been widely described, which include improved yield stability and sustainability of intensive farming, enhanced tolerance to biotic and abiotic stresses, lower use of pesticides that contaminate the soil and water, and the ability to use the desired stress-sensitive scions grafted onto rootstocks with tolerance to stresses (Zhen *et al.*, 2010; Pérez-Alfocea, 2015).

In the specific case of pepper, selecting the proper rootstock is not always achievable due to incompatibility issues, even within the same species, which results in low availability of commercial rootstocks. Consequently, the use of grafting is more restricted in pepper than in other species (*see Section 1.6.1. of the introductory chapter for more information*). However, when rootstocks are tolerant to stress and compatible with scions, fruit production is improved and economically profitable compared to ungrafted pepper plants (López-Marín *et al.*, 2016).

One way of increasing pepper genetic variability to favour the success and compatibility of grafting is to find wild and semiwild accessions inside the *Capsicum annuum* species that tolerate and grow in extreme environments, since contributing to tolerance without adding any negative effect on the scion is the only requirement for rootstocks (King *et al.*, 2010; Penella and Calatayud, 2018). To this end, we decided to explore the degree of tolerance to water and salt stress of several *C. annuum* accessions from the COMAV-UPV collection (Valencia, Spain) (*Chapter 2*). Among all the studied parameters, photosynthesis and stomatal conductance were proposed as the most accurate ones to explain biomass differences, as reported in previous works (Rouphael *et al.*, 2012; Penella *et al.*, 2013, 2014b). However, other parameters emerged as useful tools to determine tolerance, such as ψ_s and ψ_w, or accumulation of Na^+ in the case of salt stress. In this screening experiment, A31 and A34 were the accessions classified as tolerant to water and salt stress, respectively, which have added genetic diversity to the selected tolerant accessions from previous works (Penella *et al.*, 2013, 2014b). All of them were included in a breeding programme to obtain new tolerant hybrid rootstocks. Then, many of these hybrids were tested agronomically under real-field conditions of

water scarcity and high concentration of salts, obtaining significant and positive results compared to ungrafted scions or grafted onto tolerant accessions (data not shown in the present document).

Not only was important to find new genetic diversity but also to unravel the mechanisms of tolerance to salt and water stress of ungrafted accessions and grafted pepper plants. Thus, among all the tolerant rootstocks already selected in previous screening experiments, in this doctoral thesis we decided to thoroughly study one accession (A25) and one hybrid (NIBER®) previously categorised as tolerant to salt and water stress when used as rootstocks (Calatayud *et al.*, 2016; Penella *et al.*, 2016). One of the main objectives of the developed studies was to elucidate the physiological mechanisms of tolerance that scions aquire when grafted onto tolerant rootstocks, as well as the scion-rootstock interactions. Within this objective, A25 was studied as rootstock under suboptimal water conditions (*Chapter 3*) and hybrid NIBER® was studied as rootstock under salt stress conditions (*Chapter 4*), using a commercial variety as scion. Additionally, accession A25 was also compared with A6, previously described as sensitive to salt stress (Penella *et al.*, 2013), with the aim of clarifying the main molecular pathways differentially expressed in A25 responsible for tolerance to high salinity stress, through a transcriptomic approach (*Chapter 5*).

Focusing firstly on the impact of salt addition on plants, an important effect is the differential transport and accumulation of ions within the plant, mainly Na^+ and Cl^- (Isayenkov and Maathuis, 2019); the negative effects of salts are ameliorated by the improvement of ion homeostasis mechanisms, which are pivotal to reach tolerance and to maintain growth and development (Huang *et al.*, 2013). With the aim of determining the effects of both ions in pepper plants, we decided to measure them in salt stress experiments. In all of them, both grafted and ungrafted pepper plants, Na^+ was the main ion responsible for toxicity. Regarding Cl^-, a greater concentration with respect to Na^+ was required to provoke the same photosynthetic and oxidative damage. Similar results have been found in previous studies in both grafted and ungrafted pepper plants (Chartzoulakis and Klapaki, 2000; Penella *et al.*, 2015, 2017).

Another important consequence of NaCl toxicity is the competition between Na^+ and K^+ for binding sites and transporters, which results in a decline in the concentration of K^+ (Isayenkov and Maathuis, 2019); this is why we also measured this ion in the salt stress assays of this document. Indeed, we have herein demonstrated that better K^+ homeostasis under salt stress conditions was unfavourable for Na^+ accumulation in plants and, thus, reduced its phytotoxic effects, as other authors have already demonstrated in other species (Stępień and Kłbus, 2006; Fan *et al.*, 2011). Specifically, the ability to maintain or increase the concentration of K^+ and decrease the Na^+/K^+ ratio of ungrafted A25 accessions and scions grafted onto NIBER® rootstocks, respectively, were thus signs of tolerance. Although Na^+/K^+ decreased when NIBER® was used as rootstock, the higher ratio in the roots with respect to the leaves in all plant combinations studied suggests that Na^+ was accumulated mainly in the roots, as Rouphael *et al.* (2012) have

already demonstrated in grafted melon plants. In the specificic case of A25, where a transcriptomic research was conducted, we additionally found the up-regulation of potassium transporter AKT1 signalling, highly related to K^+ transport, after comparing high-salt concentration and control conditions. We found the activation of CBL9 and the inactivation of HAI3 putative genes, in both cases related in previous studies to the degree of activation of AKT1 (Xu *et al.*, 2006; Lee *et al.*, 2007; Wu *et al.*, 2018).

Abiotic stresses, either by water deficit or by the phytotoxic effect of salt ions, also have negative effets on photosynthesis, which limit plant growth and development, as well as other important processes in plants (He *et al.*, 2009; López-Marín *et al.*, 2017). Due to its importance, we monitored the gas exchange parameters in all the studies carried out in this document. Overall, we detected a better preservation in tolerant pepper plants, directly related to the maintenance of plant growth, as other authors have already detected in pepper (Penella *et al.*, 2014a; López-Marín *et al.*, 2017).

In the specific case of high salinity conditions, Na^+ concentration in aerial organs has been previously demonstrated to have detrimental effects on photosynthetic activities and disrupt chloroplast integrity (Badawi *et al.*, 2004; Munns and Tester, 2008). As a result, it limits the transport of Na^+ to the leaves and favours its release and accumulation in non-toxic organelles, which are pivotal to maintain photosytnhesis and stomatal conductance (Munns and Tester, 2008). The obtained results, presented in this document, confirm that the negative effects on photosynthetic parameters are largely aggravated due to the harmful effects of the accumulation of Na^+ to toxic levels. In the ungrafted accession A25 (tolerant to salinity) treated with high salt concentration, a greater proportion of Na^+ was accumulated in the roots with respect to A6 seedlings (sensitive to salinity). However, the accumulation of this ion in the leaves was similar in both accessions. After comparing differentially expressed genes between A25 and A6, we confirmed that the impact of Na^+ toxicity on the leaves was unequal in both cases. Up-regulation of the BASS1 and TIL putative genes in A25 would have resulted in the symport extrusion of both pantoate and Na^+ out of the chloroplast and the protection of this organelle and chlorophyll *b*, respectively, as Huang *et al.* (2018) and Abo-Ogiala *et al.* (2014) demonstated in *Arabidopsis thaliana* seedlings. Additionally, up-regulation of the OCT4 putative gene in A25 would have accumulated it into non-toxic organelles as vacuoles, as has been previously reported (Küfner and Koch, 2008). However, other up-regulated putative genes in the A25 accession after the comparison of salt and control conditions contributed to maintaining photosynthesis. We proposed the putative genes FIB1A, SPDS3 and JAR1, since previous studies have already demonstrated that they help in the protection of chloroplast and photosynthetic machinery and in the prevention of chlorophyll degradation (Yang *et al.*, 2006; Ahmad *et al.*, 2013; Khoshbakht *et al.*, 2018). Furthermore, in line with the results obtained by Stępień and Kłbus (2006) in *Cucumis sativus*, we suggest that the reduced Na^+/K^+ ratio in both leaves (from the scion) and roots (from NIBER®) in grafted plants, with respect to other plant combinations, reduced the phytotoxic effect of Na^+ on tissues,

which, in turn, improved photosynthesis and stomatal conductance. Consequently, all these improvements enhanced plant growth and development.

It is equally important to study the negative effects of suboptimal conditions of water availability on photosynthesis, although grafting onto tolerant rootstocks can improve both gas exchange and marketable yield, as has been previously confirmed (Penella *et al.*, 2014b; López-Marín *et al.*, 2017). Specifically, in the water stress experiments developed herein, we detected that the drop of photosynthesis (A_N) was mainly related to stomatal limitations, since stomatal conductance (g_s) and A_N diminished in a coordinated relationship. However, we detected the lowest decrease of such parameters in the tolerant accession A31 and the scion grafted onto the A25 tolerant rootstock. Indeed, non-stomatal limitations were not affected in tolerant plants, since we did not find significant decreases in instantaneous carboxylation efficiency (A_N/C_i) or accumulation of substomatal concentration of CO_2 (C_i). Nevertheless, the regulation of stomatal aperture, which involves multiple mechanisms, is generally very important in the maintenance of photosynthesis under water stress conditions in many species (Dodd and Ryan, 2016).

One of the main regulation mechanisms of such stomatal aperture is mediated by the control of abscisic acid (ABA) (Fernando and Schroeder, 2016). Accumulation of this hormone is induced in different tissues under abiotic stresses, such as salt and water deficit, and it causes stomatal closure in photosynthetic tissues, prevents excessive transpiration and improves water use efficiency (Liu *et al.*, 2016). However, if ABA homeostasis cannot be regulated after a long term period of stress, gas exchange is reduced and may restrict growth and development (Sreenivasulu *et al.*, 2012; Ryu and Cho, 2015). Consequently, monitoring the behaviour of this hormone in plants can elucidate new mechanisms of tolerance in pepper plants, as was detected in some of the salt stress experiments conducted in this thesis. Firstly, in the experiment where NIBER® was used as rootstock, we observed a strong correlation between greater ABA concentration in both leaves and roots and the decrease in g_s for all plant combinations studied. Specifically, when NIBER® was used as rootstock, ABA concentration was lower in leaves and, thus, the stomatal closure was also lower (i.e., increase of g_s), as has been previously noted in other tolerant plants (He and Cramer, 1996). Additionally, the drop of ABA was observed to depend on the rootstock, since reciprocal grafting (i.e., using NIBER® as scion) exhibited levels similar to those of ungrafted and self-grafted plants. On the other hand, the A25 ungrafted accession, after comparing salt and control conditions and A25 with sensitive accessions (A6), differentially expressed a series of putative genes (i.e., CAMTA5 or ERD4) that were linked to a misregulation of the ABA-signalling pathway. In fact, several studies have already demonstrated that the overexpression of such genes may confer plants tolerance to abiotic stresses and improve their biomass (Liu *et al.*, 2009; Pandey *et al.*, 2013). We also found that improved aerial and root biomass in the tolerant accession A25 was linked to the up-regulation of a series of putative genes related to cell expansion and

division and the starch mobilisation pathway (i.e., CDC2, expansins or BAM1), previously described as essential for biomass maintenance (Valerio *et al.*, 2011; Marowa *et al.*, 2016; Qi and Zhang, 2020). Consequently, we can conclude that a disrupted ABA signalling or concentration could be related to greater tolerance.

In addition to the discussed changes in carbon assimilation, drought and salinity stresses may affect several nitrogen (N) metabolism stages (Feller and Vaseva, 2014; Penella *et al.*, 2014a), which are tightly regulated by photosynthesis and stomatal closure (Kaiser and Huber, 2001). Tolerance is reached through the modulation of key enzymes related to N metabolism, which are important for biomass and production (Xu and Zhou, 2006). Among all of them, nitrate reductase (NR), the enzyme measured herein, is relevant in nitrate assimilation, although it is sensitive to water scarcity or increments of salt concentration in the water or the soil (Penella *et al.*, 2014a, 2015). Therefore, the maintenance of the activity of such enzyme in the leaves of the scion grafted onto A25 or onto NIBER® was considered as a sign of tolerance, related to higher g_s and A_N preservation. In the case of non-tolerant grafted combinations, NR in the leaves stopped its activity almost completely. However, the observed higher NR activity in the roots compared with that in the leaves was a consequence of the inhibition of the transfer of NO_3^- to the leaves, which can be limited by lower transpiration, as has been previously described in the literature (Lexa and Cheeseman, 1997; Penella *et al.*, 2014a).

Gas exchange and growth maintenance also requires the preservation of water content in plant cells and water uptake flow. Different mechanisms are available in plants to maintain water content and improve tolerance, which mainly depend on the type of stress, the duration of the stress and the studied species. Among these, the accumulation of compatible osmolytes and controlling the water uptake rate are the most common factors (Colla *et al.*, 2010; Anjum *et al.*, 2012). Therefore, in our experiments, we focused on measuring a possible compatible osmolyte, the proline, and water movement by the water (ψ_W) and osmotic (ψ_s) potential. Comparing all the experiments developed herein, where ψ_s and ψ_W were measured, the major changes were found under salt stress rather than water stress conditions, although changes were found under both stresses. Likewise, under salt stress conditions, it was observed that the main compatible osmolyte responsible for the change in ψ_s was not proline, but the accumulation of Na^+ in roots in the tolerant accessions and grafting combinations studied. Such ion has been described in the literature to be accumulated inside non-toxic organelles, in order to avoid its toxic accumulation in the roots (Zhang *et al.*, 2014) and to function as an osmolyte that decreases ψ_s without extra energy cost, unlike proline (Munns, 2002).

Concerning the maintenance of water flow under suboptimal water conditions, improving water relations was not a relevant trait in the case of the screening for tolerant accessions, due to the small changes found in both ψ_s and ψ_W with respect to the control plants. However, the tolerant accession A25 used as rootstock conferred an

improved water status to the scion by the accumulation of solutes, which led to greater maintenance of RWC and ψ_W with respect to the control conditions. As a result, A25 would have conferred the scion a better osmotic adjustment, as has been previously described (Penella *et al.*, 2014a). On the other hand, ungrafted and self-grafted plants significantly decreased water uptake, which was not compensated by the accumulation of compatible osmolytes (low ψ_s), since both RWC and ψ_W decreased.

The inhibition of gas exchange and the restriction of water uptake exacerbate the negative conditions of plants by increasing the oxidative damage, as a result of the imbalance between reactive oxygen species (ROS) generation and scavenging (Miller *et al.*, 2010; Zulfugarov *et al.*, 2011). Therefore, to evaluate the mechanisms that tolerant rootstocks used to reduce the oxidative stress, we developed two experiments with grafted plants to determine ROS damage and detoxification by the evaluation of the damage in lipid membranes, the synthesis of hydrogen peroxide (H_2O_2), the capacity to activate different antioxidant molecules (e.g., phenols) and total radical scavenging capacity. We concluded that different strategies were developed in grafted tolerant pepper plants to cope with oxidative stress after abiotic stresses started.

On the one hand, under water stress conditions, we found lesser peroxidative damage of lipid membranes in plants grafted onto A25, which was accompanied by a lower accumulation of H_2O_2, ROS scavenging capacity and phenol content in the scion. Consequently, both oxidative damage and the antioxidative mechanisms were not up-regulated compared to ungrafted or self-grafted plants. Diminished photosynthetic capacity has been associated in previous studies with oxidative damage, since impaired CO_2 fixation rate and stomatal closure enhance ROS production (Hu *et al.*, 2010). Our results coincide with this fact, since plants grafted onto the A25 accession, with a better photosynthetic capacity, maintained lower levels of oxidative damage, which is a sign of tolerance. On the contrary, ungrafted and self-grafted plants showed impaired gas exchange and promoted ROS generation; despite the increase of ROS scavenging and phenol content, such increase was not enough to prevent the oxidative damage under water deficit conditions, resulting in severe biomass reduction.

A different strategy was proposed under salt stress conditions, where the reduction of lipid peroxidation was associated with improved ROS scavenging and increased phenol content when the scion was grafted onto the tolerant rootstock NIBER®. The accumulation of H_2O_2 observed in this plant combination was not associated with more oxidative damage but was suggested to play a role as a secondary messenger, helping in the activation of the antioxidant machinery. In fact, similar functions for H_2O_2 have already been proposed in rice (Formentin *et al.*, 2018) and wheat (Liu *et al.*, 2020) under salt stress conditions. Regarding proline, its role as a compatible solute has not been stated herein, since the accumulation we found in all plant combinations was not enough to cause a drop in ψ_s, as was mentioned above. However, the rise of the concentration of proline when NIBER® was used as rootstock with respect to other plant combinations suggests that it played other relevant roles. In this sense, several authors

have attributed diverse functions, such as enzyme-stabilizing agent, peroxidative damage reduction or photosynthetic damage prevention (Demir and Kocaçalişkan, 2001; Ashraf *et al.*, 2008; Huang *et al.*, 2009), suggesting analogous functions herein. Lastly, although polyamine content was not measured in the present study, the up-regulation of related genes (SPDS3) in the ungrafted A25 accession when salt stress and control conditions were compared suggests that polyamine was playing a relevant role in the tolerance to oxidative damage, as has been reported in previous studies (Khoshbakht *et al.*, 2018), although further information would be necessary to confirm it.

Finally, it is a fact that grafting per se can modify and improve scion characteristics, as has been broadly described in the literature (Orsini *et al.*, 2013; Ren *et al.*, 2018). Therefore, we always introduced self-grafted plants as a double control to separate the grafting effects from the tolerant rootstock effects. In all our experiments presented in this document, we demonstrated that the use of self-grafted plants improves scion characteristics under stress, despite the fact that these plant combinations were not vigorous enough to confer tolerance to the scion. Under water deficit conditions, self-grafted plants improved water uptake by better RWC and ψ_w, C and N balance and ROS detoxification. In the case of salt stress conditions, even if they improved Na^+/K^+ and NR activity, they were severely affected, since the photosynthetic and biomass attributes decreased to the levels of the ungrafted plants. Consequently, cutting and joining two plants together modifies a series of pathways that confer defence improvement to the scion. In the literature, hormone signalling transduction, starch and sucrose metabolism (Ren *et al.*, 2018; Zhang *et al.*, 2019) and stomatal movement (Orsini *et al.*, 2013) have been modified under grafting conditions. However, using vigorous rootstocks significantly improves fruit production, biomass and tolerance compared to self-grafted plants (Estañ *et al.*, 2004; Liu *et al.*, 2014), as we also demonstrated in the present document.

To summarise all the above mentioned, we demonstrated that tolerance to salt and water stresses by using tolerant rootstocks is a complex phenomenon that implies the interconnection of multiple pathways to reach the state of tolerance. Although different mechanisms are important, the maintenance of photosynthesis and stomatal conductance, the drop of ABA content, the regulation of N metabolism and the differential expression of genes related to such parametes were proposed to be associated with biomass preservation in grafted pepper plants and ungrafted accessions. Likewise, increasing water uptake and flow was described as important mechanisms to improve tolerance. In the specific case of salt stress, the reduced transport of Na^+ to aerial organs by preferential accumulation in rootstocks was proposed as an advantage, since it prevented the toxic accumulation of this ion in photosynthetic organs and organelles and favoured the osmotic adjustment. All these factors led to the reduction of the oxidative damage in grafted pepper plants and the assignment of a new role for H_2O_2 as a signalling molecule under the salt conditions studied here. All these mechanisms mentioned here allowed accessions and grafted plants to cope with salt and water stress.

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Chapter 7

Final Conclusions

The knowledge that we have obtained in this doctoral thesis can be summarised in the following key points:

1. New pepper accessions have been found to be tolerant to water and salt stresses, which can be added to the genetic pool of the previously selected tolerant accessions to generate hybrids to be used as rootstocks.
2. Tolerance to salt stress in grafted pepper plants or accessions is achieved by limitation of Na^+ transport to leaves, as well as more efficient transport and accumulation of K^+ in roots and leaves, which are essential to accomplish ion homeostasis.
3. In grafted and ungrafted plants tolerant to water or salt stress, photosynthesis is more conservative and constitutes an essential parameter to determine tolerance.
4. The tolerant pepper rootstocks show lower ABA concentrations in leaves under salt stress, and the genes related to ABA signalling are misregulated, which leads to enhanced stomatal aperture, transpiration and, thus, more biomass.
5. Hydrogen peroxide presents a dual role in tolerant grafted plants under water scarcity and high salinity conditions: on the one hand, it contributes to oxidative damage and, on the other hand, it works as a secondary messenger with a considerable number of antioxidant signalling functions.
6. The drop in osmotic potential found in tolerant accessions and grafted plants to salinity and water scarcity is not linked with the increase in proline as a compatible osmolyte. The increase in proline is associated with a protective role.
7. Studying the molecular mechanisms of tolerance in tolerant pepper rootstocks under salt stress is used to confirm the previously found agronomical and physiological behaviour, and to unravel new molecular mechanisms hardly explored to date. All the differentially expressed genes were linked with hormonal signalling, growth and development, photoprotection, regulation of ion transporters and ROS detoxification.

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