



Article

Improving the Salt Tolerance of “Old Limachino Tomato” by Using a New Salt-Tolerant Rootstock

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Abstract: Salinity is a major constraint limiting the yield of tomatoes. However, grafting strategies may help to overcome the salt toxicity of this important horticultural species if appropriate rootstocks are identified. The present study aimed to test a new rootstock, JUPAFORT1, obtained by crossing the glycophyte *Solanum lycopersicum* (cv. Poncho Negro) with the halophyte wild-related species *Solanum chilense* to improve the salinity tolerance of the Chilean tomato landrace Old Limachino Tomato (OLT). Intact OLT plants were exposed to 0, 80, or 160 mM of NaCl for 21 days at the vegetative stage and compared with self-grafted (L/L) and Limachino plants grafted on JUPAFORT1 rootstock (L/R) under a completely randomized design. JUPAFORT1 increased OLT scion vigor in the absence of salt but did not significantly increase fresh weight under stress conditions. However, JUPAFORT1 confers to the scion an anisohydric behavior contrasting with the isohydric behavior of L and L/L plants as indicated by measurements of stomatal conductance; L/R plants were able to maintain their metabolic status despite a slight decrease in the leaf's relative water content. JUPAFORT1 rootstock also enabled the maintenance of photosynthetic pigment concentrations in the scion in contrast to L and L/L plants, which exhibited a decrease in photosynthetic pigments under stress conditions. L/R plants encountered oxidative stress at the highest stress intensity (160 mM of NaCl) only, while L and L/L plants suffered from oxidative damage at a lower dose (80 mM of NaCl). L/R plants behaved as includer plants and did not sequester Na⁺ in the root system, in contrast to L and L/L, which behaved as excluder plants retaining Na⁺ in the root system to avoid its translocation to the shoots. The expression of genes coding for ion transporters (*HKT1.1*, *HKT1.2*, *LKT1*, *SKOR*, *SOS2*, and *SOS3*) in the root system was not modified by salinity in L/R. In contrast, their expression varied in response to salinity in L and L/L. Overall, L/R plants exhibited higher physiological stability than L/L or L plants in response to an increasing NaCl dose and did not require additional energy investment to trigger an adaptive response to salinity. This suggests that the constitutive salinity tolerance of the halophyte *S. chilense* was maintained in the interspecific rootstock. JUPAFORT1 issued from *S. lycopersicum* × *S. chilense* may thus improve salt-stress resilience in OLT tomatoes. Additional studies are required to identify the molecular components involved in the root-to-shoot signaling pathway in this promising material.

Keywords: halophyte; NaCl; oxidative stress; *Solanum chilense*; tomato landrace; wild tomato



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1. Introduction

Salinity is one of the main factors limiting plant growth and productivity in cultivated species. Tomato is a glycophyte species considered to be highly salt sensitive. Further, salinity affects almost all aspects of plant physiology and biochemistry, such as plant growth, plant water and oxidative status, mineral nutrition, photosynthesis, and hormonal profile [1,2]. Improving the salinity tolerance of crops thus requires selecting an adequate combination of several physiological traits that influence the plant's adaptive responses to the environment at all stages of development and ultimately contribute to yield maintenance [1,2]. It is, however, likely that some genes controlling these physiological traits are no longer present in modern commercial cultivars of domesticated glycophytes, which were selected in the past for their high yield potential under non-saline conditions [3,4].

In tomatoes grown under salinity stress, long-term damage has been linked to the low water potential of the external medium hampering water absorption by the plant and the excessive accumulation of Na^+ and Cl^- within the leaves. Photosynthesis is one of the most sensitive processes affected by saline stress. Indeed, salinity reduced the net photosynthetic rate as a consequence of a decrease in stomatal conductance (g_s) and alteration of the intercellular CO_2 concentration (C_i) of plants [5]. Moreover, salinity reduces PSII efficiency by altering the photosystem II (PSII) complex, causing primary charge separation in PSII, damage pigment-protein complexes of chloroplast thylakoid membranes, and a decrease in PSII electron transport quantum yield [1,3]. Because salinity severely limits photosynthetic CO_2 fixation, the rate of light energy absorption by photosynthetic pigments frequently exceeds the rate of its consumption in the chloroplast. Excess energy can accelerate PSII damage through the generation of reactive oxygen species (ROS) such as superoxide ions (*O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (*OH^-) [6]. These highly toxic and reactive compounds can damage DNA, proteins, chlorophyll, and membrane structures [1,2].

Some plants have selected adaptative strategies to cope with high salt levels in soil. Among them, the limitation of Na^+ absorption by the roots and translocation to the shoot on the one hand, as well as antioxidative mechanisms in photosynthetic organs on the other hand, are undoubtedly the most efficient strategies [1]. Because they share a similar ionic radius, Na^+ absorption by K^+ transporters often leads to K^+ deficiencies, and the maintenance of K^+ versus Na^+ selectivity is paramount for salt-exposed plants [7,8].

Membrane transporters for K^+ flux identified in plants are generally classified into three channels and three transporter families based on phylogenetic analyses. The first group includes shaker-type K^+ channels and two-pore K^+ channels, such as non-selective cation channels (NSCCs) [9]. The second group consists of the KUP/HAK/KT (potassium uptake permease, high-affinity K^+ transporters, and K^+ transporters, respectively) family of transporters, and the third group consists of the HKT (high-affinity K^+ transport) family of transporters and K^+/H^+ antiporters [10]. The SOS (Salt Overly Sensitive) pathway is also associated with two important processes that lead to salinity tolerance. One is a root cell-based mechanism that relies on the flux of Na^+ back into the soil solution. The second is the control of Na^+ unloading in the xylem, which has been confirmed in Arabidopsis, tomato, rice, and barley [10,11]. Three distinct proteins are involved in the SOS pathway: SOS1 is a plasma membrane Na^+/H^+ antiporter, SOS2 is a serine/threonine protein kinase, and SOS3 is a calcium-binding protein regulating SOS1 activity [10,12,13].

As far as tolerance to secondary oxidative stress is concerned, plants rely on a large set of endogenous non-enzymatic and enzymatic antioxidants. Ascorbate and glutathione assume key functions in the Asada cycle and contribute to limiting oxidative damage [14]. Ascorbate peroxidase (APX; EC 1.11.1.11), superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2), glutathione-S-transferase (GST; 2.5.1.18), dehydroascorbate reductase (DHAR; EC 1.8.5.1), and monodehydroascorbate reductase (MDHAR) are important enzymes directly involved in ROS detoxification [1,15,16].

Most studies on salt's impact on cultivated tomatoes deal with modern commercial cultivars. However, some neglected local varieties now receive considerable attention

concerning the high quality of produced fruits from both organoleptic and nutritional points of view. This is especially the case of the promising “OLT Limachino tomato” (OLT) [17], which has been recently rediscovered in the Limache basin of Central Chile and exhibits attractive sensory and health properties. The plant is well adapted to the local environment, does not require high amounts of pesticides or fertilizers, and has great potential for sustainable agriculture. The consumers’ demand for this high-quality product is ever-increasing. However, OLT is unfortunately sensitive to NaCl [18], and there is therefore considerable interest in increasing its mean level of salinity tolerance.

Since roots are the first organs in contact with salinity, using adapted rootstock is a convenient strategy to limit salt accumulation and improve Na⁺ tolerance in tomatoes [6,19,20]. He et al. [6] stated that alleviating salinity-induced growth inhibition in tomato plants grafted on a Chinese commercial rootstock would be associated with improved scion antioxidant capacity and a concomitant improvement in photosynthesis. On the other hand, some authors investigated the mechanisms involved in the effects of grafting on yield in tomatoes and reported that the salinity tolerance conferred by some grafting combinations was related to the ability of the rootstock to reduce the rates of salt ion uptake and transport to the shoot [19,20]. The selection of the rootstock is nevertheless a challenging task and requires a combination of numerous root-related characteristics. Local salt-tolerant varieties and wild-related halophyte plant species might be used for this purpose. The tomato cv. Poncho Negro was ancestrally developed and established in the extreme conditions of Northern Chile (Arica and Parinacota region). It is tolerant to mineral toxicities (salinity and boron) [21,22] and biotic stress, specifically to *Pseudomonas syringae* pv tomato attack [23]. On the other hand, numerous data are available for some close-related wild halophyte species [24–27] and interspecific crosses with cultivated *S. lycopersicum* [19,28]. Introgression lines from numerous wild species have been successfully performed. Paradoxically, only a few data are available for the wild halophyte *Solanum chilense*. This species is native to the Atacama Desert and can grow at salinity levels similar to those found in seawater. The fascinating ability of *S. chilense* to adapt and cope with saline environments has been underexploited, mainly due to the self-incompatibility inducing cross-pollination of this wild species, the small size of produced fruits, and other traits that are undesirable to incorporate into breeding programs [24,29–36]. These authors, however, also demonstrated that the halophyte *S. chilense* behaves as an includer and does not restrict Na⁺ translocation from the roots to the shoots, while the salt-resistant glycophyte cultivars of cultivated *S. lycopersicum* behave as excluder, sequestering Na⁺ in the roots and thus avoiding its translocation to the shoot. Previous studies [24,29–36] have confirmed that the wild relative tomato *S. chilense* is tolerant to salinity and can cope with high endogenous Na⁺ levels. In this case, hormonal signals issued from the roots may trigger efficient salt-tolerance mechanisms in the shoots [37,38]. However, to the best of our knowledge, no data are available on the use of interspecific hybrids issued from *Solanum lycopersicum* × *Solanum chilense* for rootstock selection, especially considering that this cross is difficult to conduct considering the self-incompatible nature of the wild parent.

Interspecific crosses successfully performed between *Solanum lycopersicum* cv. Poncho Negro (accession SLY0001) and wild halophyte *Solanum chilense* (donor accession LA 4170) allowed us to recently select, for the first time to the best of our knowledge, a promising F1 rootstock material (JUPAFORT1; INIA), which was cloned in vitro [39]. The purpose of the present study was to assess the Na⁺ distribution in the OLT/JUPAFOR scion/rootstock combination in relation to mineral status, the management of oxidative stress and photosynthetically related parameters in the shoots, and the expression of genes coding for the Na⁺/K⁺ transporter in the roots.

2. Material and Methods

The experiment was conducted in greenhouses at the INIA-La Cruz Regional Center located in La Cruz, Region of Valparaíso (32° 59' LS; 71° 10' LW, 105 m.a.s.l.). The trials

were conducted during the vegetative stage of plant development in the spring–summer season of 2021 and 2022.

2.1. Plant Material and Growing Conditions

The tomato genotype (*Solanum lycopersicum* Mill.) cv. known, in English, as Old Limachino tomato (OLT, accession SLY074) [40,41], was used as a scion, while the rootstock (R) used is a F1 hybrid clonal material (INIA JUPAFORT1). This rootstock material was previously created at the INIA' premises by crossing a cultivated tomato *Solanum lycopersicum* (salinity-resistant tomato genotype cv. called Poncho Negro, accession SLY0001) with a wild halophytic tomato *Solanum chilense* (*Solanum chilense* Dun., donor accession LA 4170). It was propagated in vitro through a protocol developed at INIA that optimizes the micropropagation process for grafting [39]. One F1 plant (JUPAFORT1) was selected and clonally propagated in vitro so that all the rootstocks were genetically identical. Clonal rootstock seedlings (R) maintained in vitro were then acclimatized in greenhouse conditions to perform the grafting process with the OLT plants at the three true leaves stage. OLT seeds were sown and seedlings were allowed to grow inside a growth chamber (Plant Growth Incubator 487 Liter, Laboratory Instruments-MRC) in four plumavit containers filled with a perlitevermiculite mixture (1: 3 v/v), regularly moistened for three weeks with half-strength modified Hoagland solution containing 5 mM of KNO₃, 1 mM of NH₄H₂PO₄, 0.5 mM of MgSO₄, and 5.5 mM of Ca(NO₃)₂; 25 µM of KCl, 10 µM of H₃BO₃, 1 µM of MnSO₄, 1 µM of ZnSO₄, 0.25 µM of CuSO₄, and 10 µM of Na₂MoO₄; and 50 mg L⁻¹ of Fe-EDDHA [40].

In this study, three groups of OLT plants were considered: (1) ungrafted plants (L), (2) self-grafted (L/L), and (3) plants grafted with the clonal rootstock (L/R). When the OLT plants were used for grafting, the scion contained three true leaves. The grafted plants (L/L and L/R) were obtained using a manual splice-grafting procedure and were secured with a silicone clip. All plants were then transplanted into 1.5 L pots when they had three to four fully unfolded true leaves. Seven days after stress application (scions exhibiting 4–5 true leaves), a nutrient solution without NaCl was used as a control treatment (EC = 3 dS m⁻¹), while solutions containing 80 (EC = 9 dS m⁻¹) and 160 (EC = 14 dS m⁻¹) mM of NaCl were applied every 3 days until the end of the experiment, 21 days after treatment (DAT). The first experimental factor was the rootstock (R), while the second factor was the salinity level. Each treatment included six replicates (six plants) arranged in a completely randomized design. The natural light intensity at the top of the canopy averaged 500 µmol m⁻² s⁻¹ (photosynthetic photon flux density, PPFD). The air temperature ranged between 25 °C and 32 °C during the day and approximately 18 °C at night. The relative humidity was maintained at 85 ± 5% at night and 45 ± 5% during the day. The environmental conditions were permanently recorded using a Hobo MX2301A temperature/RH Data Logger; data were transferred to the HOBO connect Monitoring Application.

2.2. Measurements

2.2.1. Plant Growth and Water Status

The fresh (FW) and dry weight (DW) of leaves, stems, and roots were estimated at 21 DAT (days after stress imposition) on six plants per treatment. The dry weight was considered after 48 h in an oven at 80 °C. To evaluate vegetative growth, the plant height (length of the dominant axis), basal diameter, and number of leaves were also measured at 1, 7, 14, and 21 DAT. The number of leaves was counted by recording all leaves on the main stem with blades longer than 1 cm.

The leaf's water potential was measured before sunrise (Ψ_{w_p}) and at midday (Ψ_{w_n}) using a Scholander-type pressure chamber (PMS Instrument Co., Orlando, FL, USA) [29] on the sixth fully expanded leaf (counted from the base). The ylem pressure potential (Ψ_{w_x}) was quantified using the same chamber after conditioning leaves for two hours in dark bags to eliminate leaf transpiration [42]. The relative water content (RWC) evaluated before

sunrise (RWC_p) and at midday (RWC_n) was determined according to Martínez et al. [42] and expressed in %:

Relative Water Content at predawn (RWC_p)

$$RWC_p(\%) = \left(\frac{FW_p - DW_p}{TW_p - DW_p} \right) \times 100$$

Relative Water Content at noon (RWC_n)

$$RWC_n(\%) = \left(\frac{FW_n - DW_n}{TW_n - DW_n} \right) \times 100$$

where FW_p and FW_n are the fresh weight at predawn and noon, respectively, TW_p and TW_n are the turgid weight at predawn and noon, respectively measured after 24 h of saturation (when leaf weight reached a plateau) on deionized water at 4 °C in the dark, and DW is the dry weight determined after 48 h in an oven at 80 °C.

The leaf's water content on dry matter before dawn (WC_{DWp} , g/g) and at midday (WC_{DWn} , g/g) were determined using the method developed by Martínez et al. [29]. These measurements were performed on six plants per plant group and treatment at 21 DAT.

2.2.2. Leaf Stomatal Conductance, Chlorophyll Fluorescence, and Pigment Concentrations

The leaf stomatal conductance (g_s) was measured at midday on the sixth fully expanded leaf using a porometer (Decagon devices, SC1, NE Hopkins Ct., Pullman, WA, USA). The chlorophyll content index was estimated using a portable chlorophyll meter (Chlorophyll Content Meter Opti-Sciences Model CCM-200 Plus GPS) on the same leaf. The basal (F_0) and maximal fluorescence (F_m) and maximum PSII yield (F_v/F_m) of chlorophyll fluorescence were determined as previously detailed [43] using a chlorophyll *a* fluorescence monitoring system (FMS2, Hansatech, Norfolk, UK) on the same leaf. These measurements were performed on six plants per plant group and treatment at 6, 13, and 20 DAT.

The chlorophyll *a* and *b* and carotenoid concentrations were determined using extracts of 100 mg of pre-cooled mortar-ground fresh leaf material in 8 mL of 80% *v/v* cold acetone. The absorbance of the extracts was read at 663.2, 646.8, and 470 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan), and the concentrations of chlorophyll *a*, chlorophyll *b*, and carotenoids were calculated according to Lichtenthaler [44]. These measurements were performed on the sixth fully expanded leaf of six plants per plant group and treatment at 21 DAT.

2.2.3. Oxidative Damage and Total Antioxidant Capacity

The malondialdehyde (MDA) content was measured according to Heath and Packer [45] using a Shimadzu UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan)–Metler Toledo, Zaventem, Belgium. The antioxidant capacity at the leaf level was measured using two methods: (i) ferric-reducing antioxidant power (FRAP) and (ii) a DPPH (2,2-diphenyl 1-picrylhydrazyle) assay. The FRAP was measured based on the methodology of Benzie and Strain [46] with slight modifications [40,41]. The DPPH was determined using the colorimetric method based on work by Brand-Williams et al. [47]. These measurements were performed on the sixth fully expanded leaf of six plants per plant group and treatment at 21 DAT.

2.2.4. Non-Enzymatic Antioxidant Compounds

Non-enzymatic antioxidant compound concentrations were measured on the sixth fully expanded leaf of six plants per plant group and treatment at 21 DAT.

Total polyphenols were measured spectrophotometrically using the adapted colorimetric assay with a Folin–Ciocalteu reagent and reading absorbance at 765 nm [48] in reference to a standard curve established with gallic acid.

Ascorbate quantification was performed according to Kampfenkel [49]. A 0.25 g leaf tissue sample was homogenized with 4 mL of trichloroacetic acid and left for 15 min on ice. The samples were then centrifuged for 5 min at $10,000\times g$ at $4\text{ }^{\circ}\text{C}$, and the supernatant was recovered. To determine the total ascorbate, oxidized ascorbate (DHA) was reduced to AsA with DL-dithiotreitol (DTT), and excess DTT was removed with NEM. For the determination of reduced ascorbate, 0.2 mL of the supernatant, 0.4 mL of 0.2 M phosphate buffer with a pH of 7.4, 0.4 mL of Milli-Q water, 1 mL of 10% *w/v* TCA, 0.8 mL of 85% H_3PO_4 , 0.8 mL of 4% dipyridyl, and 0.4 mL of 3% FeCl_3 were used. The solutions to determine the reduced ascorbate and total ascorbate were incubated for 1 h at $37\text{ }^{\circ}\text{C}$, and the absorbance was read in a spectrophotometer at a wavelength of 525 nm. The determination of oxidized ascorbate (DHA) was estimated by subtracting the total minus reduced ascorbate values.

$$\text{Oxidized Ascorbate (DHA)} = \text{total ascorbate (ASAT)} - \text{Reduced Ascorbate (AsA)}$$

To determine reduced glutathione (GSH) and total glutathione (total GSH), 0.2 g of frozen leaf tissue was extracted and derivatized using *ortho*-phthalaldehyde (OPA) [50]. The extraction was performed as previously detailed [51]. The derivatives were separated on a reverse-phase HPLC column with a fluorescence-detected acetonitrile gradient system. A volume of 5 μL of the sample was injected into a Shimadzu HPLC system coupled with a Piramide C18 column (125×4.6 mm internal diameter; 5 μm particle size) (Macherey-Nagel, Düren, Germany). The derivatives were eluted in acetonitrile gradient in 50 mM of sodium acetate buffer (pH 6.2) at $30\text{ }^{\circ}\text{C}$ at a flow rate of 0.7 mL min^{-1} . Detection was performed with a Shimadzu RF-20 fluorescence detector at an emission wavelength of 420 nm and an excitation wavelength of 340 nm. GSH was quantified using a calibration curve with standard solutions from 0.0625 to 50 μM . Oxidized glutathione (GSSG) was determined by subtracting the GSH from the Total GSH content.

2.2.5. Antioxidant Enzyme Activity

Approximately 0.5 g of fresh leaf tissue (from the sixth fully expanded leaf of six plants per plant group and treatment at 21 DAT) was ground in liquid nitrogen and then homogenized with 4 mL of 50 mM phosphate buffer containing 0.1 mM EDTA, 1% *w/v* polyvinylpyrrolidone (PVP), 1 mM of ascorbate, 5% *w/v* glycerol, and 1 M NaCl. Subsequently, the samples were centrifuged at $10,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. After the supernatant was recovered, the samples were stored at $-80\text{ }^{\circ}\text{C}$ until the determination of enzyme activities and protein quantification. The activity of glutathione reductase (GR; EC 1.6.4.2) was quantified by NADPH consumption ($\epsilon = 6.22, \text{mM}^{-1} \text{cm}^{-1}$) at 340 nm for 5 min [52]. The ascorbate peroxidase activity (APX; EC 1.11.1.11) was determined by the ascorbate consumption at 265 nm ($\epsilon = 13.7 \text{mM}^{-1} \text{cm}^{-1}$) for 30 s [53]. Enzyme activity was expressed as $\text{mmol AsA min}^{-1} \text{mg}^{-1}$ protein. Catalase activity (EC 1.11.1.6) was determined by the consumption of H_2O_2 at 240 nm for 5 min [53]. Dehydroascorbate reductase activity (DHAR; EC 1.8.5.1) was measured at 265 nm for 3 min following the absorbance change resulting from ascorbic acid formation [53]. Glutathion-S-transferase activity (EC 2.5.1.18) was measured by the increase in oxidized glutathione ($\epsilon = 9.6, \text{mM}^{-1} \text{cm}^{-1}$) via the absorbance change at 340 nm for 5 min [53]. Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was measured using the method developed by Miyake and Asada [54]. The total superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to Beyer and Fridovitch [55], modified by Yu et al. [56]. Each enzyme activity was analyzed in triplicate. Protein quantification was performed spectrophotometrically at 595 nm according to the Bradford method [57] with Bio-Rad (Hercules, CA, USA) assay protein BIORAD using BSA for calibration.

2.2.6. Ion Concentration

For Na^+ and K^+ quantification, tissues from six plants (root, stem, and leaf) per plant group and treatment at 21 DAT were considered. For each sample, *c.a.* 50 mg of dry matter was digested in 4 mL of 0.5% nitric acid at $80\text{ }^{\circ}\text{C}$. After complete evaporation, residues

were dissolved with HNO_3 (68%) + HCl_{cc} (1:3 *v/v*) and incubated under gentle agitation of 100 rpm or 48 h. The solution was then filtered with Whatman 70 mm Grade 1, and the filtrate was used to determine the Na^+ and K^+ concentrations via flame emission with an atomic absorption spectrometer (Shimadzu AA-680 atomic absorption spectrometer (Shimadzu Ltd., Kyoto, Japan)).

2.2.7. Differential Gene Expression in Roots

The expression of seven genes involved in Na^+ and K^+ transport mechanisms associated with tomato salinity tolerance was analyzed in the root system by means of qRT-PCR in three plants per plant type and treatment. For this purpose, specific primers suitable for qPCR were designed for the following genes: [1] putative Na^+ transporters HKT1.1 (*HKT1.1*) and [2] HKT1.2 (*HKT1.2*), [3] potassium channel SKOR-like (*SKOR*), [4] potassium channel (*LKT1*), [5] plasmalemma Na^+/H^+ antiporter (*SOS1*), [6] calcineurin B-like interacting protein kinase (*SOS2*), and [7] calcium sensor calcineurin B-like (*SOS3*) (Supplementary Table S1). Since the clonal rootstock used in this study was an interspecific hybrid between *S. lycopersicum* and *S. chilense*, highly transferable primers were designed between the two species. The efficiency of amplifying a single amplicon between 64 and 199 bp was tested on a root transcriptome of the *S. lycopersicum* × *S. chilense* hybrid using in silico PCR. Roots from treated and control plants were collected 21 days after initiation of the salt treatment (21 DAT), quickly washed, and immediately frozen in liquid nitrogen and stored at -80°C . The total RNA from each sample was then obtained using the RNeasy Plant Mini Kit from Qiagen [58]. The qPCR assay was performed with the Gotaq@Probe qPCR Master Mix Kit using an Agilent Real Time PCR kit (AriaMX Base Unit Agilent Technologies). The genes coding for elongation factor 1-alpha (*EF1aF*) and Actin (actin) were used as references. The method described by Pfaffl [59] was used to perform all relative expression calculations. Samples were run in triplicate, including three inserts as negative. Data are presented as the mean of the three replicates $\pm$ standard deviation.

2.3. Statistical Analysis

Physiological, biochemical, gene expression, and biomass data were analyzed using a two-way analysis of variance (ANOVA), and the multiple range test was used for mean comparisons at a significance level of $p < 0.05$. The statistical model was defined based on fixed effects and hierarchical ranking criteria. The grafting type and salinity concentration (NaCl concentration) were considered as the main effects, as well as their interactions. When the ANOVA was significant at $p \leq 0.05$, Duncan's multiple range test was used to compare the means of each variable under study. All analyses were performed in RStudio version 3.0.2 (R Development Core Team, 2012; available online at <http://www.R-project.org>; accessed on 2 October 2022; the software was obtained from INIA, Chile). The plotting program was generated using GraphPad Prism (version 8.0 GraphPad software, San Diego, CA, USA).

3. Results

3.1. Plant Growth

In the absence of NaCl , the leaf FW was higher in L/R plants compared to L and L/L plants (Figure 1A). In response to salinity, it significantly decreased only in L/R plants in response to 160 mM. Leaf DW exhibited the lowest value in L/L and remained unaffected by salinity. Similarly, the stem FW (Figure 1C) was higher in L and L/R than in L/L in control conditions. Salinity had no significant effect on the stem FW or stem DW (Figure 1D), whatever the considered grafting type. The root FW (Figure 1E) of L/L exhibited a low value compared to other grafting types in the absence of stress. The salinity increased the root FW in self-grafted plants L/L, but did not impact the root DW, which remained similar in all treatments.

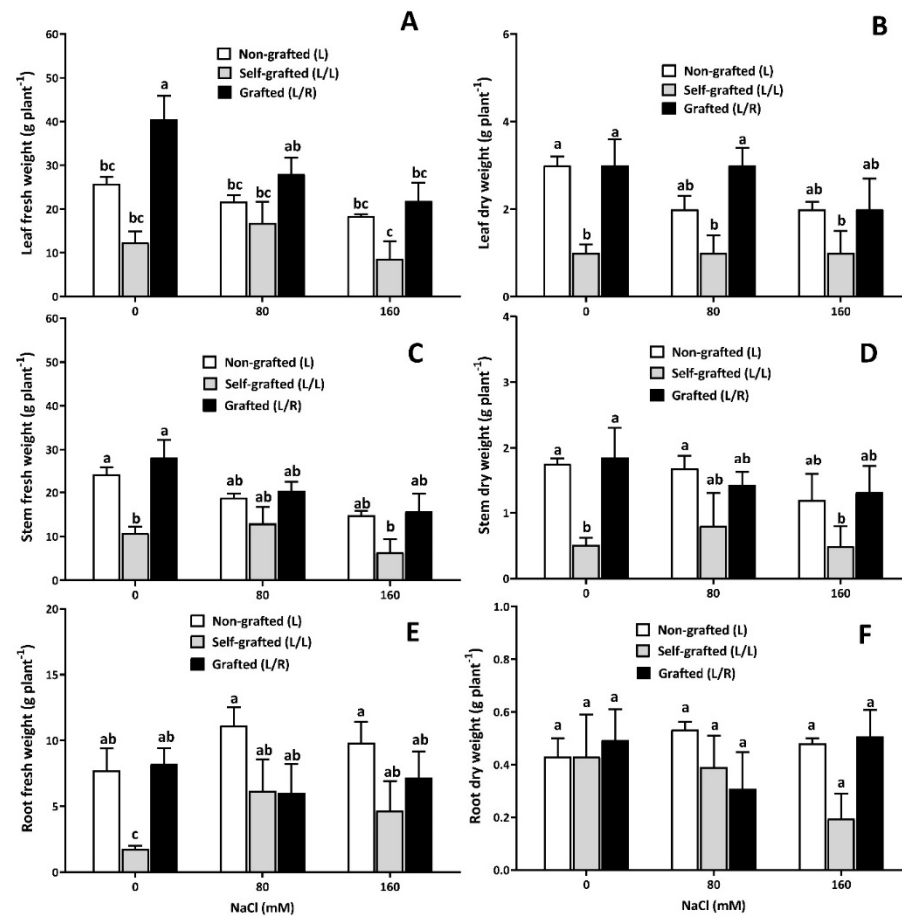


Figure 1. Plant growth-related properties as a function of NaCl dose. Fresh and dry weights of leaves (A,B), stems (C,D), and roots (E,F) (g plant⁻¹) for plants corresponding to treatments L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown under three salinity treatments: 0 mM of NaCl; 80 mM of NaCl, and 160 mM of NaCl for 21 days (DAT). Each value is the mean of six replicates, and vertical bars are S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and grafting types.

The plant height was significantly reduced in L plants 7 days with 160 mM of NaCl and after 21 days with 80 mM of NaCl (Figure 2A). L/L were smaller than L plants (Figure 2B), and 80 mM of NaCl unexpectedly increased the plant height, while 160 mM of NaCl decreased it compared to 0 mM of NaCl. The plant height was only slightly affected by salinity in L/R plants (Figure 2C). No effect of grafting type or salinity was observed regarding the stem diameter (Figure 2D–F). The number of leaves in L plants was reduced by salinity after 14 days (Figure 2G). In L/L, 80 mM of NaCl increased the number of leaves starting on day 7 (Figure 2H), while plants exposed to 160 mM of NaCl produced the lowest number of leaves. The leaf production was not affected by NaCl in L/R plants, except for a slight decrease in the number of leaves recorded for L/R plants exposed to 160 mM on day 7 and day 21 (Figure 2I).

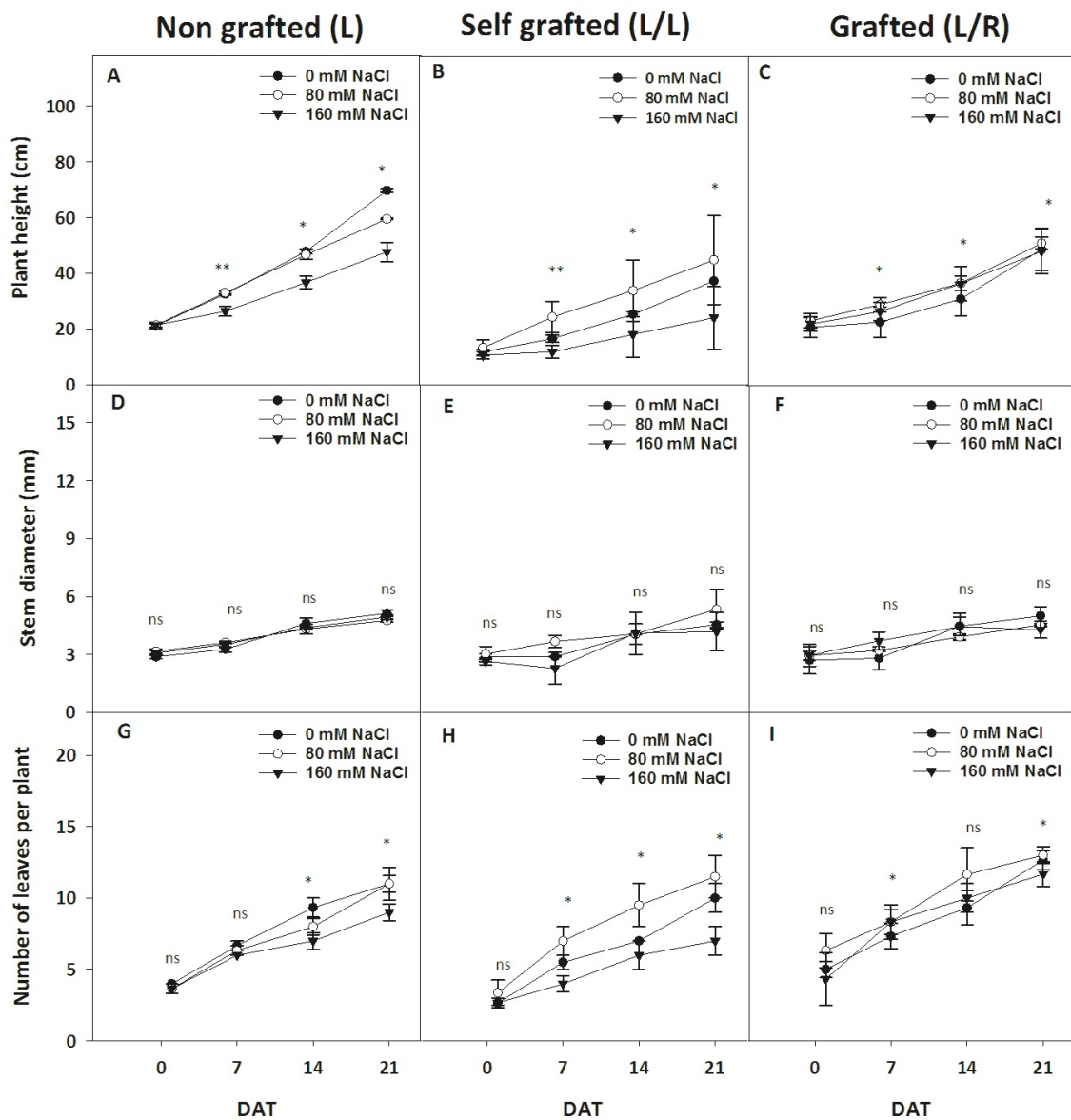


Figure 2. The evolution of plant morphological parameters during exposure to different NaCl doses. The plant height (cm; A–C), basal stem diameter (mm; D–F), and number of leaves per plant (G–I) for plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown under three salinity treatments: 0 mM of NaCl; 80 mM of NaCl, and 160 mM of NaCl for 1, 7, 14, and 21 days after beginning treatment (DAT). Each value is the mean of six replicates, and vertical bars are S.E. Multifactorial ANOVA test: (*) $p \leq 0.05$, and $p \leq 0.01$ (**), by date; ns: non significant.

3.2. Plant Water Status and Photosynthesis-Related Parameters

The plant water status-related parameters are presented in Table 1. Ψ_{wp} and Ψ_{wx} remained unaffected by salinity in the three plant groups. Ψ_{wn} increased in response to 160 mM of NaCl in L/L and L/R. Ψ_{wn} was globally higher in L/R than in L/L plants, regardless of the salt treatment. RWC_p decreased in response to 160 mM of NaCl in the three plant groups, and self-grafted L/L was the only combination affected by 80 mM of NaCl. RWC_n decreased in response to 80 and 160 mM of NaCl in L plants, while it decreased only at the highest dose in L/L and L/R plants. WC_{DWp} and WC_{DWn} remained unaffected by salinity and were similar for the three plant groups.

Table 1. The leaf water potential at predawn (Ψ_{w_p} , MPa), the leaf water potential at noon (Ψ_{w_n} , MPa), the xylem pressure potential (Ψ_{w_x} , MPa), the leaf relative water content at predawn (RWC_p , %), the leaf relative water content at noon (RWC_n , %), the leaf water content on dry matter at predawn (WC_{DWp} , g/g), and the leaf water content on dry matter at noon (WC_{DWn} , g/g) in non-grafted (L), self-grafted (L/L), and grafted on INIA JUPAFOR1 rootstock (L/R) in shoots of Old Limachino tomato plants grown under 0, 80, or 160 mM of NaCl for 21 days. Each value is the mean of six replicates $\pm$ S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and grafting types.

Water status variable	Non-Grafted (L)			Self-Grafted (L/L)			Grafted (L/R)		
	NaCl (mM)			NaCl (mM)			NaCl (mM)		
	0	80	160	0	80	160	0	80	160
Ψ_{w_p} (MPa)	-0.46 ± 0.06 a	-0.37 ± 0.10 a	-0.24 ± 0.03 a	-0.33 ± 0.03 a	-0.29 ± 0.05 a	-0.37 ± 0.11 a	-0.39 ± 0.08 a	-0.34 ± 0.08 a	-0.26 ± 0.06 a
Ψ_{w_n} (MPa)	-0.45 ± 0.07 ab	-0.51 ± 0.09 a	-0.49 ± 0.09 a	-0.53 ± 0.07 a	-0.56 ± 0.12 a	-0.33 ± 0.06 b	-0.33 ± 0.05 b	-0.35 ± 0.06 b	-0.22 ± 0.01 c
Ψ_{w_x} (MPa)	-0.31 ± 0.04 a	-0.40 ± 0.09 a	-0.36 ± 0.08 a	-0.39 ± 0.06 a	-0.42 ± 0.06 a	-0.59 ± 0.10 a	-0.43 ± 0.06 a	-0.31 ± 0.05 a	-0.44 ± 0.15 a
RWC_p (%)	93.63 ± 0.94 a	84.76 ± 2.43 abc	79.26 ± 1.97 c	91.74 ± 2.40 ab	82.05 ± 2.21 c	82.03 ± 5.69 c	91.76 ± 2.32 ab	86.83 ± 2.62 abc	81.68 ± 2.59 c
RWC_n (%)	82.90 ± 3.00 a	80.00 ± 1.25 b	73.60 ± 0.84 b	82.17 ± 5.59 a	80.67 ± 1.59 a	72.39 ± 2.09 b	78.38 ± 3.48 a	78.18 ± 2.39 a	72.54 ± 3.22 b
WC_{DWp} (g/g)	10.17 ± 0.43 a	8.86 ± 0.33 a	8.50 ± 0.17 a	10.78 ± 0.74 a	9.42 ± 0.68 a	9.96 ± 1.79 a	10.37 ± 1.31 a	10.22 ± 1.50 a	11.82 ± 0.41 a
WC_{DWn} (g/g)	9.23 ± 0.87 a	8.76 ± 0.32 a	9.27 ± 1.51 a	8.71 ± 0.54 a	8.77 ± 0.76 a	9.72 ± 0.23 a	7.76 ± 1.02 a	9.25 ± 0.81 a	9.85 ± 2.61 a

The stomatal conductance (g_s) and chlorophyll index are shown in Figure 3. In L plants, g_s increased up to 14 DAT and thereafter decreased up to 21 DAT at 80 mM and 0 mM of NaCl, while such a pattern was less pronounced at 160 mM. In contrast, g_s decreased at 14 DAT in L/L plants and then increased during the following days up to 21 DAT at 0 and 160 mM of NaCl. At 80 mM of NaCl, g_s remained constant until 7 DAT, increased between 7 and 14 DAT, and decreased between 14 and 21 DAT (Figure 3B). For the L/R treatment, g_s remained constant regardless of the NaCl dose until 14 DAT, but a significant increase was recorded at 21 DAT for plants exposed to 160 mM of NaCl (Figure 3C). Figure 3D–F show that the chlorophyll index of L and L/R plants increased over time. It was also observed that the chlorophyll index increased progressively starting at 14 DAT for 80 mM of NaCl for L and L/L plants. In the case of L/R plants, a significant increase in the presence of 160 mM of NaCl was recorded at 21 DAT.

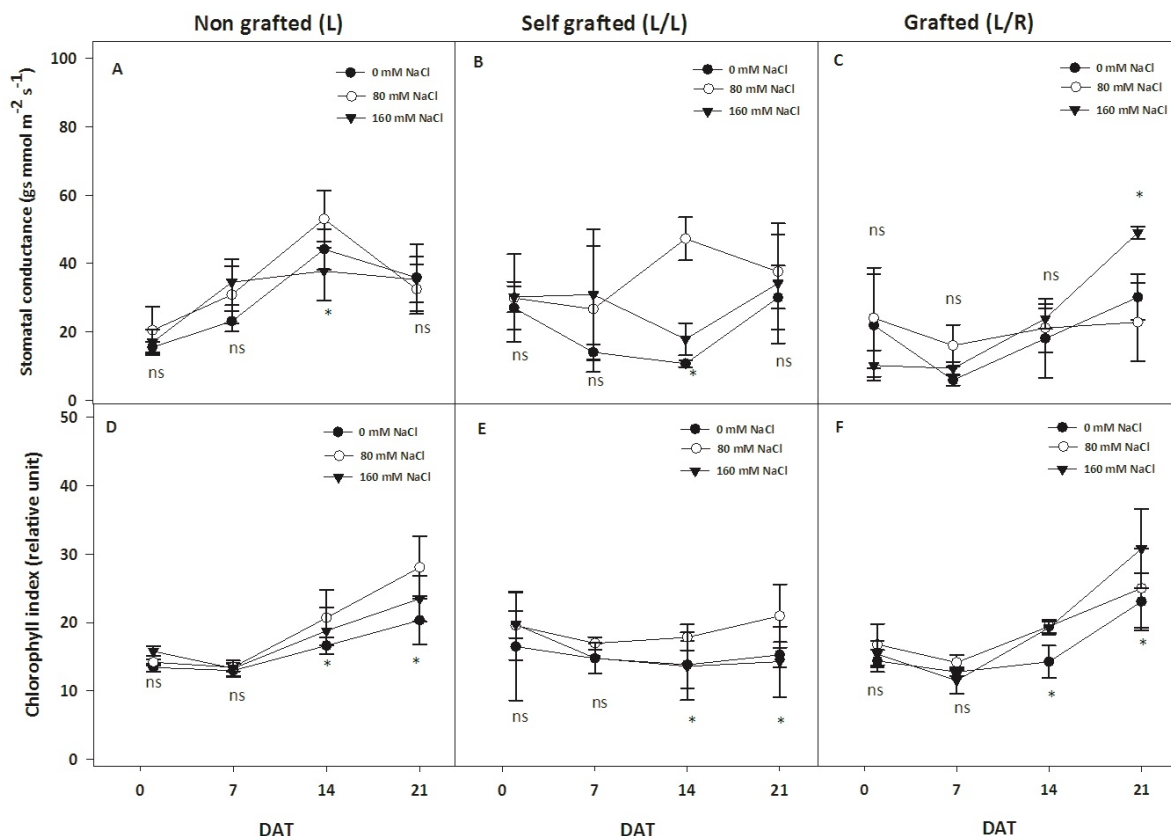


Figure 3. The evolution of the stomatal conductance and chlorophyll content index during exposure to different NaCl doses. Stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$ A–C) and chlorophyll index (%; D–F) for plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown under three salinity treatments: 0 mM of NaCl; 80 mM of NaCl, and 160 mM of NaCl at 1, 7, 14, and 21 days after beginning treatment (DAT). Each value is the mean of six replicates, and vertical bars are S.E. Multifactorial ANOVA test: (*) $p \leq 0.05$, by date. ns: non significant.

All fluorescence-related parameters showed high stability and remained unaffected by the type of plants or salinity level, except at 20 DAT, where a reduction in F_m and F_v/F_m was observed, especially in L/L plants at 160 mM of NaCl (Figure 4E). In control conditions, the Chl *a* content was the highest in L/R plants (Figure 5). It slightly increased in response to 80 mM of NaCl in L/L plants and in response to 160 mM of NaCl in L plants (Figure 5A). The three plant groups had similar Chl *b* content in control conditions (Figure 5B) and 80 mM of NaCl, but it decreased in response to 160 mM of NaCl in L/L only. The Chl *a/b* ratio was the lowest in L/L plants in the control treatment, and it increased for all three

plant groups at 80 and 160 mM of NaCl (Figure 5C). The carotenoid content was higher in L/L plants compared to L/R plants under control conditions. Salinity increased the carotenoid content in all plant groups at 80 mM of NaCl (Figure 5D). It then remained constant at 160 mM of NaCl, except for the carotenoid content of L/L plants, which slightly decreased at 160 mM of NaCl compared to 80 mM of NaCl.

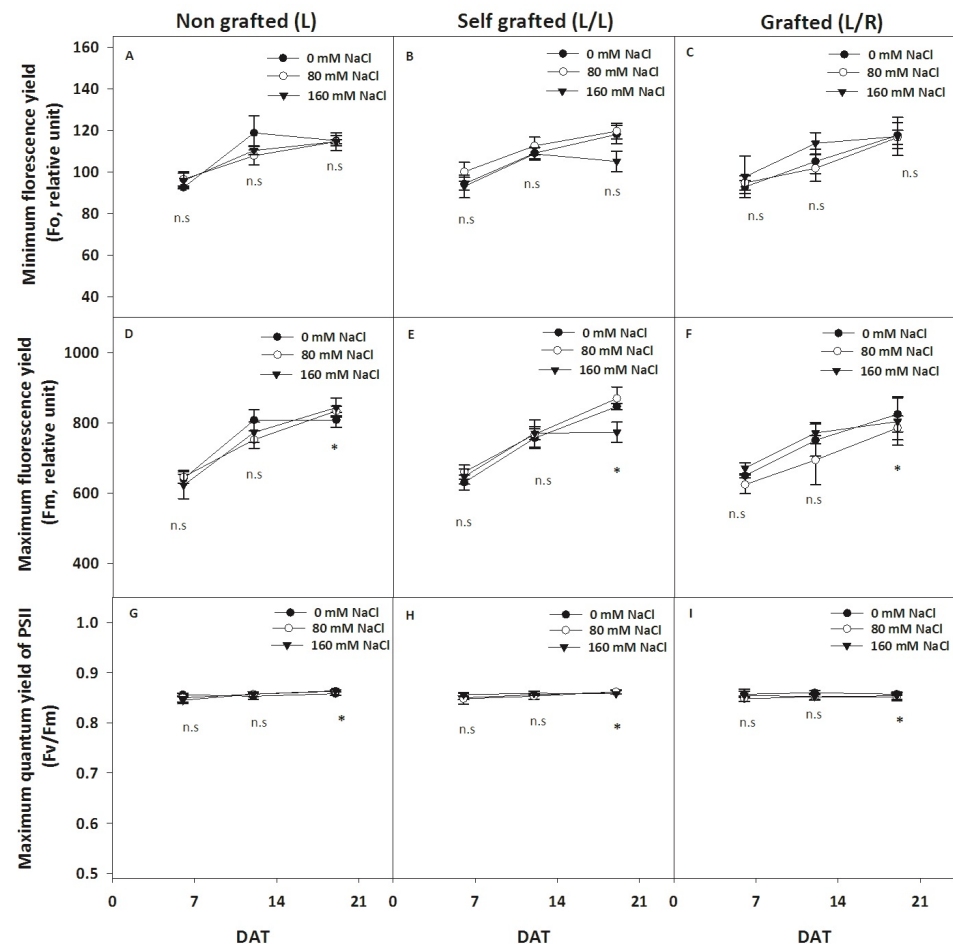


Figure 4. The evolution of chlorophyll fluorescence-related parameters during exposure to different NaCl doses. The basal (F_0 , relative unit; A–C) and maximum (F_m , relative unit; D–F) fluorescence and maximum PSII yield (F_v/F_m , G–I) in leaves for plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown under three salinity treatments: 0 mM of NaCl; 80 mM of NaCl, and 160 mM of NaCl at 6, 13, and 20 days after the beginning of the treatment (DAT). Each value is the mean of six replicates, and vertical bars are S.E. Multifactorial ANOVA test: (*) $p \leq 0.05$, n.s, non significant.

Under control conditions, the total protein content was higher in L/L and L/R than in L plants (Figure 6A). Under salinity, this variable remained unaffected in L/R plants, while the total protein content increased in both L and L/L plants, especially at the highest NaCl concentration (160 mM) (Figure 6A).

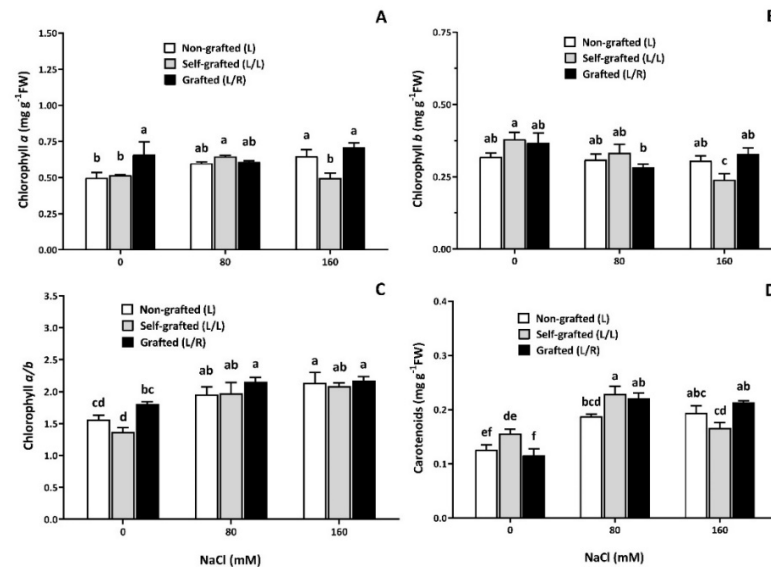


Figure 5. The pigment concentration as a function of the NaCl dose. Chlorophyll (Chl) a (A) and b (B) contents, chlorophyll a/b ratio (C), and carotenoids (D) for plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown for 21 days under three salinity treatments: 0 mM of NaCl; 80 mM of NaCl, and 160 mM of NaCl. Each value is the mean of six replicates, and vertical bars are S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and grafting types.

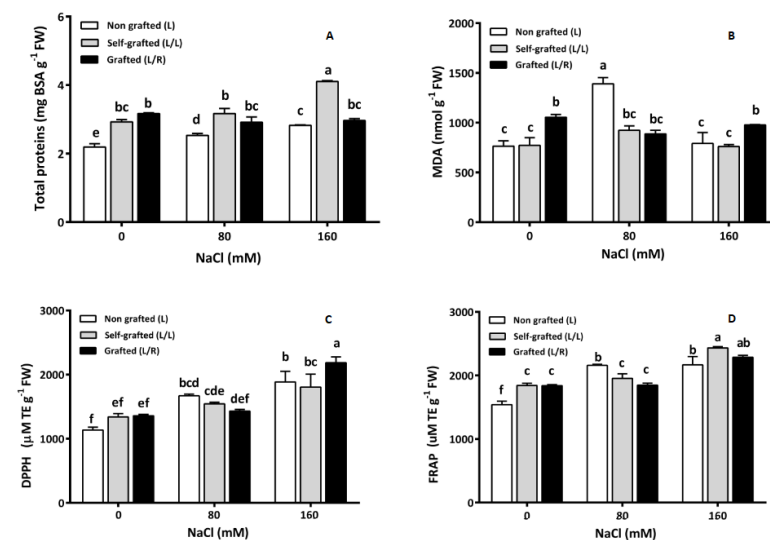


Figure 6. The total proteins (mg g⁻¹ FW; (A)), malondialdehyde (MDA; nmol g⁻¹ FW, (B)), and antioxidant capacity: DPPH (2,2-diphenyl 1-picrylhydrazyle, (C)) and FRAP (ferric-reducing antioxidant power) (mM Trolox equivalent TE.g⁻¹ FW) in leaves for plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown for 21 days under three salinity treatments: 0 mM of NaCl; 80 mM of NaCl, and 160 mM of NaCl. Each value is the mean of six replicates, and vertical bars are S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and grafting types.

3.3. Management of Oxidative Stress

In the absence of NaCl, the MDA (Figure 6B) content was the highest in L/R plants and remained constant in the presence of salt. MDA also remained unaffected by salt in L/L, while it exhibited a maximal value in response to 80 mM of NaCl in L. The antioxidant

capacity, assessed by DPPH and FRAP, is shown in Figures 6C and 6D, respectively. Salinity increased DPPH in all plant groups, with a more pronounced effect at 160 mM of NaCl. At this dose, the highest value was recorded for L/R plants, while activity did not differ among plant groups at 0 and 80 mM of NaCl. L plants showed the lowest FRAP value in the control treatment; 80 mM of NaCl increased FRAP values in L plants only, while the highest salt concentration (160 mM of NaCl) also increased it in L/L and L/R.

The polyphenol concentration did not vary with the plant groups at 0 and 80 mM of NaCl. When the plants were exposed to salinity stress, L and L/L plants progressively increased their polyphenol concentrations. They exhibited the highest value at 160 mM of NaCl, whereas this concentration remained stable in L/R plants (Figure 7).

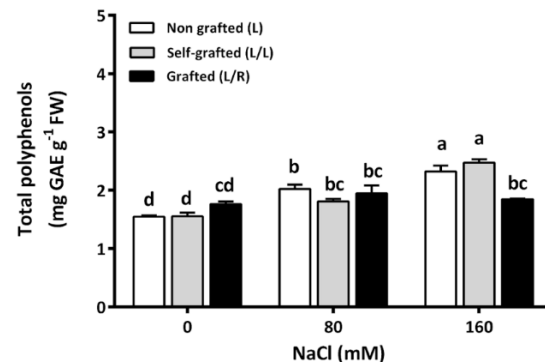


Figure 7. The leaf total polyphenols concentration (mg GAE (gallic acid equivalent) g^{-1} FW) for plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown for 21 days under three salinity treatments: 0 of NaCl; 80 of NaCl, and 160 of NaCl). Each value is the mean of six replicates, and vertical bars are S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and grafting types.

The ascorbic acid (AsA) concentration (Figure 8A) was higher in the non-saline treatment than in the saline treatments. In control conditions, L/R had a lower AsA concentration than L plants, while no differences among plant groups were recorded under salinity. L plants showed the lowest DHA (Figure 8B) concentration compared to the two other plant groups under control conditions. At 80 of NaCl, no differences were observed among plant groups. At high salinity (160 of NaCl), L/R exhibited higher values than L plants (Figure 8B). Moreover, DHA increased between 80 and 160 of NaCl in L/R plants, while it remained constant in L and L/L plants (Figure 8B). Figure 8C shows that L plants under non-saline conditions had the highest AsA/DHA ratio, which strongly decreased in response to 80 and 160 mM of NaCl. For the other plant groups, the AsA/DHA ratio remained constant, whatever the salinity level. For all plant groups, the total ascorbic acid content decreased between 0 and 80 of NaCl and then remained stable up to 160 mM of NaCl (Figure 8D).

The reduced glutathione (GSH) concentration was similar for all plant groups under control conditions (Figure 9A). The GSH increased in L/R plants exposed to 80 of NaCl, while it remained constant in L and L/L. At high salinity (160 mM), GSH increased in all plants and was higher in L/L and L/R than in L plants. The concentration of oxidized glutathione (GSSG) (Figure 9B) was lower in L/L plants than in other plants in the absence of NaCl. In response to salinity, GSSG increased in L/L and L/R plants and exhibited the highest value in L/R plants exposed to 160 of NaCl. The highest GSH/GSSG ratio (Figure 9C) was recorded in L/L plants in the absence of salt. Under salinity, an increase in this ratio was observed in L plants at 160 of NaCl. On the contrary, a significant reduction in GSH/GSSG was observed in L/L plants at 80 and 160 mM of NaCl. The GSH/GSSG ratio remained constant in L/R plants, regardless of the salinity level. At 0 of NaCl, the total glutathione concentration (Figure 9D) was similar in all plant groups. Under salinity,

the total GSH increased in L/R plants at 80 mM of NaCl and 160 mM of NaCl. In L and L/L plants, the total GSH increased at 160 mM only (Figure 9D).

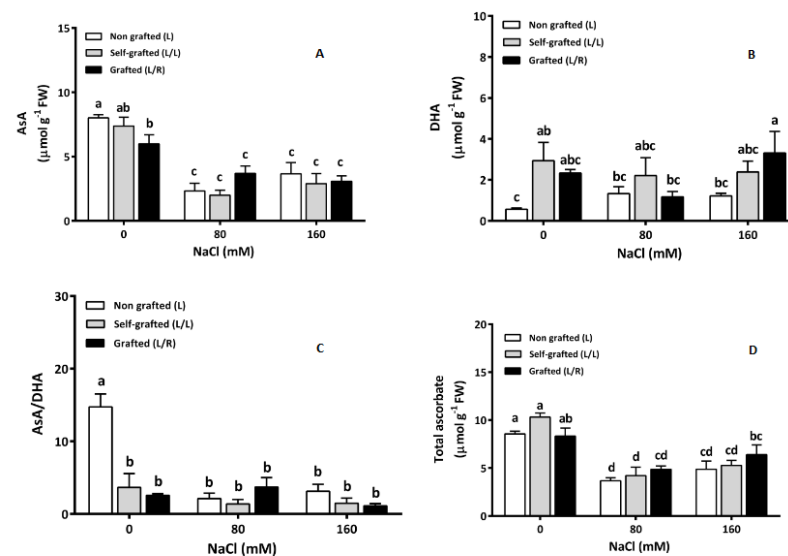


Figure 8. Reduced ascorbic acid (AsA; $\mu\text{mol g}^{-1} \text{FW}$; (A)) and dehydroascorbate (DHA; $\mu\text{mol g}^{-1} \text{FW}$; (B)), AsA/DHA (C) and total ascorbate ($\mu\text{mol g}^{-1} \text{FW}$; (D)) in leaves for plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown for 21 days under three salinity treatments: 0 mM of NaCl; 80 of NaCl, and 160 of NaCl. Each value is the mean of six replicates, and vertical bars are S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and grafting types.

The GR activity (Figure 10A) was higher in L than in L/L and L/R under control and 80 mM NaCl conditions. All plant groups exhibited similar GR activities at 160 of NaCl. L/R plants were the only ones for which the GR enzyme activity was not reduced by salinity. The APX activity (Figure 10B) under control conditions was higher in L/L and L/R plants, and it slightly increased at 80 of NaCl for L plants only. APX activities still increased at 160 of NaCl for L and L/R plants, while it remained constant in L/L plants at all NaCl doses. In the absence of salt, the CAT activity (Figure 10C) was similar for all plant groups. It increased in L and L/L plants at 80 mM and 160 mM of NaCl but only increased in L/R plants at 160 mM of NaCl.

The DHAR activity under control conditions and at 160 of NaCl was higher in L plants compared to the other plant groups (Figure 10D). At 80 mM, DHAR was higher in L plants than in L/R plants (Figure 10D). Increasing salinity caused an increase in DHAR in all plant groups, reaching higher values in L plants. The GST enzyme activity for the control treatment and the two salinities was higher in the L plants compared to the L/L and L/R grafted plants (Figure 10E). The GST activity increased at 80 of NaCl for L/L plants, while it remained stable in L/R plants at all salinity levels. The MDHAR activity (Figure 10F) was lower in L plants under control conditions than in the other plant groups. At 80 of NaCl, the MDHAR activity was the lowest in L/R plants and the highest in L plants, while the lowest value was found in L/L plants at 160 of NaCl (Figure 10F). Once again, the MDHAR activity in L/R remained constant at all salinity levels. Under control conditions, the SOD enzyme activity was higher in L and L/L than in L/R plants (Figure 10G). Under salinity, an increase in SOD activity was only observed in L/R plants at 160 of NaCl (Figure 10G).

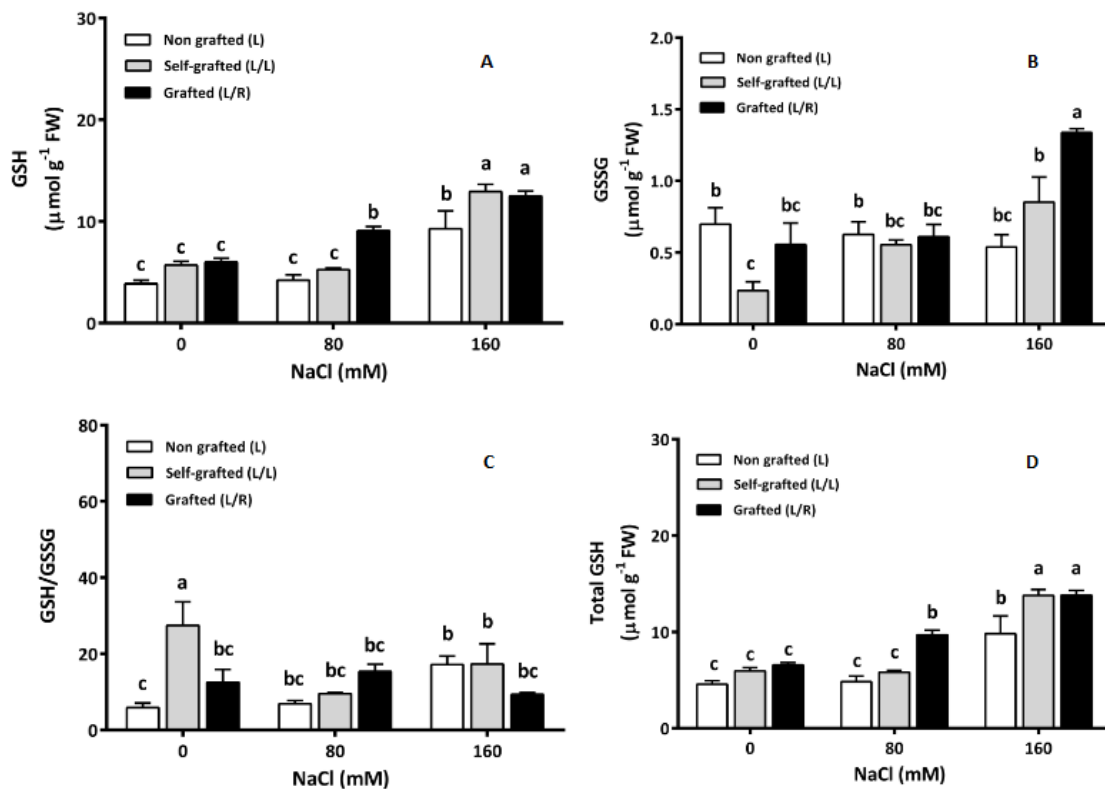


Figure 9. Reduced glutathione (GSH; $\mu\text{mol g}^{-1}$ FW; (A)) and oxidized glutathione (GSSG; $\mu\text{mol g}^{-1}$ FW; (B)) concentrations, GSH/GSSG (C), and total GSH concentrations ($\mu\text{mol g}^{-1}$ FW; (D)) in the leaves of plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown for 21 days under three salinity treatments: 0 of NaCl; 80 of NaCl, and 160 of NaCl. Each value is the mean of six replicates, and vertical bars are S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and grafting types.

3.4. Na^+ and K^+ Concentrations

The effects of the plant group and salt stress on mineral content (Na^+ and K^+) in roots, stems, and leaves are presented in Table 2. Under salinity, all plant groups showed significantly higher Na^+ levels compared to the control treatment, with the highest Na^+ concentrations being observed in L/L plants. The Na^+ content was slightly higher in leaves and stems of L/R compared to L plants, while an inverse trend was recorded for roots at 160 of NaCl. This trend was consistently observed across independent experiments but remained insignificant from a statistical point of view. Salinity induced a decrease in the K^+ concentration in leaves and stems, but as far as L plants are concerned, such a decrease was insignificant for leaves. L/L and L/R plants exhibited similar K^+ concentrations at 160 of NaCl. In leaves, the highest Na^+/K^+ ratio was observed in L/L plants exposed to 160 of NaCl. In L and L/R plants, the leaf Na^+/K^+ ratio increased between 0 and 80 of NaCl and then remained stable between 80 and 160 of NaCl. In the stem, the Na^+/K^+ ratio increased at 80 of NaCl for all plant groups and then remained stable up to 160 of NaCl. In the roots, the Na^+/K^+ ratio increased at 80 of NaCl and then remained stable up to 160 of NaCl in L and L/L plants, while it still increased in L/R plants, reaching the maximal value for L/R roots exposed to 160 of NaCl.

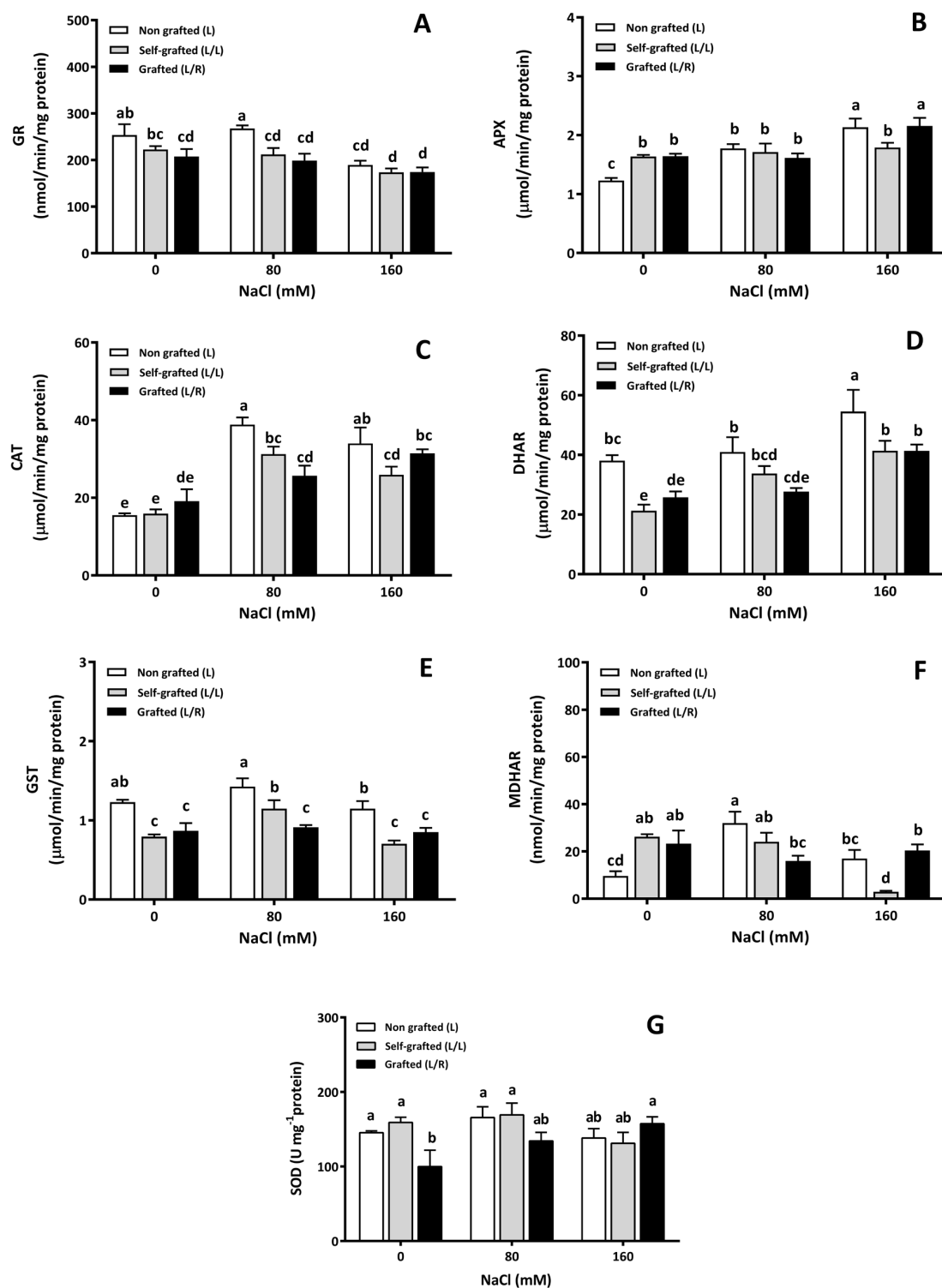


Figure 10. The antioxidant enzyme activity as a function of different NaCl doses. The enzymatic activity of antioxidant enzymes (glutathione reductase (GR; (A)), ascorbate peroxidase (APX; (B)), catalase (CAT; (C)), dehydro-ascorbate reductase (DHAR; (D)), glutathione-S-transferase (GST; (E)), monodehydroascorbate reductase (MDHAR; (F)), and superoxide dismutase (SOD; (G))) in leaves of plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown for 21 days under three salinity treatments: 0 of NaCl; 80 of NaCl, and 160 of NaCl. Each value is the mean of six replicates, and vertical bars are S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and grafting types.

Table 2. Potassium (mmol K⁺ g^{−1} DW) and sodium (mmol Na⁺ g^{−1} DW) concentration and sodium-to-potassium ratios (Na⁺ /K⁺) in the leaf, stem, and root of non-grafted (L), self-grafted (L/L), and grafted on INIA JUPAFOR1 rootstock (L/R)) Old Limachino tomato plants grown under 0, 80, or 160 of NaCl for 21 days. Each value is the mean of six replicates ± S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan’s test) between treatments and grafting types.

		Non-Grafted (L)			Self-Grafted (L/L)			Grafted (L/R)		
		NaCl (mM)			NaCl (mM)			NaCl (mM)		
		0	80	160	0	80	160	0	80	160
Leaf	Na ⁺ (mmol g ^{−1} DW)	0.07 ± 0.01 b	0.54 ± 0.10 a	0.67 ± 0.10 a	0.09 ± 0.03 b	0.67 ± 0.22 a	0.86 ± 0.09 a	0.08 ± 0.01 b	0.61 ± 0.16 a	0.79 ± 0.27 a
	K ⁺ (mmol g ^{−1} DW)	1.29 ± 0.1 abc	1.02 ± 0.04 cde	0.99 ± 0.06 cde	1.39 ± 0.10 ab	1.08 ± 0.12 bcd	0.74 ± 0.17 e	1.40 ± 0.07 a	1.03 ± 0.06 cd	0.93 ± 0.1 cde
	Na ⁺ /K ⁺	0.06 ± 0.02 c	0.52 ± 0.09 b	0.70 ± 0.12 b	0.08 ± 0.03 c	0.71 ± 0.19 b	1.30 ± 0.27 a	0.06 ± 0.01 c	0.63 ± 0.21 b	0.87 ± 0.32 b
Stem	Na ⁺ (mmol g ^{−1} DW)	0.09 ± 0.01 b	0.70 ± 0.13 a	0.77 ± 0.09 a	0.09 ± 0.01 b	0.79 ± 0.07 a	1.03 ± 0.19 a	0.13 ± 0.02 b	0.74 ± 0.19 a	0.93 ± 0.31 a
	K ⁺ (mmol g ^{−1} DW)	2.20 ± 0.14 b	1.95 ± 0.07 c	1.91 ± 0.10 c	2.46 ± 0.18 b	1.88 ± 0.06 c	1.85 ± 0.08 c	2.78 ± 0.23 a	1.93 ± 0.11 c	1.83 ± 0.11 c
	Na ⁺ /K ⁺	0.04 ± 0.00 b	0.35 ± 0.06 a	0.41 ± 0.06 a	0.04 ± 0.01 b	0.43 ± 0.05 a	0.57 ± 0.05 a	0.05 ± 0.01 b	0.40 ± 0.13 a	0.49 ± 0.14 a
Root	Na ⁺ (mmol g ^{−1})	0.08 ± 0.02 c	0.45 ± 0.03 ab	0.67 ± 0.07 a	0.04 ± 0.01 c	0.56 ± 0.11 ab	0.73 ± 0.08 a	0.14 ± 0.02 c	0.43 ± 0.02 ab	0.59 ± 0.02 ab
	K ⁺ (mmol g ^{−1})	0.49 ± 0.08 a	0.48 ± 0.06 a	0.69 ± 0.07 a	0.51 ± 0.09 a	0.83 ± 0.31 a	0.64 ± 0.13 a	0.65 ± 0.17 a	0.45 ± 0.17 a	0.37 ± 0.16 a
	Na ⁺ /K ⁺	0.22 ± 0.07 c	1.06 ± 0.19 b	1.08 ± 0.18 b	0.09 ± 0.03 c	0.90 ± 0.26 b	1.28 ± 0.25 ab	0.28 ± 0.06 c	1.09 ± 0.20 b	1.66 ± 0.17 a

3.5. Gene Expression

The gene expression in roots was considered for six of the seven transporters selected in silico: the Na⁺ transporter HKT1.1 (*HKT1.1*), Na⁺ transporter HKT1.2 (*HKT1.2*), potassium channel SKOR-like (*SKOR*), potassium channel (*LKT1*), calcineurin B-like interacting protein kinase (*SOS2*), and calcium sensor calcineurin B-like (*SOS3*) (Figure 11). The expression of *SOS1* could not be reliably detected due to its very low threshold cycle. The expression of gene coding for the Na⁺ transporter HKT1.1 (Figure 11A) was significantly reduced by salinity in L/R plants compared to L/L plants, which did not show statistically significant changes in response to salinity. Similarly, *HKT1.1* expression was not significantly affected by salinity in L plants. The expression of *HKT1.2* (Figure 11B) was increased by salinity only in L/L plants, with the highest value reported at 160 of NaCl. The relative expression of the *SOS2* gene showed a significant increase in L plants at 80 mM of NaCl but was unaffected by salinity in L/L and L/R (Figure 11E).

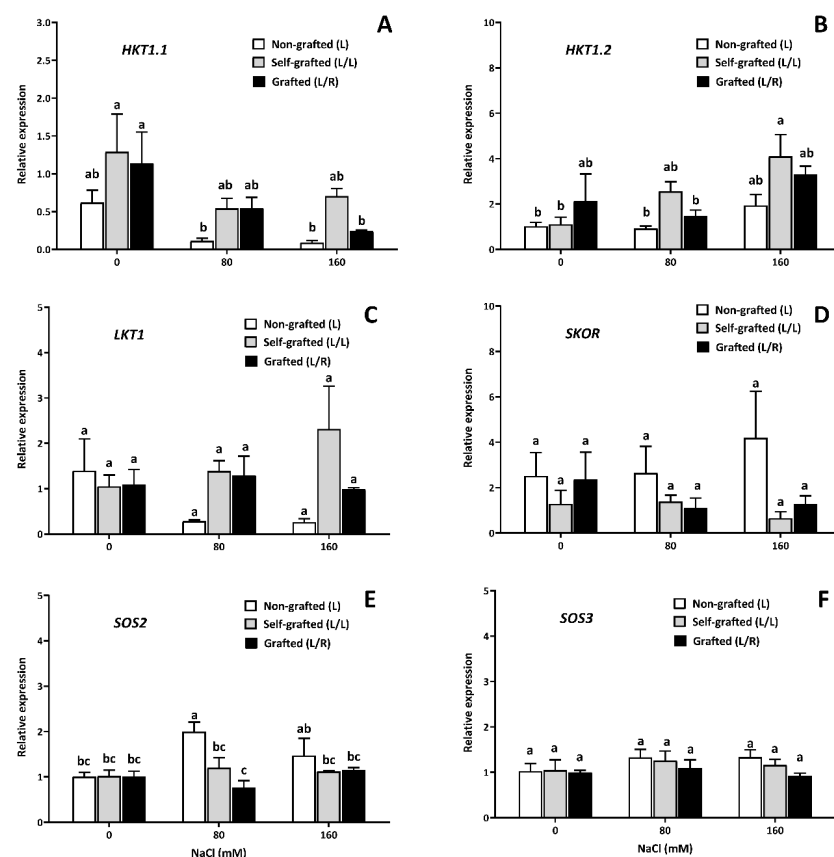


Figure 11. The expression of gene coding for the ion transporter as a function of the NaCl dose. The gene expression in the roots (relative units) of the Na⁺ transporter HKT1.1 (*HKT1.1*, (A)), Na⁺ transporter HKT1.2 (*HKT1.2*, (B)), potassium channel SKOR-like (*SKOR*, (D)); potassium channel (*LKT1*, (C)), calcineurin B-like interacting protein kinase (*SOS2*, (E)), and calcium sensor calcineurin B-like (*SOS3*, (F)) for plants corresponding to treatment L (ungrafted Old Limachino tomato), L/L (self-grafted Old Limachino tomato), and L/R (grafted Old Limachino tomato on INIA JUPAFORT1 rootstock) grown under three salinity treatments: 0 of NaCl, 80 of NaCl, and 160 of NaCl at 21 days after beginning treatment (DAT). Each value is the mean of three replicates, and vertical bars are S.E. Lowercase letters represent differences ($p \leq 0.05$ Duncan's test) between treatments and types of graftings.

On the other hand, the genes *LKT1* (Figure 11C), *SKOR* (Figure 11D), and *SO3* (Figure 11F) did not show significant changes in their relative expression due to salinity and remained statistically similar in all plant groups.

4. Discussion

4.1. Effect of JUPAFORT1 Rootstock on Growth and Plant Water Status under Salinity

Since the fresh biomass in leaves and stems was significantly higher in L/R plants under control conditions compared to L and L/L plants (Figure 1A), the interspecific rootstock JUPAFORT1 was able to induce a strong growth stimulation under these conditions. On the other hand, an opposite effect was observed in L/L plants, which showed the lowest growth in the control treatment and at the extreme salinity level (Figure 1A). These different responses in the absence of salt were similar to those previously found on the effects of the Durinta F1 cultivar grafted on Beaufort rootstock (interspecific, De Ruiter) [60]. Our results suggest that JUPAFORT1 is a vigorous clonal rootstock for OLT plants under non-saline conditions.

The poor response of L/L plants in terms of their growing capacity suggests a strong weakening of their vigor due to the effect of cutting in OLT plants. The leaf, stem, and root FW were indeed lower in L/L than in L plants (Figure 1). It is known that mechanical damage such as cutting may induce a transient burst of ethylene, which could inhibit growth. However, our data indicate that the JUPAFORT1 rootstock in L/R treatment was able to overcome such inhibition in the absence of salt. Salinity, however, reduced the leaf weight in L/R plants, while stems and roots were hardly affected. These results indicate that the vigorous vegetative growth obtained under control conditions (e.g., commercial F1 hybrids) using rootstocks is not necessarily maintained under saline conditions. This contrasts with data obtained in other studies for other rootstocks, which demonstrated that the good performances obtained using vigorous rootstocks under control conditions may be transferable to saline conditions. This deleterious impact on leaf growth in L/R plants was not recorded for root growth, which remained unaffected by salinity. The JUPAFORT1 rootstock is issued from a cross between a salt-tolerant cultivar of *S. lycopersicum* (Poncho Negro) and the wild halophyte relative *S. chilense*. Such a decrease in the shoot/root ratio is frequently reported in halophytes [2,4] and might be regarded as an attempt to reduce evaporative surface under water stress conditions and to save energy for triggering salt-adaptive mechanisms [61,62].

In terms of the OLT plant height, our results indicate that the JUPAFORT1 rootstock tended to reduce it (Figure 2A,C) under both control and saline conditions. This indicates that JUPAFORT1 may reduce the internode elongation of OLT plants independently of salinity. *Solanum chilense* is known to have a bushy growing behavior with a higher number of ramifications than its cultivated counterpart. It is also well established that internode elongation depends on the hormonal profile of the plant, and *S. chilense* was reported to strongly differ from *S. lycopersicum* [31–33], suggesting that root phytohormones translocated from the root to the shoot may drastically modify the plant's growing habit. Similarly, this property of JUPAFORT1 could be translated into a higher production potential per axis in L/R plants. In contrast, the stem diameter was similar in all groups and remained unaffected by salinity. *Solanum chilense* exhibits a narrow stem that hampers its direct use as rootstock and justifies preliminary crosses with *S. lycopersicum*. The present work demonstrates that JUPAFORT1 exhibited a larger stem than its wild parent, allowing for efficient grafting processes.

L/R plants were mainly affected by two water status variables under salt stress conditions, namely, an increase in the midday water potential (Ψ_{w_n}) and a decrease in the midday relative leaf water content (RWC_n). The first hypothesis to explain such a striking rise of Ψ_{w_n} in L/R plants under extreme-salinity conditions could be an increase in the osmotic potential (Ψ_s) resulting from increased solute dilution by increased water entry into the cells as a consequence of succulence adaptive processes. A second hypothesis could be a change in the turgor potential due to greater cell wall elasticity, which contributes to salinity tolerance [1,63]. It has to be mentioned that the relative content (RWC) was affected by salinity but that the actual water content (WC) estimated on a dry weight basis was not, which supports the second hypothesis. The maintenance of metabolic activity despite a decrease in the relative water content is relevant from a “tolerance strategy” rather than

an “avoidance strategy”, which would require expensive osmotic adjustment processes or precocious stomatal closure compromising photosynthetic activity [1,64]. The increase in Ψ_{wn} at 160 mM compared to 80 mM in R/L was not associated with a significant decrease in leaf and stem fresh or dry weights, suggesting that R/L tolerated the water component of salt stress. Regarding the effect of JUPAFORT1 on the stomatal conductance of OLT plants, the results suggest that this variable was affected only at the end of the cycle (21 DAT) and under extreme-salinity conditions. This stability of stomatal conductance observed in L/R plants suggests that these plants were less affected by salt stress than the other two treatments (Figure 3A–C), which aligns with previous results [65]. It is also noteworthy that the highest stress intensity (160 of NaCl) at 21 DAT increased rather than decreased g_s value in L/R plants, suggesting that those plants displayed a typical anisohydric behavior and maintained their capacity of CO₂ fixation at high salinities. At the same time, L and L/L still behaved as isohydric plants [66].

4.2. Effect of JUPAFORT1 on the Content of Photosynthetic Pigments, Protein, and MDA in the Plant under Saline Conditions

The basal fluorescence (F_0) of Chl *a* in the leaves of L/R plants was not altered by the salinity stress. In contrast, the maximal fluorescence (F_m) was only marginally modified at the end of the experiment for the highest NaCl dose. Since a similar behavior was recorded for L and L/L plants, it may be assumed that the JUPAFORT1 rootstock did not afford specific advantages regarding the light phase of photosynthesis. Using other rootstock issued from *S. lycopersicum*, other authors observed that PSII efficiency in dark-adapted leaves at 50 DAT, measured as the F_v/F_m ratio, was 5% higher in high-vigor grafting combinations. In contrast, in low-vigorousness grafting combinations, the values were similar to those of control plants under 75 of NaCl [27]. Such a difference in the F_v/F_m ratio was not recorded in the present work.

At the highest stress intensities, Chl*a*, Chl*b*, and carotenoid concentrations were higher in L/R plants than in L/L. Although the higher pigment content might be regarded as a consequence of leaf growth inhibition, leading to an “accumulating” effect, a positive impact of R rootstock on pigment stability could not be ruled out. The JUPAFORT1 rootstock may help stressed plants avoid or overcome salt-induced senescence, which could also result from the specific hormonal status conferred by the halophyte parents in relation to ethylene, salicylic acid, and polyamine synthesis [31–33]. Ethylene is a well-known stress-induced senescing hormone, and a recent study using Poncho Negro as rootstock for Limachino scion reported great ethylene production in L/R plants than in L/L plants [23]. The fact that the pigment concentration remained higher in our L/R plants than in L/L plants provides a different picture and suggests that under high-salt-stress conditions, *S. chilense* used in the present work in combination with Poncho Negro may confer specific properties to JUPAFORT1. This might be related to the high level of salicylic acid synthesis reported for *S. chilense* [31–33] since salicylic acid is frequently reported as an antagonist of ethylene action [37]. This hypothesis is reinforced by the fact that in terms of the protein and MDA content, L/R plants showed no variation under salinity, while L plants exhibited a strong increase in MDA in response to 80 of NaCl. This suggests that JUPAFORT1 confers a degree of protection to OLT plants against salt stress, preventing damages caused by ionic toxicity at the level of proteins and cell membranes [14].

4.3. Effect of JUPAFORT1 on Plant Antioxidant Capacity and Activity under Salinity Conditions

Salt-tolerant glycophytes may cope with transient or episodic high doses of salt and can trigger adaptive processes to cope with NaCl. Most of these processes are induced by stress, and such induction has an energetic cost [52,62]. In contrast, halophyte species are often permanently exposed to salinity, as is the case for *S. chilense* in the Atacama desert, and their high level of tolerance relies on constitutive properties that do not imply additional energetical investment in the presence of NaCl [2,4,12]. It is noteworthy that numerous properties related to the management of secondary oxidative stress remained constant in

L/R plants at all salinity levels (MDA and polyphenol concentrations, AsA/DHA and GSH/GSSG ratios, GR, GST, and MDHAR activities), while those properties fluctuated depending on the NaCl dose in L and L/L plants. This suggests that the JUPAFORT1 rootstock was able to confer to OLT scion the ability to display constitutive regulation of properties such as the Asada cycle [16]. It is of special interest to notice that, in our case, the halophytic properties should involve the roots since *S. chilense* was used for rootstock obtention. At the same time, the scion is still issued from a glycophyte species. However, the homeostasis of the redox status was clearly observed in the shoots, and this suggests that some messages sent by the roots may directly influence the scion behavior.

DPPH and FRAP antioxidant capacities in L/R plants increased under extreme-salinity conditions, while they already increased in response to moderate salinity (80 of NaCl) in L plants. This suggests that L/R plants mainly encountered oxidative stress requiring antioxidant mechanisms only at very high stress intensities. This hypothesis is supported by MDA concentrations, which strongly increased at 80 of NaCl in L but not in L/R plants. As the polyphenol (antioxidant compound) content of L/R plants remained constant under salinity conditions, this variable would not explain the antioxidant capacity of these plants at 160 mM. It is known that the main non-enzymatic antioxidant molecules involved in the ascorbate–glutathione cycle are ascorbate and glutathione [14,16,67]. Ascorbate (vitamin C, AsA) is one of the most important non-enzymatic antioxidant compounds and has been detected in most plant cells, organelles, and apoplasts. The AsA content in L/R plants exposed to salinity was lower than that in the control condition. Similarly, the total ascorbate in L/R plants decreased at 80 of NaCl and even to a lower extent at 160 of NaCl. This suggests that ascorbate was not the major contributor to the above-mentioned increase in antioxidant capacity in stressed plants.

GSH is a tripeptide (alpha-glutamyl cysteinyl glycine) found in cellular compartments such as the cytosol, chloroplasts, endoplasmic reticulum, vacuoles, and mitochondria. It has been described to act directly as an antioxidant in reducing most stress-generated free radicals [16]. In our study, all plant groups showed the same GSH content under control conditions (Figure 9A), but NaCl increased the GSH content in L/R plants at both 80 of NaCl and 160 of NaCl, indicating a positive correlation between salinity and the GSH content in these plants (Figure 9A). L/R plants also exhibited the highest GSSG concentration in response to the highest NaCl dose, indicating that GSH oxidation is directly involved in managing the stress situation that total GSH may contribute to the highest antioxidant capacity recorded by FRAP and DPPH activity in salt-treated L/R plants. In addition, the change in the GSH/GSSG ratio during H₂O₂ degradation is important for signaling processes through the redox pathway [68]. It has been suggested that the GSH/GSSG ratio indicates cellular redox balance and may be involved in ROS sensing [16,67–69]. L/R plants showed no change in this ratio under salinity conditions, while the ratio was modified (although in the opposite direction) in L and L/L plants. This, once again, suggests that the JUPAFORT1 rootstock helps the scion to maintain the oxidation state of glutathione under both control and salinity conditions.

Under salinity, SOD activity was only increased in response to extreme salinity, suggesting that JUPAFORT1 activates a protective mechanism against leaf oxidation in the OLT scion under salt stress by reducing O₂*. This type of increase in SOD activity in response to salinity was also observed in the tomato landraces Hail 747 and Hail1072 and is considered a crucial mechanism against secondary oxidative stress [70]. Similarly, CAT activity was increased in L/R plants under extreme-salinity conditions, in contrast to L and L/L plants, in which CAT activity increased at 80 of NaCl (Figure 10C). The lower CAT activity in L/R plants could indicate that this type of transplant is less altered in its redox state (GSH/GSSG), as the tissues show lower oxidation with stable MDA levels and slight changes in PSII Chl *a* fluorescence. This might also be related to the anisohydric behavior of the scion in this combination, which reduces the risk of photorespiration resulting from stomatal closure.

GST enzyme activity can be induced by various environmental stimuli. For example, increased GST levels maintain cellular redox homeostasis and protect organisms against oxidative stress [15,66,71]. Although many GST genes have been cloned and analyzed in various plant species, including tomatoes, the effect of tomato rootstocks on GST enzyme activity has rarely been investigated. Our results showed that this activity was lower in both L/L and L/R plants than in L plants (Figure 10E). Under salinity, this activity remained unaffected in L/R plants. These results suggest once again that JUPAFORT1 helps to maintain homeostatic conditions in stressed plants, thus conferring tolerance to salinity through the stabilization of antioxidative enzymes.

Within the AsA-GSH glutathione cycle, the activities of the four enzymes involved in this cycle (APX, MDHAR, DHAR, and GR) were measured. The enzyme ascorbate peroxidase (APX) is part of the isoenzyme family of hem peroxidases found in different plant organs [66,72]. Under control conditions, APX activity was higher in both L/L and L/R plants. Under salinity, this activity increased in plants at high salinity, in contrast to L plants, in which APX increased at intermediate salinities (Figure 10B). A similar trend was observed for DHAR (Figure 10D). This once again corroborates our hypothesis that oxidative stress triggering APX activation occurred at lower salinity in L than in L/R and that JUPAFORT1 thus efficiently contributes to reducing secondary oxidative stress in L scion.

4.4. Effect of JUPAFORT1 on Plant Na⁺ and K⁺ Levels under Salinity Conditions and Expression of Genes Coding for Ion Transporters

JUPAFORT1 was obtained after crossing the wild halophyte *S. chilense*, which behaves as an includer, [31–33,35,36], and the salt-resistant cv. Poncho Negro from the halophyte *S. lycopersicum*, which behaves as an excluder [21]. No significant difference was recorded for the shoot Na⁺ concentration between the three groups of plants at both 80 and 160 mM. However, the Na⁺ in root/Na⁺ leaf ratios were always lower for L/R plants than other groups, suggesting that L/R plants were less efficient in root Na⁺ sequestration. Such “including” behavior is often reported for halophyte plant species, especially those that can sequester Na⁺ in salt glands [73]. The specificity of our grafting system is that the root system is influenced by the halophytic origin of the rootstock. At the same time, Na⁺ accumulation occurs in the aerial part of the glycophyte scion.

Further, plant salinity resistance is not only a function of the Na⁺ total concentration but also depends on the Na⁺ distribution between cell compartments. Na⁺ may contribute to osmotic adjustment if it is correctly sequestered in vacuoles through the NHX transporter. It may also be less detrimental if correctly extruded by the SOS system in the apoplasm, thus avoiding accumulation in the cytosol [1]. Na⁺ transport is known to be, at least partly, mediated by HKT transporter proteins that contribute to salinity tolerance in several species, including tomatoes [7,74]. These proteins are localized in the xylem and phloem of roots and shoots and play a role in Na⁺ exclusion from the xylem [8]. Some reports question the assumption that Na⁺ exclusion leads to improved salinity tolerance, while others reported the opposite [75].

In our investigation, the expression level of the *HKT1.1* gene was the same in all plant groups under non-saline conditions. However, this level was reduced in the roots of L/R plants under high salinity (Figure 11A). On the other hand, the *HKT1.2* level was also the same in the three plant groups under control conditions. Under salinity, this level was not modified in L/R plants, but it was particularly modified in L/L plants at 160 of NaCl (Figure 11B). These results suggest that JUPAFORT1 maintained Na⁺ flux to the top of the plant, confirming its includer behavior. However, this increase in toxicity may be mitigated by the ability of JUPAFORT1 to produce greater succulence at the leaf level. Since the level of *HKT1.2* was increased under salinity in L/L plants, they transported a higher level of Na⁺ into stems and leaves, thus increasing their toxicity. These results are similar to those reported in wild tomato species under salinity conditions [74]. On the other hand, L and L/R plants did not show changes in the gene expression of this transporter (*HKT1.2*) in

roots (Figure 11B), as previously reported in roots of *S. lycopersicum* plants under saline conditions [35,36].

On the other hand, the SOS pathway is involved in Na^+ extrusion in plants [13,76], and SOS2 plays a role in Na^+ and K^+ fluxes to the aerial part of the plant [77]. In the case of the SOS2 gene, our research provided evidence that salinity only increased gene expression in the roots of L plants, which could be related to the low level of the Na^+/K^+ ratio in the plant (leaves and stems) compared to L/L plants (Figure 11E). Although SOS1 expression was not detected in our study, SOS2 upregulation may suggest that Na^+ was excluded from the roots of L plants by the SOS pathway, which could contribute to the excluder properties of the domesticated *S. lycopersicum*.

However, despite the increased gene expression of the SOS pathway in the roots of L plants, the Na^+ content in this organ increased at the highest salinity, suggesting that the exclusion process ensured by SOS2 may be saturated in L plants, as previously observed for SOS1 in tomato under salinity [78]. Concerning the SOS3-SOS2 complex, no changes in these two genes were observed in the roots of L/R plants under salinity, suggesting that JUPAFORT1 does not activate the ion-exclusion system in the roots of these plants through the plasma membrane Na^+/H^+ antiporter [79,80]. In this regard, several studies have observed that tomato SOS3 is mainly expressed in roots [80] and that the SOS3-SOS2 complex can activate the Na^+/H^+ antiporter of Arabidopsis *AtSOS1* [80,81]. It is noteworthy that in L/R plants, gene expression was characterized in the root system for all six genes studied (*HKT1.1*; *HKT1.2*; *LKT1*; *SKOR*; *SOS2*, and *SOS3*), and all of them, except *HKT1.1*, showed no expression changes under salinity. This suggests, once again, that the expression of the vast majority of these genes is constitutive in these L/R plants and that there is no need for them to activate additional transcription that will require energy input [61,62]. Therefore, the gene expression results at the root level suggest that JUPAFORT1 confers increased salinity tolerance to OLT plants. However, we should keep in mind that JUPAFORT1 is a hybrid resulting from the cross between a salt-tolerant glycophyte (*S. lycopersicum* cv. Poncho Negro) and the halophyte *S. chilense*. Hence, regulation of the SOS pathway in this type of material should be rather complex since the physiological importance of this pathway is not the same for excluding glycophytes and including halophytes.

5. Conclusions

Our study shows that the clonal rootstock JUPAFORT1, derived from an interspecific cross between wild halophyte *Solanum chilense* and the landrace Poncho Negro of cultivated glycophyte *Solanum lycopersicum*, confer to the Old Limachino scion some typical halophyte properties helping it to cope with salinity. These properties could be related to regulating the plant water status and the constitutive maintenance of the oxidative stress defense system at the foliar level, including non-enzymatic antioxidants (mainly glutathione) and enzymatic antioxidants, which exhibited higher stability when the L scion was grafted on the rootstock. The expression of genes coding for ion transporters and the extrusion system (*HKT1.1*, *SOS2*, and *SOS3* genes) was not modified in the root of JUPAFORT1 rootstock, which is in line with the behavior of the includer halophyte parent, *S. chilense*. This is the first time that such an interspecific hybrid rootstock issued from *S. lycopersicum* × *S. chilense* was tested, and our work demonstrates that JUPAFORT1 may improve the behavior of salt-affected scion. Further works are required to identify the molecular and biochemical components of the root-to-shoot signaling pathway. This information is key for future breeding programs on salinity-tolerant rootstocks. These innovative results at the vegetative level also motivate the study of the influence of JUPAFORT1 at the reproductive stage in terms of flowering, fruit formation, and yield-related parameters. Finally, a comparison with commercial rootstock is required to validate the interest of JUPAFORT1 for salt tolerance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10080780/s1>, Table S1. Selected primers for genes involved in Na⁺ and K⁺ transport mechanisms associated with salinity tolerance in plants to be analyzed by qPCR.

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