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

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**1 Abstract**

Climatic change, intensive agriculture, and worsening water quality induce abiotic stress conditions for plants. Among these factors, salinity stress is a limit factor for plant growth. Therefore, the purpose of this study was to analyze the phytohormones role and oxidative metabolism in response to salt stress of two genotypes of tomato cv. Grand Brix and cv. Marmande RAF, the crops was carried out in a growth chamber. Salinity stress reduces biomass and relative growth rate (RGR) in both genotypes, this effect being greater in cv. Marmande RAF. These results, together with main stress indicator response, the O2.-, indicate that cv. Marmande RAF is more sensitive to Saline stress. Grand Brix showed less oxidative stress, because it presented greater detoxification of the O2.-, due to SOD enzyme activity induction and greater antioxidant capacity. Furthermore, Grand Brix has a better hormonal profile adapted to salt stress resistance, the accumulation of IAA, GA4 and CKs and their beneficial role against oxidative stress could make the difference between resistance and sensitivity to salt stress. On the other hand, a lower ACC concentration, ethylene precursor, combined with a greater O2.- detoxification in the cv. Grand Brix could play a fundamental role in tolerance to saline stress. Besides, an increase in ABA levels promotes better stomatal closure, better photosynthesis control and a lower rate of water loss. This data could be essential to select plants with greater resistance to saline stress.

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

**2 Introduction**

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

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

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

Cytokinins (CKs) such as trans-zeatine (tZ) and isopentenyl adenine (iP), are also known to alleviate the adverse effects of salinity on plant growth (Fahad et al. 2015). It has been observed that an increase of the CKs can decrease the damages caused by the ROS, this can help you to be more tolerant of stress (Pogány et al. 2004). Ghanem et al. (2011) underwent two varieties of tomato to salt stress and they showed that the concentration of CKs in the plants increase in the leaves in saline treatment. These authors concluded that a greater accumulation of CKs, could improve the resistance to salt stress by delay leaf senescence, which would improve maintaining stomatal conductance.

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

ABA is known as a hormone that increases their concentration in stressed plants and is key to coordinate stress responses. In maize plants subjected to salt stress, the most resistant hybrid to salt stress had higher ABA concentration. These authors describe how increasing ABA could benefit to plants under salt stress conditions, inducing a lower rate of transpiration, so the tissues accumulate fewer Na+ (Zörba et al. 2013). ABA is also closely related to H2O2 and Ca2+ ion, both of which interact in the stomatal closure/opening process. In addition, H2O2 stimulates the accumulation of ABA. Finally, the accumulation of H2O2 stimulates the accumulation of ABA, this ABA promotes the stomatal closure thus avoiding the loss of water that can cause saline stress (Mittler and blumwald 2015).

Ethylene interacts with other hormones (GA and ABA) in homeostasis processes (Rzewuski and Sauter 2008). It accumulates alongside ROS. The ethylene and O2.- in plants under abiotic stress are the main cause of programmed cell death. The ethylene and O2.- in plants under abiotic stress behaves as an indicator of stress. An increase in the hormone jasmonic (JA) can lead to an increase in ethylene levels (Overmyer et al. 2003).

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

In this context, considering the importance of phytohormones and role oxidative metabolism in plant resistance to saline stress, we investigate here the response of oxidative metabolic process and phytohormones concentrations in two tomato genotypes submitted to salinity stress. The aim is to determine whether the oxidative metabolism and hormonal profile are determinant to define the cultivar with the strongest tolerance to saline stress. Also, understand how different hormones and oxidative metabolism are related with salt stress tolerance.

**3 Materials and Methods**

3.1 Plant material and treatments

Seeds of *Solanum lycopersicum* cv. Gran brix and *Solanum lycopersicum* cv. Marmande RAF (Saliplant S.L., Spain) were germinated and grown for 30 days in cell flats of 3 cm × 3 cm × 10 cm filled with a perlite mixture substratum. The flats were placed on benches in an experimental greenhouse located in Southern Spain (Saliplant S.L., Motril, Granada). After 30 days, the seedlings were transferred to a growth chamber (Department of plant physiology, University of Granada) under the following controlled environmental conditions: Relative humidity 60-80%; Day/night temperatures 28/19 °C; 16/8 h photoperiod at a photosynthetic photon flux density (PPFD) of 350 *μ*mol m−2s−1 (measured at the top of the seedlings with a 190 SB quantum sensor, LI-CORInc., Lincoln, Nebraska, USA). Under these conditions, the plants were grown in hydroponic cultivation in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) of 3 L volume, 8 plants/tray. Throughout the experiment the plants were treated with a growth solution made up of 4 mM KNO3, 3 mM Ca(NO3)2·4H2O, 2 mM MgSO4·7H2O, 6 mM KH2PO4, 1 mM NaH2PO4·2H2O, 2 µM MnCl2·4H2O, 1 µM ZnSO4·7H2O, 0.25 µM CuSO4·5H2O, 0.1 µM Na2MoO4·2H2O, 5 µM Fe-chelate (Sequestrene; 138 FeG100) and 50 µM H3BO3, pH 5.5–6.0.

3.2 Experimental design

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

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

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

3.4 Determination of the concentration of promoters and indicators of oxidative stress (MDA, H2O2 and O2.-), lipoxygenase (LOX) activity

For the extraction of MDA, 0.1 g of frozen leaf material was grounded, in 1 mL of buffer 50 mM (0.07% of NaH2PO4.2H2O and 1.6% of Na2HPO4.12H2O). The extract was centrifuged to 20000 g for 25 min. Subsequently, an aliquot of supernatant was mixed in test tubes with 4 mL of trichloroacetic acid 20% containing 0.5% of thiobarbituric acid. The resulting mixture was heated to 95°C for 30 minutes. Then it was rapidly cooled in an ice bath. The absorbance of the supernatant was measured at 532 nm. The value for the non-specific absorption at 600 nm was subtracted from the reading obtained at 532 nm, (Fu and Huang 2001).

Leaf H2O2 concentration was measured colorimetrically according to Mukherjee and Choudhuri (1983). 0.1 g of frozen leaf material was grounded in cold acetone. An aliquot of 1 mL of the extract was mixed with 200 µL of 0.1% titanium dioxide H2SO4 to 20% (v:v) and the mixture was centrifuged at 6000 g for 15 minutes. The intensity of the yellow colour of the supernatant was measured at 415 nm. The concentration of H2O2 was calculated from a standard curve of H2O2.

The O2.- concentration in the leaves was measured colorimetrically according to Barrameda-Medina et al. (2014). 0.1 g of frozen leaf material was grounded and 300 µL of phosphate buffer 50 mM was added. The mixture was centrifuged at 10000 g for 15 min. From the mixture, 250µL of the supernatant were caught. Then, buffer phosphate 50 mM and 250 µL of hydroxylamine 10 mM were added to it. The mix was incubated for 20 minutes at 25ºC. Subsequently, 60 µL of the supernatant were caught and 180 µL of sulfonyl acid 17 mM and 180 µL of α-1-Naphthylamine 7 mM were added and the mixture was incubated for 1h at room temperature. When the incubation was finished, the colour intensity was measured at 530 nm. The O2.- concentration was calculated from a standard curve of O2.-.

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

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

The SOD activity was measured in accordance with Yu et al. (1998), by means of a test based on the inhibition of the Nitro photochemical reduction tetrazolium (NBT). The absorbance of the sample was measured at a wavelength of 560 nm. The SOD activity was expressed in units (U) min-1 mg-1 protein, where one unit corresponds to the amount of the enzyme required to cause inhibition of 50% of the reduction of the NTB.

CAT activity was determined as described by Badiani et al. (1990), through the analysis of the consumption of H2O2 (39.4 molar extinction coefficient mM-1cm-1) at a wavelength of 240 nm for 3 minutes.

The assay of enzymes APX and GR was performed according to Rao et al. (1996). The APX activity was determined by recording the change in absorbance at 290 nm for 3 minutes of a reaction mixture containing buffer potassium phosphate 100 mM (pH 7.5), 0.5 mM of AsA, 0.2 mM H2O2 and 0.75 mL of enzyme extract. On the other hand, the GR activity was measured following the oxidation of NADPH at 340 nm for 3 minutes in a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 2 mM Na2-EDTA, 0.2 mM NADPH, 0.5 mM GSSG and 0.75 mL of enzyme extract.

GSHPx activity was determined as described by Edwards *et al.* 1996, through the analysis of the consumption of H2O2 (39.4 molar extinction coefficient mM-1cm-1) at a wavelength of 240 nm for 3 minutes.

3.6 Determination of the concentration of protein in the plant extracts

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

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

For the extraction and quantification of the AsA reduced the method followed was described by (Law et al 1992). This method is based on the reduction of Fe3+ to Fe2+ by AsA in acid solution. 0.5 g frozen leaf material was ground in 5 mL of metaphosphoric acid 5% (w/v) and was subsequently centrifuged at 16000 g for 15 minutes. After 0.2 mL of supernatant was added to a tube with 0.5 mL buffer phosphate sodium 150 mM (pH 7.5) and 0.1 mL of distilled water. The mixture was shaken and incubated at room temperature and in darkness for 10 minutes. Then, 0.1 mL of N-etilmaleimida 0.5% (w/v), 0.4 mL of orthophosphoric acid to 44% (v/v), 0.4 mL of 2, 2´-bipiridil to 4% (w/v) in ethanol 70% and 0.2 mL of FeCl3 to 3% (w/v) were added. Then the test tubes were shaken and incubated at 40°C and in darkness for 40 minutes. Finally, the absorbance measured at 525 nm against a standard curve of AsA followed the same procedure above.

For the extraction and quantification of the GSH reduced, the method followed was described by Gronwald et al. (1987). 0.2 g frozen leaf material was ground in 1 mL of HCl 0.2 M and was centrifuged at 16000 g for 10 minutes. Then 500 μl of the supernatant was caught and 500 μl of sodium phosphate buffer (pH 7.5) was added. An aliquot of 25 μl was extracted and 90 μl sodium phosphate buffer, 10 μl EDTA 10 mM, 10 μl of NADPH 10 mM, 10 μl DTNB 6 mM, 35 μl of distilled water and 10 μl of GR 10 UD/mL were added. The GSH Reduced concentration was measured at 412nm.

3.8 Determination of the antioxidants test

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

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

3.9 Hormone extraction and analysis

IAA, GA4, CKs (tZ and iP), ethylene precursor ACC, ABA, SA and JA concentrations were analysed as in Ghanem et al. (2008) with some modifications. The GA1, GA3 and Riboside concentrations were analysed too, these hormones concentrations were not found in both genotypes, data not revealed. Briefly, 30 mg of homogenized dry material was dropped in 0.5 ml of cold (-20°C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20000 g, 15 min) and re-extracted for 30 min at 4ºC in an additional 0.5 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and part of plant pigments and evaporated at 40ºC under vacuum either to near dryness or until the organic solvent was removed. The residue was dissolved in 1 ml methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through Millex nylon membrane filters 13 mm diameter of 0.22 µm pore size (Millipore, Bedford, MA, USA). Next, 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using heated electrospray ionization (HESI) interface. The mass spectra were determined using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each component analysed (1, 10, 50, and 100 µg l-1) and corrected for 10 µg l-1 deuterated internal standards. Recovery percentages ranged between 92 and 95%.

3.10 Determination of Na+ and K+ ions

The samples were mineralized by wet digestion according to Wolf (1982). To carry this out, 0.2 g of freeze-dried leaves were ground and mineralized with 98% H2SO4 and H2O2 to 30% at 300 °C. K+ and Na+ were analyzed by ICP-OES.

3.11 Statistical analysis

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

**4 Results**

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

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

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

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

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Biomass (g-1 FW) | | RGR (g g-1 day-1) | |
| Grand Brix | Marmande | Grand Brix | Marmande |
| Control | 8.55±0.04a† | 8.47±0.13a | 0.14±0.00a | 0.14±0.01a |
| Salinity | 5.83±0.13b | 4.66±0.13b | 0.11±0.01b | 0.09±0.01b |
| P-value | \*\*\*§ | \*\*\* | \*\*\* | \*\*\* |
|  |  |  |  |  |
| Grand Brix | 7.22a‡  6.56b  \*\*\*  0.30 | | 0.13a  0.11b  \*  0.01 | |
| Marmande |
| P-value |
| LSD0.05 |

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

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Na (mg g-1DW) | | K (mg g-1DW) | | Na/K (mg g-1DW) | |
| Grand Brix | Marmande | Grand Brix | Grand Brix | Grand Brix | Marmande |
| Control | 3.29±0.28b† | 2.70±0.01b | 24.39±0.48a | 22.33±0.17a | 0.14±0.01b | 0.12±0.00b |
| Salinity | 18.78±0.07a | 11.25±0.21a | 16.34±0.11b | 15.26±0.19b | 1.15±0.00a | 0.74±0.01a |
| P-value | \*\*\*§ | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
|  |  |  |  |  |  |  |
| Grand Brix | 11.03a‡  6.98b  \*\*\*  1.28 | | 20.37a  18.80b  \*\*\*  0.59 | | 0.65a  0.43b  \*\*\*  0.07 | |
| Marmande |
| P-value |
| LSD0.05 |

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

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

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

4.2 Promoters and indicators of oxidative stress

Our results showed a decline in the O2- concentration in the salinity treatment in both genotypes with respect to control (Figure 1A; P<0.001). The foliar H2O2 concentration increased in the salinity treatment in Marmande RAF and decreased in Grand Brix (Figure 1B; P<0.05).

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

In general, on comparing the two genotypes, we found that Marmande RAF had higher concentrations of O2- and H2O2 (Figure 1A1; P<0.001; Figure 1B; P<0.05), whereas LOX activity and MDA concentration did not differ between genotypes (Figure 1C1 and 1D1; P>0.05)

4.3 Antioxidant enzyme systems

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

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

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**Figure 1:** Effect of saline treatment in the concentration of O2.-(µg g-1 FW) (A), H2O2(µg g-1 FW) (B), LOX (Abs min-1 mg-1 protein) (C) and MDA (µmol g-1 FW) (D) in plants of Solanum lycopersicum cv. Grand Brix and cv. Marmande RAF. The columns Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1, B1, C1 and D1 Values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0.05). Levels of significance are represented by NS (non-significant) P> 0.05; \* P< 0.05; \*\* P< 0.01 and \*\*\* P< 0.001 relative to the control.

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

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | SOD (U mg-1protein) | | | CAT (Abs min-1 mg-1 protein) | | | | APX (Abs min-1 mg-1 protein) | | | | GR (Abs min-1 mg-1 protein) | | | | GSHPx (Abs min-1 mg-1 protein) | | | |
|  | Grand Brix | | Marmande | | Grand Brix | | Marmande | | Grand Brix | | Marmande | | Grand Brix | | Marmande | | Grand Brix | | Marmande | |
| Control | | 2.50±0.08b† | | 1.91±0.04b | | 0.04±0.00a | | 0.02±0.00a | | 0.03±0.00b | | 0.03±0.00b | | 0.02±0.00a | | 0,02±0,00a | | 0,01±0,00b | | 0,01±0,00b | |
| Salinity | | 3.38±0.05a | | 2.63±0.04a | | 0.01±0.00b | | 0.01±0.00b | | 0.04±0.00a | | 0.07±0.00a | | 0.02±0.00a | | 0,02±0,00a | | 0,02±0,00a | | 0,02±0,00a | |
| P-valor | | \*\*\*§ | | \*\*\* | | \*\*\* | | \*\*\* | | \*\*\* | | \*\*\* | | NS | | NS | | \*\*\* | | \*\*\* | |
|  | |  | |  | |  | |  | |  | |  | |  | |  | |  | |  | |
| Grand Brix | | 2.85a‡ | | | | 0.02a | | | | 0.05b | | | | 0.02a | | | | 0.02b | | | |
| Marmande | | 2.05b | | | | 0.02a | | | | 0.07a | | | | 0.02a | | | | 0.03a | | | |
| P-valor | | \*\*\* | | | | NS | | | | \*\*\* | | | | NS | | | | \*\*\* | | | |
| LSD0.05 | | 0.15 | | | | 0.00 | | | | 0.01 | | | | 0.00 | | | | 0.00 | | | |

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

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

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

4.4 Non-enzymatic antioxidant systems

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

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

**Table 6:** Effect of saline and alkaline treatments in the concentration of AsAtot, AsAred and AsAtot/AsAred ratio in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Total AsA (mg g-1 FW) | | Reduced AsA (mg g-1 FW) | | AsAtot/AsAred (mg g-1 FW) | |
| Grand Brix | Marmande | Grand Brix | Marmande | Grand Brix | Marmande |
| Control | 0.09±0.00b† | 0.10±0.00a | 0.01±0.00b | 0.02±0.00a | 5.88±0.18b | 7.15±0.09b |
| Salinity | 0.12±0.00a | 0.10±0.00a | 0.02±0.00a | 0.02±0.00a | 7.36±0.21a | 7.74±0.22a |
| P-valor | \*\*\*§ | NS | \*\*\* | NS | \*\*\* | \*\*\* |
|  |  |  |  |  |  |  |
| Grand Brix | 0.11a‡ | | 0.03a | | 5.91a | |
| Marmande | 0.10b | | 0.03a | | 6.10a | |
| P-valor | \*\* | | NS | | NS | |
| LSD0.05 | 0.00 | | 0.00 | | 0.42 | |

**Table 7:** Effect of saline and alkaline treatments in the concentration of GSHtot, GSHred and GSHtot/GSHred ratio in plants of *Solanum lycopersicum* cv. Grand Brix and cv. Marmande RAF

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Total GSH (mg g-1 FW) | | Reduced GSH (mg g-1 FW) | | GSHtot/GSHred (mg g-1 FW) | |
| Grand Brix | Marmande | Grand Brix | Marmande | Grand Brix | Marmande |
| Control | 0,27±0,00a† | 0,25±0,00a | 0,18±0,00a | 0,18±0,01a | 1,47±0,01a | 1,46±0,01a |
| Salinity | 0,12±0,00b | 0,11±0,00b | 0,08±0,01b | 0,08±0,00b | 1,28±0,01b | 1,39±0,02b |
| P-valor | \*\*\*§ | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\* |
|  |  |  |  |  |  |  |
| Grand Brix | 0,20a‡ | | 0,13a | | 1,38b | |
| Marmande | 0,18b | | 0,13a | | 1,43a | |
| P-valor | \*\* | | NS | | \*\*\* | |
| LSD0.05 | 0,01 | | 0,01 | | 0,04 | |

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

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

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

4.5 Antioxidant test

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

**Gráfico

Descripción generada automáticamente**

**Figure 2:** Effect of saline treatment in the antioxidant test DPPH expressed as % g-1 FW (A), Reducing power expressed as% of ascorbic acid (1 mM) equivalent activity g-1 FW (B), FRAP expressed as mg Fe(SO4) g-1 FW (C) and TEAC expressed as mmol Trolox equivalent (TE) g-1 FW. (D) in plants of Solanum lycopersicum cv.Grand Brix and cv.Marmande RAF. The columns Values are mean ± standard error (n= 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1, B1, C1 and D1 Values are means (control + salinity n= 18) and differences between means were compared using LSD test (P= 0.05). Levels of significance are represented by NS (non-significant) P> 0.05; \* P< 0.05; \*\* P< 0.01 and \*\*\* P< 0.001 relative to the control.

4.6 Phytohormone profile

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

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

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

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | IAA (ng g-1) | | tZ (ng g-1) | | iP (ng g-1) | | GA4 (ng g-1) | |
| Grand Brix | Marmande | Grand Brix | Marmande | Grand Brix | Marmande | Grand Brix | Marmande |
| Control | 0.86±0.14b† | NF | 379.72±1.12b | 921.31±32.00b | 0.28±0.04b | 2.46±0.11b | NF | NF |
| Salinity | 1.38±0.08a | NF | 715.25±7.98a | 1213.41±25.49a | 0.74±0.03a | 6.87±0.21a | 0.26±0.14a | NF |
| P-value | \*§ | - | \*\*\* | \*\* | \*\*\* | \*\*\* | - | - |
|  |  |  |  |  |  |  |  |  |
| Grand Brix | -  -  -  - | | 547.48b‡  1067.36a  \*\*\*  47.38 | | 0.51b  4.66a  \*\*\*  1.51 | | -  -  -  - | |
| Marmande |
| P-value |
| LSD0.05 |

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

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ABA (ng g-1) | | ACC (ng g-1) | | JA (ng g-1) | | SA (ng g-1) | |
| Grand Brix | Marmande | Grand Brix | Marmande | Grand Brix | Marmande | Grand Brix | Marmande |
| Control | 369.29±9.47b† | 507.74±0.81b | 18.55±2.76a | 3.70±0.23b | 580.12±16.66a | 70.87±2.53a | 4048.37±40.92b | 650.19±10.42b |
| Salinity | 602.56±0.58a | 568.02±1.56a | 9.33±2.60b | 5.56±1.18a | 345.03±10.18b | 80.39±4.82a | 14338.3±99.75a | 1797.71±13.82a |
| P-value | \*\*\*§ | \*\*\* | \*\* | \* | \*\*\* | NS | \*\*\* | \*\*\* |
|  |  |  |  |  |  |  |  |  |
| Grand Brix | 485.92a‡  537.88a  NS  66.03 | | 13.94a  4.63b  \*\*  5.96 | | 462.58a  75.63b  \*\*\*  94.73 | | 9193.35a  1223.95b  \*\*\*  3448.91 | |
| Marmande |
| P-value |
| LSD0.05 |

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

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

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

**5 Discussion**

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

The decline in biomass and RGR under salinity stress, are related to Na+ accumulation and K+ deficit, as these alter the basic physiological processes for the plants such as photosynthesis (Li et al. 2010). In our experiment, the salinity treatment resulted in the greatest accumulation of Na+ (an increase of 470% in the cv. Grand Brix 318% and an increase of in the cv. Marmande RAF; Table 2). Works such as Li et al. (2010) have also reported a greater accumulation of Na+ in the salinity treatments in alfalfa plants. In addition, works as Gong et al. (2013) and Wang et al. (2011) in tomato or Li et al. (2014) in rice have found a decline in the concentration of K+ when the plants were grown with saline treatments. This agrees with our results in which the saline treatment in both genotypes registered the lowest K+ concentrations (Table 2). Finally, our results a priori appear to indicate that the cv. Grand Brix should be more affected by the salinity treatment since this genotype accumulates more Na+ and the Na+/K+ ratio is greater than the cv. Marmande RAF; however, the growth data indicate that the more sensitive genotype was the cv. Marmande RAF (Table 2). This may be due to the strategies such as Na+ compartmentalization in the vacuoles or the immobilization of this ion (Li et al. 2010), which could be reinforced in the cv. Grand Brix and therefore might be less affected by Na+ accumulation than the cv. Marmande RAF.

ROS accumulate under different types of stress and for different reasons, as for example under alteration of the photosynthetic machinery, the excess electrons produced are transferred to O2 molecules and these produce ROS (e.g. H2O2 and O2-) (Çakmak 2005). In our experiment O2- levels are decreased in saline treatment. In our experiment, the O2.- was reduced by 42% in Grand Brix and by 22% in Marmande RAF, and H2O2 was reduced by 13% in Grand Brix and increased by 36% in Marmande RAF. This could be due to the enzyme SOD, but it has also been shown that hormones such as auxins can help in the detoxification of ROS. Therefore in Grand Brix the increase in the concentration of auxins (Table 3) could help explain this O2- reduction (Noctor et al. 2015). The increase in ROS promotes lipid peroxidation, a process that can determine LOX activity and the MDA concentration, which is a subproduct of this peroxidation (Zhou and Zhao 2004). The increases in ROS and lipid peroxidation have been associated with salinity stress in tomato (Gong et al. 2013; Gong et al. 2014b). In our experiment, we found significant increases, especially in the MDA concentrations (Figure 1D P<0.001) under stress conditions. Sánchez-Rodríguez et al. 2010 showed in the cultivars most sensitive to water stress a ROS accumulated and an increased in the MDA concentration. ROS and MDA concentration have been defined as good stress indicators (Gong et al 2014a). So, the Figure 1 corroborates that Marmande RAF is more sensitive to this stress than Grand Brix.

Greater SOD activity under salinity stress has been shown by different authors in tomato plants and in Iris lactea (Wang et al. 2008; Gong et al. 2013; Gong et al. 2014b). Our results coincide with the findings of these authors, and the higher SOD activities in the salinity treatment (Table 4) explain the low O2- levels (Figure 1A). Also, it bears highlighting that a comparison of the two genotypes indicates that Grand Brix induces more SOD and is more stress resistant. These results suggest that the detoxification of the ion O2- by SOD activity is a key process in the identification of the toxicity of saline stress and in the possible generation or selection of plants with greater resistance to this stress.

The other antioxidant enzymes (CAT, APX, GR, and GSHPX) have also been shown to increase in activity in tomato plants and Iris lactea subjected to this type of ionic stress (Wang et al. 2008; Gong et al. 2013; Gong et al. 2014b). For APX and GSHPx enzymes, our results coincide with the results of these authors (Table 4). However, contrary to our expectations, Marmande RAF presented greater activity for APX and GSHPx enzymes, and this would explain the low H2O2 accumulation in these plants. Furthermore, would indicate that the enzymatic detoxification of H2O2 is not determinant for assessing the tolerant genotype. In our experiment, the H2O2 levels are elevated in the saline treatment (Figure 1) causing damage by oxidative stress. But it has been observed that an increase of CKs can decrease the damages caused by the ROS. Grand Brix presents higher levels of CKs (Table 3), this can help to present greater tolerance to stress (Pogány et al. 2004).

In short, Marmande RAF was affected by the stress treatment (Table 1), which could be due to greater O2- accumulation, this ROS could be the main cause of the oxidative stress. Grand Brix registered greater SOD activity (Table 4) and therefore would be protected from the damage caused by this ROS, making it the more tolerant genotype against this type of stress.

The levels of non-enzymatic antioxidants are indicators of the redox state of the plant and responsible in part for resistance to stress. Previous studies show that under salinity stress, the levels of AsA and GSH increase (Gong et al. 2013). In this sense, the concentrations of the different forms of AsA and GSH in our work behave in a different way (Table 5 and 6). AsA is used to detoxify H2O2 together with APX activity, and therefore its increases coincide in Grand Brix (Tables 4 and 5). Sánchez-Rodríguez et al. 2010 showed high AsA concentrations in the most resistant cultivar. The decrease in GSH in both genotypes with the applications of salinity treatment (Table 5) could be due to the GSH is being used for other ends and because other compounds under low molecular weight are being generated that could intervene also in the resistance to this type of stress (Khaliq et al. 2015). In any case, it bears emphasizing that, AsAtot and GSHtot concentrations were higher in Grand Brix, which proved more resistant to saline stress in our work (Tables 5 and 6).

The current literature shows how CAT activity increases under saline stress. However, in our experiment the CAT activity declines (Table 5). This decrease may be due to an enzymatic inhibition, however, the accumulation of H2O2 is not very high (Figure 1). It could be because the H2O2 detoxification is carried out by other ways such as the APX and GSHPx enzymes or by compounds such as AsA.

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

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

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

Rapid accumulation of GAs is a characteristic of plants exposed to abiotic stresses, and it is an important phytohormone that can impart stress tolerance including salinity. In our experiment, the GA4 was only detected in cv. Grand Brix in saline treatment, while in control plants and in the cv. Marmande RAF was at a too low concentration to be detected (Table 3). Therefore, we assume that there is an increase in GA4 concentration in this treatment which can increase the resistance to salinity in cv. Grand Brix as above mentioned. Iqbal and Ashraf (2013) submitted two cultivars of wheat to salt stress and an external GAs application. They observed that treatments with GAs subjected to salt stress registered higher biomass than untreated plants. Therefore, they concluded that GAs could reduce the detrimental effect of ABA to be antagonists. Whereby, this suggests that the increase of GA4 concentration in the saline treatment of cv. Grand Brix could increase its tolerance to salt stress.

In short, the IAA, tZ and GA4 concentrations hormones increase the resistance to salt stress, maintaining the growth of the plant. The cv. Grand Prix presents the greatest increases in the concentrations of these hormones, being the most resistant to salt stress genotype.

ABA and ethylene hormones have long been regarded as stress hormones with speciﬁc roles in regulating tolerance and adaptation to salt stress (Amjad et al. 2014). Amjad et al. (2014) submitted two genotypes of tomato to salt stress, their results showed that ABA concentration increased in both genotypes, by saline treatment, but increased more in the salinity tolerant genotype. For ethylene concentration, both genotypes increased equally in saline treatment. In our experiment ABA concentration increased in the same way, increasing more in the saline treatment cv. Grand Brix (63% in the cv. Grand Brix and 12% in the cv. Marmande RAF; Table 4). Amjad et al. (2014) also relate a higher ABA concentration with greater efficiency in water use and better stomatal regulation, possibly improving salt stress. ACC (ethylene precursor) concentration decreases by 50% in cv. Grand Brix while increasing 50% for cv. Marmande RAF (Table 4). Sharp and LeNoble (2002) showed as an increase in ethylene concentration reduces plant biomass because it promotes programmed cell death and leaf senescence. These authors also show an interaction between ethylene and ABA, where the ABA inhibits ethylene action and synthesis. In our experiment a lower concentration of ethylene combined with a greater O2.- detoxification in the cv. Grand Brix could play a fundamental role in tolerance to saline stress (Overmyer et al. 2003). Besides, an increase in ABA levels promotes better stomatal closure and better control of photosynthesis and a lower rate of water loss.

The H2O2 stimulates the ABA accumulation (Table 4). This accumulation of ABA promotes stomatal closure thus avoiding the loss of water that can cause saline stress, and generating better osmotic adjustment and therefore better resistance to stress. (Mittler and blumwald 2015).

JA and SA hormones are mainly related to the stress by pathogens, however they have other functions related to abiotic stresses and interact with other hormones. For example, JA is known to favour the accumulation of ABA and CKs (Avalbaev et al. 2016), and also is involved in the ACC synthesis (Dar et al. 2015), also the JA has a close relationship with the SA. When SA has a very high concentration acts as a negative allosteric effector of JA and this can reduce their synthesis and concentration in the plant (Singh and Gautam 2013). This may be the reason why the JA concentration decreases in the saline treatment cv. Grand Brix (Table 4). SA is also similarly related to increased concentrations of ABA and CKs (Shakirova et al. 2003). Our results agree with those of the authors (Table 3 and 4). On the other hand, SA has been linked to improve the response of plants against oxidative stress, such as caused by salt stress (Singh and Gautam 2013). These results further supports that the cv. Grand Brix is more tolerant to salinity, because the hormone SA concentration increased by 254% in the cv. Grand Brix (Table 4).

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

**6 Conclusion**

This work leads us to conclude that Na+ accumulation, the ion K+ reduction and especially the O2- increase could be the main causes of the toxic effects of the stress studied. On the other hand, cv. Grand Brix has a better hormonal profile adapted to salt stress resistance. Hormones related to growth reveal differences between genotypes that could be key for resistance to salt stress. The comparative study of the two genotypes indicated that cv. Grand Brix, despite accumulating a higher Na+ concentration than did cv. Marmande RAF, showed less oxidative stress, as it showed greater detoxification of the ion superoxide with an induction of the enzymatic activity SOD and greater antioxidant capacity. Furthermore, the accumulation of IAA, GA4 and CKs and their beneficial role against oxidative stress could make the difference between resistance and sensitivity to salt stress. On the other hand, the ABA and tZ increase, and ACC decreased concentrations could also mark the resistance in the cv. Grand Brix. In conclusion, our results reveal that O2- accumulation could be the key factor determining the toxic effect of saline stress, and therefore its detoxification is essential to develop and/or select plants with stronger resistance to such abiotic stress in plants. Furthermore, the key hormones in the saline stress tolerance could be IAA, tZ, GA4, ABA and ACC. although would be necessary more specific studies.

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