Menadione Sodium Bisulphite (MSB): beyond seed-soaking. Root pretreatment with MSB primes salt stress tolerance in tomato plants

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Abstract

Salinity and drought are considered significant abiotic plant stressors with major impact on plant development that causes serious agricultural yield losses. Amongst the strategies to face this problem, the use of compounds capable of inducing abiotic stress tolerance is still little explored. Menadione sodium bisulphite (MSB), a water-soluble vitamin K_3 derivative, was previously shown to prime salt stress tolerance when Arabidopsis seeds were pre-soaked with this compound. However, this method has some technical problems regarding seed storage and longevity. In order to overcome these handicaps, we assessed the effect of supplying MSB to roots to prime the response to salinity stress, analysing the effect of two NaCl concentrations (100 and 150 mM). We selected tomato plants, the most economically important horticultural crop, as our biological model. In this new system, MSB primes salt tolerance in tomato plants by improving net photosynthesis, regulating stomatal aperture and maintaining water balance. Furthermore, MSB induces a faster proline accumulation and ion homeostasis by up-regulating several ion transporter genes, and increases antioxidant activity. As a result, a clear positive effect on plant growth was observed, indicated by the relative growth rate (RGR), These findings again highlight the potential usefulness of MSB as a priming agent for enhancing crop tolerance in the field under adverse environmental conditions.

Key words

Abiotic stress, menadione sodium bisulphite, priming, salt stress, tomato

1. Introduction

Global warming is possibly the most important problem faced by agricultural production in the present century, especially due to intensified and wider-reaching abiotic stresses (Bellard et al., 2012). Among these factors, drought and soil salinity cause substantial losses in most important crops (Shrivastava and Kumar, 2015). Environmental conditions are expected to worsen in many regions in the near future and it has been estimated that more than 50% of arable land may be salinized by the year 2050 (Jamil et al., 2011). Soil salinity alone is one of the most devastating agents, causing reductions in quality and productivity of crops (Shahbaz and Ashraf, 2013).

Various strategies have been described to cope with salinity stress under field conditions, such as the use of more tolerant transgenic lines (Hirayama and Shinozaki, 2010). However, salt tolerance is not conferred by a single gene only (Munns and Tester, 2008) and European countries now greatly limit the use of transgenic plants. These strategies are moreover highly criticized due to poor evaluation methodology under field conditions (Ashraf et al., 2008) and besides, constitutive expression of a specific transgene usually leads to a decrease in yield (Heil and Baldwin, 2002). Therefore, it would be highly desirable that defence genes were expressed only under stress conditions. Plants have numerous defence strategies to bear stress. Amongst them is priming, usually defined as genetic or biochemical modifications induced by a first stress exposure that lead to enhanced resistance to a future stress (Conrath et al., 2015). For this reason, priming treatments constitute fruitful strategies in combatting salinity, since the defence arsenal in primed plants remains dormant until the stress triggers them. Interestingly, priming does not involve greater fitness costs under optimum growth conditions (Van Hulten, et al., 2006).

Several vitamins have been tested as priming agents that can increase plant resistance to different unrelated stresses (Boubakri et al., 2016). Among them, previous work by our research group has demonstrated that menadione sodium bisulphite (MSB), a water-soluble menadione (vitamin K_3) derivative previously

reported as a plant growth regulator (Rama-Rao, et al.,1985), is capable of priming Arabidopsis against biotic (Borges et al., 2009; Carrillo-Perdomo et al., 2016) and abiotic stresses (Jiménez-Arias et al., 2015a,b). These previous studies suggest that MSB produces a slight oxidative burst that develops a reactive oxygen species (ROS) dependent signalling network. This induces an accumulation of latent defence proteins such as ROS-scavenging and transcription factors, resulting in a primed state and an enhanced stress response (Borges et al., 2014). Finally, we have patented several practical applications of MSB in agriculture, including the induction of plant tolerance to salt stress (Borges et al., 2010) and several MSB-based formulations have been marketed for crop protection.

Soaking seeds in MSB has shown successful results against salt stress (Jiménez-Arias et al., 2015 a,b), and against biotic stress via foliar spray (Borges et al., 2009; Carrillo-Perdomo et al., 2016) and root application (De Nisi et al., 2006). In Arabidopsis this priming effect against salinity stress involves a modification of the proline accumulation dynamics after salt addition (Jiménez-Arias et al., 2015a), caused by epigenetic changes in genes controlling proline metabolism (Jiménez-Arias et al., 2015b). Thus, soaking seeds with MSB seems to be a potentially beneficial treatment for use under field conditions. Despite promising results obtained in inducing salt tolerance, from a practical point of view this method may however have some technical problems regarding seed storage. Indeed, reduced seed longevity (Chiu et al., 2002) and loss of the primed state with storage have already been reported (Sliwinska and Jendrzejczak, 2002). For this reason, this type of application is sometimes very limited (Paparella et al., 2015). Nevertheless, the advantages of priming induced by MSB through root treatment to improve salinity tolerance have not been studied in depth. Soil salinity adversely affects the yield of a wide variety of crops. including tomato, which, in economic terms, is the world's most important horticultural crop (Bergougnoux, 2014). It was chosen for this reason.

The main goal of this study is to assess the effect of MSB application to roots in tomato plants exposed to salt stress and to establish some of the components

involved in regulating plant responses to NaCl. The results obtained highlight the potential of MSB as priming agent for improving salt tolerance in the tomato and other horticultural crops.

2. Material and Methods

2.1. Plant material, treatments and experimental design.

Tomato (*Solanum lycopersicum*) plants var. Robin were provided by a local plant nursery. Plantlets with two leaves were used. Roots were accurately washed with water and placed in a hydroponic system. The containers were 4 L PVC boxes accommodating 30 holders with one plant each. Plants were cultivated in a growth chamber at 22°C, 16 h light (150-200 µmol m^{-2} s^{-1}) and 60-70% relative humidity. The solution used was: 1.25 mM KNO_3, 0.5 mM KH_2PO_4, 0.75 mM $MgSO_4$ x $7H_2O$, 0.75 mM Ca $(NO_3)_2$ x $4H_2O$, 50 µM H_3BO_3, 10 µM $MnSO_4$ x H_2O, 2 µM $ZnSO_4$ x $7H_2O$, 1.5 µM $CuSO_4$ x $5H_2O$, 0.075 µM $(NH_4)_6Mo_7O_{24}$ x $4H_2O$, 44.8 µM Sequestrene® (Syngenta, USA). After transplanting to the hydroponic conditions, plants had two days of adaptation using half the concentration of the described nutritive solution. After the two days, plants were treated by replacing the solution with distilled water (treatment DW) as a control or 1.3 mg/L of MSB (M5750; Sigma-Aldrich, St. Louis, MO, USA) diluted in water for 24 hours (treatment MSB). After this, the treatment solution was removed and the plants were kept 24 hours in half-concentrated nutrient solution, at this point the salinity experiment began by adding 100 (9.7 mS/cm) and 150 mM (13.5 mS/cm) NaCl to the nutrient solution. Salt addition established four more experimental conditions: plants exposed to 100 and 150 mM of NaCl (Salt1 and Salt2 treatments, respectively), and plants exposed to both treatments (MSB-Salt1 and MSB-Salt2 treatments, respectively). Salt exposure experiments were sampled at times indicated in the legends of each figure or table. Three independent experiments were carried out and data shown represent the average of 30 plants per treatment in growth experiments. For the rest of measures 18 plants per treatment were used, with the exception of staining protocols where 10 leaves per experimental treatment were used and for

transcriptional studies were 4 replicates with 2 plants from each experimental treatment.

2.2. Growth measurements

Plants were cut into roots, stems and leaves. First, we measured the total surface area of leaves using the Petiole LTD smartphone application, then plants were dried in a hot-air oven at 70 ºC for 72 hours, and dry weight (DW) was determined for all plant sections separately. The relative growth rate (RGR) was calculated using the spreadsheet provided by Hunt (2002).

2.3. Determination of chlorophylls, proline, total carbohydrates

Fresh leaves (50 mg) were immediately ground in liquid nitrogen and chlorophylls extracted with ice-cold acetone/water 85% (v/v). The extract was then centrifuged at 15,000 g for 5 minutes and supernatant kept at -20 ºC until the chlorophyll pigments were quantified according to Porra (2002).

Proline content was determined as in Bates et al. (1973) with minor modifications. Approximately 100 mg fresh tissue was used. Proline concentration was determined from a standard curve, and calculated on a fresh weight basis.

Total carbohydrate determinations were performed by the phenol-sulphuric acid method (Dubois et al., 1956) using a multiplate protocol set out by Masuko et al. (2005). Total carbohydrate was calculated on a fresh weight basis using L-glucose as standard.

2.4. Na^+, K^+ and Ca^{2+} content

Tomato plants were harvested, rinsed with deionized water and dried in a hot air oven at 70 ºC for 3 days. Dry ground tissues (100 mg) were decomposed with HNO_3 (8 ml) in a microwave digestion unit (Millestone mls, 1200). The Na^+, K^+ and Ca^{2+} contents were determined using an atomic absorption spectrometer (SpectrAA 220FS; Varian, Springvale, Australia).

2.5. Gas exchange and chlorophyll fluorescence measurements

169 The gas exchange of leaves was measured using an Infrared Gas Analyzer,
170 LCpro-SD (BioScientific Ltd., England). The photosynthetic photon flux density
171 (PPFD) was set at 1,000 μmol m^{-2} s^{-1} after optimization with a light curve. The
172 cuvette air flow rate was 500 mL min^{-1}. Net photosynthetic rate, stomatal
173 conductance, and transpiration were recorded simultaneously. Water use efficiency
174 (WUE) was calculated as the ratio between net photosynthesis and transpiration.
175 Chlorophyll fluorescence measurements were as described by Jiménez-Arias et al.
176 (2015a)

177 ***2.6. Antioxidant capacity and lipid peroxidation***

178 The non-enzymatic antioxidant status of plant tissues was assayed according to
179 the Oxygen Radical Absorbance Capacity (ORAC) method developed by Gillespie
180 et al. (2007). The assay is performed in a microplate assessed with a 96-well multi-
181 detection plate reader (Fluorstar Omega CBMC LABTECH, Germany). ORAC was
182 calculated on a fresh weight basis using Trolox as antioxidant standard. Catalase,
183 ascorbate peroxidase and glutathione reductase were analysed following the
184 protocols optimized by Elavarty and Martin (2010). Lipid peroxidation was
185 determined by measuring the amount of malondialdehyde (MDA) according to the
186 method of Hodges et al. (1999).

187 ***2.7. Analysis of relative electrolyte leakage and relative water content.***

188 Most apical leaves were used to determine Relative Water Content (RWC). This
189 was accomplished by excising twenty 1-cm diameter discs for each treatment. All
190 leaf discs were weighed immediately, providing a measure of fresh mass (FM).
191 After weighing, the discs were soaked in deionized water for 24 h and then
192 weighed again to obtain a fully turgid mass (TM). Finally, the discs were dried at
193 85°C and weighed to obtain a dry mass (DM). The leaf RWC was calculated as
194 follows: RWC = (FM − DM) / (TM− DM).

195 Electrolyte leakage was used to evaluate membrane permeability in the leaves. It
196 was determined with an electrical conductivity (EC) meter. Plants were collected
197 from each treatment group and samples taken from the most apical leaves. A 1-

198 cm^2 segment was cut out at random from each leaf, washed three times with
199 distilled water in order to remove surface contaminants and then placed individually
200 in stoppered vials containing 10 mL of distilled water. The vials were incubated at
201 25ºC on a shaker (100 rpm) for 24 h. EC of the bathing solution was measured
202 after incubation (EC1). Then the same vials with leaf samples were placed in an
203 autoclave at 120ºC for 20 min and the second measurement of electrical
204 conductivity (EC2) was taken after cooling the solution to room temperature.
205 Electrolyte leakage was calculated as EC1/EC2 and expressed as percent (Lutts et
206 al., 1995).

207 ***2.8. In situ localization of H_2O_2***

208 Leaves from each treatment at different times were excised and immersed in a 1%
209 solution of 3,3′-diaminobenzidine (DAB) in 10 mM MES buffer (pH 6.5), vacuum-
210 infiltrated for 5 min and then incubated at room temperature for 3 h in the absence
211 of light. Leaves were illuminated (1h) until the appearance of brown spots
212 characteristic of the reaction of DAB with H_2O_2. Leaves were bleached by
213 immersing in boiling ethanol to visualize the brown spots. To verify the specificity of
214 precipitates, before staining with DAB some leaves were immersed for 2 h in
215 solutions containing the H_2O_2 scavenger 1 mM ascorbate (ASC). H_2O_2 deposits
216 were quantified by scanning spots from leaf pictures and the number of pixels was
217 quantified with the ImageJ v1.51r software (National Institutes of Health). The
218 results were expressed as a percentage of the ratio between spots area and total
219 leaf area, to compensate for differences in leaf size.

220 ***2.9. In situ localization of superoxide ions***

221 Leaves from each treatment at different times were excised and immersed in a
222 0.1% solution of nitroblue tetrazolium (NBT) in 50 mM K-phosphate buffer (pH 6.4),
223 containing 10 mM Na-azide. They were vacuum-infiltrated for 3 hours and then
224 illuminated until blue spots appeared, characteristic of blue formazan precipitates.
225 Leaves were bleached by immersing in boiling ethanol. As negative controls before
226 staining with NBT, leaves were immersed in 1 mM tetramethylpiperidinooxy, an

$O_2^{\cdot-}$ scavenger, for 3 h. Superoxide deposits were quantified by scanning spots from leaf pictures as mentioned above.

2.10. Quantification of abscisic acid (ABA) by HPLC

ABA quantification was performed by an adapted procedure depicted in Supplementary Fig.3, which was based on three previously reported methods (Nakurte et al., 2012; Munné-Bosch et al., 2011 and Li et al. 2011). The following instruments and reagents were employed: Shimatzu Europa modular High Performance Liquid Chromatograph (SPD-M 10AVP diode-array detector, PF 10A XL fluorescence detector, LC-10A two pumps, CTO-10A column oven and SIL-10A auto injector). Milli-Q ultrapure water purification system. Standard substance of ABA was purchased to Sigma-Aldrich company, methanol was chromatographic pure of VWR Chemical company, acetic acid was analytical pure from MERCK KGaA and water used in the experiment was ultrapure water.

2.12. Microscopic determinations of stomatal aperture

Five plants from each treatment were sampled and epidermis from the undersides of two leaves of each plant was taken after 3 and 7 days of salt addition and was observed in five different fields of view. Stomatal aperture was calculated using one random stoma from each field of view studied, 50 stomata were measured for each experimental condition.

2.13. Relative quantification of gene expression by RT-qPCR

Relative quantification of mRNA levels was carried out as described by Borges et al.,(2009). Results of gene expression of each experimental time-point were analysed as four independent biological replicas from 100 mg of roots, stem or leaves. Internal references for normalization of mRNA quantification were SGN-U314153 (CAC) and SGN-U346908 (Expressed) described by Expósito-Rodríguez et al., (2008). Amplification primers for these references and analysed genes are shown in Supplementary Table 1.

2.14. Statistical procedure

Statistical analyses for growth experiments were performed by one-way ANOVA and the significance of differences between experimental groups was calculated using a Tamhane post-hoc test. Additionally the other parameters were analysed using a T-Student test. All statistical tests were performed with IBM-SPSS20 software.

3. Results

3.1. Root treatment with MSB increases growth and prevents water loss under salinity stress.

Before starting the study, we tested the effect of different MSB concentrations on plant growth (data not shown), and finally 1.3 mg/L was used for all assays.

Root treatment with MSB increased plant dry weight after 4 days but after 7 days DW and MSB-treated plants reached similar weights (Table 1). However, comparison of dry weights after salt addition in DW and MSB plants revealed that MSB led to higher dry weight values, most notably after 7 days of salt treatment (42% for 100 mM and 29% for 150 mM NaCl). The RGR values, used as a growth index, showed non-significant differences between DW and MSB plants in the absence of salt during the 7 days of treatment. Nevertheless, RGR was reduced by 36% and 64% in untreated salt-stressed plants at 100 and 150 mM NaCl respectively. In contrast, in MSB-treated plants the reduction was considerably lower, 7 and 47% in 100 and 150 mM NaCl, respectively. This resulted in an increase in tolerance of about 81% for 100 mM NaCl treatment and 27% for 150 mM NaCl (Table 1). Figure S1 illustrates the effect of NaCl and MSB on tomato growth. The analyses of relative water content (RWC) showed that salinity promoted a reduction in RWC, while in MSB-treated plants the RWC was 28% and 42% higher in 100 mM and 150 mM NaCl, respectively, in comparison to MSB-untreated plants 7 days after salt exposure (Table 1).

3.2. MSB alleviates salt-induced inhibition in photosynthesis

To assess the effect of MSB treatment on photosynthesis parameters, measurements were performed every 24 hours after salt addition. As shown in Fig. 1, plants exposed to 100 mM NaCl reduced their photosynthesis rate after 2 days, independently of being pretreated or not with MSB (Fig. 1), reaching their minimum levels after 3 days. However, in MSB-treated plants the drop in photosynthetic activity was significantly less acute. Plants exposed to 150 mM NaCl showed the same behaviour but starting one day after salt insult (Fig. 1). Growth data (Table 1) are consistent with the photosynthesis rates (Fig. 1) within each experimental group.

MSB was capable of ameliorating the drops in stomatal conductance from 3 days and 2 days in plants exposed to 100 and 150 mM NaCl respectively (Fig. 1). After 3 days, MSB plants submitted to these two NaCl concentrations had the maximum difference in stomatal conductance between plants treated with MSB or not (Fig. 1). Concerning water use efficiency (WUE), it was interesting that the plants were able to adapt their water balance after 3 days of salinity stress, with similar WUE levels to DW and MSB plants (Fig. 1).

In order to confirm stomatal conductance data, we performed direct measurements of stomatal aperture (Fig. 2). After 7 days of stress, salt clearly affected stomatal aperture (Fig. 2) in plants untreated with MSB, which was negatively correlated with NaCl concentration. However, MSB-primed plants exposed to salt did not have significantly different values compared to DW or MSB plants. After 7 days (Fig. 2), the stomatal aperture behaviour was similar to that at 3 days, except for MSB-Salt1, which reached lower and significant values.

Total carbohydrate data presented in Table 1 are consistent with respective photosynthesis rates. Both salt concentrations reduced the amount of carbohydrates in plants, but MSB treatment buffered the reduction by about 28% and 42% in 100 and 150 mM NaCl, respectively (Table 1).

3.3. MSB protects plant photosynthetic machinery

To check the correct functioning of the photosystems in plants, we analysed chlorophyll fluorescence (Fv/Fm). Figure 3 shows the reduction in Fv/Fm induced by NaCl; the highest reduction was observed in plants treated with 150 mM NaCl (Fig. 3). For both NaCl concentrations, a biphasic effect was observed between 1-3 days and 4-7 days. MSB significantly reduced the effect of 100 mM and 150 mM NaCl. In addition, at 96 hours after salt addition (150 mM NaCl) the Fv/Fm ratio dropped but MSB buffered this drop and increased these values until non-stressed levels were reached, while in untreated plants exposed to salt these values continued to fall. These data are consistent with the total chlorophyll amounts (Table 1) and suggest that MSB is capable of protecting these pigments from degradation caused by salt stress imposed by the addition of 150 mM NaCl to the hydroponic medium.

3.4. MSB enhances antioxidant potential of plant

To assess if the MSB treatment was able to manage the increased levels of ROS as a consequence of salt stress conditions imposed on tomato plants, we analysed several antioxidant enzymes involved in ROS detoxification such as catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR).

Plants treated with MSB only showed a difference in CAT activity in comparison with DW control after 7 days, showing a six-fold increase in its activity (Fig. 4). Both salt concentrations significantly increased CAT activity only after 4 days, but the response was higher in MSB-primed plants (Fig. 4).

MSB increased APX activity, with a maximum difference of 4.5-fold over control after 4 days (Fig. 4). Moreover, only Salt2 plants reached MSB activity levels after 7 days. Salt1 and Salt2 only showed significant changes with respect to DW control in APX activity after 7 days, while MSB-treated plants submitted to salt had on average 2.5 fold more APX activity than their counterparts (Fig. 4).

The GR activity increased slightly, but significantly, after 1 day in the three experimental groups treated with MSB (Fig. 4). These differences remain after 7

342 days of salt stress, except for plants exposed to 100 mM NaCl, which reached its
343 maximum GR activity at 4 days.

344 Furthermore, salt stress induced a significant increase in non-enzymatic
345 antioxidant capacity, which was higher in MSB-treated plants (Fig. 4), although the
346 differences were progressively reduced during the experimental period. Moreover,
347 MSB seems to raise antioxidant capacity under unstressed conditions (Fig. 4).

348 Histochemical localization of H_2O_2 using DAB is shown in Table 2 and
349 Supplementary Fig. 2 (A-C). After one day of salt stress, MSB, MSB-Salt1 and
350 MSB-Salt2 plants show 28, 19 and 42% reduction in H_2O_2 comparing DW-treated,
351 Salt1 and Salt2 plants, respectively (Table 2). These differences became greater
352 after 4 days of salinity stress with a 55% and 83% reduction at 100 and 150 mM,
353 while without NaCl the values were very similar (Table 2). After 7 days, H_2O_2
354 accumulation was very similar in MSB-primed and unprimed plants, except in those
355 exposed to 150 mM NaCl. In these, MSB reduced the accumulation of H_2O_2 to
356 77% compared with the NaCl-treated plants (Table 2). Histochemical localization of
357 $O_2^{\cdot-}$ revealed the opposite trend to H_2O_2. Plants exposed to MSB and salt-stress
358 showed an increase in superoxide radical accumulation in leaves, compared to the
359 MSB-untreated salt stressed plants (Table 2) and Supplementary Fig. 2 (D-F).

360 In order to test if priming plants with MSB ameliorates oxidative damage to the
361 plasma membrane induced by salinity stress, the percentage of electrolyte leakage
362 and lipid peroxidation were measured after salt addition. Indeed, treatment with
363 MSB reduced electrolyte leakage and lipid peroxidation at both salt concentrations
364 (Table 3).

365 ***3.5. MSB regulates ion homeostasis under salt stress.***

366 We analysed the expression of several genes involved in ion homeostasis 4 days
367 after salt exposure in roots, stem and leaves. At this time point, DW and MSB-
368 treated plants did not show significant differences in the expression of any
369 analysed genes (Fig. 5).

Significant differences induced by salt exposure in expression of the tomato gene encoding the Na+/H+ antiporter 2 (*NHX2*) were only detected in leaves, but with different trends in plants treated or not treated with MSB (Fig. 5). While salt stress induced a decrease in *NHX2* expression in unprimed plants, reaching a two-fold downregulation in the Salt2 group, it had the opposite effect in plants treated with MSB.

Expression of the (Sodium/potassium)/proton exchanger 4 (*NHX4*) gene was upregulated by salt stress both in roots and stems, without significant differences between MSB-primed and unprimed plants. Nevertheless, treatment with MSB prevented significant decrease in the NHX4 gene expression, induced by salt exposure in leaves (Fig 5).

In contrast, expression of the plasmalemma Na^+/H^+ antiporter (*SOS1*) gene in roots was not altered in any experimental group. Both salt concentrations increased *SOS1* expression in stems, but in MSB-primed plants the response was notably higher (Fig 5). In leaves, opposite behaviour was again observed in primed and unprimed plants. In this plant tissue, as with *NHX2* gene, expression was downregulated in unprimed plants but upregulated by salt in plants pre-treated with MSB.

Finally, expression of the gene *HKT1,2*, which encode a plasmalemma Na^+/H^+ antiporter, showed the greatest change in expression, in relative terms, as a response to salt insult (Fig. 5). This increase in *HKT1,2* expression was greater in MSB-primed plants in all analysed tissues, especially in roots, reaching 22-fold upregulation.

The values of ion concentrations after 7 days of salt stress (Table 4) indicate an MSB-dependent increase in K^+ concentration in leaf and stem at both NaCl concentrations assayed. Plants treated with MSB and exposed to 100 mM NaCl showed 16% and 26% higher K^+ concentrations in leaves and stem respectively, the differences being even higher in leaves in response to 150 Mm NaCl. Interestingly, MSB prevented the accumulation of Na^+ in leaf tissue, with a 30%

reduction at both NaCl concentrations (Table 4). The Na^+/K^+ ratio in leaves was approximately half that in MSB treated plants for both salt concentrations (Table 4). In addition, MSB showed a 28% higher Ca^{2+} concentration in stems and 19% less in root tissues, as compared with untreated plants after the addition of 100 mM NaCl. However, there were no significant differences in leaf tissues. Besides, plants treated with MSB and exposed to 150 mM NaCl showed 10, 27 and 5% less Ca^{2+} in leaf, stem and root tissues, compared with untreated plants (Table 4).

3.6. MSB promotes changes in the kinetics of proline and ABA accumulation under salinity stress

Proline levels were increased in all experimental groups submitted to salt stress, as early as 1 day after addition of NaCl to the nutrient solution (Fig. 6). At the end of the assay (7 days), proline levels were similar for the same NaCl concentration, but MSB-primed plants reached these levels sooner (4 days).

Abscisic acid (ABA) is an important component in plant response to salinity. To determine the effect of MSB on ABA production in response to NaCl addition, a time-course analysis of this hormone was carried out. As shown in Fig. 7, salt exposure increased ABA levels compared to the corresponding controls but, in general, primed plants exhibited significantly higher hormone concentration. Seven days after salt exposition, ABA levels were considerably lower than at the beginning of the experiment.

4. Discussion

Nowadays, one of the most important challenges is to feed a growing population that will reach 9 billion people by 2050 (Tilman et al., 2013), in a scenario where climate change is predicted to cause a dramatic reduction in the area available for agriculture (Rosenzweig et al., 2014). Indeed, to complicate this predicted situation, frequent extreme weather events are expected during the 21st century. Among them, heavy precipitation, heat waves, and rising sea level, with resulting floods, drought, and salinity as the most critical consequences (Mba et al., 2012).

Currently, salinity stress alone affects approx. 20% of irrigated land and reduces crop yields significantly (Negrão et al., 2017). Moreover, the increase in salinity of agricultural land is expected to result in up to 50% loss of cultivable lands by the middle of the 21st century (Mahajan and Tuteja, 2005).

Soil salinity stress affects plants in two phases. 1) Around the roots it impedes water extraction from the soil. 2) Inside plants, it can be toxic (Munns and Tester, 2008). The first (osmotic stress) affects plant-water relations through stomatal closure and leads to growth inhibition (Munns and Termaat, 1986). The second phase (ionic stress) is governed by a specific ion-dependent response to salinity involving toxic accumulation of ions in the shoot. This particularly affects old leaves, causing premature senescence of plants and finally reduction in yield, or even plant death (Munns and Tester, 2008). Plants have evolved three distinct strategies to face salt stress: 1) Tolerance to osmotic stress; 2) Na^+ exclusion from leaf blades; 3) Tissue tolerance to Na^+ (Negrão et al., 2017).

Photosynthesis and cell elongation are the main processes affected by salt stress in early growth stages (Chaves et al., 2009). This behaviour was clearly observed in the present work (Fig. 1). Salinity considerably reduced net photosynthesis and stomatal conductance, which have a clear negative effect on plant growth (Fig. S1), as indicated by the RGR and total carbohydrate measured in plants submitted to both salt concentrations assayed (Table 1). As shown in Fig. 1, net photosynthesis and stomatal conductance were reduced by NaCl. However, MSB treatment prevents the negative effect of NaCl on growth, as measured in dry weight, RGR and total carbohydrate content (Table 1). However, this effect was dependent on NaCl concentration, being more effective at moderate salt concentrations. The protective role of MSB may be related to the improvement in photosynthesis parameters and stomatal conductance (Fig. 1). In this sense, the severe reduction in photosynthesis caused by NaCl would be due to the disturbances in gas exchange because of stomatal closure, which would reduce CO_2 availability. In turn, the positive effect of MSB is related to stomatal regulation during salt stress. Net photosynthesis rates are positively correlated with stomatal

458 conductance under optimal and osmotic stress conditions, thus effective stomatal
459 control promotes rapid growth and tolerance to osmotic stress (Haworth et al.,
460 2018). Comparison of water-use efficiency (WUE) values in presence of NaCl (Fig.
461 1C) reveals that, in general, MSB did not have a significant effect in WUE, despite
462 MSB-primed plants showing higher stomatal conductance and aperture (Fig. 1B,
463 2), suggesting that MSB has a protective effect on water balance under salinity
464 conditions, as indicated by higher RWC percentages (Table 1).

465 One of the most studied mechanisms that plants use to cope with osmotic
466 imbalance caused by salt stress is the accumulation of compatible low molecular
467 weight osmolytes, such as the amino acid L-proline (Szabados and Savouré,
468 2010). The cytoplasmic and intercellular accumulation of proline is able to protect
469 cells from damage, acting as both a radical scavenger and an osmoprotective
470 agent (Szabados and Savouré, 2010). Under our experimental conditions, MSB
471 promotes and accelerates proline accumulation, (Fig. 6). A similar effect was
472 observed in two previous studies using Arabidopsis seeds pre-soaked with MSB
473 and exposed to salinity (Jiménez-Arias et al., 2015 a,b). In those studies, MSB
474 showed a priming effect, improving tolerance against salinity stress in Arabidopsis
475 plants. It was also demonstrated that this process involves epigenetic changes in
476 the promoter region of genes controlling proline metabolism, in such a way that
477 leads to the upregulation of *PYRROLINE-5-CARBOXYLATE SYNTHETASE1* gene
478 *(P5CS1)* involved in proline biosynthesis, and the downregulation of *EARLY*
479 *RESPONSIVE TO DEHYDRATION 5* gene*,* (*ERD5)* involved in proline
480 degradation, giving rise to proline accumulation (Jiménez-Arias et al., 2015b). In
481 addition to its role as an osmolyte, it has been suggested that proline may be
482 beneficial to maintain the electron-chain of photosynthesis and respiration. Proline
483 acts as a sink to drain away any excess reductants, providing the NAD^+ and
484 $NADP^+$ necessary to sustain those processes (Kishor et al., 2005), which would
485 explain the positive effect of MSB on net photosynthesis (Fig. 1A) and on the
486 Fv/Fm ratio (Fig. 3).

Concerning the effect of MSB on ion concentration, the changes observed in accumulation and translocation of Na^+ and K^+ are noteworthy, under both 100 and 150 mM NaCl concentrations, giving rise to a reduction in Na^+/K^+ ratio, particularly in leaves (Table 4). A plasma membrane Na^+/H^+ antiporter activity has been demonstrated in tomato plants, identified as the protein SlSOS1, which is involved in Na^+ extrusion in tomato plants (Olías et al., 2009). De Nisi (2006) demonstrated that MSB application within a hydroponic system increased H^+-ATPase activity in tomato roots. The H^+-ATPase generates the proton motive force across the plasma membrane necessary to activate most of the ion and metabolite transport (Morsomme and Boutry, 2000). The role of other Na+ transporters such as HKT1,2, recently reported to modulate Na+/K+ homeostasis, cannot be ruled out in tomato under saline conditions (Jaime-Pérez et al., 2017). Indeed, MSB treatment was able to activate *SOS1* and *HKT1,2* expression in our experimental model Fig. 6 C), especially HKT1*,2* which has been reported as a key gene in Na^+ extrusion from the aerial parts of tomato plants under salt stress (Jaime-Pérez et al., 2017). MSB treatment also induced the up-regulation of *SOS1* gene in stem and leaf tissues (Fig. 6). We hypothesized that MSB could extend the half-life of *SOS1* mRNA, because stress-induced *SOS1* mRNA stability is mediated by reactive oxygen species (Chung et al., 2007), and MSB is a notable superoxide generator (Sun et al., 1999). In our conditions, expression of *NHX4* gene was downregulated in leaves by salt addition, and MSB priming prevented this (Fig. 6 B). Jaime-Pérez et al., (2017) concluded that the combination of HKT1,2, SlSOS1 and NHX4 proteins are required to regulate Na^+ and K^+ concentrations. Our data about gene expression, in combination with the analysis of ion concentrations, suggest that MSB-priming improved ion homeostasis machinery under salt stress.

It is well known that salt stress is associated with increased production of ROS, which in turn may provoke an extended oxidative stress, resulting in peroxidation of essential macromolecules, particularly lipids, thus affecting the plasma membrane (Meloni et al., 2003). After 1 day of salt exposure (Table 2), significant ROS accumulation (O_2^- and H_2O_2) is observed in tomato leaves, although ROS decrease progressively until the end of the experiment. MSB accelerates the drop

518 in hydrogen peroxide content, compared to salt-treated plants (Table 2). It should
519 be noted that MSB-treated plants accumulated higher levels of superoxide ions
520 during the trial (Table 2). This is not surprising because MSB is a well-known
521 superoxide ion generator (Sun et al., 1999). MSB appears to promote priming by
522 activating the expression of key genes involved in a ROS-dependent signalling
523 network (Borges et al., 2014). One of them is the gene encoding the transcription
524 factor ZAT12, which is required for ascorbate peroxidase (*APX*) expression under
525 stress (Rizhsky et al., 2004). Herein, we present evidence of a higher activity of
526 APX in MSB-treated plants during the seven days of the experiment (Fig. 4), which
527 together with increased catalase and glutathione reductase activities at higher salt
528 doses and enhanced total antioxidant capacity (Fig. 4) improve the efficiency in
529 eliminating H_2O_2 (Table 2). This strengthened antioxidant response can explain the
530 higher levels of chlorophylls at higher salt concentration (Table 1), the stability of
531 the Fv/Fm ratio (especially at 150 mM NaCl, Fig. 3), and the reduced damage to
532 plasma membranes as indicated by lower values of electrolyte leakage and lipid
533 peroxidation (Table 3).

534 Many previous works have focussed on the role of abscisic acid (ABA) and other
535 hormones in abiotic stresses such as drought and salinity (Vishawakarma et al.,
536 2017). Some contradictory results have been published regarding whether ABA
537 levels affect these stress responses or not, and their role as a modulator of
538 stomatal closure, conductance and transpiration (Jakab et al., 2005; Wan and Li,
539 2006; Shaw et al., 2016). Moreover, another 'dogma' subject to debate is the role
540 of ABA as a plant growth inhibitor, in contrast to the latest studies that address its
541 role as growth promoter (Humplík et al., 2017). In general, we detected increased
542 ABA levels for at least four days of NaCl exposure in MSB-treated plants (Fig. 7).
543 In addition, 3 days after salt addition a significantly higher level of stomatal
544 conductance in MSB-pretreated plants was found (Fig. 2). Under our experimental
545 conditions, higher values of this hormone could activate the ABA-dependent
546 biosynthesis of proline (Szabados and Savouré, 2010), enabling MSB-treated
547 plants to more rapidly adjust to osmotic imbalance caused by salt stress. However,

548 ABA-independent mechanisms for regulating proline accumulation have also been
549 reported in conditions of low water-potential stress (Sharma and Verslues, 2010).

550 In summary, MSB seems to promote tolerance to NaCl by acting at different levels:
551 Activating important genes for ion homeostasis and antioxidant defences,
552 increasing photosynthesis rate, and improving osmotic and water balance.
553 However, whether MSB has a direct effect on each of these processes or exerts an
554 indirect action needs to be addressed in future studies. A hypothetical model of
555 MSB action is proposed in Fig. 8.

556 Susceptibility or tolerance of plants to high salinity is the result of a coordinated
557 action of many stress-responsive genes (Munns and Tester, 2008). This
558 complexity hinders the design of transgenic strategies in crops to overcome salinity
559 stress (Ashraf et al., 2008). The priming strategy to enhance stress tolerance
560 consists of stimulating and accelerating the plants' defences to face further
561 adverse conditions sooner than unprimed plants (Bruce et al., 2008), avoiding
562 undesirable fitness costs (Van Hulten et al., 2006). Previous work by our group,
563 using an MBS seed-soaking treatment, demonstrated that this compound is
564 capable of inducing salinity tolerance in Arabidopsis plants (Jimenez-Arias et al.,
565 2015 a,b). However, this approach has some problems related to seed viability,
566 hampering its use in the field (Paparella et al., 2015). The present study provides
567 evidence for the protective effect of MSB against salt stress, using root treatment.
568 This highlights its potential use as a priming agent for enhancing crop plant
569 tolerance under adverse environmental conditions, with practical applications in
570 agriculture.

5. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Acknowledgements

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7. References

Ashraf, M., Athar, H.R., Harris, P.J.C., Kwon, T.R. 2008, Some prospective strategies for improving crop salt tolerance. Adv. Agron. 97, 45-110.

Bates, L.S., Waldren, R.P., Teare, I.D. 1973, Rapid determination of free proline for water-stress studies. Plant Soil 39, 205-207.

Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W., Courchamp, F. 2012, Impacts of climate change on the future of biodiversity. Ecol. Lett. 15, 365-377.

Bergougnoux, V. 2014, The history of tomato: from domestication to biopharming. Biotecnol. Adv. 32, 170-189.

Borges, A.A., Jiménez-Arias, D., Expósito-Rodríguez, M., Sandalio, L.M., Pérez, J.A. 2014, Priming crops against biotic and abiotic stresses: MSB as a tool for studying mechanisms. Front. Plant Sci. 5, 642.

Borges, A.A., Borges-Pérez, A., Jiménez-Arias, D., Expósito-Rodríguez, M., Martín-Rodríguez, V., Luis-Jorge, J.C. 2010. Use of menadione for boosting the tolerance of plants against salinity stress. Patent WO2010/018281.

Borges, A.A., Dobon, A., Expósito-Rodríguez, M., Jiménez-Arias, D., Borges-Pérez, A., Casañas-Sánchez, V., et al., 2009., Molecular analysis of menadione-induced resistance against biotic stress in Arabidopsis. Plant Biotechnol. J. 7, 744-762.

Boubakri, H., Gargouri, M., Mliki, A., Brini, F., Chong, J., Jbara, M., 2016. Vitamins for enhancing plant resistance. Planta 244, 529-543.

Bruce, T.J.A., Matthes, M.C., Napier, J.A., Pickett, J.A., 2007, Stressful ‘‘memories’’ of plants: evidence and possible mechanisms. Plant Sci. 173, 603-608.

Carrillo-Perdomo, E., Jiménez-Arias, D., Aller, A., Borges, A.A., 2016, Menadione Sodium Bisulphite (MSB) enhances the resistance response of tomato leading to repel mollusk pests. Pest Manag. Sci. 72, 950-960.

Chaves, M.M., Flexas, J., Pinheiro, C., 2009, Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann. Bot-London 103, 551-560.

Chiu, K.Y., Chen, C.L., Sung, J.M., 2002. Effect of priming temperature on storability of primed sh-2 sweet corn seed. Crop Sci. 42, 1996-2003.

Chung, J.S., Zhu, J.K., Bressan, R.A., Hasegawa, P.M., Shi, H., 2008., Reactive oxygen species mediate Na+-induced SOS1 mRNA stability in Arabidopsis. Plant J. 53, 554–565

Conrath, U., Beckers, G.J.M., Langenbach, C.J.G., Jaskiewicz, M.R., 2015, Priming for enhanced defense. Ann. Rev. Phytopathol. 53, 97-119.

De Nisi, P., Manzotti, P., Zocchi, G., 2006, Effect of Vitamin K3 on plasma membrane-bound H+-ATPase and reductase activities in plants. Plant Sci. 170, 936-941.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956, Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356.

Rizhsky, L., Liang, H.,Mittler, R., 2003, The water-water cycle is essential for chloroplast protection in the absence of stress. J. Biol. Chem. 278,38921–38925.

Elavarthi, S., Bjorn, M., 2010, Spectrophotometric Assays for Antioxidant Enzymes in Plants, in: Sunkar, R., (ed), Plant Stress Tolerance, Methods in Molecular Biology 639. Springer Science+Bussines Media, pp.273-280.

Expósito-Rodríguez, M., Borges A.A., Borges Pérez A, Pérez J.A., 2008, Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol. 8, 131.

Gillespie, K.M., Chae, J.M., Ainsworth, E.A., 2007, Rapid measurement of total antioxidant capacity in plants. Nat. Protoc. 2, 867-870.

Haworth, M., Marino, G., Cosentino, S.L., Brunetti, C., De Carlo, A., Avola, G., et al., 2018, Increased free abscisic acid during drought enhances stomatal sensitivity and modifies stomatal behaviour in fast growing giant reed (Arundo donax L.). Environ. Exp. Bot. 147, 116-124.

Heil, M., Baldwin, I.T. 2002, Fitness costs of induced resistance: emerging experimental support for a slippery concept. Trends Plant Sci. 7, 61-67.

Hirayama, T., Shinozaki, K. 2010., Research on plant abiotic stress responses in the post-genome era: past, present and future. Plant J. 61, 1041-1052.

Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K. 1999., Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207, 604-611.

Humplík, J.F., Bergougnoux, V., VanVolkenburgh, E., 2017, To stimulate or inhibit? That is the question for the function of abscisic acid. Trends Plant Sci. 22, 830-841.

Hunt, R., Causton, D.R., Shipley, B., Askew, A.P., 2002, A Modern Tool for Classical Plant Growth Analysis. Ann. Bot-London 90, 485-488.

Jaime-Pérez, N., Pineda, B., García-Sogo, B., Atares, A., Athman, A., Byrt, C.S., et al., 2017, The sodium transporter encoded by the HKT1;2 gene modulates

sodium/potassium homeostasis in tomato shoots under salinity. Plant Cell Environ. 40, 658-671.

Jakab, G., Ton, J., Flors, V., Zimmerli, L., Métraux, J., Mauch-Mani, B., 2005, Enhancing Arabidopsis Salt and Drought Stress Tolerance by Chemical Priming for Its Abscisic Acid Responses. Plant Physiol. 139, 267-274.

Jamil, A., Riaz, S., Ashraf, M., Foolad, M.R., 2011, Gene expression profiling of plants under salt stress. Crit. Rev. Plant Sci. 30, 435-458.

Jiménez-Arias, D., Pérez, J.A., Luis, J.C., Martín-Rodríguez, V., Valdés-González, F., Borges, A.A., 2015a., Treating seeds in menadione sodium bisulphite primes salt tolerance in Arabidopsis by inducing an earlier plant adaptation. Environ. Exp. Bot. 109, 23-30.

Jiménez-Arias, D., Borges, A.A., Luis, J.C., Valdés-González, F., Pérez, J.A., 2015b, Priming effect of menadione sodium bisulphite against salinity stress in Arabidopsis involves epigenetic changes in genes controlling proline metabolism. Environ. Exp. Bot. 120, 23-30.

Kishor, P.B.K., Sangam, S., Amrutha, R.N., Laxmi, P.S., Naidu, K.R., Rao, K.R.S.S., et al., 2005, Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. Curr. Sci. 88, 424-438.

Lutts, S., Kinet, J.M., Bouharmont, J. 1995, Changes in plant response to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. J. Exp. Bot. 46, 1843-1852.

Mahajan, S., Tuteja, N., 2005, Cold, salinity and drought stresses: an overview. Arch. Biochem. Biophys. 444, 139-58.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimurac, S., Lee, Y.C., 2005, Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. Anal. Biochem. 339 (2005) 69-72.

Mba, C., Guimaraes, E., Ghosh, K., 2012, Re-orienting crop improvement for the changing climatic conditions of the 21st century. BMC Agric. Food Secur. 1, 7.

Meloni, D.A., Oliva, M.A., Martinez, C.A., Cambraia, J., 2003, Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ. Exp. Bot. 49, 69-76.

Morsomme, P., Boutry, M., 2000, The plant plasma membrane H+-ATPase: structure, function and regulation. Biochim. Biophys. Acta 1465, 1-16.

Munns, R., Termaat, A. 1986. Whole Plant Responses to Salinity. Australian J. Plant Physiol. 13, 143-160.

Munns, R., Tester, M., 2008, Mechanisms of Salinity Tolerance. Ann. Rev. Plant Biol. 59, 651-681.

Negrão, S., Schmöckel, S.M., Tester, M., 2017, Evaluating physiological responses of plants to salinity stress. Ann. Bot-London 119, 1-11.

Olías, R., Eljakaoui, Z., Li, J., De Morales, P.A., Marín-Manzano, M.C., Pardo, J.M., Belver, A. 2009., The plasma membrane Na+/H+ antiporter SOS1 is essential for salt tolerance in tomato and affects the partitioning of Na+ between plant organs. Plant, Cell Environ. 32, 904-916.

Paparella, S., Araújo, S.S., Rossi, G., Wijayasinghe, M., Carbonera, D., Balestrazzi, A., 2015, Seed priming: state of the art and new perspectives. Plant Cell Rep. 34, 1281-1293.

Porra, R.J., 2002, The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth. Res. 73, 149-156.

Rama-Rao, A.V., Ravichandra, K., David, S.B., Ranade, S., 1985, Menadione sodium bisulphite: a promising plant growth regulator. Plant Growth Regul. 3, 111-118.

Rosenzweig, C., Elliott, J., Deryng, D., Ruane, A.C., Müller, C., Arneth, A., et al., 2014, Assessing agricultural risks of climate change in the 21st century in a global gridded crop model intercomparison. PNAS 111, 3268-3273.

Shahbaz, M., Ashraf, M., 2013, Improving salinity tolerance in cereals. Critical Rev. Plant Sci. 32, 237-249.

Sharma, S., Verslues, P.L., 2010, Mechanism independent of abscisic acid (ABA) or proline feed-back have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. Plant Cell Environ. 33, 1838-1851.

Shaw, A.K., Bhardwaj, P.K., Ghosh, S., Roy, S., Saha, S., Sherpa, A.R., et al., 2016, β-aminobutyric acid mediated drought stress alleviation in maize (Zea mays L.) Environ. Sci. Pollut. Res. 23, 2437-2453.

Shrivastava, P., Kumar, R., 2015, Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J. Biol. Sci. 22, 123-131.

Śliwińska, E., Jendrzejczak, E., 2002, Sugar-beet seed quality and DNA synthesis in the embryo in relation to hydration–dehydration cycles. Seed Sci. Technol. 30, 597-608.

Sun, Y.L., Zhao, Y., Hong, X., Zhai, Z.H., 1999, Cytochrome c release and caspase activation during menadione-induced apoptosis in plants. FEBS Lett. 462, 317-321.

Szabados, L., Savouré, A., 2010, Proline: a multifunctional amino acid. Trends Plant Sci. 15, 89-97.

Tilman, D., Balzer, C., Hill, J., Befort, B.L., 2011, Global food demand and the sustainable intensification of agriculture. PNAS 108, 20260-20264.

Van Hulten, M., Pelser, M., van Loon, L.C., Pieterse, C.M., Ton, J., 2006, Costs and benefits of priming for defense in Arabidopsis. PNAS 103, 5602-5607.

Vishwakarma, K., Upadhyay, N., Kumar, N., Yadav, G., Singh, J., Mishra, R.K., et al., 2017, Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Front. Plant Sci. 8, 161.

Wan, X-R., Li, L., 2006, Regulation of ABA level and water-stress tolerance of Arabidopsis by ectopic expression of a peanut 9-cis-epoxycarotenoid dioxygenase gene. Biochem. Bioph. Res. Co. 347, 1030-1038.

Table 1

Changes in different physiological variables in normal and salt-stress conditions. Data show the mean values plus their standard deviation.

Experimental group	Dry weight		7 days			
	4 days	7 days	RGR	RWC	Total Chlorophyll	Total Carbohydrate
DW	103.8±(3.2)a	142.31±(4.9)a	0.14±(0.006)a	87.29±(7.5)a	1.4±(0.1)a	31.31±(3.3)a
Salt1	75.49±(2)b	94.78±(2.7)b	0.09±/0.004)b	67.03±(9.5)b	1±(0.1)b	26.05±(2.2)b
Salt2	64.08±(1.9)c	70.70±(1.6)c	0.05±(0.005)c	59.92±(9.9)c	0.50±(0.1)c	19.78±(1.9)c
MSB	122.48±(2.7)d	144.78±(2.8)a	0.15±(0.006)a	90.31±(5.5)a	1.47±(0.1)a	32.73±(3.4)d
MSB-Salt1	103.04±(1.6)a	134.13±(2)d	0.14±(0.009)a	84.47±(9.3)a	0.99±(0.1)b	31.89±(0.4)d
MSB-Salt2	77.64±(1.6)b	91.17±(2.7)b	0.08±(0.003)b	78.4±(8.5)a	0.66±(0.04)d	28.45±(3.7)a

Data show the mean values plus their standard deviation. RGR Relative Growth rate ($g.g^{-1}.day^{-1}$). RWC relative water content (%). Total chlorophyll mg/mg plant fresh weight) and total carbohydrate (µg/mg plant fresh weight). Values for the same time-point followed by different letters are significantly different at $p<0.05$,

Table 2

Quantification of in situ localization of H_2O_2 and O^{2-} in leaves of MSB-treated and untreated tomato plants under salt-stress conditions

		Experimental group		
1 Day	H_2O_2	**DW** 65.7±(4.8)a	**Salt1** 85.3±(4.7)a	**Salt2** 88.3±(2)a
		MSB 47±(8.7)b	**MSB-Salt1** 69.3±(5.4)b	**MSB-Salt2** 51±(6)b
	O_2^-	**DW** 15.8±(7.1)a	**Salt1** 3.3±(2.5)a	**Salt2** 16.3±(3.9)a
		MSB 23±(7.3)a	**MSB-Salt1** 32.4±(6.4)b	**MSB-Salt2** 36.2±(7.3)b
4 Day	H_2O_2	**DW** 42±(1.7)a	**Salt1** 64.8±(4.5)a	**Salt2** 55.7±(3)a
		MSB 36.7±(8)a	**MSB-Salt1** 29±(7.2)b	**MSB-Salt2** 9.3±(0.4)b
	O_2^-	**DW** 12.1±(1.9)a	**Salt1** 3.2±(4.5)a	**Salt2** 14.6(±3)a
		MSB 1.8±(0.3)b	**MSB-Salt1** 22.2±(6.6)b	**MSB-Salt2** 12.8±(8.5)a
7 Day	H_2O_2	**DW** 2.8±(2.3)a	**Salt1** 7.5±(2.2)a	**Salt2** 13.2±(2.3)a
		MSB 1.5±(0.5)a	**MSB-Salt1** 3.9±(1.9)a	**MSB-Salt2** 3±(1.5)b
	O_2^-	**DW** 5.7±(3.7)a	**Salt1** 5.2±(4.6)a	**Salt2** 4.6±(3.4)a
		MSB 7.7±(2.5)a	**MSB-Salt1** 10±(7)a	**MSB-Salt2** 16.9±(2.2)b

Data show the mean values plus their standard deviation. Values within the same time point and salt concentration, followed by different letters are significantly different at $p<0.05$.

Table 3

Damage measures using different variables of MSB-treated and untreated tomato plants after 7 days under salt-stress conditions.

Experimental groups	Electrolyte leakage	Lipid peroxidation
Salt1	52.05±(5.96)a	1.55±(0.13)a
Salt2	63.37±(2.17)b	2.93±(0.3)b
MSB-Salt1	36.13±(8.17)c	1.15±(0.13)c
MSB-Salt2	48.09±(7.81)a	0.96±(0.37)c

Data show the mean values plus their standard deviation. Values within the same time-point followed by different letters are significantly different at $p<0.05$.

Table 4

Changes in ion content of MSB-treated and untreated tomato plants after 7 days under salt-stress conditions.

Experimental group	Tissue	Na^+	K^+	Na^+/K^+	Ca^{2+}
Salt1	Leaf	37.3±(0.3)a	4±(0.05)a	9.4	2.28±(0.002)a
	Stem	17.5±(0.5)a	10.3±(0.2)a	1.7	1.29±(0.0001)a
	Roots	20.2±(0.2)a	6±(0.02)a	3.4	1.01±(0.0001)a
MSB-Salt1	Leaf	26.3±(0.4)b	4.6±(0.4)b	5.7	2.13±(0.0001)a
	Stem	17.7±(0.4)b	13.1±(0.2)b	1.3	1.81±(0.0001)b
	Roots	19.3±(0.2)b	6.3±(0.03)a	3.05	0.81±(0.001)b
Salt2	Leaf	75.3±(0.6)a	3.4±0.1)a	19.3	3.91±(0.04)a
	Stem	55.4±(0.5)a	9.3±(0.1)a	6.0	1.81±(0.01)a
	Roots	36.4±(0.3)a	7.6±(0.1)a	4.8	1.73±(0.01)a
MSB-Salt2	Leaf	54.5±(0.4)b	4.9±(0.1)b	10.9	3.53±(0.01)b
	Stem	46.1±(0.4)b	9.9±(0.1)b	4.7	1.33±(0.001)b
	Roots	37.6±(0.4)b	9.1±(0.8)b	4.1	1.64±(0.01)b

Data show the mean values plus their standard deviation. Values within the same salt-concentration and tissue followed by different letters are significantly different at $p<0.05$.

Figure legends

Fig. 1. Analysis of several photosynthetic parameters in MSB-treated and untreated tomato plants under normal and salt stress conditions. Discontinuous lines represent the average values of DW and MSB pre-treated plants without salt stress. Values for the same time-point followed by different letters are significantly different at $p<0.05$.

Fig. 2. Analysis of stomatal aperture under optical microscopy values of MSB-treated and untreated tomato plants under normal and salt stress conditions. Data obtained after 3 days and 7 days of salt exposure are shown. Values for the same time-point followed by different letters are significantly different at $p<0.05$.

Fig. 3. Analysis of chlorophyll fluorescence values of MSB-treated and untreated tomato plants under normal and salt stress conditions. Values for the same time-point followed by different letters are significantly different at $p<0.05$.

Fig. 4. Antioxidant status of MSB-treated and untreated tomato plants under normal and salt stress conditions. Enzymatic and non-enzymatic activities were analysed. Values for the same time-point followed by different letters are significantly different at $p<0.05$.

Fig. 5. Expression analysis of genes involved in ion homeostasis in roots, stem and leaves of MSB-treated and untreated tomato plants under salt stress conditions. Levels of the different mRNA species were relativized to DW control. Bars with different letters in the same tissue are significantly different at $p<0.05$. *NHX2*: Na+/H+ antiporter 2; *NHX4*: (Sodium/potassium)/proton exchanger 4; *SOS1*: plasmalemma Na+/H+ antiporter; *HKT1.2*: sodium transporter HKT1.2.

Fig. 6. Kinetics of proline accumulation in leaves of MSB-treated and untreated tomato plants under normal and salt stress conditions. Values for the same time-point followed by different letters are significantly different at $p<0.05$.

Fig. 7. Kinetics of ABA accumulation in leaves of MSB-treated and untreated tomato plants under normal and salt stress conditions. Values for the same time-point followed by different letters are significantly different at $p<0.05$.

Fig. 8. Hypothetical schema of the effects of MSB on defence mechanisms against salt stress in tomato plants. MSB promotes tolerance to NaCl by acting at different levels: Na^+/K^+ homeostasis; antioxidant defences; photosynthesis rate; osmotic and water balance. Abbreviations: H^+-ATPase. plasmalemma-bound H^+-ATPase; SlSOS1. tomato plasma membrane Na^+/H^+ antiporter protein.

Supplementary Fig. S1. Visual appearance of MSB-treated and untreated tomato plants under normal and salt stress conditions.

Supplementary Fig. S2. Effects of MSB on the in situ accumulation of H_2O_2 and superoxide radicals. H_2O_2 (A-C panel) and superoxide radical (D-F panel) in tomato plant leaves under normal and salt-stress conditions. A and D show leaves at 1 day after salt addition; B and E at 4 days after salt addition; and C and F at 7 days hours after salt addition. The average and standard deviation of three independent experiments of each experimental group are indicated in brackets.

Supplementary Fig. S3. Summary of the extraction protocol and analysis of ABA in tomato plants.

Supplementary Table 1. Amplification primers for gene expression analyses.

Fig. 1.

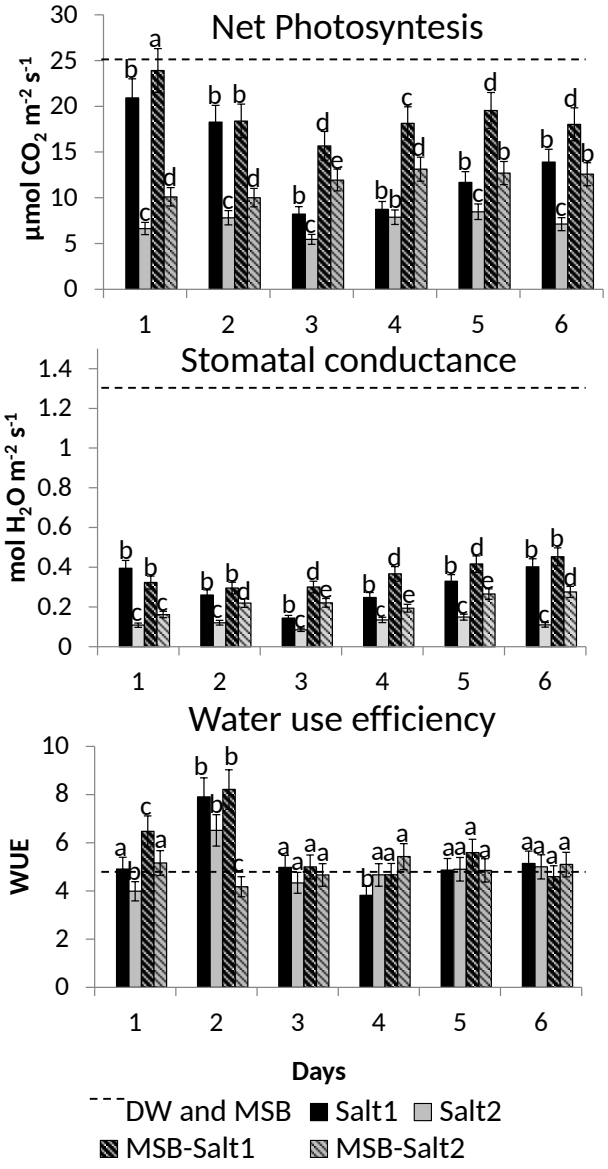

Net Photosyntesis
µmol CO2 m-2 s-1
Stomatal conductance
mol H2O m-2 s-1
Water use efficiency
WUE
Days
DW and MSB
Salt1
Salt2
MSB-Salt1
MSB-Salt2

Fig.2.

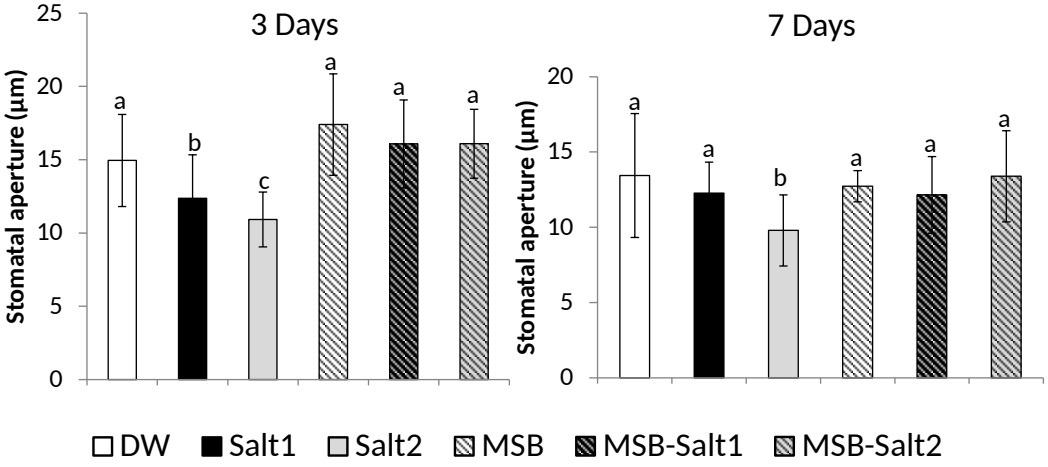

3 Days
7 Days
Stomatal aperture (µm)
Stomatal aperture (µm)
25
20
15
10
5
0
20
15
10
5
0
a
b
c
a
a
a
a
a
b
a
a
a
DW
Salt1
Salt2
MSB
MSB-Salt1
MSB-Salt2

Fig. 3

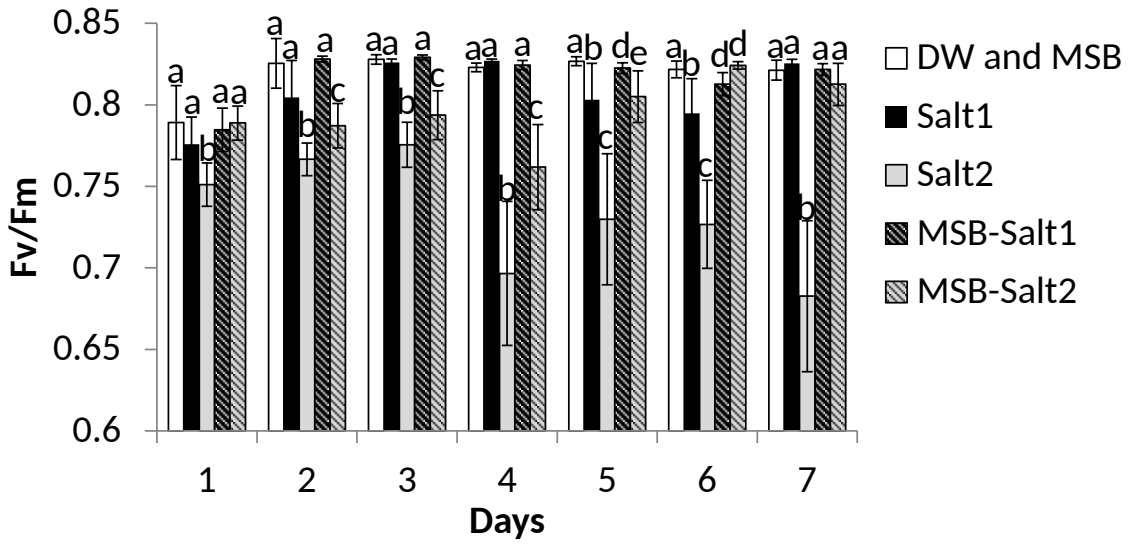

0.85
0.8
0.75
0.7
0.65
0.6
Fv/Fm
1
2
3
4
5
6
7
Days
DW and MSB
Salt1
Salt2
MSB-Salt1
MSB-Salt2

Fig. 4

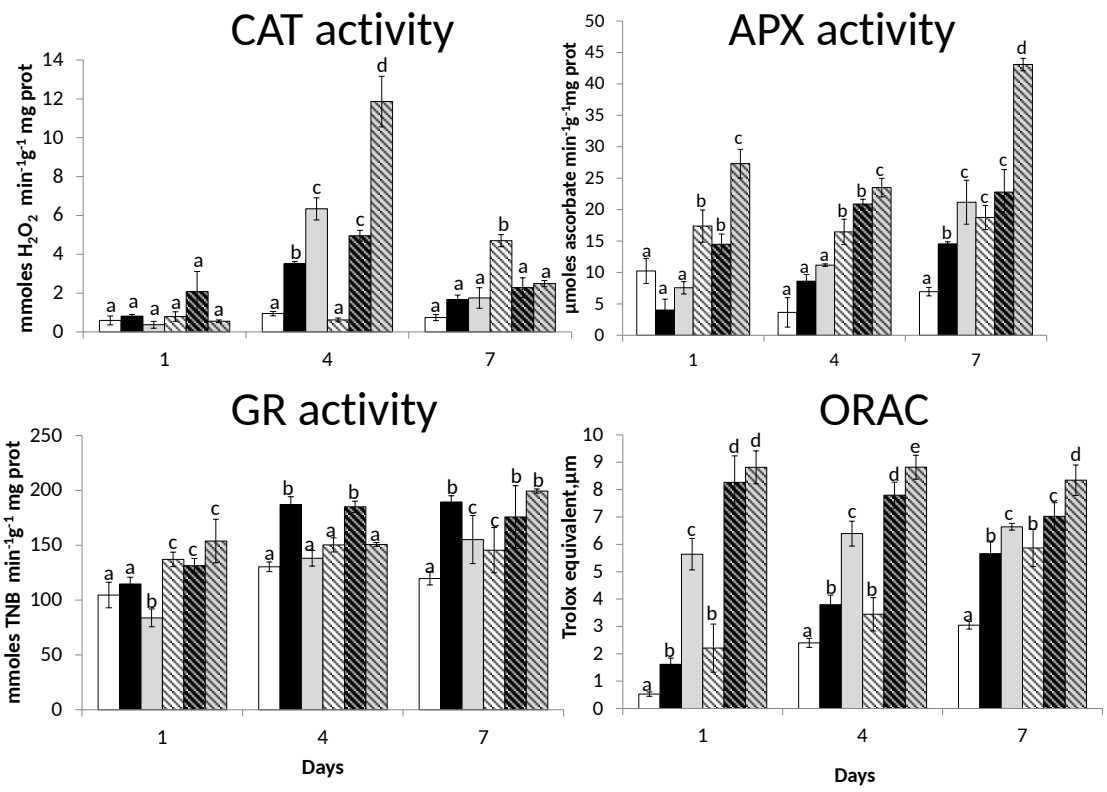

CAT activity
mmoles H_2O_2 $min^{-1}g^{-1}$ mg prot
APX activity
µmoles ascorbate $min^{-1}g^{-1}$mg prot
GR activity
mmoles TNB $min^{-1}g^{-1}$ mg prot
ORAC
Trolox equivalent,µm
Days
Days

Fig.5

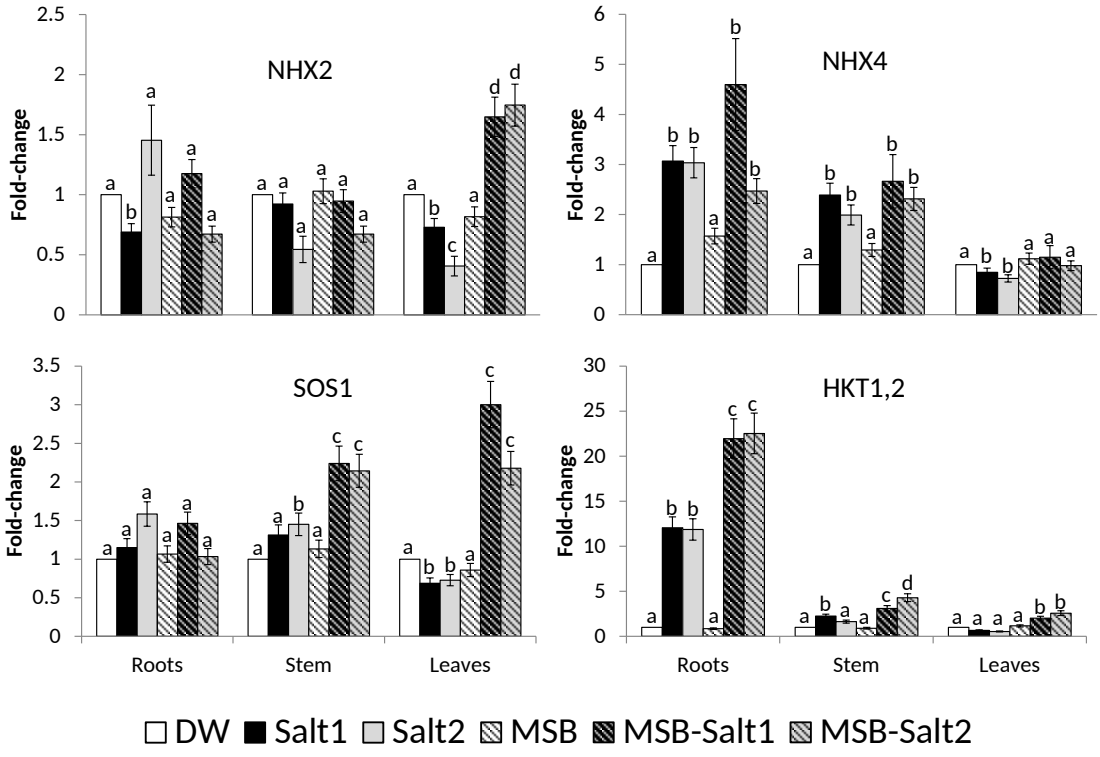

NHX2
NHX4
SOS1
HKT1,2
Fold-change
Roots
Stem
Leaves
DW
Salt1
Salt2
MSB
MSB-Salt1
MSB-Salt2

Fig.6

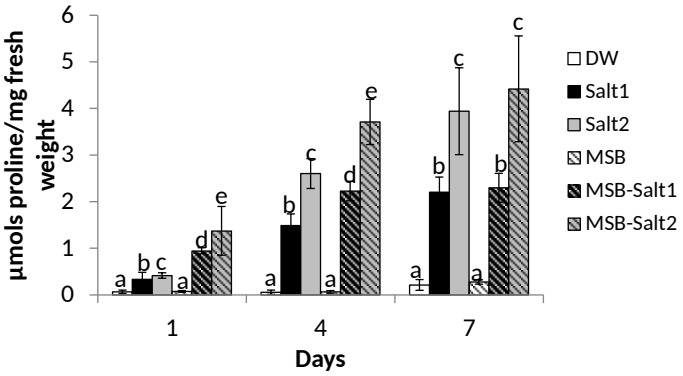

µmols proline/mg fresh weight
6
5
4
3
2
1
0
a
b c
a
d
e
a
b
c
a
d
e
a
b
c
a
b
c
1
4
7
Days
DW
Salt1
Salt2
MSB
MSB-Salt1
MSB-Salt2

Fig. 7

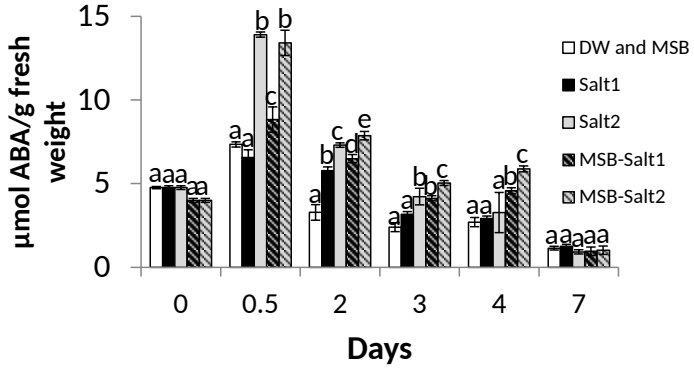
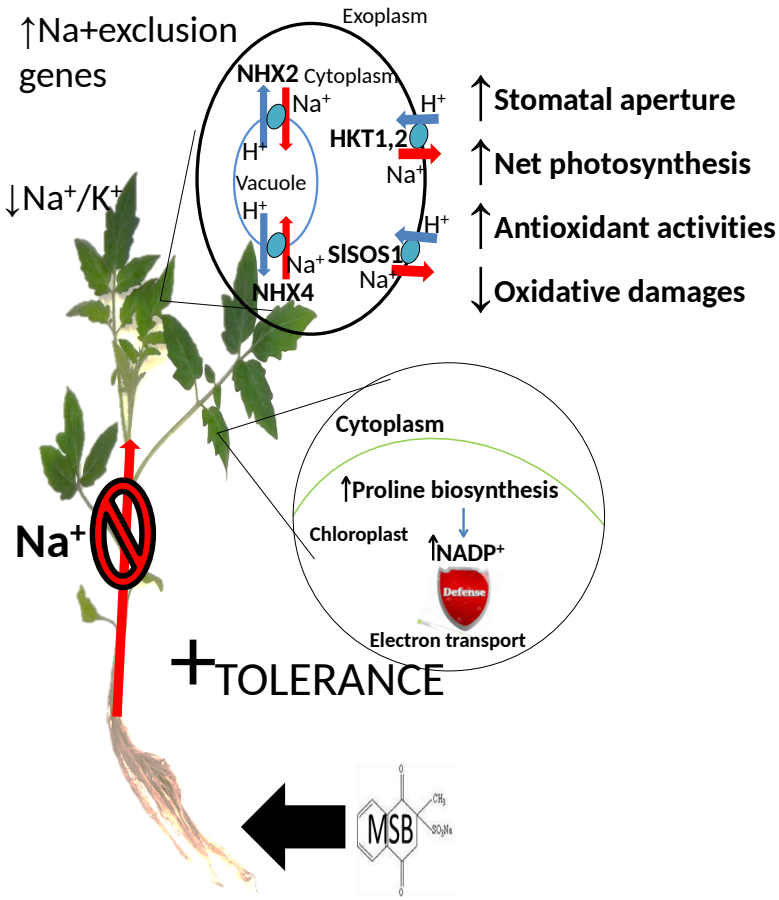
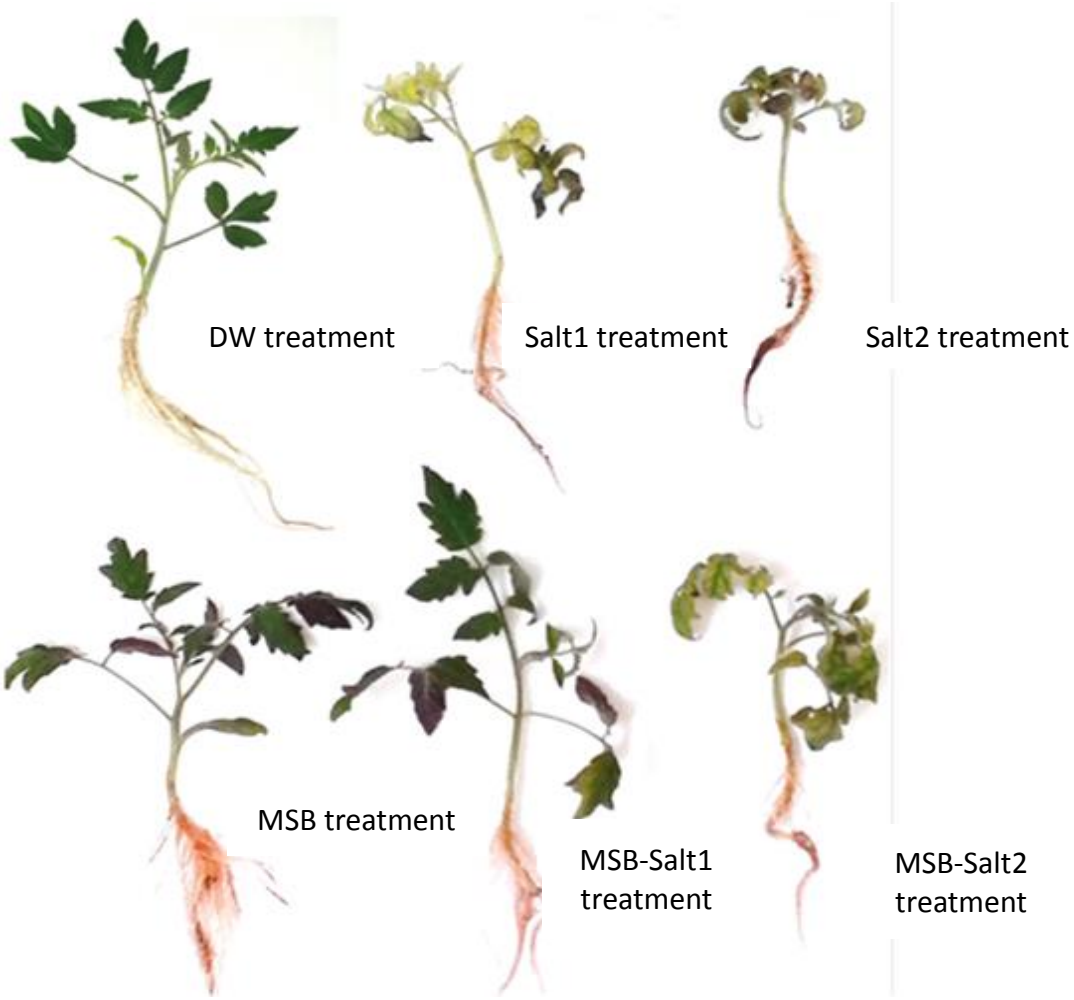
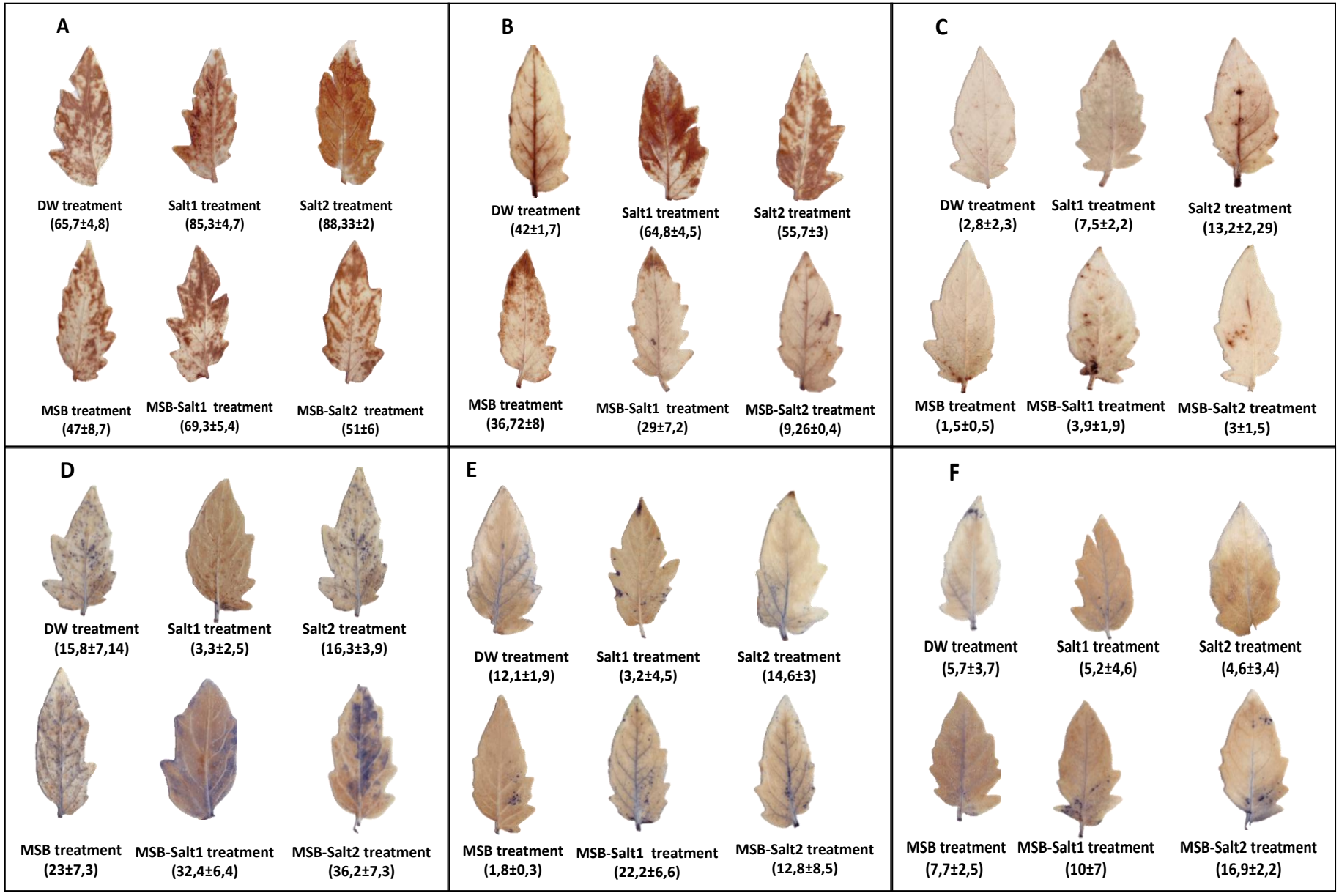

µmol ABA/g fresh weight
15
10
5
0
0
0.5
2
3
4
7
Days
DW and MSB
Salt1
Salt2
MSB-Salt1
MSB-Salt2

Fig. 8


↑Na+exclusion genes
↓Na+/K+
Exoplasm
NHX2
Cytoplasm
Na+
H+
Vacuole
H+
Na+
NHX4
HKT1,2
H+
Na+
SlSOS1
H+
Na+
↑Stomatal aperture
↑Net photosynthesis
↑Antioxidant activities
↓Oxidative damages
Cytoplasm
↑Proline biosynthesis
Chloroplast
↑NADP+
Defense
Electron transport
Na+
+TOLERANCE
MSB

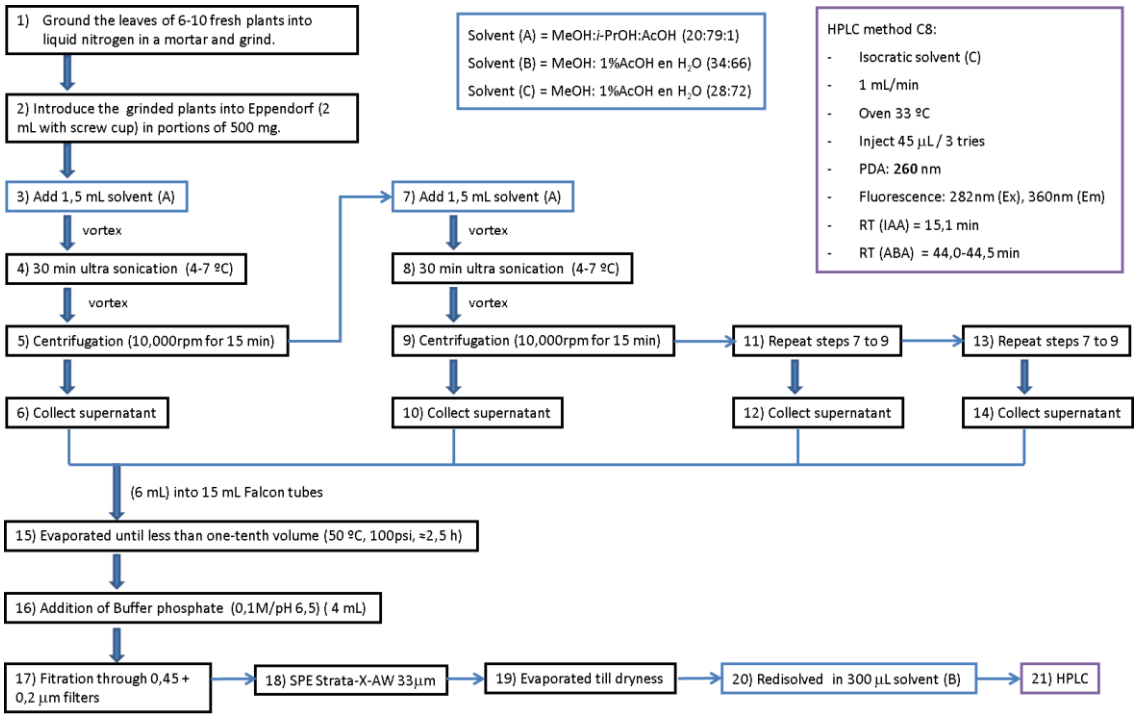
Supplementary Figure S1




A
DW treatment (65,7±4,8)
Salt1 treatment (85,3±4,7)
Salt2 treatment (88,33±2)
MSB treatment (47±8,7)
MSB-Salt1 treatment (69,3±5,4)
MSB-Salt2 treatment (51±6)
B
DW treatment (42±1,7)
Salt1 treatment (64,8±4,5)
Salt2 treatment (55,7±3)
MSB treatment (36,72±8)
MSB-Salt1 treatment (29±7,2)
MSB-Salt2 treatment (9,26±0,4)
C
DW treatment (2,8±2,3)
Salt1 treatment (7,5±2,2)
Salt2 treatment (13,2±2,29)
MSB treatment (1,5±0,5)
MSB-Salt1 treatment (3,9±1,9)
MSB-Salt2 treatment (3±1,5)
D
DW treatment (15,8±7,14)
Salt1 treatment (3,3±2,5)
Salt2 treatment (16,3±3,9)
MSB treatment (23±7,3)
MSB-Salt1 treatment (32,4±6,4)
MSB-Salt2 treatment (36,2±7,3)
E
DW treatment (12,1±1,9)
Salt1 treatment (3,2±4,5)
Salt2 treatment (14,6±3)
MSB treatment (1,8±0,3)
MSB-Salt1 treatment (22,2±6,6)
MSB-Salt2 treatment (12,8±8,5)
F
DW treatment (5,7±3,7)
Salt1 treatment (5,2±4,6)
Salt2 treatment (4,6±3,4)
MSB treatment (7,7±2,5)
MSB-Salt1 treatment (10±7)
MSB-Salt2 treatment (16,9±2,2)

Supplementary Figure S 3. Summary of the extraction protocol and analysis of ABA in tomato plants.

Schematic extraction diagram to analyze plant material using HPLC.



Bibliography:

1) Ilva Nakurte, Anete Keisa, Nils Rostoks: **Development and Validation of a Reversed-Phase Liquid Chromatography Method for the Simultaneous Determination of Indole-3-Acetic Acid, Indole-3-Pyruvic Acid, and Abscisic Acid in Barley (Hordeum vulgare L.).** *Journal of Analytical Methods in Chemistry* Volume **2012**, Article ID 103575, 6 pages.
2) Maren Müller, Sergi Munné-Bosch: **Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry**. *Plant Methods* **2011** 7:37.
3) Yi Tang, Li Wang, Chao Ma, Ji Liu, Bin Liu, Huanxiu Li: **The Use of HPLC in Determination of Endogenous Hormones in Anthers of Bitter Melon.** *Journal of Life Sciences* **2011**, *5*, 139-142.

Supplementary Table 1. Amplification primers for gene expression analyses

Oligos	**Sequence 5´-3´**
SlHKT1.2f	TGAGCTAGGGAATGTAATAAACG
SlHKT1.2r	AGAGAGAAACTAACGATGAACC
SlSOS1f	TCGAGTGATGATTCTGGTGG
SlSOS1r	ATCACAGTGTGGAAAGGCT
LeNHX2f	CCTTTGAGGGGAACAATGG
LeNHX2 r	CATCTTCATCTTCGTCTCC
LeNHX4f	TGGTGGGCAGGTTTGATGAGAG
LeNHX4r	TGTGGTGGCAGCAGGAGACTTA
LeCACf	CCTCCGTTGTGATGTAACTGG
LeCACr	ATTGGTGGAAAGTAACATCATCG
LeEXPRESSEDf	GCTAAGAACGCTGGACCTAATG
LeEXPRESSEDr	TGGGTGTGCCTTTCTGAATG