Effects of salicylic acid on hormonal cross talk, fatty acids profile, and ions homeostasis from salt-stressed safflower

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ABSTRACT
It has been shown that salicylic acid (SA) acts as an endogenous signal molecule responsible for inducing stress tolerance. The aim of the present work is to investigate the effect of sodium chloride (0, 100, and 200 mM) and exogenous SA (1 mM) on some biochemical and molecular responses of safflower. Results revealed that K⁺, Ca²⁺, indol-3-acetic acid (IAA), and gibberellic acid (GA) contents decreased under salinity however, Na⁺ content, and SOS1 and NHX1 genes expression increased. Further, palmitic and oleic acids contents decreased, while stearic, linoleic and linolenic acids content increased under salinity. Exogenous SA had a positive effect on K⁺, Ca²⁺, IAA, and GA contents, but decreased Na⁺ content. In addition, SA induced expression of SOS1 and NHX1 genes in all plants. Our data indicate that SA helps safflower to better cope with salinity. The results provide new insights to mechanisms that help regulate salinity resistance in safflower. SA may be considered as a foliar application to ameliorate salinity effects, due to its low price and availability.

Highlights
• SA helps safflower plants to better cope with saline conditions by the expression of SOS1 and NHX1
• SA regulates various aspects of plant responses to salt stress through signaling cross-talk with other hormones
• Exogenous SA in salt-stressed safflower showed a large increase in desaturation of fatty acids in membrane

Introduction
Climate change is one of the major challenges of our time and the socio-economic consequences are alarming. It is predicted that global climate change will alter environmental parameters such as rainfall distribution with e.g. less rainfall in some regions, which in turn may increase the salinity of soils. As a consequence of increased soil salinity, plant nutrient availability reduces, and therefore, growth and productivity decreases (Zahedi et al. 2012). Salinity is also a significant problem in safflower (Carthamus tinctorius L.). Productivity in arid and semi-arid areas. Safflower is a multi-purpose crop grown for its purpose crop grown for its

To the best of our knowledge, there is no information on how to improve tolerance to salt stress in plants. However, SA has been suggested as potential mechanisms of salt tolerance in plants (Ashraf et al. 2010). SA regulates various aspects of plant responses to stress through extensive signaling cross-talk with other hormones (Jayakannan et al. 2013). The ability of exogenous SA to enhance antioxidant protection, increase the accumulation of osmolytes, and maintain optimum Na⁺/K⁺ ratio under saline conditions has been suggested as potential mechanisms of salt tolerance in plants (Ashraf et al. 2010; Hayat et al. 2010). These qualities make SA an ideal chemical to increase resistance to salt stress in plants.

Our previous works indicated that SA minimizes the negative effects of salt stress by improving growth parameters, accumulation of compatible solutes, and increasing antioxidant activity, and therefore, could be used for partial amelioration of salt stress in safflower (Shaki et al. 2017, 2018). To the best of our knowledge, there is no information...
available so far about the effect of SA on ions homeostasis, hormonal cross-talk and fatty acids compositions in salt-treate
ted safflower plants. Thus, the working hypothesis for this
study was that beneficial effects of SA during salt stress may
be related to up-regulation of sodium/proton transporters
genes and consequent effects on intracellular ionic homeosta-
sis of Na⁺ and K⁺, as well as desaturation of fatty acids in
membrane. Consequently, the aim of this work was to inves-
tigate the impact of SA on some key physiological parameters
in safflower, as well as genes expression. Revealing the mech-
isms underlying salt tolerance of safflower, which is
mediated by SA, might provide a basis to improve safflower
growth and productivity in saline areas.

Materials and methods

Plant cultivation and chemical treatments

Seeds from safflower plants were sown in Tref peat in a green-
house with a 15 h light/9 h dark photoperiod. Seedlings were
transferred to plastic pots (15 cm in diameter, 15 cm deep)
without salicylic acid (SA) (1 mM) for 21 days. Half-strength
Hoagland’s nutrient solution (pH 6.8–7) used as a nutrient
media (Hoagland and Arnon 1950). The SA was dissolved
dissolved in distilled water and approximately 3 ml of the solution
in liquid nitrogen before being stored at

Sampling for gene expression was done 24 and 48 h after
the last treatment and leaves were immediately frozen in
liquid nitrogen before being stored at −70°C. The final har-
est was performed after 21 days of treatment for biochemical
analysis and leaves were sampled, fresh leaf samples from
each plant were stored at −70°C until performing biochemi-
cal analysis.

Determination of Na⁺ content

The oven-dried leaves (0.5 g) were digested with 2 ml of
H₂SO₄ according to the method of Wolf (1982). Sodium in
the digests was determined with a flame photometer (JEN-
WAY PEP 7).

Determination of K⁺ and Ca²⁺ contents

Samples were finely ground, and oven-dried leaves (0.1 g)
were digested with 5 ml H₂SO₄ in digestal system (Nyomora
et al. 1997). The concentrations of K⁺ and Ca²⁺ were analyzed
by ICP-ODS (Vista-MPX).

RNA extraction

Frozen tissue was ground to a fine powder in liquid nitrogen
using a mortar and pestle. Then, total RNA was extracted
before cDNA synthesis according to the manufacturer’s
instructions.

cDNA synthesis

Three micrograms of total RNA was reverse transcribed into
complementary DNA (cDNA) using Revert Aid™ Reverse
Transcriptase (Fermentas, Germany), oligo dT18 and ran-
don hexamer primers (MWG, Germany) in a total volume of
20 µl reaction mixture, according to the manufacturer’s
instructions.

Primer design

The primer pairs for NHXI and SOSI genes were designed
using PRIMER EXPRESS software (Applied Biosystems). The
house keeping gene actin was used as the standard
for checking the quantity and quality of cDNA and/or
RNA templates. Primers used for qRT-PCR are listed in
Table 1.

RT-qPCR analysis

The relative expression level was quantified in comparison
with the house keeping gene b-actin as an internal control
(Buyuk et al. 2016). Quantitative real-time PCR was per-
formed using Applied Biosystems 7500 Real-Time PCR
System (Applied Biosystem/MDS SCIEX, Foster City, CA,
USA), with 10 ng cDNA, 10 µl of SYBR Green I master
mix (Takara, Shiga, Japan), and 200 nM of forward and
reverse primers up to final reaction volumes of 20 µl.
The PCR was performed by an initial denaturation at
95°C for 5 min, followed by 40 cycles of denaturation at
95°C for 10 s, annealing at 60°C for 30 s, and extension
at 72°C for 30 s. The specificity of the PCR products was
examined by melting curve analysis, restrictionendonu-
clidean digestion followed by 12% polyacrylamide gel el-
trophoresis. The genes expression level was expressed
relative to the appropriate control. Serial dilutions of
cDNA were examined to obtain a standard curve for
each primer pair.

Determination of indol-3-acetic acid (IAA)

The method for determination of IAA production was
described by Malik and Singh (1980). Fresh leaf tissue
(0.1 g) was extracted in 3 ml ethanol 96%. The IAA concen-
tration was determined using UV–Vis spectrophotometer at
353 nm.

Determination of gibberellic acid (GA)

Determination of GA was based on the method described by
Berrios et al. (2004). Fresh leaf tissue (0.1 g) was extracted in
3 ml ethanol 96%. The absorbance of the solution was
measured by spectrophotometer at 254 nm. The concen-
tration of GA in the sample was determined using a linear
regression equation of the standard graph.

Extraction and analysis of fatty acids

Samples (1 g) were extracted with chloroform: methanol
(2:1 v/v) following the modified procedure of Bligh and
Dyer (1959). Gas chromatography (GC-17A Shadguz) with DB-Wax column (30 m long, 0.25 mm diameter, and flame ionization detector) was used for fatty acid profile determination. To evaluate the efficiency of the desaturation pathway during salt treatment, the desaturation ratios from oleic to linoleic (ODR: oleic desaturation ratio) and from linoleic to linolenic acid (LDR: linoleic desaturation ratio) were calculated as follows: 

\[
ODR = \frac{(% \text{C18:2} + % \text{C18:3})}{(% \text{C18:1} + % \text{C18:2} + % \text{C18:3})} \times 100
\]

\[
LDR = \frac{(% \text{C18:3})}{(% \text{C18:2} + % \text{C18:3})} \times 100
\]

The magnitude of desaturation ratios represents the amount of substrate which is successfully desaturated from C18:1 to C18:2 and C18:3, and thus measure the desaturating enzymes’ activities (Mondal et al. 2010).

**Statistical analysis**

The experiment was laid out in a completely randomized design (CRD) with three replications. Each data point was the mean of three replicates (n = 3) in each group. Statistical calculations were performed with SPSS (version 18). Tests for significant differences were conducted using analysis of variance (ANOVA) with Duncan’s multiple range tests at the 0.05 level of confidence. The principal component analysis (PCA) and hierarchical cluster analysis (HCA) were performed using the XLSTAT (version 2018.7) and CIMminner, respectively.

**Results**

The effects of increasing level of NaCl on some biochemical and molecular parameters of safflower were determined at 21 days after the start of treatments. Na⁺ accumulation in safflower plants increased when the plants were exposed to salt stress (Table 2). Exogenous SA on salt-stressed plants reduced Na⁺ content about 37.56% in comparison with controls. Further, Na⁺ stress reduced K⁺ and Ca²⁺ contents in plants. However, exogenous SA treated plants had higher K⁺ content under saline and non-saline conditions. In salt-stressed plants, Ca²⁺ and palmitic acid were found on the left lower side of the biplot. Also, Ca²⁺ and palmitic acid were observed on the right lower side of the biplot. The induction of SOS1 gene expression in salt-treated plants was greater after 48 h. NHX1 gene expression also increased in plants under salt stress (Figure 1(b)) and SA treatment enhanced the amount of expression in all plants. This increase was greater 48 h after the last treatment in salt-treated safflower plants. There was no significant difference between control plants after 24 and 48 h.

Salt stress reduced IAA content in the leaves of safflower in 200 mM NaCl-treated plants when compared with controls (Figure 2). Exogenously applied SA increased IAA content in only salt-stressed plants. The highest amount of IAA content was detected at 200 mM NaCl-treated plants under SA application. Salinity also reduced GA content in salt-treated plants. Exogenous SA increased GA content in both salt-stressed and unstressed plants. This increase was most pronounced in 200 mM NaCl-treated plants, which was almost 2-fold higher than the amount in leaves without SA application.

The fatty acid profiles displayed great quantitative differences in safflower plants (Table 3). Plants showed a remarkable increase in stearic acid (18:0), followed by linoleic (C18:2) and linolenic acids (18:3), but decrease in palmitic (C16:0) and oleic acids (C18:1) under salinity. In terms of saturated fatty acids, exogenous SA increased palmitic acid content in only 100 mM NaCl-treated plants, and decreased stearic acid content in all plants in comparison with controls. Also, in terms of unsaturated fatty acids, exogenous SA increased oleic acid content in all salt-treated plants. However, SA application decreased linoleic acid content in both untreated and salt-treated plants, as well as linolenic acid content in only 200 mM NaCl-treated plants.

In general, NaCl treatment resulted in a remarkable increase of unsaturated fatty acids in safflower. The same pattern was also evidenced by the increase of ODR in salt-treated plants, which reflects the higher efficiency of the desaturation system from linoleic to linolenic acid.

Principal component analysis indicated that principal component 1 (F1) described 64.36% of total variation and principal component 2 (F2) described 25.06% (Figure 3) with a cumulative percentage of 89.42%. PCA allowed for easy visualization of the complex data. The contributors to the principal component of F1 and F2 were compared. LDR, NHX1, SOS1, and linolenic acid were grouped together with positive loading on the right upper side of the biplot. Further, Na⁺, ODR, stearic acid, and linoleic acid were observed on the right lower side of the biplot. Also, Ca²⁺ and palmitic acid were found on the left lower part, whereas K⁺, GA, oleic acid, and IAA were grouped on the left upper part of the biplot. In addition, HCA

**Table 1. Primer sequences used for RT-qPCR in this study.**

| Gene | Product | Forward primer (5’–3’-reverse) | Reverse primer (5’–3’) |
|------|---------|---------------------------------|-----------------------|
| Actin | Actin   | TGCTTGACCAGCTCATCGTGTTGG       | TCCTGCCGACCCATCTGATTT |
| SOS1  | Na⁺/H⁺ antiporter | CACGTTCCAAGAAGGGCGCTGAT   | CACGGCTTGGGCTTCATTGG  |
| NHX1  | Na⁺/H⁺ antiporter | TCGGGGAGGGCTGGTGAAT         | CACCAAAAAACCCGGCTCAG |

**Table 2. Effects of NaCl (0, 100, 200 mM) and salicylic acid on ions content at 21 days after the start of treatments in safflower.**

| Treatments | Parameters (mg/g D.W.) | Na⁺ content | K⁺ content | Ca²⁺ content |
|------------|------------------------|-------------|------------|--------------|
| −SA        | 0                      | 12.31 ± 0.71 d| 18.32 ± 0.72 b| 17.96 ± 0.64 b|
|            | 100                    | 24.22 ± 0.80 b| 15.21 ± 0.65 c| 16.41 ± 0.55 c |
|            | 200                    | 34.29 ± 0.57 a| 8.01 ± 0.68 d| 11.33 ± 0.46 e |
| +SA        | 0                      | 12.29 ± 0.92 d| 22.11 ± 0.44 a| 17.68 ± 0.53 b|
|            | 100                    | 16.26 ± 0.58 c| 19.88 ± 0.51 b| 19.12 ± 0.72 a|
|            | 200                    | 21.41 ± 0.66 b| 14.53 ± 0.78 c| 14.58 ± 0.88 d|

Notes: The groups are −SA (plants with no salicylic acid treatment), +SA (plants sprayed with 1 mM salicylic acid every other day). Data are the means ±SE.
Figure 1. RT-qPCR analyses of A. SOS1 and B. NHX1 genes transcript of safflower plants treated with salinity and SA after 24 and 48 h. The groups are −SA (plants with no SA treatment) and +SA (plants sprayed with 1 mM SA every other day). B-Actin was used as an endogenous control to normalize the data for input RNA difference between the various samples. Columns indicate mean ± SE.

Figure 2. Effects of salinity and exogenous SA on content of auxin (IAA) and gibberellic acid (GA) in safflower plants at 21 days after the start of treatments. The groups are −SA (plants with no SA treatment) and +SA (plants sprayed with 1 mM SA every other day). Columns indicate mean ± SE.
which is a method of cluster analysis, indicated a hierarchy of clusters (Figure 3). This suggests that these parameters had a positive correlation among themselves, and SA had a positive effect on parameters on the upper side of the biplot.

**Discussion**

Our previous work indicated that growth parameters which were followed by measuring fresh and dry weight of safflower plants were remarkably inhibited under different NaCl concentrations. However, the application of SA improved the negative effect of salinity by increasing plant growth especially in 200 mM NaCl-treated plants (Shaki et al. 2017). In the current study, some biochemical and molecular parameters were investigated to better understand the effects of exogenous application of SA on safflower plants in saline conditions. In this investigation, the accumulation of Na⁺ increased in plants due to salt stress, whereas the levels of K⁺ and Ca²⁺ decreased. However, the application of SA lowered the accumulation of Na⁺ content accompanied by an enhanced accumulation of K⁺ and Ca²⁺ in plants (Table 2). However, the mechanism by which SA accomplished this decline in Na⁺ and enhanced K⁺ and Ca²⁺ accumulation needs further research. It is well established that salt tolerance is commonly characterized to enhance Na⁺ exclusion and increase absorption of K⁺ to maintain optimum K⁺/Na⁺ ratio (Malezkadeh 2015). As Ashraf and Harris (2004) assumed, this ratio might be a valid selection criterion for assessing the salinity resistance of different species of plants. Accordingly, potassium acquisition from soil is a critical process for salt tolerance of plants. Our results can be explained in the light of some previous reports in which it has been found that salt stress increased the accumulation of Na⁺ and reduces that of K⁺ and Ca²⁺ in some plant species (Habib et al. 2012; Malezkadeh 2015). Other findings on Arabidopsis indicated an increase in the electrolyte leakage, which is mainly related to K⁺ efflux from plant cells, under osmotic stress. However, reduction of that was observed by the application of SA on plants (Jayakannan et al. 2013). It seems that the ability of SA to ameliorate the negative effects of salt stress on growth may be due to a decrease of electrolyte leakage and the increase of accumulation of ions in plants.

Maintenance of ion homeostasis in the cytosol is important for plants in stress conditions. Salinity has been reported to increase the activity and gene expression of Na⁺ transporters such as SOS1 and NHX1 in cells (Chen et al. 2010). Previous studies indicated that salt acclimation in other plant species occurred with Na⁺ transportation into the apoplast and/or its sequestration into the vacuoles, which may further modulate the ion homeostasis in the cytosol (Chen et al. 2010; Katschnig et al. 2015). According to our results, it seems like SA functions in defense system by reducing toxic Na⁺ in cytosol following the increase in SOS1 and NHX1 genes expression. Overall, our results provide the first evidence, to our knowledge, that SA plays a role in enhancing salt tolerance of safflower by increasing salt secretion through increased expression of the SOS1 gene. Moreover, SA could induce increased Na⁺ sequestration into the vacuoles via increasing the expression of NHX1 gene. SA-modulated activity of Na⁺ transporters is closely correlated with the salt tolerance of safflower.

There are some evidence on the role of endogenous auxin in salt stress but, much more information is needed on salt-induced changes in the synthesis and metabolism of auxin. In this experiment, the IAA content decreased at a severe concentration of NaCl (Figure 2). Supporting our results, Naqvi et al. (1986) reported a decrease in IAA content in etiolated Zea mays coleoptiles under salinity. Similarly, IAA content was found to decrease with decreasing water potential in Triticum aestivum plants (Rubin et al. 2002). Further, SA resulted in accumulation of IAA in safflower plants. IAA is known to increase cell wall extensibility (Cleland 1981), and therefore, enhanced levels of IAA under SA treatment may increase leaf cell extensibility, which is involved in maintaining growth under conditions of transiently reduced hydration due to salt stress. Supporting this idea, Li et al. (2003) reported that increased levels of IAA due to exogenous gibberellinic acid delayed the biosynthesis of lignin and induced more growth in Myrica rubra plants.

Our results clearly indicate that overall gibberellin levels in safflower plants were affected by NaCl stress. Similarly, it has been proved that desiccating excised lettuce leaves indicated a rapid decline in gibberellin-like activity (Aharoni et al. 1977). Further, exogenous application of GA reduced the adverse effects of salinity on the growth and productivity of rice (Prakash and Prathapesan 1990), Sorghum (Amzallag et al. 1990), Sorghum (Amzallag et al. 1990), and soybean (Hamayun et al. 2010). It is also reported that SA may have a role in some of the physiological processes associated with GA, since the exogenous application of SA was able to improve seed germination in Arabidopsis thaliana under salt stress conditions (Alonso-Ramirez et al. 2009). These data support the idea that SA can have an important role in GA biosynthesis and action and that some of the physiological effects of this hormone may be mediated by GA. In summary, our results show the existence of cross-talk between these two hormones in stress conditions, showing another junction in the complex mechanism of hormone interactions.

Salinity also modified fatty acids composition in safflower, which is considered to be very critical in stress tolerance (Azachi et al. 2002). In this experiment, increased content of linoleic and linolenic acids was observed under salinity. Further, a redirection of the lipidic metabolism towards the synthesis of unsaturated fatty acids was obtained (Table 3).

**Table 3. Effects of NaCl (0, 100, 200 mM) and salicylic acid on fatty acids content at 21 days after the start of treatments in safflower.**

| Fatty acids   | 0 mM −SA | +SA         | 100 mM −SA | +SA         | 200 mM −SA | +SA         |
|---------------|----------|-------------|------------|-------------|------------|-------------|
| Palmitic acid | 16.22 ± 0.72a | 16.41 ± 0.81a | 12.96 ± 0.98c | 13.28 ± 0.68b | 12.41 ± 0.74d | 12.28 ± 0.77d |
| Stearic acid  | 1.91 ± 0.12d | 1.66 ± 0.11e | 2.41 ± 0.26a | 2.11 ± 0.23c | 2.62 ± 0.48a | 2.23 ± 0.14b | 2.09 ± 0.16c |
| Oleic acid    | 6.83 ± 0.62d | 6.77 ± 0.91a | 6.22 ± 0.54d | 6.52 ± 0.44c | 5.46 ± 0.72e | 5.66 ± 0.68b |
| Linoleic acid | 10.09 ± 0.82d | 9.88 ± 0.74a | 11.83 ± 0.89b | 10.44 ± 0.91c | 12.25 ± 1.11a | 10.03 ± 0.92d |
| Linolenic acid| 23.63 ± 1.01d | 23.57 ± 1.04d | 24.97 ± 0.94c | 24.89 ± 0.71c | 30.18 ± 1.08a | 28.34 ± 0.82b |
| ODR          | 83.16 | 83.17 | 85.54 | 84.42 | 88.60 | 85.21 |
| LDR          | 70.08 | 70.46 | 67.85 | 70.45 | 71.13 | 73.86 |
Thus, such increase in unsaturated fatty acids contents could be related to the importance of maintaining a high degree of unsaturation to control membrane fluidity crucial for proper functions of the plasma membrane (Azachi et al. 2002).

Supporting our results, López-Pérez et al. (2009) reported increased unsaturation index in *Brassica oleracea* plants under salt stress. Similarly, increased level of unsaturation was observed in *A. thaliana* under drought stress (Gigon et al. 2004). They showed that drought stress caused an increase in linolenic acid content, which could result from the activation of desaturase activities. In addition, salt-induced harmful effects on fatty acids composition were alleviated by SA. Exogenous SA caused considerable change in key saturated and unsaturated fatty acids of safflower. It can be assumed that SA, to some extent, modulates stress impacts on fatty acids compositions of safflower. Similarly, the role of SA in amelioration of stress effects on fatty acids profile of sunflower (*Helianthus annuus* L.) was investigated by other researchers, which can support our results (Noreen and Ashraf 2010; Ebrahimian and Bybordi 2012).

The PCA and HCA grouping allows certain parameters to be identified as those responsible for plant behavior changes under stress conditions. PCA is used to extract the important information from a multivariate data table and to express this information as a set of few new variables called principal components. The information in a given data set corresponds to the total variation it contains. To investigate the contributors to the principal component, the loadings in F1 and F2 were compared (Figure 3). It is suggested that parameters with vectors in the same directions had a positive correlation among themselves, and a negative correlation with other parameters, indicating that impacts of salinity on these parameters may be different from the effects on the other measured parameters. Further, it was observed that SA had a positive effect on most measured parameters under 100 and 200 mM NaCl treatments.

**Conclusion**

Taken together, our data revealed that SA, to some extent, helps safflower plants to cope with saline conditions. It is supported by the expression of the key genes responsible in ion homeostasis, such as *SOS1* and *NHX1*, as well as the desaturation of fatty acids in membrane. Further, the results indicate the existence of cross-talk between plant hormones, showing another junction in the complex mechanism of hormone interactions. Our results provide new insights towards identifying the underlying mechanisms of salt tolerance in safflower. Further, SA may be considered as a foliar application to ameliorate salinity effects, due to its low price and availability.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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