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Central role of salicylic acid in resistance of safflower (*Carthamus tinctorius* L.) against salinity

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ABSTRACT

The effects of salicylic acid (SA) on growth parameters and enzyme activities were investigated in salt-stressed safflower (*Carthamus tinctorius* L.). Twenty-five days after sowing, seedlings were treated with NaCl (0, 100, and 200 mM) and SA (1 mM), and were harvested at 21 days after treatments. Results showed that some growth parameters decreased under salinity, while malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content, phenolic compounds, and some enzyme activities increased. SA application increased some growth parameters, MDA and H_2O_2 content, and enzyme activities except catalase (CAT), which was different from the other enzymes and SA significantly reduced CAT activity in plants. These results suggest that SA-induced tolerance to salinity may be related to regulation of antioxidative responses and H_2O_2 level. Our study suggested that the resistant safflower can direct reactive oxygen species from a threat to an opportunity by using SA. Therefore, exogenous application of SA played this role through regulation of the antioxidant system.

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KEYWORDS
Safflower; salicylic acid; salinity; resistance

Introduction

Salinity is an environmental factor that limits crop production and soil fertility in many areas in the world (Silveira et al. 2001; Khan and Panda 2007; Aftab et al. 2011). It causes deficiency of some nutrients and an increase in Na^+ levels in plants (Grattan and Grieve 1998; Ramezani et al. 2012; Zahedi et al. 2012). Salinity is a significant problem in safflower (*Carthamus tinctorius* L.) production in many areas in the world. Safflower is a herbaceous plant that belongs to Asteraceae family (Sadeghi et al. 2013). It has been widely cultivated for its flowers and oil (Işigigür et al. 1995). Safflower is known as a moderately salt-tolerant plant (Bassil and Kaffka 2002). Its salt tolerance is more than that exhibited by some other oilseed plants, but like the majority of the cultivated plants, its growth and yield decrease depending on the salinity level.

Plants generally protect themselves against salinity by many strategies such as the production of reactive oxygen species (ROS), synthesis of defense proteins, and accumulation of some secondary metabolites (Sorahinobar et al. 2016). Several factors associated with salinity can lead to an increase in ROS formation, which can damage membrane lipids, nucleic acids, and proteins (Liang et al. 2003; Patade et al. 2011). On the other hand, specific levels of ROS are essential and lead to an increase in antioxidative protection (Stevens et al. 2006; Vital et al. 2008).

To prevent from damaging effects of ROS, plants possess antioxidative mechanisms to scavenge excess ROS in plant cells. Several antioxidative enzymes, including superoxide dismutase (SOD), peroxidase (POX), and polyphenol oxidase (PPO), are involved in detoxification of ROS (Zhang and Kirkham 1996; Lee and Lee 2000). Additionally, in order to protect against overproduction of ROS, plants synthesize some low-molecular compounds, such as ascorbate and phenolic compounds (Kim et al. 2007; Kováčik and Bačkor 2007). Phenolic compounds serve as the potent non-enzymatic antioxidant and therefore extinguish oxidative free radicals in plant cells (Rice-Evans et al. 1996; Grassmann et al. 2002). The phenylpropanoid pathway is one of the important pathway in plant cells. Phenylalanine ammonia-lyase (PAL) is another important enzyme in plant cells, which acts in this pathway and is involved in the synthesis of compounds such as phenolics and lignin (Hemm et al. 2004).

Manipulation of crop production with some chemical compounds or growth regulators has a main role in development of plants. The plant yield under stress condition can be increased by exogenous application of some growth regulators such as salicylic acid (SA). SA, a well-known signaling messenger, is able to reduce symptoms of several environmental stresses in plant tissues (Horváth et al. 2007; Hayat et al. 2010). It acts as a protector against various stresses and has a key role in defense mechanism in plants (Klessig and Malamy 1994; Gunes et al. 2007). It has been shown that SA is amongst the most important compounds involved in plant resistance against salinity.

Safflower (*C. tinctorius* L.) is an important oilseed plant whose growth and development can be affected by salt stress. Here, we performed a study to compare the effects of exogenous application of SA on safflower plants to distinguish the effects of some physiological and biochemical parameters in response to salinity and to assess the possibility of improving salt tolerance in safflower plants.

Materials and methods

Plant cultivation and chemical treatments

Seeds of safflower (*C. tinctorius* L.) CV. Goldasht were obtained from the Seed and Plant Improvement Institute of

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Karaj, Iran. Seeds were surface sterilized for 5 min in 10% sodium hypochlorite solution and then in 96% ethanol for 1 min and thoroughly washed with distilled water. Seeds were sown in Tref peat in a greenhouse with a 15 h light/9 h dark photoperiod at 27 ± 2°C temperature. Plastic pots were filled with perlite and seedlings were thinned to 5 per pot 25 days after sowing. Each pot was considered as one replicate and there were four replicates for each treatment.

Sodium chloride (0, 100, and 200 mM) and SA (1 mM) (Aftab et al. 2011) were applied for 21 days during vegetative growth of plants. Each pot was treated with different salt concentration with 100 ml of half-strength Hoagland's nutrient solution (pH = 6.8–7.0) at alternative days (Hoagland and Arnon 1950). The nutrient solution was replaced every alternate day with fresh one. A foliar spray of SA (Aftab et al. 2011) was applied uniformly to the plants three times (at 1-week intervals), using an atomizer. The final harvest was performed after 21 days of treatment and leaves were collected. Five plants per treatment were used for analyses in all the experiments. Then they were oven-dried at 60°C for 3 days for the determination of dry weight (five replicates per treatment). Besides, fresh leaf samples from plants were stored at −70°C until the biochemical analysis.

SA quantification by HPLC

According to the method of Wen et al. (2005), sample preparation for extraction and quantification of SA was performed. Treated and control leaf tissues (1 g) were extracted with methanol/water/trifluoroacetic acid (TFA) (50:50:0.1) mixed solvent, and the volume of the turbid fluid was adjusted to 10 ml. The mixture was centrifuged at 3000 rpm for 5 min, and filtered through a nylon filter. Three replications (per each treatment) were used for the estimation of total SA content. Chromatographic separations were performed on an Agilent 1200 series high-performance liquid chromatography (HPLC), including a quaternary pump and a degasser equipped with a G1321A fluorescence detector and a G1315D diode array detector. Separation process was carried out on a C18 column (250 9 4.6 mm, with 5.0 m particle size) from Waters Company (Massachusetts, USA). The flow rate of the mobile phase was kept at 0.5 ml/min. Phase A was water containing 0.02% TFA, and phase B was methanol containing 0.02% TFA. The column temperature was controlled at 25°C. Injection volume was 10 µl and samples were detected at 305 nm (Wen et al. 2005).

Determination of malondialdehyde content

Malondialdehyde (MDA) content in this experiment was measured in relation to thiobarbituric acid (TBA) reactive substances (Heath and Packer 1968). Leaf samples (0.2 g) of plants were homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged for 20 min at 10,000×*g*. To 1 ml of the aliquot of the supernatant, 4 ml of 20% (TCA) containing 0.5% TBA was added. The mixture was heated at 95°C for 30 min and was quickly chilled in an ice bath. After that the mixture centrifuged at 10,000×*g* for 15 min. The absorbance of the mixture was evaluated at 532 and 600 nm. The value for non-specific absorption at 600 nm was then subtracted from that of 532 nm. The concentration of MDA content was calculated by using an absorption coefficient of 155 mM^{-1} cm^{-1}.

Determination of hydrogen peroxide content

For determination of H_2O_2 (hydrogen peroxide) levels, plant materials (0.5 g) were homogenized in 1 ml of 0.1% (w/v) TCA and centrifuged for 15 min at 12,000×*g* (Velikova et al. 2000). An estimated 0.5 ml of the extract was added to 1 ml of 1 M potassium iodide (KI) and 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0). Eventually, the absorbance was recorded at 390 nm and then H_2O_2 content was calculated using a standard curve.

Determination of antioxidant enzymes' activity

Plant leaf tissues were homogenized at 4°C in 1 M Tris-HCl (pH 6.8) to estimate different enzyme activities. The Tris-HCl buffer contained 5 mM 1.4 dithiotheritol (DTT), 0.5 mM NaCl, and 5 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 13,000×*g* (J2-21 M, Beckman, Palo Alto, USA) for 30 min at 4°C. The obtained supernatant was kept at −70°C and used for enzyme assays and protein determination. A UV–Vis spectrophotometer (UV-160, Shimadzu, and Tokyo, Japan) was used for detecting enzyme activity. Protein was determined according to the Bradford assay (Bradford 1976), using bovine serum albumin (BSA) as standard.

Estimation of SOD (EC 1.15.1.1) activity was performed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) in a reaction mixture (Giannopolitis and Ries 1977). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.5), 75 µM NBT, 75 µM riboflavin, 0.1 mM EDTA, 13 mM L-methionine, and 0.1 ml of enzyme extract. For 18 min, the reaction mixture was irradiated and absorbance was recorded at 560 nm against the nonirradiated blank. One unit of SOD was defined as the amount of enzyme, which caused 50% inhibition of NBT reduction under the assay condition, and the results were reported in the [Unit mg^{-1} (protein)].

The reaction mixture for POX (EC 1.11.1.7) activity measurement comprised 4 ml of 0.2 M acetate buffer (pH 4.8), 0.2 ml of 20 mM benzidine, 0.4 ml of H_2O_2 (3%), and 50 µl of enzyme extract (Abeles and Biles 1991). The increase in absorbance was recorded at 530 nm. The POX activity was defined as 1 µM of benzidine oxidized per min per mg protein [Unit mg^{-1} (protein)].

The reaction mixture for PPO (E.C. 1.14.18.1) activity measurement contained 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.8), 0.2 ml of 20 mM pyrogallol and 50 µl of enzymes extract at 40°C (Raymond et al. 1993). The increase in absorbance was recorded at 430 nm. The PPO activity was defined as 1 µM of pyrogallol oxidized per minutes per mg protein [unit mg^{-1} (protein)].

Ascorbate peroxidase (APX; EC 1.11.1.1) activity was measured using the reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate, and 10 µl protein extract in a total volume of 1 ml. (Jebara et al. 2005). The reaction was initiated by the addition of H_2O_2, and the concentration of oxidized ascorbate was measured by a decrease in absorbance at 290 nm for 1 min. The concentration of oxidized ascorbate was calculated by using the molar extinction coefficient (2.8 mM^{-1} cm^{-1}). The results were expressed as 1 µM of ascorbate oxidized per minutes per mg protein [Unit mg^{-1} (protein)].

Total catalase (CAT; EC 1.11.1.6) activity was assayed from the H_2O_2 decomposition rate as measured by a decrease

in absorbance at 240 nm (Aebi 1984). The reaction mixture comprised 0.625 ml of 50 mM potassium phosphate buffer (pH 7.0), 75 µl H_2O_2 (3%), and 10 µl of protein extract. CAT activity was expressed as units (1 mol of H_2O_2 decomposed per minutes per mg protein [Unit mg^{-1} (protein)].

Determination of PAL activity

PAL activity was measured based on the rate of cinnamic acid production in reaction (Ochoa-Alejo and Gómez-Peralta 1993). One milliliter of the extraction buffer, 0.4 ml of double distilled water, 0.5 ml of 10 mM L-phenylalanine, and 20 µl of enzyme extract was incubated at 37°C for 1 h. Then, 0.5 ml of HCl (6 M) was added to the solution and the product was extracted using 5 ml of ethyl acetate. Thus, the extracting solvent removed by evaporation. The residue was suspended in 3 ml of NaOH (0.05 M) and the cinnamic acid concentration in it was calculated with absorbance measured at 290 nm with a spectrophotometer. The results were expressed according to each unit of PAL activity that is equal to 1 µmol of cinnamic acid produced per minutes.

Scavenging ability on DPPH radical

In order to free radical scavenging activity measurement, leaf tissue (0.1 g) was extracted in 1 ml methanol 80% (Shimada et al. 1992). Then 0.1 ml of plant extract was added to 3.9 ml of 80 ppm of 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution. The mixture was vigorously shaken and then was allowed to sit at room temperature for 30 min in the dark. The absorbance was recorded at 517 nm and corresponds to the extract ability to reduce the radical DPPH to the yellow-colored diphenylpicrylhydrazine. The free radical scavenging activity was calculated using the following equation:

$$\text{Inhibition of DPPH radical (\%)} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right] \times 100.$$

Total phenolic concentration

Total phenolics were extracted with 80% (v/v) methanol at 70°C water bath for 3 h (Niknam and Ebrahimzadeh 2002). The suspensions of methanolic extraction were filtered, the methanol was removed by vacuum distillation, and the aqueous solutions were used for quantitative determination. Total phenolics were assayed using the Folin–Ciocalteu reagent (Singleton and Rossi 1965), which was slightly modified (Ranganna 1986). An aliquot of 250 µL of extract was added to 2 ml distilled water, 250 µL folin reagent, and 0.5 ml Na_2CO_3 (7%). The solution was adjusted with distilled water to a final volume of 3 ml and thoroughly mixed. After 30 min the absorbance was recorded at 760 nm. Aqueous solutions of gallic acid (0–200 µg ml^{-1}) were used as standards for plotting working curve and leaf total phenolic concentration was expressed as GAE in µg g^{-1} FW.

Statistical analysis

Figure 1 Statistical analysis was performed with a randomized complete block design. Experiments were repeated three times, with three replications in each group. Tests for significant differences among treatments were conducted using analysis of variance (ANOVA) using SPSS (version 18) with Duncan's multiple range tests and P values $\leq .05$ are considered to be significant.

Results and discussion

In the present study, some physiological parameters were investigated to better understand the effects of exogenous application of SA in safflower under salinity. Plant adaptations to salinity are affected by several environmental factors. In our experiment, growth parameters which were followed by measuring FW and DW were remarkably inhibited under different NaCl concentrations in *C. tinctorius* L. and were severe at the concentration of 200 mM. (Figure 1). Reduction of growth under stress have been previously observed in different studies (Jaleel et al. 2007; Shaheen et al. 2013; Merati et al. 2014).

Application of SA in plants improved the negative effect of salinity by increasing leaf growth, especially in 200 mM NaCl-treated plants. It was found that SA treatment had more effect on stress tolerance at severe stress conditions. Promoting effect of SA on growth aspects has been reported in many other crop species (Fariduddin et al. 2003; Shakirova et al. 2003; Khodary 2004; El-Tayeb 2005; Dicko et al. 2006). The increase of growth induced by SA may be due to the induction of antioxidant function and metabolic activity that increase plant tolerance (Wang and Li 2006). It was also reported that SA application in plants is concomitant with the accumulation of active oxygen species (Gunes et al. 2007).

HPLC analysis showed that SA content in leaves of salt-stressed plants (both 100 and 200 mM NaCl) was significantly higher in comparison with controls (Figure 2). Following SA treatment of plants, their SA content was significantly induced, in both salt-stressed and unstressed plants, as compared to the controls. Its content increased about 2 fold in all SA-treated plants.

It was found that salinity causes a significant increase of MDA production in safflower (Figure 3(a)). Exogenous application of SA showed an increase in MDA content in salt-stressed plants but there was no significant difference between 100 and 200 mM NaCl treatments after SA application.

For determination of ROS scavenging capacity, H_2O_2 levels of plants were estimated under stress conditions. Basal H_2O_2 content in salt-stressed plants was higher compared to unstressed plants (Figure 3(b)). Both salt treatments showed a significant increase in H_2O_2 at 21 days. Plants treated with SA enhanced H_2O_2 production significantly in comparison with controls. Peroxidation of lipid in cell membranes under oxidative stress conditions reflects oxidative damage induced by free radicals (Demiral and Türkan 2004). H_2O_2 triggers many defense responses and is involved in SA accumulation in plants (Leon et al. 1995). As reported in other studies (Harfouche et al. 2008; Chao et al. 2010; Wang and Liu 2012), similarly an increase in H_2O_2 level was observed in the present study following SA treatment. Moreover, it was suggested that the promotion of H_2O_2 accumulation by SA is related to the inhibition of enzymes responsible for H_2O_2 scavenging or an increase in SOD activity (Kang et al. 2003; Krantev et al. 2008; Chao et al. 2010; Hayat et al. 2010). This might be a reason for the general increase of H_2O_2 content in plants (Figure 3(b)).

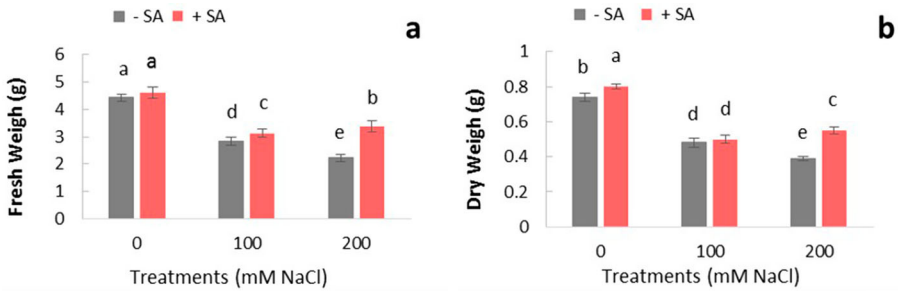

Figure 1. Effects of NaCl (0, 100, and 200 mM) and SA on growth parameters at 21 days after treatments in *C. tinctorius* L. The groups are −SA (plants with no SA treatment), +SA (plants sprayed with 1 mM sodium salicylate three times a week for every other day). Data are the means ± SE. Means with different letters indicate a significant difference at $P \leq .05$ using Duncan's multiple range test.

The differences in antioxidant enzyme activities are depicted in Table 1. During the experimental period, SA treatment caused more induction of these activities except CAT in plants. In salt-stressed plants, all enzyme activities were significantly increased. The highest induction of enzyme activity was observed in PPO in 200 mM NaCl-treated plants with SA application. In our study, results for CAT activity were different from the other enzymes. SA treatment significantly reduced CAT activity in plants especially in 200 mM NaCl treatment.

Plants respond to stress by increasing the antioxidant activity to restore the cellular equilibrium between production and scavenging of ROS (Salah et al. 2011; Bano et al. 2014). Decreased antioxidant activity can lead to overproduction of ROS and lipid peroxidation of cell membranes, which would result in harmful ion leakage.

SOD protects cells from oxidative stress by converting the destructive superoxide radical into molecular oxygen and H_2O_2, which are less dangerous (Scandalios 1993). Hence, it would decrease the risk of hydroxyl radical formation from superoxide. Subsequently, H_2O_2 molecules are degraded by CAT and POX (Xu et al. 2013). According to our results, activity of SOD in safflower was enhanced after exposure to salinity (Table 1). Similarly, increased activity of SOD was reported in *M. pulegium* under drought stress (Hassanpour et al. 2012), and in *Zea mays* (Kaya et al. 2013) and *M. pulegium* L. (Merati et al. 2014) under salt stress. Hence, it is plausible that the increase in SOD activity can be an attempt to overcome the oxidative stress. A similar result was presented in wheat plants treated with SA (Sorahinobar et al. 2016).

POX can catalyze the oxidation of some compounds such as lignin, suberin, and phenolics in the cell wall and also has a main role in removing of H_2O_2 from cytosol and chloroplasts (Jbir et al. 2001; Dicko et al. 2006). These are effective in construction and lignification of the cell wall in stress conditions. Plants increase lignin synthesis in the cell wall in stress condition in order to maintain water (Garcia et al. 1997). Increasing the POX activity induced by salinity was reported in rice (Demiral and Türkan 2004), safflower (Hosseini et al. 2010; Karray-Bouraoui et al. 2011), *Artemisia annua* L. (Aftab et al. 2011), and pennyroyal (Merati et al. 2014). Similar findings were observed in *A. annua* L. treated with exogenous SA in salt-stressed plants (Aftab et al. 2011). In our study, the activities of POX as an ROS scavenger increased in SA-treated plants compared to controls; hence, it could provide a mechanism for enhanced resistance of safflower plants in salinity.

PPO is an enzyme responsible for oxidation of phenolic compounds. PPO activity considerably increased under salinity especially at 200 mM NaCl in safflower (Table 1). Increased PPO activity may reduce the phenolics, thereby protecting the content of Indole-3-acetic acid (IAA) (Merati et al. 2014), and this can enhance cell wall growth. The observation that PPO activity was affected by the application of SA in both salt-stressed and unstressed plants supported the idea that its response can be SA dependent. A similar result was also reported in chamomile plants using exogenous application of SA (Kováčik et al. 2009).

APX and CAT are two other enzymes responsible for removing H_2O_2 from cells (Dewir et al. 2006). In our experiment, APX activity significantly increased under different NaCl concentrations and more induced by SA treatment but as mentioned, results for CAT activity were different. SA treatment significantly reduced CAT activity in plants under 200 mM NaCl treatment (Table 1). H_2O_2 is an integral component of cell signaling cascades (Mittler 2002; Pastori and Foyer 2002). Similar findings were presented in other plants treated with SA (Kováčik et al. 2009; Sorahinobar et al. 2016). It seems that APX and CAT regulations serve to limit excessive H_2O_2 accumulation in the cells.

PAL is a key enzyme in the phenylpropanoid pathway. It should be noted that an increase in PAL activity may be related to the plant defense system through biosynthesis of some metabolites, such as SA, phenols, and lignin in defense pathways (Mandal et al. 2009). This study demonstrated that PAL activity was induced by salinity, while this increase was stronger in plants treated by SA in comparison with controls. Furthermore, SA-induced PAL activity in plants suggests a positive feedback in SA production.

To better understand the underlying mechanisms of resistance against salinity, the activity of PAL was investigated as a key enzyme in the production of SA. Salinity significantly

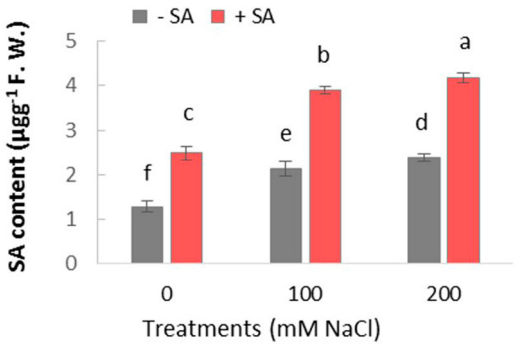

Figure 2. Effect of salinity (0, 100, and 200 mM NaCl) and exogenous application of on SA content in leaves of safflower plants at 21 days after treatments. The groups are −SA (plants with no SA treatment) and SA (plants sprayed with 1 mM sodium salicylate three times a week for every other day). Columns indicate mean ± SE. Means with different letters indicate a significant difference at $P \leq .05$ using Duncan multiple range test.

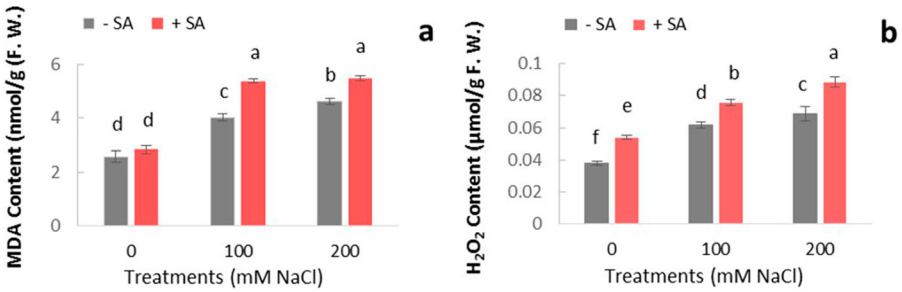

Figure 3. Effect of salinity (0, 100, and 200 mM NaCl) and exogenous application of SA on content of (a) MDA and (b) H_2O_2 in leaves of safflower plants at 21 days after treatments. The groups are −SA (plants with no SA treatment) and +SA (plants sprayed with 1 mM sodium salicylate three times a week for every other day). Columns indicate mean ± SE based on three replicates. Means with different letters indicate a significant difference at $P \leq .05$ using Duncan multiple range test.

increased PAL activity in salt-stressed and unstressed plants (Figure 4). However, no significant difference was observed between 100 and 200 mM NaCl treatments. SA treatment significantly increased PAL activity in both salt-stressed and unstressed plants. This increase was more pronounced in plants treated with SA, under 200 mM NaCl salinity.

Besides the enzymatic antioxidant system, induction of non-enzymatic antioxidant compounds in plants was observed. Our result showed that significant changes in DPPH radical scavenging activity occurred in response to salinity (Figure 5(a)). Besides, SA treatment increased DPPH radical scavenging activity in both salt-stressed and unstressed plants in comparison with controls. This increase in 200 mM NaCl treatment plants was about two-fold of controls.

In our experiment, the phenolic contents significantly increased under salinity (Figure 5(b)). The highest amount of phenolic compounds was observed in plants under 200 mM NaCl with application of SA. Phenolic compounds are potent inhibitors of oxidative stress in cells (Rice-Evans et al. 1996). The key step in the biosynthesis of phenolics, which is converting phenylalanine to trans-cinnamic acid, is controlled by PAL. Phenolics can also cooperate with POX in H_2O_2 scavenging in the cells. According to our results, accumulation of phenolic compounds in salt-stressed plants treated with SA can play an important role in resistance against salinity. With regard to DPPH-free radical scavenging activity and phenolics content in response to salinity, SA-treated plants in comparison with controls are more potent in controlling ROS production.

The rise in phenolic compounds after treatment with SA may be due to increased PAL activity, as PAL was reported to be associated with the synthesis of phenolic compounds via phenylpropanoid pathway (Hahlbrock and Scheel 1989). Our results are also in accordance with the study on pennyroyal plants (Hassanpour et al. 2012). They observed that higher levels of phenolics are associated with higher levels of antioxidant enzyme activity. Since, the phenolic compounds have an antioxidative role in plants; therefore, the simultaneous increase in phenolics level and DPPH-free radical scavenging activity suggests that the increase in free radical scavenging activity might be due to the increase in phenolics level which is induced by exogenous SA application.

Conclusion

Salinity tolerance is associated with the activity of some antioxidant enzymes and with the accumulation of non-enzymatic antioxidant compounds (Asada 1999). From our findings, it can be concluded that the exogenous application of SA in combination with salinity ameliorated stress effects in *C. tinctorius* by improving antioxidant enzymes and regulation of H_2O_2 level in plant cells. Our study provides an overview of the salt-stressed safflower interaction by analysis of enzymatic and non-enzymatic antioxidative pathways and some enzymes such as PAL, which is involved in the production of signaling molecules, such as SA. In addition, our

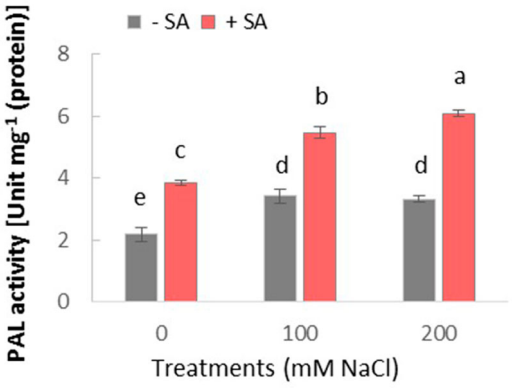

Figure 4. Effect of salinity (0, 100, and 200 mM NaCl) and exogenous application of SA on PAL activity in leaves of safflower plants at 21 days after treatments. The groups are −SA (plants with no SA treatment) and +SA (plants sprayed with 1 mM sodium salicylate three times a week for every other day). Columns indicate mean ± SE based on three replicates. Means with different letters indicate a significant difference at $P \leq .05$ using Duncan's multiple range test.

Table 1. Specific activity of five antioxidative enzymes in safflower treated with NaCl (0, 100, and 200 mM) and SA treatment at 21 days after treatments.

Treatments		Antioxidant enzymes activity [U mg^{-1}(protein)]				
SA	NaCl (mM)	SOD	POX	PPO	APX	CAT
−SA	0	0.033 ± 0.001 f	0.028 ± 0.008 e	0.026 ± 0.002 l	0.044 ± 0.008 g	0.022 ± 0.007 d
	100	0.044 ± 0.005 e	0.039 ± 0.003 c	0.046 ± 0.001 g	0.062 ± 0.003 d	0.034 ± 0.008 c
	200	0.046 ± 0.004 e	0.040 ± 0.002 c	0.058 ± 0.004 e	0.069 ± 0.002 c	0.033 ± 0.011 c
+SA	0	0.048 ± 0.011 e	0.038 ± 0.007 c	0.038 ± 0.000 h	0.050 ± 0.007 f	0.023 ± 0.002 d
	100	0.053 ± 0.003 d	0.047 ± 0.010 b	0.056 ± 0.003 e	0.068 ± 0.010 c	0.031 ± 0.000 c
	200	0.060 ± 0.000 c	0.055 ± 0.011 a	0.082 ± 0.006 b	0.074 ± 0.011 b	0.025 ± 0.003 d

Notes: The groups are −SA (plants with no SA treatment) and +SA (plants sprayed with 1 mM sodium salicylate three times a week for every other day). Data are the means ± SE based on three replicates. Means with different letters indicate a significant difference at $P \leq .05$ using Duncan multiple range test.

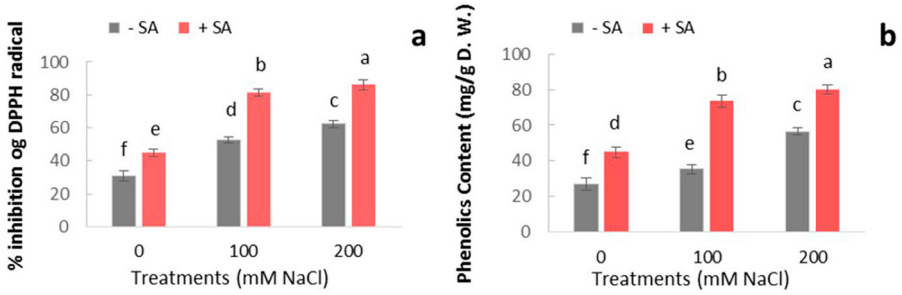

Figure 5. Effect of salinity (0, 100, and 200 mM NaCl) and exogenous application of SA on (a) DPPH scavenging activity and (b) total phenolics content in leaves of safflower plants at 21 days after treatments. The groups are −SA (plants with no SA treatment) and +SA (plants sprayed with 1 mM sodium salicylate three times a week for every other day). Columns indicate mean ± SE based on three replicates. Means with different letters indicate a significant difference at $P \leq .05$ using Duncan multiple range test.

results showed that there are different physiological and biochemical response patterns in stress condition in SA-treated plants. The present findings suggest that these differences are probably associated with salinity resistance. It is indicated that a central role for the SA signaling pathway in activating safflower response against salt stress is possible. The current results can provide new insights to better realizing the responsible mechanisms to regulate salt stress resistance in *C. tinctorius*.

Finally, our study suggested that the resistant safflower plants can direct ROS from a threat to an opportunity by using some key regulators such as SA. We propose that exogenous application of SA in plants played this role through regulation of the antioxidant enzymes. Therefore, these components can be considered to ameliorate salinity effects in safflower, due to low price and their availability. Further work on the signaling systems and gene expression of enzymes involved in salt stress is required to obtain more information about how SA treatment ameliorate salinity effects in *C. tinctorius* plants.

Disclosure statement

No potential conflict of interest was reported by the authors.

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