Effects of exogenous salicylic acid on antioxidative responses, phenolic metabolism and photochemical activity of strawberry under salt stress

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Abstract

To further clarify the specific photochemical mechanisms of salicylic acid (SA)-mediated adaptation to salt stress, this experiment was conducted to examine the effect of SA (100 and 500 μM) on photosynthesis, antioxidative capacity and phenolic metabolism in strawberry plants under salt stress (50 mM). The results showed that high SA had a negative effect on strawberry plants as reduced leaf dry weight in plants that exposed to 500 μM SA under non-salt stress conditions. Salt stress negatively affected the leaf dry weight, whereas foliar spray of 100 μM SA alleviated the salt-induced inhibitory effects on the plant growth. Salt stress caused a significant decrease in photosystem performance index (PI\text{abs}); however, plants exposed to salt stress after SA pretreatment conserved their photosynthetic electron transport rate, compared with NaCl-alone treated plants, relating to the improvement of water-splitting complex on the donor side of PSII (F_v/F_o). The application of 100 μM SA in saline condition also increased the accumulation of soluble sugars like trehalose (Tre). Lipid peroxidation was observed in plants subjected to salinity stress, as evidenced by higher malondialdehyde (MDA) levels. In contrast, foliar spray of SA at a concentration of 100 μM promoted catalase (CAT) activity as well as phenolic content, thus reducing MDA and, consequently oxidative damage to membranes. Hence, foliar application of SA at 100 μM was effective in alleviation of salt stress in strawberry by improving PSII functioning, induction of compatible osmolytes and phenol metabolism, and mitigating membrane damage.

Keywords: phenolic content; photosystem performance index; salicylic acid; salt stress; strawberry


Introduction

Environmental stresses such as salinity directly impact plants’ growth and productivity through osmotic effects and ion specific effects, and by causing oxidative stress and enhancing the production of reactive oxygen species (ROS) (Hasanuzzaman et al., 2013; Jiang et al., 2017). This elevation of ROS production can damage biomolecules of the cell including membrane lipids, proteins, DNA and photosynthetic pigments, resulting in significant reduce in photosynthetic capacity (Munns and Tester, 2008; Ashraf et al., 2016; Jiang et al., 2016). Therefore, it is necessary to expand methods and strategies to

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alleviate deleterious effects of salt stress on plants.

Hence, there are intensive efforts to enhance plant tolerance to salinity and to other environmental stressors. Among these approaches, exogenous substances, such as melatonin (Wei et al., 2014; Jiang et al., 2016), silicon and selenium (Diao et al., 2014; Feng et al., 2013; Gengmao et al., 2015), polyamine (Shu et al., 2015), and nitric oxide (Zhang et al., 2006) have been employed to increase plant salt tolerance. Salicylic acid (SA) is a signal molecule for modifying plant responses to stress. Numerous studies have indicated that exogenous addition of SA can mitigate toxicity symptoms induced by salinity stress in many plant species (Ashraf et al., 2010; Hayat et al., 2010) by regulation of ROS balance (Lee et al., 2010), via maintaining photosynthetic capacity (Hao et al., 2012; Miura and Tada, 2014) as well as controlling K homeostasis, Na uptake and Na redistribution (Ashraf et al., 2010; Hayat et al., 2010).

Strawberry plant is considered to be extremely sensitive to salts (Saied et al., 2005; Jamalian et al., 2013), and soil salinity reduces its growth and productivity (Pirlak and Esitken, 2004). It has been reported that foliar spray of SA can protect strawberry plants by alteration of mineral uptake under salt stress (Karlidag et al. 2009). However, the information regarding the effects of exogenous SA supply on photosynthesis, antioxidative capacity and phenolic metabolism of salt-stressed strawberry plants remains elusive. To address this issue, we examined in some detail the biochemical mechanisms by which SA affects the plant growth, PSI function, antioxidative system and phenolic metabolism in NaCl-treated strawberry (Fragaria × ananassa) to understand the mechanism of plant response to exogenous SA under salt stress, which may be important for sustainable strawberry production, especially in salinity areas. In addition, we try to introduce the useful way to utilize the rapid chlorophyll a fluorescence method for monitoring effects of SA on strawberry photosynthetic performance under salt stress.

Materials and Methods

Plant material and treatments

Strawberry (Fragaria × ananassa Duch. cv. ‘Gaviota’) plants of similar size and development stage were grown in plastic pots filled with a mixture of perlite and coco peat (1:1) and then watered with half-strength Hoagland solution. The nutrient solution contained NH₄H₂PO₄ (115.03 g/l), KNO₃ (101.10 g/l), Ca(NO₃)₂·4H₂O (236.15 g/l), MgSO₄·7H₂O (246.47 g/l), KCl (3.728 g/l), H₃BO₃ (1.546 g/l), MnSO₄·H₂O (0.845 g/l), ZnSO₄·7H₂O (0.575 g/l), CuSO₄·5H₂O (0.125 g/l), H₃MoO₄·H₂O (0.09 g/l), FeEDTA (9.31 g/l). Plants were kept in a greenhouse located near the city of Malekan, NW Iran (46°6′ E and 36°46′ N) with day/night temperature of 22-26/16-18 °C, relative humidity of 65-68% and daily photon flux density of about 400-450 µmol m⁻² s⁻¹ throughout the experimental period. For pretreatments, salicylic acid (SA) was dissolved in absolute ethanol then added drop wise to water (ethanol/water: 1/1000 v/v), and then sprayed in the morning with a compression sprayer of 1 L capacity. Control plants were sprayed with ethanol/water (without SA) in absence of NaCl. At day 7 after SA pretreatment, SA-pretreated and non-pretreated plants were subjected to 50 mM NaCl in the nutrient solution. To avoid osmotic shock, NaCl was gradually added to the nutrient solution through two steps to achieve the final concentration of 50 mM. The solution was renewed every four days. Control plants were irrigated with the nutrient solution without NaCl. The pH of the nutrient solution was controlled by a pH meter and adjusted to 6. After 10 days of salt treatment, plants were harvested for morphological and physiological determinations. Leaves were washed with distilled water, blotted dry on filter paper and after determination of fresh weight (FW) were dried for 48 h at 70 °C for determination of dry weight (DW).

Chlorophyll a fluorescence measurement

Chlorophyll a fluorescence transient (OJIP transients) were evaluated with a Packet-PEA chlorophyll fluorimeter (Plant Efficiency Analyser, Hansatech Instruments Ltd., King’s Lynn, Norfolk, PE 32 1JL, England) in dark-adapted leaves for at least 20 min, using the JIP-test to analyses chlorophyll a fluorescence rises. Some groups of measured and calculated parameters using the
JIP-test (Strasser et al. 2004) were described in the following section.

- $F_v/F_m$, the maximum PSII photochemical efficiency, namely the maximum quantum yield of primary photochemistry. Where $F_m$ or $F_{max}$ is maximal chlorophyll fluorescence intensity measured when all photosystem II (PSII) reaction centers are closed, $F_v$ is variable chlorophyll fluorescence ($F_m-F_o$), $F_o$ is minimal fluorescence (all PSII RCs are assumed to be open), respectively.
- $F_o/F_v$, the efficiency of the water-splitting complex on the donor side of PSII. Where $F_v$ is variable chlorophyll fluorescence ($F_m-F_o$) and $F_o$ is minimal fluorescence (all PSII RCs are assumed to be open), respectively.
- $\Phi_{PSII}$, the performance index that is calculated as: $(RC/ABS) \times (\psi_{PSII}/(1-\psi_{PSII})) \times (\psi_{o}/(1-\psi_{o}))$, where, RC is for reaction center; ABS is for absorption flux; $\psi_{PSII}$ is for maximal quantum yield for primary photochemistry; and $\psi_{o}$ is for the quantum yield for electron transport.

**Determination of total carotenoids, chlorophyll a and b, soluble protein, soluble sugar and trehalose content**

The leaf concentration of chlorophyll and carotenoids was assayed according to Lichtenthaler and Wellburn (1983). After centrifugation at 1000 rpm for one minute, supernatants were used for determination of photosynthetic pigments, and the absorbance was read at 400-700 nm on spectrophotometer. Leaf concentrations of chlorophylls and carotenoids were calculated as:

$$\text{Chl a} = 15.65 \times \text{A666} - 7.340 \times \text{A653}$$
$$\text{Chl b} = 27.05 \times \text{A653} - 11.21 \times \text{A666}$$
$$\text{Total carotenoids} = 1000 \times \text{A470} - 2.860 \times \text{Ca} - 129.2 \times \text{Cb}/245$$

Soluble proteins were assayed according to Bradford (1976) using a commercial reagent (Sigma) and BSA (Merck) as standard. Proline was quantified by the method of Bates et al. (1973). Leaf samples from each group were homogenized in 3% (w/v) sulphosalicylic acid and the homogenate was centrifuged at 3,000g for 20 min. Mixture was boiled for 1 h in water bath after addition of acid ninhydrin and glacial acetic acid. Reaction was then stopped by ice bath, and then absorbance at 520 nm was recorded. Proline (Sigma) was used for production of a standard curve. The soluble sugar was assayed using the anthrone method (Magne et al. 2006). The homogenate was centrifuged, and the supernatant was mixed with anthrone reagent and incubated for 10 min at 100 °C. After cooling, the absorbance was measured at 625 nm. Glucose (Merck) was used for production of a standard curve. Trehalose content was quantified according to Li et al (2014) with minor modifications. Samples were homogenized in 5 ml of 80% (v/v) hot ethanol, after centrifugation at 11,500g for 2 min, supernatant was dried at 80 °C followed by re-suspension in 5 ml distilled water. Mixture was boiled for 10 min after addition of 0.2 N H$_2$SO$_4$, and reaction was then stopped by ice bath. Anthrone reagent was added to the above mixture and boiled for 10 min to develop a color, and then cooled again. The absorbance was recorded at 630 nm and Tre concentration was evaluated as µmol g$^{-1}$ FW using a standard curve developed with Tre.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble sugars (mg g$^{-1}$ FW)</th>
<th>Trehalose (µM g$^{-1}$ FW)</th>
<th>Starch (mg g$^{-1}$ FW)</th>
<th>Proline (µg g$^{-1}$ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Salinity</td>
<td></td>
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<tr>
<td>0 µM SA</td>
<td>10.6±1.73</td>
<td>2.79±0.31</td>
<td>3.19±0.49</td>
<td>12.7±0.46</td>
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<tr>
<td>100 µM SA</td>
<td>12.8±1.08</td>
<td>3.33±0.26</td>
<td>2.84±0.63</td>
<td>13.8±2.76</td>
</tr>
<tr>
<td>500 µM SA</td>
<td>13.1±1.14</td>
<td>3.40±0.31</td>
<td>2.44±0.23</td>
<td>15.6±0.29</td>
</tr>
<tr>
<td>0 µM SA</td>
<td>11.3±0.53</td>
<td>2.84±0.70</td>
<td>3.40±0.28</td>
<td>15.9±2.37</td>
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<tr>
<td>+Salinity</td>
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<tr>
<td>100 µM SA</td>
<td>13.2±0.30</td>
<td>3.68±0.33</td>
<td>2.28±0.10</td>
<td>15.2±0.40</td>
</tr>
<tr>
<td>500 µM SA</td>
<td>13.7±0.18</td>
<td>3.59±0.27</td>
<td>2.54±0.40</td>
<td>26.1±5.60</td>
</tr>
</tbody>
</table>

Table 1
Effects of SA on the concentration of soluble sugars, trehalose, starch and proline in strawberry plants under salt stress. Measurements were performed 10 d after salt treatment. Data of each column indicated by the same letter are not significantly different ($P < 0.05$, Tukey test). Values are the mean ± SD.
Assay of phenylalanine ammonia-lyase (PAL) activity and related metabolites

To determine PAL activity, formation of cinnamic acid was recorded by spectrophotometry at 290 nm according to modified method of Zucker (1965). One unit (U) of PAL activity was defined as the amount of the enzyme that produced 1 nM cinnamic acid per h. Total phenolic content was quantified by the method of Velioglu et al. (1998). Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh weight. Total flavonoid content was assessed using the method adapted by Meda et al. (2005). Briefly, 5 ml of 2% aluminum chloride (AlCl₃) in methanol was mixed with the same volume of leaf extracts (0.02 mg/ml). Absorption readings at 415 nm were taken after 10 minutes against a blank sample without AlCl₃. The total flavonoid content was calculated using a standard curve of quercetin and expressed as mg quercetin equivalent (QE)/100 g extract. Anthocyanin content was estimated according to the method of Krizek et al. (1993) using HCl-methanol solvent (1: 99, v:v), and the amount of anthocyanin was ranked from the absorbance at 550 nm.

Assay of antioxidative enzymes and related metabolites

The activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) were evaluated according to methods described elsewhere (Habibi and Hajiboland 2012). Lipid peroxidation was determined from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid (Sigma) at 532 nm. MDA levels were quantified from a 1, 1, 3, 3-tetraethoxypropane (Sigma) standard curve. The hydrogen peroxide (H₂O₂) content in the leaves was calculated according to the method of Velikova et al. (2000). The content of H₂O₂ was given on a standard curve.

Statistical Analysis

Experiments were undertaken in complete randomized block design (RBD). Statistical analysis was carried out using Sigma Stat (3.5) with Tukey test (P < 0.05). Chlorophyll fluorescence data were analyzed and conducted using the PEA Plus V1.10 software.

Results

Under non-saline conditions, leaf biomass of plants was increased by foliar application of SA at 100 μM. In contrary, foliar application of SA at 500 μM reduced leaf dry weight in strawberry plants under non-salt stress conditions (Fig. I). While root growth was not changed by salt stress, the leaf dry weight of salt-stressed plants decreased as compared with control (Fig. I). This decrease in leaf dry weight was mitigated by exogenously applied SA at the concentration of 100 μM. The concentration of compatible solute
of strawberry leaves was not negatively affected by salinity (Table 1). In this study, under salt stress, the application of SA at 100 μM caused substantial increases in the total soluble sugars and trehalose contents while decreasing starch in the leaves. Although foliar spray of SA at concentration of 500 μM caused a significant increase in proline accumulation under salinity stress, its levels were not affected by foliar spray of SA at concentration of 100 μM. Under saline conditions, leaf concentration of chlorophyll b was decreased by foliar application of SA at 500 μM (Fig. II). There were no significant differences in the chlorophyll a contents between the treatments with and without SA under NaCl stress. However, compared with the non-SA-treated plants, the carotenoids content increased under salt stress.

Results showed that the flavonoid and anthocyanin concentration as well as the PAL activity remained unchanged under salt stress with or without SA treatment (Fig. III). However, exogenously SA at concentration of 100 μM effectively improved the leaf phenolic content under both salt-stress and non-saline conditions. Under salt stress, PAL activity decreased by 45% in 500 μM SA-treated plants, compared with control plants (Fig. III). The maximum quantum yield of photosystem II (Fv/Fm) did not change significantly with NaCl, compared with the non-salt-treated strawberry (Fig IV). However, the photosystems performance index (Plap) and the water-splitting complex on the donor side of PSII (as inferred from Fv/Fo) exhibited significant decreases under salt-stressed conditions. Notably, SA pretreatment alleviated these adverse effects of salt stress. Under non-saline conditions, SA pretreatment at concentration of 500 μM enhanced chlorophyll fluorescence in the I–P part of the induction curve (Fig. V). Salt stress resulted in a significant decrease in the in the I–P part of the induction curve of plants (Fig. VI). However, when plants are subjected to salt stress in the presence of SA, no obvious large decrease in the I–P part of the induction curve was observed. No significant differences in SOD activity were observed among plants grown with 100 and 500 μM SA and without SA application under saline or non-saline conditions (Fig. VII). As showed in Fig. VII, SA activated the CAT enzyme in plants grown with NaCl, particularly at 100 and 500 μM.
concentrations. More importantly, NaCl increased CAT activity by 34%, and 100 μM SA increased its activity by 67% in salt-treated plants. However, an obvious increase in CAT activity was not detected in SA-treated plants grown without NaCl. Salt stress caused severe damage to the strawberry plants, determined by the MDA accumulation. However, SA pretreatment at concentration of 100 μM minimized the MDA accumulation, which was attributed to the high activity of the CAT enzymes.

Discussion
SA pretreatment improved growth during salt stress

Strawberry is considered a salt-sensitive plant; therefore, its growth and yield are severely decreased by salinity (Saied et al., 2005; Pirlak and Esitken, 2004). In this study, leaf biomass was significantly diminished in strawberry plants grown with 50 mM NaCl. However, the growth inhibition under salt stress was mitigated by the application of SA at the concentration of 100 μM. This result was consistent with the findings of Jini and Joseph (2017), who found that the application of SA improved the growth and yield of rice under salt stress conditions, and Jayakannan et al. (2013) proved that SA pretreatment improved shoot growth of Arabidopsis during salt stress. Thus, we found that a foliar spray of SA at concentration of 100 μM was more effective than at concentration of 500 μM SA, which could mitigate negative effects of salt stress on strawberry shoot growth.

SA pretreatment altered compatible solute concentration during NaCl stress

Previous studies have shown that SA causes a significant increase in soluble sugars accumulation (Chandra et al., 2007; Jini and Joseph, 2017). In this current study, under salt stress, the application of 100 μM SA increased soluble sugars accumulation while proline contents were not affected by foliar spray of SA at concentration of 100 μM. This might be an important mechanism for trehalose to maintain

Fig. IV. Effects of SA on the maximum quantum yield of PSII ($F_{v}/F_{m}$), the Performance Index ($P_{I_{abs}}$), and the efficiency of the water-splitting complex on the donor side of PSII ($F_{v}/F_{o}$) in strawberry under salt stress. Bars indicated with the same letter are not significantly different ($P < 0.05$, Tukey test). Values are means ± SD ($n = 4$).

Fig. V. Effects of SA on the chlorophyll $a$ fluorescence induction curve of strawberry under non-saline conditions.
metabolic equilibrium to improve stress tolerance under salinity, which the osmoprotective role of trehalose decreased the need for plants to accumulate other osmolytes like proline under stress conditions. In addition, SA pretreatment increased the leaf concentrations of soluble sugars like trehalose under saline conditions, indicating that fluctuation in the concentration of trehalose was an adaptative strategy leading to tolerance to salt stress.

**SA pretreatment alleviated the adverse effects of salt stress on the PSII functioning**

Photosynthesis is one of the primary processes affected by salinity (Jiang et al., 2017). In this study, salt stress inhibited photosystems performance index (PIabs), with decrease in the water-splitting complex on the donor side of PSII (as inferred from \(F_{v}/F_{o}\)), which might be related to the negative effect of salt stress on this fraction of electron transport chain of PSII. Moreover, salt stress caused a significant reduce in the \(I-P\) part of the fluorescence rise curve of plants. This reduction of \(I-P\) part of the induction curve may be associated to the damage to the reaction center resulting in lower photochemistry activity (Kalaji et al., 2011, 2016). This study revealed that the application of SA significantly increased the photochemical activity of photosynthesis in strawberry plants grown under salt stress. The improvement of photosynthesis in response to SA has also been described in *Helianthus annuus* (Noreen et al., 2017) and *Dianthus superbus* (Ma et al., 2017) under salt stress. Interestingly, 100 μM SA-treated plants showed higher leaf phenolic content than non-SA-treated plants under salt stress. Since phenolics accumulations modulate the ROS level and maintain photosynthetic capacity (Xu and Rothstein, 2018), this higher phenolic accumulation can enhance the antioxidant capacity and radical-scavenging activity (Elguera et al., 2013) to prevent membrane lipid peroxidation (Chu et al., 2010) as well as counteract the adverse effect of salt stress on the structure of the photosynthetic apparatus. In addition, the application of SA to salt-treated plants at a concentration of 100 μM induced the accumulation of carotenoids. This higher carotenoid accumulation may participate in...
maximal photochemical efficiency of PSII, leading to higher photochemical efficiency of PSII (Habibi and Ajory, 2015; Habibi, 2017). Thus, the application of SA improved the photochemical activity of strawberry grown under salt stress, leading to an improved salt tolerance.

SA was an alleviant for the oxidative stress effects caused by NaCl

In the current experiment, the greater accumulation of MDA, an important indicator of membrane lipid peroxidation ion leakage (Shen et al., 2014; Jiang et al., 2017), was observed under salt stress condition. Under salt stress, a clear increase in CAT activity was detected in 100 μM SA-treated plants compared with non-SA-treated plants. CAT is one of the most effective antioxidant enzymes in scavenging excess ROS and preventing oxidative damage (Feng et al., 2013). These observations were consistent with the findings of Shen et al. (2014) and Ma et al. (2017), who reported that SA significantly increased the activities of antioxidant enzymes under salt stress. In this study, under salt stress, increase in the CAT activity levels correlated with a significant reduction in the damage to cell membranes in SA-treated plants compared with non-SA-treated plants. Thus, the application of SA at a concentration of 100 μM stimulated the antioxidant defense systems to reduce oxidative damage to membranes under salt-stressed conditions.

Conclusion

Our results revealed that strawberry is a salt-sensitive plant, and exogenous supply of SA could mitigate the detrimental effects of salinity on the growth and photosynthesis of strawberry, through the improvement in photosynthetic capacity by the preservation of water-splitting complex on the donor side of PSII, the activation of the antioxidant defense system and phenolic metabolism to prevent ROS damage under salinity stress. Thus, this study provides evidence that low SA application influences phenolic metabolism and PSII functioning in NaCl-treated strawberry plants, which may provide more information to enhance the antioxidant defense system in salinity areas.

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