Foliar 2,3-dihydroporphyrin iron (III) spray confers ameliorative antioxidation, ion redistribution and seed traits of salt-stressed soybean plants

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Abstract

Dihydroporphyrin iron (III) chelates (also known as DHFe) have a role in plant growth regulation under normal and stressful conditions. In the present study, using Glycine max cultivars Jackson (the salt-sensitive) and Lee68 (the salt-tolerant) as the experimental materials, the physiological and molecular events contributing to the ameliorative effects of foliar DHFe spray on seedling growth; leaf photosynthetic parameters; reactive oxygen species (ROS) content; antioxidant enzyme activity; Na⁺, K⁺ and Cl⁻ contents; and seed traits of soybean plants under control or salt-stressed conditions were investigated. The results showed that foliar spraying DHFe solution on soybean seedlings under NaCl treatment can significantly increase the leaf osmotic potential (ψs) and relative water content (RWC); reduce the Na⁺ and Cl⁻ contents and Na⁺/K⁺ ratio; and simultaneously enhance the leaf antioxidant enzymes (CAT and APX, POD and SOD) activity, together with the mitigated ROS damage (lower H₂O₂ and MDA contents). Thus, it can apparently restore the salt stress-inhibited growth and photosynthetic capacity of Jackson and Lee68 seedlings, of which the salt-sensitive cv. Jackson displayed more pronounced effects. This may be related to the fact that, except for DHFe as a kind of antioxidant, foliar application of DHFe could enhance the transcription levels of GmCLC1 in roots and leaves and those of GmSOS1 in roots of Jackson plants under salt stress. When continuously cultivated to maturity, foliar spraying DHFe under salt stress could improve, to a certain extent, the seed traits (including the numbers of pods and seeds and seeds dry weight per plant) of both soybean cultivars. This approach may also provide a valuable theoretical basis and technical guidance for future practical application of DHFe as a type of plant growth promoter for the chemical regulation of foliar DHFe spray in mitigating salt injury to soybean and other crops under saline cultivation conditions.

Keywords: Antioxidant enzymes, foliar spraying DHFe, photosynthesis, salt treatment, seeds yield, soybean.
1. Introduction

It is estimated that more than 20% of the world’s agricultural land and 50% of its cropland are facing salinization (Zhang et al., 2011). Salt stress is one of the most serious environmental factors limiting agricultural crop growth and productivity worldwide, and thus, salinization of water and soils in major agricultural areas, particularly in arid and semi-arid regions, has become a major concern for global food production (Ondrasek et al., 2011; Shi et al., 2015). Salt stress can cause ionic stress, osmotic stress, and nutritional imbalance and produce more reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$) and superoxide radical (O$_2^{-}$), which leads to lipids peroxidation, proteins oxidation, impairment of nucleic acids, inhibition of enzyme activities, and activated programmed cell death. These issues ultimately result in reduced photosynthetic efficiency, accelerated senescence, decreased yield and quality, and even death by changing the physiological, biochemical and molecular levels of the plants (Rady, 2016). NaCl is often the major molecule causing salt stress on plants or crops in agricultural practice because the high concentrations of Na$^+$ and Cl$^-$ that can accumulate within the plant cells not only possess ionic toxicity but also cause a water deficit condition and ionic imbalance or deficiency (such as K$^+$) of others nutrients (Munns and Tester, 2008; Guo et al., 2017). As we know, Na$^+$ efflux across the plasma membrane is mainly attributable to the salt overly sensitive 1 (SOS1) Na$^+$/$\text{H}^+$ antiporter. Numerous transgenic studies have suggested that SOS1 as a Na$^+$ efflux transporter may be considered as the most important functional protein for the acquirement of a salt tolerance trait for different plant species (Nie et al., 2015). Cl$^-$ uptake, transport and compartmentation are mediated by chloride channels (CLCs), which are mostly distributed in the tonoplast of cell vacuoles, for soybean as an example, Gm-CLC1 has been shown to locate at the tonoplast and play a crucial role for Cl$^-$ uptake and compartmentation in the vesicles and for Cl$^-$/salt tolerance under saline conditions (Wong et al., 2013; Wei et al., 2016). The improvement of crop salt tolerance and saline soil bioremediation is currently one of the most important research projects in the process of sustainable agriculture development. There are several ways to improve salt tolerance of crops, including the chemical regulation of exogenous application of certain plant growth substances, traditional plant breeding and modern transgenic plants.

A widely studied chlorophyll derivative, chlorophyllin (CHL), the sodium-copper salt and water-soluble analogue of the ubiquitous green pigment chlorophyll, has been shown to possesses higher antioxidant ability than AsA, GSH, mannitol, and tert-butanol at equimolar concentrations for protecting mitochondria, inhibiting the mutagenicity of various chemicals in bacteria or mice (Kamat et al., 2000). For example, Fe-CHL, a novel hydroxyl radical scavenger in mammals, could promote wheat root growth by increasing superoxide dismutase (SOD) and peroxidase (POD) activities and intracellular nitric oxide (NO) generation while decreasing the indole acetic acid (IAA) oxidase activity (Tong et al., 2010). Dihydroporphyrin iron (III) chelates (DHFe), as one kind of specific chlorophyll derivative, can be formed by chelating pyropheophorbide, purpurin, and dihydtoporphyrin, serving as main ligands and different acid radicals or hydroxy radicals serving as axial ligands (X) with the transitional trivalent iron ions. High contents of organic matter somehow prevent crystallisation and oxidation trends of mineral species, such as iron (III) (Pizarro et al., 2017). DHFe species have been shown to possess plant growth regulatory activity. Additionally, our previous work has suggested that foliar DHFe spray
displays ameliorative effects on seedling growth and seed yield of salt-stressed rapeseed plants by significantly reducing Cl⁻ content in the above-ground plant parts and enhancing leaf antioxidant enzyme activity, resulting in the decrease of ROS level and improvement of photosynthesis (Cao et al., 2016).

Soybean, which comes from China and is one of the major crops in China, is the world’s most widely planted cereal leguminous crop and also the main source of plant proteins and fats for human agricultural production and daily life (Zhang et al., 2011). During soybean planting and production, salt, drought, waterlogging and other adverse environmental stresses often occur, leading to suppression of plant growth and development and decreased yield and quality (Hasanuzzaman et al., 2016). Therefore, seeking a simple and effective measure for improving soybean stress tolerance by chemical regulation is both very meaningful in theory and necessary in practice. In our earlier works, measures such as seed soaking by soybean isoflavones (Wu et al., 2011; Tian et al., 2014) and foliar spraying of methanol (Wei et al., 2015) enhanced soybean tolerance to salt or drought stress. However, the effects of DHFe on soybean plants under salt stress and the related physiological and molecular events are still unknown. In this study, we chose soybean cv. Jackson (the salt-sensitive) and cv. Lee68 (the salt-tolerant) as the experimental materials, and the ameliorative effects of foliar DHFe spraying on soybean salt injury and its mechanisms were analysed by comparing the changes in plant growth, leaf photosynthesis parameters, Na⁺ and Cl⁻ contents and Na⁺/K⁺ ratio, activities of antioxidant enzymes and ROS levels, transcriptional pattern of GmCLC1 and Gm-SOS1, and related seed traits. Our objectives were to further elucidate the physiological and molecular mechanisms in the mitigating effects of DHFe application on soybean salt injury and to provide a reliable theoretical basis and technical guidance for future practical application of DHFe as plant growth regulators for chemical regulation of salt tolerance in many other crops.

2. Materials and Methods

2.1. Plant materials and growth conditions

Experimental soybeans were G. max (L.) Merr. cv. Jackson (the salt-sensitive) and cv. Lee68 (the salt-tolerant). Seeds were sterilized with 0.1% (w/v) HgCl₂ for 5 min, rinsed with deionized water, soaked in deionized water for 8 h, then germinated at 25°C in dark. The germinated seeds (each with approximately 0.5–1 cm epicotyl) were sown in plastic pots (11 cm deep and 9 cm in diameter) containing vermiculite and filled with 1/2 Hoagland solution, then cultured in indoor greenhouse at 25 ± 2°C/18 ± 2°C (day/night). The photoperiod was about 14 h/10 h (day/night).

2.2. Experimental design

(1) The seedlings test: According to our previous work as Cao et al. (2016), when the first trifoliate leaves appeared, the seedlings of cv. Jackson and cv. Lee68 were randomly divided into 4 groups. The first was continually cultured in 1/2 Hoagland solution and sprayed with deionized water (Control). The second was treated with 1/2 Hoagland sprayed with 0.01 mg kg⁻¹ DHFe (DHFe). The third was treated with 1/2 Hoagland+130 mmol L⁻¹ NaCl and sprayed with deionized water (NaCl). The fourth was treated with 1/2 Hoagland+130 mmol L⁻¹ NaCl and sprayed with 0.01 mg kg⁻¹ DHFe (NaCl+DHFe). For each treatment, 6 pots in one plastic container, 5 plants/pot, there are 30 plants in total. These spraying treatments (15 mL each time) were conducted in the afternoon and repeated every other day. The culture solutions were renewed
every 3 d, and after 10 d, the plants were analysed for leaf osmotic potential ($\psi_s$), relative water content (RWC), maximum quantum efficiency of PSII photochemistry (Fv/Fm), photosynthetic parameters (Pn, Gs, Ci and Tr), contents of Na$^+$, K$^+$, Cl$^-$, malondialdehyde (MDA) and $H_2O_2$, $O_2^-$ generation rate, and activity of antioxidant enzymes (APX, CAT, SOD, and POD). In addition, the transcriptional patterns of the $GmCLC1$ and $GmSOS1$ genes were compared in the roots and leaves of Jackson and Lee68 seedlings (including the above treatments of NaCl and NaCl+DHFe) within the 0, 3, 6, 12, 24 and 48 h salt stress.

(2) The analysis test of seed-related traits: These measurements were conducted during the entire growth duration for soybeans grown in 24-cm-deep and 15-cm-diameter pots. For this group, the steps included seed germination, seedling cultivation, salt solution treatment, and foliar spray of DHFe that were conducted the same as the above groups. However, the growing medium was changed to peat and sand (1:1, w/w) compost. Soybean plants were grown in outdoor glass greenhouse under natural lighting conditions to maturity for seed survey data.

2.3. Determination of leaf $\psi_s$, RWC, Fv/Fm and photosynthetic parameters

Leaf $\psi_s$ was measured as our previous method (Zhou and Yu 2010). Pieces of the most recent fully expanded leaf were placed in centrifuge tubes that were sealed and frozen in liquid nitrogen, and $\psi_s$ was determined by an automatic freezing-point depression osmometer (FM-8P type, Shanghai, China) on sap extracted from the frozen leaf samples by pressing in the injector. 

$$\psi_s = -nRT \quad (n = \text{mosmol L}^{-1}, \text{the value was read directly from the osmometer; } R = 0.008314 \text{ L MPa mol}^{-1} \text{ K}^{-1}, \text{and } T(K) = \text{ambient temperature}).$$

To evaluate leaf RWC, 50 leaf discs (5 mm in diameter) from each of the group plants were weighed to determine fresh weight (FW), hydrated to full turgidity by being floated in deionized water for 24 h at 4°C and weighed again to determine the turgid fresh weight (TW). Dry weight (DW) was determined by drying for 10 min at 105$^\circ$C and 48-72 h at 80°C till constant weight. RWC was then calculated as $[(FW−DW)/(TW−DW)] \times 100\%$ (Hu et al., 2016). Leaf Fv/Fm was measured at room temperature with a plant efficiency analyzer (Handy PEA Fluorometer, Hansatech Instruments, UK). The first pair of unifoliate leaves was dark-adapted for 30 min using Handy-PEA leaf clips. The flux density of incident photosynthetic active radiation (PAR) was 3,000 µmol m$^{-2}$ s$^{-1}$. Fv/Fm was read directly. Net photosynthetic rate (Pn), intercellular CO$_2$ concentration (Ci), stomatal conductance (Gs) and transpiration rate (Tr) were measured using a portable photosynthetic system (LI-6400, LI-COR Inc., USA). The measurements were done by maintaining air temperature at 28°C, CO$_2$ concentration at 382-385 µmol mol$^{-1}$ and light intensity at 1,000 µmol·m$^{-2}·$s$^{-1}$ photosynthetic photo flux density (PPFD) (Tian et al., 2014).

2.4. Assay of Na$^+$, K$^+$, Cl$^-$

The contents of K$^+$ and Na$^+$ were determined as Wei et al. (2015) with minor modifications. Soybean seedlings were fully rinsed in distilled water after 105 °C fixing for 5 min and were dried to constant weight at 80 °C. Dry matter was ground and screened with a 60-mesh sieve, then 100 mg for each sample were put into the tube (25 mL) and added 20 mL of deionized water, boiled together for 2 h, then filtered. Deionized water was added to make a final volume of 50 mL. K$^+$ and Na$^+$ contents were estimated using flame spectrophotometer (FP640 type, Shanghai Precision & Scientific Instrument Co., China). Measurements were calibrated using NaCl or KCl solutions of known con-
centrations, while Cl⁻ content was measured by spectrophotometry (Zhou and Yu, 2009).

2.5. Measurements of MDA, \( \text{H}_2\text{O}_2 \) contents and \( \text{O}_2^- \) generation rate

MDA content was measured according to the method described as Wei et al. (2015). The leaf samples (0.3 g) were homogenized in 3 mL 5% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 12,000×g for 10 min, then 2 mL of supernatant was mixed with 2 mL of 0.67% (w/v) thiobarbituric acid (TBA) in 5% (w/v) TCA and incubated at 100°C for 30 min. After centrifuging the optical density was measured at 450, 532 and 600 nm, respectively. The amount of MDA was calculated from the following formula:

\[
C = 6.45(A_{532} - A_{600}) - 0.56A_{450},
\]

where \( C \) represents the concentration of MDA in supernatant and expressed as \( \mu \)mol·L⁻¹, \( A_{532} \), \( A_{600} \) and \( A_{450} \) represent the absorbance values at 532, 600 and 450 nm, respectively. The final MDA content was expressed as nmol·g⁻¹ FW.

The content of \( \text{H}_2\text{O}_2 \) was measured following the method of Liu et al. (2013). Leaf tissues (0.2 g) were homogenized with 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath, and then at 12,000 g for 15 min at 4°C. Then, 0.5 mL of the supernatant was added to 0.5 mL 10 mmol L⁻¹ phosphate buffer (pH 7.0) and 1 mL 10 mmol L⁻¹ KI. The absorbance of the mixture was then read at 390 nm. The amount of \( \text{H}_2\text{O}_2 \) was calculated from a standard curve.

The generation rate of \( \text{O}_2^- \) was determined according to the method of Qiu et al. (2014). Fresh leaves (1.0 g) were homogenized in 2 mL of 50 mmol L⁻¹ phosphate buffer (pH 7.8) and were then centrifuged at 12,000 g for 10 min. The supernatants (1 mL) were mixed with 0.9 mL of 50 mmol L⁻¹ phosphate buffer (pH 7.8), 0.1 mL of 10 mmol L⁻¹ hydroxylamine chlorhydrate, and then were incubated at 25°C for 30 min. After 30 min, 1 mL of the abovementioned culture solution was added to 1 mL of 17 mmol L⁻¹ sulphanalidine and 1 mL 7 mmol L⁻¹ a-naphthylamine at 25°C for 20 min. The absorbance was measured at 530 nm and \( \text{O}_2^- \) generation rate was calculated from a standard curve of NaNO₂.

2.6. Antioxidant enzyme assays

Enzyme extractions were performed according to the method of Li et al. (2011) with slight modifications. Fresh leaves (0.2 g) were homogenized in a mortar and pestle with 2 mL of 50 mmol L⁻¹ ice-cold phosphate buffer (pH 7.0) containing 1 mmol L⁻¹ EDTA·Na₂ and 0.5% PVP (w/v). The homogenate was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was used as the enzyme extract for assays of APX, CAT, POD, and SOD activities. All extraction operations were carried out at 4°C.

The APX activity was assayed in a reaction mixture of 3 mL containing 100 mmol L⁻¹ phosphate (pH 7.0), 0.5 mmol L⁻¹ AsA, 0.1 mmol L⁻¹ \( \text{H}_2\text{O}_2 \), and 0.1 mL enzyme extract. The reaction was started by adding enzyme extract to the mixture. Enzyme activity was quantified by following the decrease in absorbance at 290 nm for 3 min (Jiang and Zhang 2002). One unit (U) of APX activity was defined as an absorbance change of 0.01 units min⁻¹.

The CAT activity was assayed spectrophotometrically at 240 nm in a 3-mL reaction mixture containing 0.1 mL of enzyme extract, 100 mmol L⁻¹ phosphate buffer (pH 7.0), 0.1 μmol L⁻¹ EDTA, and 0.1% \( \text{H}_2\text{O}_2 \). The decomposition of \( \text{H}_2\text{O}_2 \) was measured by following the decrease in absorbance at 240 nm for 3 min and quantified by its molar extinction coefficient (39.4 mmol L⁻¹ cm⁻¹). One unit of CAT activity was defined as a change in absorbance of 0.1 U min⁻¹ caused by the addition of the enzyme extract (Qiu et al., 2014).

The POD activity was measured with guaiacol as the substrate according to Liang et al. (2003) with some
modifications. The reaction mixture (3 mL) consisted of 100 mmol L⁻¹ sodium acetate buffer (pH 5.4), 0.25% guaiacol, 0.75% H₂O₂, and 100 mL enzyme extract. The increase in absorbance due to oxidation of guaiacol was measured at 470 nm for 1 min. One unit of POD activity was defined as a change in absorbance of 0.1 U min⁻¹.

The SOD activity was assayed using the photochemical nitroblue tetrazolium (NBT) method (Li et al., 2011). The reaction mixture contained 100 mmol L⁻¹ phosphate buffer (pH 7.8), 130 mmol L⁻¹ methionine, 750 μmol L⁻¹ NBT, 20 μmol L⁻¹ riboflavin, 0.1 mmol L⁻¹ EDTA·Na₂, 505 μL deionized water, and 80 μL enzyme extract in a 3 mL volume. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction monitored at 560 nm.

2.7. RNA extraction and analysis of GmCLC1 and GmSOS1 gene expression

Gene-specific primers for GmCLC1 and GmSOS1 were designed using Primer Premier software (ver. 5.0) as follows: GmCLC1-F: 5′-AGCCAGCAGTGGTTAGCTTC-3′; GmCLC1-R: 5′-TTCCGTGCAGTTCTGTAGCC-3′; GmSOS1-F: 5′-AGCACAGAACGAAGACAGCA-3′; and GmSOS1-R: 5′-GCCTGCTTGGTGAGTCAATC-3′. Total RNAs were isolated from the frozen roots or leaves of Lee68 and Jackson plants using a TRIzol reagent kit and were used for the synthesis of cDNA based on the Hieff™ First Strand cDNA synthesis Super Mix for RT-qPCR (YEASEN, Shanghai, China). The soybean tubulin gene with the forward and reverse primers 5′-ACCATCAAGACTAAGAGGACTG-3′ and 5′-AACAAAAAAGGAACGAACAAATC-3′, respectively, was used as an internal reference. qRT-PCR was performed with 384-well plates using the QuantStudio 5 Real-Time PCR System (Thermo-Fisher Scientific China, Inc., Shanghai), where 0.4 μL of primers (1 μM each), 50 ng of prepared cDNA (2 μL), 7.2 μL of ddH₂O, and 10 μL of SYBR mix were combined and brought to a final volume of 20 μL per well. PCR cycles were set up as follows: 95°C for 5 min, 95°C for 10 s, and 59°C for 20 s, with a total of 40 amplification cycles. The gene relative expression levels were normalized and calibrated according to the 2⁻ΔΔCT method (Schmittgen and Livak 2008), with the soybean tubulin gene as the internal reference, and the results were presented as the means ± SDs of three replicates.

2.8. Analysis of seed-related traits

Ten soybean plants at maturity were randomly selected, and the numbers of pods and seeds and the seed dry weight per plant were recorded.

2.9. Statistical analysis

All data were analysed and presented as the mean ± SD for each treatment (n = 3, or n = 10 for seed traits at maturity) using SPSS software ver. 19.0. The data were subjected to one-way analysis of variance (ANOVA), and the mean differences were compared using Duncan’s test (P < 0.05).

3. Results

3.1. Effects of foliar spraying DHFe on growth, leaf ψs, RWC and Fv/Fm of salt-stressed soybean seedlings.

Compared with the control, the growth of cv. Jackson and Lee68 seedlings was improved after spraying DHFe (0.01 mg kg⁻¹), and even the leaf PSII maximum photochemical efficiency (Fv/Fm) of Lee68 was enhanced by 6.60% (P < 0.05), while the leaf osmotic potential (ψs) and the relative water content (RWC) of
the two soybean seedlings were not different from each control. Under 130 mmol L⁻¹ NaCl stress, the growth of Jackson and Lee68 seedlings was obviously reduced, displaying with basal leaf yellowing, leaf $\psi_s$, RWC and Fv/Fm dropping significantly ($P < 0.05$) compared with the control, and a larger decline was observed in the salt-sensitive Jackson (4.59 times, 62.08%, and 16.09%, respectively). After foliar spraying DHFe under salt stress, the growth of two soybean seedlings, leaf colour and the abovementioned parameters were all remarkably recovered, especially the value of Fv/Fm, which was restored to control level (Figure 1).

![Figure 1](image-url)

**Figure 1.** Effects of foliar spraying of DHFe on the growth phenotype (A, from left to right, represent treatments of Control, DHFe, NaCl, and NaCl+DHFe), leaf osmotic potential ($\psi_s$, B), RWC (C) and Fv/Fm (D) of soybean cv. Jackson and cv. Lee68 seedlings under NaCl stress. Data are means ±SD of 3 replicates per treatment, different letters among the four treatments on the same soybean cultivar indicate significant difference at $P<0.05$ by Duncan’s test. The same as follows.

### 3.2 Changes in leaf $P_n$, $G_s$, $C_i$ and $T_r$ of salt-stressed soybean seedlings sprayed with DHFe

Under normal culture condition, plus foliar spraying DHFe, and except for the obvious enhancement effect on $G_s$ (increased by 38.67%) and $T_r$ (increased by 30.64%) of the Jackson seedlings, no obvious effects were displayed on the other photosynthetic parameters of Jackson and Lee68 seedlings when compared with the normal plants. However, NaCl treatment caused significant decreases ($P < 0.05$) in the above photosynthetic parameters in two soybean seedlings. If the salt-stressed Jackson and Lee68 seedlings were subsequently sprayed with DHFe, the decreased photosynthetic parameters of both soybean cultivars all evidently recovered, in comparison with the solely salt-stressed among them, and the values of $P_n$ and $C_i$ in Jackson and $G_s$, $C_i$ and $T_r$ in Lee68 approached the control levels ($P > 0.05$) (Figure 2).
3.3 Effects of foliar spraying DHFe on contents of Na\(^{+}\), Cl\(^{-}\) and Na\(^{+}/K^{+}\) ratio in leaves of NaCl-stressed soybean seedlings

Under non-saline conditions, exogenous foliar DHFe application showed no effects on contents of Na\(^{+}\), and Cl\(^{-}\) and the Na\(^{+}/K^{+}\) ratio in the leaves of Jackson and Lee68 seedlings. When exposed to NaCl stress, leaf contents of Na\(^{+}\), Cl\(^{-}\) and the Na\(^{+}/K^{+}\) ratio increased respectively by 9.15, 1.59 and 9.15 times for Jackson, and 5.25, 0.75 and 5.74 times for Lee68, in comparison with the controls. Thus, it is clear that the salt-sensitive cv. Jackson showed the larger rise. After foliar spraying DHFe under salt stress, Na\(^{+}\), Cl\(^{-}\) contents and the Na\(^{+}/K^{+}\) ratio in leaves of both soybean cultivars were obviously decreased, and all the declines in the salt-sensitive cv. Jackson reached a significant difference in contrast with the sole NaCl stress (\(P < 0.05\)) (Figure 3).

Figure 2. Changes in Pn (A), Gs (B), Ci (C), and Tr (D) of soybean cv. Jackson and cv. Lee68 seedlings under NaCl stress by foliar spraying of DHFe.
Foliar DHFe spray confers soybean NaCl tolerance

Journal of Soil Science and Plant Nutrition, 2018, 18 (4), 1048-1064

Figure 3. Effects of foliar spraying of DHFe on leaf Na⁺ (A), Cl⁻ (B) contents and Na⁺/K⁺ ratios (C) in soybean cv. Jackson and cv. Lee68 seedlings under NaCl stress

3.4. Effects of foliar spray of DHFe on MDA and H₂O₂ contents, and O₂⁻ generation rate in leaves of salt-stressed soybean seedlings

Under control conditions, MDA and H₂O₂ contents, and O₂⁻ generation rate in leaves of Jackson and Lee68 seedlings were maintained at lower levels, and no obvious influences were displayed after additional foliar DHFe application.
When Jackson and Lee68 seedlings were subjected to NaCl stress, remarkable rises in MDA and H$_2$O$_2$ contents, and $O_2^-$ generation rate were observed in the leaves in contrast with the controls ($P < 0.05$), especially for the salt-sensitive cv. Jackson (increased by 4.11, 3.29, and 1.34 times, respectively). However, if the foliar spray of DHFe subsequently accompanied NaCl treatment, the vastly raised MDA and H$_2$O$_2$ levels and $O_2^-$ production in leaves of both soybean cultivars all sharply declined. Excepting for the H$_2$O$_2$ level still significantly exceeding the controls, the MDA content and $O_2^-$ level of both soybean cultivars showed no significant difference compared to the controls ($P > 0.05$) (Figure 4).

![Figure 4](image-url)
3.5 Changes in CAT, APX, POD and SOD activities in leaves of salt-stressed soybean seedlings by foliar spraying DHFe

Slight but unapparent enhancing effects of foliar spray of DHFe on CAT, APX, POD and SOD activities emerged in leaves of the Jackson and Lee68 seedlings under normal conditions. If salt stress was applied for both soybeans, the activities of CAT, APX, POD and SOD in leaves of Jackson (the salt-sensitive) were significantly decreased by 51.82%, 22.06%, 25.96%, and 12.39%, respectively, when compared with the control, while those of Lee68 (the salt-tolerant) showed an marked rise in varying degrees. With foliar application of exogenous DHFe, coupled with salt treatment, the dropped leaf CAT, APX, POD and SOD activities in Jackson were restored to the control levels, and the CAT activity evidently exceeded the control. As for Lee68, these antioxidant enzyme activity showed a continuing rising trend (Figure 5).

![Figure 5](image)

**Figure 5.** Changes in CAT (A), APX (B), POD (C) and SOD (D) activities in leaves of soybean cv. Jackson and cv. Lee68 seedlings under NaCl stress by foliar spraying of DHFe

3.6 Effects of foliar spraying DHFe on transcriptional patterns of GmCLC1 and GmSOS1 in roots and leaves of salt-stressed soybean seedlings

During the 48 h of salt stress process, the highest relative expression (13.28 times) of GmCLC1 in the roots of the salt-sensitive Jackson seedlings without foliar spraying DHFe occurred in the NaCl treatment for 12 h. Additional foliar spraying DHFe treatment could make this peak advance to 6 h, and still maintain at these higher levels at the subsequent 12, 24 and 48 h, as respectively compared to those without foliar spraying DHFe. However, this situation was not displayed in roots of the salt-tolerant Lee68 seedlings.

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In terms of the leaves, the main performance of foliar spraying DHFe under salt stress was to significantly enhance the \( \text{GmCLC1} \) transcription levels in Jackson and Lee68 during the late process of salt treatment (e.g., 24 and 48 h) (Figure 6A). In addition, under NaCl treatment for 48 h, with or without foliar spraying DHFe, the transcriptional levels of \( \text{GmSOS1} \) in the roots of the Jackson seedlings showed first, a rise, and then, a decline (all still above the 0 h level), with the extension of salt treatment time, reaching its maximum at 12 h (11.06 times). The difference is that, foliar spraying DHFe under NaCl treatment for 48 h could still maintain the transcriptional levels of \( \text{GmSOS1} \) at the maximum level in the NaCl treatment for 24 h (10.94 times), or foliar spraying DHFe could make the enhanced \( \text{GmSOS1} \) transcription in the roots of salt-stressed Jackson plants for an extended time. However, this event was not apparent in the roots of Lee68 or the leaves of Jackson and Lee68 seedlings (Figure 6B).

**Figure 6.** Effects of foliar application of DHFe on the transcriptional patterns of \( \text{GmCLC1} \) (A) and \( \text{GmSOS1} \) (B) in leaves of soybean cv. Jackson and cv. Lee68 seedlings under the treatments of NaCl and NaCl+DHFe within the 0, 3, 6, 12, 24 and 48 h.
3.7. Effects of foliar spraying DHFe on seed-related traits of NaCl-stressed soybean plants

Under normal conditions, foliar spraying of DHFe could slightly increase in Lee68 the numbers of pods and seeds per plant, and seeds dry weight per plant, in contrast to the control ($P > 0.05$), while no rise in the abovementioned parameters was displayed for Jackson plants. Under salt stress, pods number, seeds number, and seeds dry weight per plant of Jackson and Lee68 all evidently declined in contrast with the control plants, and excepting the seeds per plant of Lee68, reached a significant level ($P < 0.05$), especially for the salt-sensitive Jackson cultivar. When DHFe was sprayed on both salt-stressed plants, pods number, seeds number, and seed dry weight per plant were obviously restored, with pods number and seeds number per plant of Lee68 recovering, and even slightly exceeding, the control level (Table 1).

### Table 1. Effects of foliar spraying of DHFe on seed-related traits of soybean under NaCl stress

<table>
<thead>
<tr>
<th>Variables</th>
<th>Seed-related traits</th>
<th>Pods per plant</th>
<th>Seeds per plant</th>
<th>Seed dry weight per plant</th>
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</thead>
<tbody>
<tr>
<td>Jackson</td>
<td>Control</td>
<td>8.5±0.5$^a$</td>
<td>13.8±3.1$^a$</td>
<td>2.26±0.71$^a$</td>
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<tr>
<td></td>
<td>DHFe</td>
<td>8.5±3.0$^a$</td>
<td>12.7±2.3$^{ab}$</td>
<td>2.19±0.48$^a$</td>
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<tr>
<td></td>
<td>NaCl</td>
<td>4.8±0.8$^b$</td>
<td>8.6±0.5$^c$</td>
<td>0.69±0.21$^b$</td>
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<tr>
<td></td>
<td>NaCl+ DHFe</td>
<td>5.5±1.01$^b$</td>
<td>10.5±1.7$^{bc}$</td>
<td>1.18±0.11$^b$</td>
</tr>
<tr>
<td>Lee68</td>
<td>Control</td>
<td>8.0±1.3$^a$</td>
<td>13.3±3.0$^{ab}$</td>
<td>1.83±0.13$^{ab}$</td>
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<td>DHFe</td>
<td>9.2±2.0$^a$</td>
<td>16.7±4.3$^a$</td>
<td>1.99±0.66$^a$</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>6.0±1.5$^b$</td>
<td>12.4±2.1$^b$</td>
<td>0.79±0.23$^c$</td>
</tr>
<tr>
<td></td>
<td>NaCl+ DHFe</td>
<td>9.0±0.9$^a$</td>
<td>14.6±1.0$^{ab}$</td>
<td>1.43±0.20$^b$</td>
</tr>
</tbody>
</table>

Note: Different letters in the same row indicate significant differences at the $P<0.05$ level by Duncan’s test.

4. Discussion

Ionic injury due to Na$^+$ and Cl$^-$ accumulation, especially in the leaves or above-ground parts of plants, cell membrane lipid peroxidation resulting from more ROS production than scavenging, growth inhibition and biomass reduction, and photosynthesis decline accompanied with the reduced yield, are the commonly physiological and morphological damages identified in plants under salt stress (Munns and Tester, 2008). The differences observed in plant stress tolerance primarily depend on its diversely congenital species or genotype, combined with various postnatal or acquired improvement measures of agronomic cultivation (including chemical regulation and stress acclimation) and genetic engineering (Tian et al., 2014; Deinlein et al., 2014). G. max cv. Jackson and cv. Lee68 are often used as the salt-sensitive and the salt-tolerant soybean materials, respectively (Wei et al., 2016). Both were adopted in this work and their salt tolerance variation was verified again. For example, under 130 mmol L$^{-1}$ NaCl treatment...
for 10 d, the seedling growth inhibition, the decreases of leaf ψs, RWC, Fv/Fm, and Pn values were significant in Jackson and Lee68 as compared to the controls, with the salt-sensitive Jackson display being more apparent (Figure 1, Figure 2A).

ROS homeostasis in plants growing in stressful environments is impaired, and the levels of major ROS, such as O₂⁻ and H₂O₂ increase, leading to cell membrane lipid peroxidation and aggravating membrane permeability damage, ultimately resulting in a remarkable rise of MDA level and huge leakage of cell contents (Miller et al., 2010). Taking the example of salt stress, maintaining high levels of antioxidant substances (including enzymes and non-enzymes) is becoming the main physiological and biochemical means for strong salt tolerance in halophytes, or as potential selection criteria for improving salt tolerance of glycophytes by chemical regulation or molecular marker assistant breeding (Ashraf, 2009; Bose et al., 2014). Many studies have suggested that some exogenous substances that affect plant growth, such as salicylic acid (SA) in mustard (Syed et al., 2011), H₂O₂ in wheat (Li et al., 2011), spermine in cucumber (Shu et al., 2013), jasmonic acid (JA) in wheat (Qiu et al., 2014), and methanol in soybean (Wei et al., 2015) can enhance the tolerance to salt stress. Foliar spray, seed soaking and soil application of plant growth regulators are the commonly used methods for managing plant growth and development under normal or stressful conditions. DHFe, a chlorophyll derivative, has good antioxidant properties. Our previous work has also suggested that DHFe can serve as a type of antioxidant plant growth promoter for the production of crops such as rapeseed, under normal or saline environments (Cao et al., 2016).

In this study, under unstressful conditions, foliar spraying DHFe to Jackson and Lee68 seedlings showed no obvious effects on leaf MDA and H₂O₂ contents, O₂⁻ generation rate, and activities of antioxidant enzymes (APX, SOD, POD, and CAT), but foliar application of DHFe to the salt-stressed Jackson and Lee68 seedlings greatly decreased the significant rises of MDA and H₂O₂ contents, and O₂⁻ generation rate to control levels (Figure 4), restoring the apparently dropped activities of APX, SOD, POD and CAT in Jackson seedlings even slightly more than control level. For Lee68 seedlings, the evidently raised activities of APX, SOD, POD and CAT continuously ascended to a higher level (Figure 5). The differences in the ROS level and antioxidant enzymes ability between Jackson and Lee68, under salt stress, with or without foliar DHFe application, are also very consistent with the discrepancy of salt tolerance of both soybean cultivars. These results further reflect the role of DHFe as a kind of antioxidant plant growth promoter for soybean production under saline environments.

Contents of K⁺, Na⁺ and Cl⁻ are important indexes to measure the degree of salt injury on plants under salt stress, and maintenance of lower Na⁺/K⁺ ratio and Cl⁻ content in plants is a vital strategy for its salt adaptation (Munns and Tester, 2008; Wei et al., 2015). Cao et al. (2016) reported that foliar DHFe application (0.01 mg kg⁻¹) could reduce Cl⁻ accumulation and increase K⁺ level in above-ground parts of rape-seed seedlings under NaCl treatment, though there was no apparent effect of DHFe application on Na⁺ contents in either above-ground parts or roots. Syed et al. (2011) reported that, foliar application of 0.5 mmol L⁻¹ SA could alleviate the negative effects of 50 mmol L⁻¹ NaCl stress on mustard by lowering leaf Na⁺ and Cl⁻ contents and enhancing growth and photosynthetic ability.

In our work, when compared with the control, Na⁺ and Cl⁻ contents, and Na⁺/K⁺ ratios were significantly increased in leaves of Jackson and Lee68 seedlings under salt stress. If foliar spraying DHFe
occurred under salt stress, the values of leaf Na⁺ and Cl⁻ contents, and Na⁺/K⁺ ratios, significantly declined in both soybean cultivars, of which the reducing effects in Jackson appeared more prominent (Figure 3). Further, we compared effects of exogenous foliar spraying DHFe on the transcriptional patterns of $GmCLC1$ and $GmSOS1$ in the leaves and roots of salt-stressed Jackson and Lee68 seedlings, and we found that foliar application of DHFe could enhance the transcription levels of $GmCLC1$ in roots and leaves and those of $GmSOS1$ in roots of the salt-sensitive Jackson plants under salt stress, whereas no obvious effects for the salt-tolerant Lee68 plants were displayed (Figure 6). This may indicate that exogenous spray of DHFe can be involved in the regulation of the process of Na⁺ and Cl⁻ transport and/or redistribution in soybean plants under salt stress. In addition, because of the relatively heavier salt injury to the salt-sensitive Jackson seedlings than the salt-tolerant Lee68 under salt stress, the ameliorative effects of an exogenous spray of DHFe on salt injury to cv. Jackson are better than those to Lee68, which can also be corroborated from the different recovery degrees of plant growth, leaf $\Psi_s$, RWC, fluorescence parameters (e.g., $F_v/F_m$) and photosynthetic parameters (such as $P_n$, $G_s$, $C_i$ and $T_r$) of both soybean cultivars (Figure 1, Figure 2). Jackson and Lee68 plants, grown in pots under salt stress with additional foliar spraying DHFe, were cultivated to the stage of maturity. Comparing the seed traits of soybean plants with the solely salt-stressed ones, they showed that the salt-inhibited pods number, seeds number, and seed dry weight per plant of both soybean cultivars recovered under foliar spraying DHFe, of which the salt-tolerant Lee68’s recovery even slightly exceeded the control level (Table 1) due to its relatively slighter salt injury. Thus, all of these findings illustrate that, in both the seedlings stage and whole growth period of the soybean, foliar spraying DHFe displayed mitigating effects on salt injury, and the mitigating degree varied within the salt tolerance of soybean cultivars.

5. Conclusions

After foliar spraying DHFe to the salt-stressed Jackson and Lee68 seedlings, leaf antioxidant enzyme activity was enhanced and conferred for reduced oxidative damage. Genes (such as $GmCLC1$ and $GmSOS1$) expression of the transport proteins for Na⁺ and Cl⁻ were positively regulated to keep lower leaf Na⁺ and Cl⁻ contents and the Na⁺/K⁺ ratio. Therefore, ROS, ionic and osmotic homeostasis were effectively maintained, which was facilitated to the improved water supply, restoring plant growth and leaf photosynthesis. Finally, the salt injuries to both soybean seedlings were alleviated, and the seed-set rate and seeds yield per plant in maturity stage were also increased. These ameliorative effects on salt injury especially showed a certain correlation with salt tolerance difference of soybean cultivars. For future study, the effects of DHFe application in soybean, rapeseed or other crops under drought, low temperature and other stressful conditions need to be further investigated to provide a more reliable theoretical basis and technical guidance for practical utilization of DHFe as a kind of plant growth promoter in the chemical regulation of crop stress tolerance.

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References


