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5-Aminolevulinic acid could enhance the salinity tolerance by alleviating oxidative damages in *Salvia miltiorrhiza*

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Abstract

S. miltiorrhiza is a Chinese medicinal plant that is widely cultivated. The root growth in *S. miltiorrhiza* are inhibited by soil salinity. Here we investigated the capability of a plant growth regulator, 5-ALA to promote the growth of *S. miltiorrhiza* under different salt stresses. Five-month old *S. miltiorrhiza* roots were uniformly irrigated with different levels of salt solution i.e. 0, 100, 200 mM NaCl. After 3 days of treatment, salt-treated *S. miltiorrhiza* plants were sprayed with different concentrations of ALA (0, 10 mg L^{-1}, 20 mg L^{-1}) on the leaves and cultured for another 7 days. Results revealed that ALA treated plants produced significantly higher biomass by sustaining leaf chlorophyll content under salt stressed. 10 mg L^{-1} ALA significantly up-regulated antioxidant enzymes activities under studied salinity treatments. Positive effects of ALA on antioxidant defense systems were also supported by a significant increase in the expression of SOD isoenzymes genes (*CSD1*, *FSD1* and *MSD2*), defense response genes (*DXS1*, *C4H, GGPPS*) and stress-related gene (*MYB36* , *MYB39*) of ALA treated plants. This study suggested that ALA can protect *S. miltiorrhiza* from salinity induced oxidative stress and injury by promoting antioxidant defense system, boosting secondary metabolic pathways and protecting photosynthetic pigments.

Keywords: 5-aminolevulinic acid (ALA); gene regulation; oxidative stress; salinity stress; *Salvia miltiorrhiza*.

Practical Application: Salinity tolerance was enhanced by spraying 5-Aminolevulinic acid on leaves in *Salvia miltiorrhiza*.

1 Introduction

Salinity, drought, cold, and extreme temperature are the major abiotic stresses which restrain the growth and development of plants (Guo et al., 2018). Among these, soil salinity has become a major yield limiting factor for cultivated crops in many parts of the world (Khan et al., 2019). In the north-western inland of China, for instance, soil salinization is universal problem because of limited rainfall, high evapotranspiration, and possible poor soil water management. Besides, saline soils occurred on the eastern coastline of China (Gengmao et al., 2014). Increased accumulation of salts in the soil has negatively impacted agricultural production (Abogadallah, 2010). Salinity could affect the functions of normal plants through osmotic stress, ion imbalance and oxidative damage (Tufail et al., 2018). Salinity stressed plants might capture these enhanced ROS by modifying the activities of antioxidant enzymes, for instance POD, APX and SOD (Gengmao et al., 2014). Some plants i.e. sunflowers and some medicinal plants have negative effects under saline soils (Liu & Shi, 2010; Guo et al., 2018). Thus, investigation of salt tolerance mechanisms of these plants are very important for the further salinity tolerance breeding.

5-aminolevulinic acid (ALA) is an important precursor for heme and chlorophyll (Ali et al., 2014; Li et al., 2019a). Thus, application of ALA could enhance the plant tolerance against various stresses. It has been reported that ALA could increase the net photosynthetic rate, chlorophyll content, antioxidant activities and inhibit the uptake and translocation of Na^+ in cotton seedlings under salinity stress (Watanabe et al., 2000). ALA could improve photosynthetic rate and chlorophyll content of melon seedlings under low light and chilling conditions (Wang et al., 2004). Application of ALA can significantly recovery the rapeseed seedlings from heavy metals induced toxicity by increasing antioxidant enzyme activities, suppressing ROS and MDA contents and recovering the ultra-structural damages (Ali et al., 2013 a, b, c; Ali et al., 2014; Ali et al., 2015).

S. miltiorrhiza, as a functional food, is one of the most extensively studied medicinal plants, which is used as human medicine and health-promotion food. (Shi et al., 2018). Tanshinone in *S. miltiorrhiza* has definite curative effect on cardiovascular diseases and neurological diseases(Hao et al., 2015; Xu et al., 2021). Recently, tanshinone has been found to have an inhibitory effect on human bladder cancer cells (Huang et al., 2020). In addition, Gengmao et al. (2014) found that the adaptive response to salt stress in *S. miltiorrhiza* was linked with its highly induced antioxidant enzyme defense systems. Guo et al. (2018) reported *Sm-miR408* in *S. miltiorrhiza* is involved in adaptative responses of salinity stress by modulating the activities of

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antioxidant enzymes i.e. SOD, POD and CAT. *S. miltiorrhiza* might maintain its growth under salinity stress by up-regulating its antioxidant system. Therefore, exploiting the induced tolerance in *S. miltiorrhiza* might become a useful tool for broadening the cultivation area of *S. miltiorrhiza* and minimizing the negative effects of salinity stress.

To date, less information is available on the use of plant growth regulators for increasing salinity tolerance in *S. miltiorrhiza*. Therefore, this study aims are (i) to reveal physio-biochemical changes induced by salinity stress in *S. miltiorrhiza*, (ii) to investigate the recovery mechanisms of exogenous application of ALA in alleviating salt induced injuries in *S. miltiorrhiza*. The study will provide insights into the positive effects of ALA on enhancing the salinity tolerance, and provide candidate genes for salinity tolerance breeding in *S. miltiorrhiza*.

2 Materials and methods

2.1 Plant growth

Healthy seeds of *S. miltiorrhiza*, cv. Tiandan 1, were obtained from Shaanxi Tasly Plant Pharmaceutical Co., Ltd, China and cultured in greenhouse of Zhejiang Sci-Tech University, Hangzhou, China.

The uniform *S. miltiorrhiza* seedlings were selected for the following experiments, after 5 months of culture. Three concentrations of salt (0, 100 or 200 mM NaCl) were used according to our preliminary experiment. To avoid stress shock, salt stress was imposed in stepwise manner (50 mM NaCl increment with each irrigation per day) to the plants in soil (Islam et al., 2018). After 3 days of salt stress treatment, the plants were sprayed with ALA (10 or 20 mg L^{-1}) and then cultured for another 7 days. The treatment combinations contained: control, 100 mM NaCl, 200 mM NaCl, 10 mg L^{-1} ALA , 20 mg L^{-1} ALA, 10 mg L^{-1} ALA + 100 mM NaCl, 10 mg L^{-1} ALA + 200 mM NaCl, 20 mg L^{-1} ALA + 100 mM NaCl, 20 mg L^{-1} ALA + 200 mM NaCl. After seven days of ALA recovery treatments, root and leaf tissues were collected for physiological and biochemical analyses with three biological replicates.

2.2 Leaf chlorophyll and carotenoid contents

Fresh leaf sample (100 mg) was used for chlorophylla (Chl a), chlorophyllb (Chl b) and carotenoid contents analyses. The leaf tissues were ground in 5 mL of acetone and ethanol mixture under dark conditions. The supernatant was used for measuring absorbance at 663 nm, 645 nm and 470 nm (Lichtenthaler, 1987).

2.3 Reactive oxygen species (ROS) and malondialdehyde (MDA)

Reactive oxygen species i.e. hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and superoxide radical ($O_2^{\cdot-}$) in different *S. miltiorrhiza* tissues were measured using a Microplate Reader (Synergy HTX). Extracellular $^{\cdot}OH$ contents were measured using an extinction coefficient of 0.28 $mm^{-1}cm^{-1}$ according to the method of Halliwell et al. (1987). Absorbance of $O_2^{\cdot-}$ was taken at 530 nm (Jiang & Zhang, 2001), and $O_2^{\cdot-}$ production rate was determined using a standard curve method (Zhou & Leul, 1999). MDA content were determined at 532 nm and 600 nm by thiobarbituric acid chromogenic method according to Zhou & Leul (1999).

2.4 Antioxidant enzyme activities

A Microplate Reader (Synergy HTX) was used for measuring antioxidant enzymes in *S miltiorrhiza* tissues. Photoredox method of nitrogen blue tetrazole was used for measuring SOD activity (Zhang et al., 2008). For POD activity measurement, guaiacol oxidation method was used from the changes in the absorbance of reaction mixture (extinction coefficient 26.6 $mm^{-1}cm^{-1}$) within 1 minute at 470 nm (Zhou & Leul, 1999). CAT activity was determined from the degradation of H_2O_2 in the reaction mixture within 1 minute at 240 nm (Aebi, 1984). APX activity was determined from the changes in absorbance of reaction mixture at 290 nm, according to the protocol of Nakano & Asada (1981).

2.5 Gene expression analyses

RNA extraction, cDNA reverse transcription and qRT-PCR methods were performed according to Xu et al. (2019). In addition, the primers were listed in Table 1 including the SOD isoenzymes genes *CSD1*, *FSD1*, *MSD2* (Han et al., 2020), the genes related to secondary metabolites, such as *DXS1*, *C4H*, *GGPPS*, *HMGR*, *PAL* in the metabolic pathway of active ingredient of *S. miltiorrhiza* (Xing et al., 2018). The genes' expressions were calculated using the method of $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001).

2.6 Statistical analysis

For morphological and physiological parameters, three biological replicates were conducted for each treatment combination. The gene expression data were recorded as the average of three biological replicates and three technical replicates. Data were analyzed using SPSS 22.0 (SPSS, Chicago, IL, USA), fisher least significant difference (LSD) test, two-way analysis of variance (ANOVA), and Duncan's multiple range test were used to determine the significant differences ($P \leq 0.05$) between individuals. The results of morphological and physiological parameters were presented as mean ± standard error (SE).

3 Results

3.1 Plant growth

In the absence of ALA treatment, *S. miltiorrhiza* plants experienced a significant reduction in plant height, root length and biomass production under salinity treatments (Table 2). 10 mg L^{-1}ALA significantly improved the growth and increased the fresh weights of leaves and roots of *S. miltiorrhiza* under different salinity stresses. For instance, ALA treated plants produced 34.21% and 30.08% higher leaf and root fresh weight, respectively, as compared to 100 mM salinity treatment alone. Whereas, higher doses of ALA (20 mg L^{-1}) had negative effects on plant growth, especially on dry weights of roots under high salt stressed plants (200 mM NaCl). For instance, dry weight of roots under the treatment of 20 mg L^{-1} ALA + 200mM NaCl

declined 31.91%, as compared to the 200mM NaCl treatment alone. This suggests that selection of an optimum level of ALA i.e. 10 mg L^{-1} is crucial for protecting *S. miltiorrhiza* plants from salinity stress injury.

3.2 Leaf pigments

Increasing NaCl concentrations in the growth media significantly reduced Chl a, Chl b and carotenoid contents of *S. miltiorrhiza* (Table 3). 10 mg L^{-1} ALA significantly increased the Chl a, Chl b, total Chlorophyll and carotenoid contents of *S. miltiorrhiza* as compared with the control, on the contrary, 20 mg L^{-1}ALA could significantly reduce chlorophyll and carotenoid contents. In addition, low dosage of ALA (10 mg L^{-1}) had obvious recovery effects on the chlorophyll and carotenoid contents of *S. miltiorrhiza* under different salinity stresses. For instance, application of 10 mg L^{-1}ALA increased leaf Chl a, Chl b and carotenoid contents of *S. miltiorrhiza* by 127.75%, 31.90% and 64.10% respectively, as compared with the 100 mM salinity stress alone. However, under the treatment of high salinity treatment of 200 mM NaCl, high dosage of ALA (20 mg $L^{-1)}$ could significantly reduce the Chla, Chlb, total and carotenoid contents of *S. miltiorrhiza* as compared to low dosage of ALA (10 mg L^{-1}).

Table 1. List of qRT-PCR primer used in present study.

Gene	Sense Primer (5' to 3')	Reverse Prime (5' to 3')	GenBank/Gene ID
Actin	GGTGCCCTGAGGTCCTGTT	AGGAACCACCGATCCAGACA	HM231319
CSD1	CGTCAGCTTCACTCAGGAGG	GCTCTTTGCCAGCAGGATTG	SMil_00004513
FSD1	CTCATTTGGGAGGGGCACAG	CGAGCGTTCTCTGGCTCATA	SMil_00006812
MSD2	TTGCTCCTGTTCGTGTTGGT	CAAGCCACACCCATCCTGAA	SMil_00025343
DXS1	CGACCAGGTAGTGCACGACG	TCATCTGAAGGAGCCATCACCAC	EU670744
C4H	CCAGGAGTCCAAATAACAGAGCC	GAGCCACCAAGCGTTCACCAA	EF377337
GGPPS	GGGGCTATTTTGGGAGGTGGAA	CAGCAGCTTGGGATACGTGGTC	FJ178784
HMGR	GCAACATCGTCTCCGCCGTCTACA	GATGGTGGCCAGCAGCCTGGAGTT	FJ747636
PAL	GGCGGCGATTGAGAGCAGGA	ATCAGCAGATAGGAAGAGGAGCACC	GQ249111
MYB36	TCTCCGATGCTGAAGAGGAC	CTTCATTGCTGCGGTTGAGA	KF059390
MYB39	ATGCCCAACCACCAACAATC	ATTTCTACGCCGGGATTTGC	KF059393

Table 2. Effects of 5-aminolevulinic acid on plant growth and biomass of *S. miltiorrhiza* under different salinity stresses.

ALA level (mg L^{-1})	NaCl level (mM)	Plant height (cm)	Root length (cm)	Leaf weight (g $plant^{-1}$)		Root weight (g $plant^{-1}$)	
				Fresh	Dry	Fresh	Dry
0	0	12.367 ± 0.379a	18.883 ± 0.535b	1.860 ± 0.120b	0.243 ± 0.031b	1.940 ± 0.120b	0.587 ± 067b
	100	11.100 ± 0.529bc	16.817 ± 1.042c	1.400 ± 0.140c	0.213 ± 0.001c	1.443 ± 0.045d	0.493 ± 0.060de
	200	10.467 ± 0.450c	16.417 ± 1.010c	1.245 ± 0.025cd	0.187 ± 0.012de	1.307 ± 0.141de	0.445 ± 0.015d
10	0	11.767 ± 0.493ab	21.313 ± 0.563a	2.080 ± 0.070a	0.288 ± 0.013a	2.120 ± 0.010a	0.668 ± 0.048a
	100	11.433 ± 0.208b	19.900 ± 1.153ab	1.879 ± 0.021b	0.250 ± 0.010b	1.877 ± 0.075bc	0.575 ± 0.022bc
	200	11.850 ± 0.550ab	19.250 ± 0.318a	1.750 ± 0.110b	0.206 ± 0.006cd	1.780 ± 0.110e	0.613 ± 0.032ab
20	0	11.000 ± 0.435bc	19.907 ± 0.969ab	1.410 ± 0.030c	0.207 ± 0.058cd	1.891 ± 0.092bc	0.510 ± 0.045cd
	100	11.300 ± 0.265bc	18.767 ± 1.150b	1.203 ± 0.176d	0.193 ± 0.006cd	1.297 ± 0.0150ce	0.405 ± 0.005e
	200	11.133 ± 0.650bc	18.750 ± 0.450b	1.277 ± 0.107cd	0.167 ± 0.015e	1.207 ± 0.012e	0.303 ± 0.006f

Each value is the mean of three replicates. Means followed by same small letters in the same column are not significantly different at $P \leq 0.05$.

Table 3. Effects of 5-aminolevulinic acid on leaf chlorophyll and carotenoid contents of *S. miltiorrhiza* under different salinity stresses.

ALA level (mg L^{-1})	NaCl level (mM)	Chlorophyll (mg g^{-1}FW)			Carotenoids (mg g^{-1} FW)
		Chl a	Chl b	Total	
0	0	0.746 ± 0.038c	0.220 ± 0.010b	0.959 ± 0.048c	0.188 ± 0.004c
	100	0.209 ± 0.008f	0.116 ± 0.003e	0.324 ± 0.010g	0.078 ± 0.004g
	200	0.175 ± 0.014g	0.066 ± 0.002f	0.241 ± 0.016h	0.070 ± 0.004h
10	0	1.246 ± 0.013a	0.353 ± 0.004a	1.600 ± 0.017a	0.302 ± 0.003a
	100	0.476 ± 0.010e	0.153 ± 0.017d	0.612 ± 0.023f	0.128 ± 0.004f
	200	0.797 ± 0.010b	0.218 ± 0.009b	1.020 ± 0.010b	0.205 ± 0.004b
20	0	0.449 ± 0.016e	0.156 ± 0.006d	0.595 ± 0.016f	0.137 ± 0.008e
	100	0.480 ± 0.024e	0.178 ± 0.004c	0.658 ± 0.027e	0.157 ± 0.003d
	200	0.607 ± 0.014d	0.125 ± 0.003e	0.731 ± 0.017d	0.160 ± 0.003d

Each value is the mean of three replicates. Means followed by same small letters in the same column are not significantly different at $P \leq 0.05$.

3.3 Lipid peroxidation and reactive oxygen species

Increasing salinity levels in the growth media significantly accelerated ROS accumulation and subsequent lipid peroxidation (MDA) in leaf and root tissues of *S. miltiorrhiza* (Table 4). In contrast, ALA (10 mg L^{-1}) significantly reduced H_2O_2 in leaf and root tissue of low salt-stressed plants by 35.44% and 12.21%, and $^{\cdot}OH$ contents by 28.04% and 22.26%, respectively, as compared with 100 mM salinity treatment alone. Similarly, lower concentration of ALA significantly reduced MDA and ROS in *S. miltiorrhiza,* except for the $^{\cdot}OH$ content in leaves under 200 mM salinity treatment. However, under low 100 mM salinity stress, higher ALA concentration i.e. 20 mg L^{-1} significantly increased MDA and $^{\cdot}OH$ levels in both leaves and roots of *S. miltiorrhiza* as compared with those applied with low dosage of ALA (10 mg L^{-1}). We concluded that low concentration of ALA (10 mg L^{-1}) could effectively reduce ROS and MDA contents of salinity-treated *S. miltiorrhiza*, as compared to higher concentrations of ALA (20 mg L^{-1}).

3.4 ROS scavenging enzymes

Changes in the activities of different antioxidant enzymes of *S. miltiorrhiza* in response to salinity and ALA treatment were shown in Figure 1. In the leaves and roots of salt-treated *S. miltiorrhiza,* CAT activity significantly decreased with the

Table 4. Effects of 5-aminolevulinic acid on contents of malondialdehyde (MDA) hydrogen peroxide (H_2O_2), hydroxyl ion ($^{\cdot}OH$) and superoxide radical ($O_2^{\cdot-}$) in *S. miltiorrhiza* under different salinity stresses.

ALA conc. (mg L^{-1})	NaCl conc. (mM)	MDA (nmol g^{-1} FW)		H_2O_2 (μmol g^{-1} FW)		$^{\cdot}OH$ (μmol g^{-1} FW)		$O_2^{\cdot-}$ (nmolmin^{-1} g^{-1} FW)	
		Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
0	0	11.470 ± 0.397c	11.237 ± 0.400d	41.790 ± 4.256d	54.290 ± 3.337de	14.128 ± 0.684b	8.840 ± 0.242d	14.585 ± 0.246c	3.539 ± 0.196d
	100	12.215 ± 0.172b	12.621 ± 0.394bc	63.013 ± 2.020b	64.223 ± 2.233c	16.198 ± 0.303a	11.444 ± 0.273c	18.114 ± 0.057a	6.356 ± 0.236c
	200	13.259 ± 0.442a	14.005 ± 0.387a	70.484 ± 0.512a	94.027 ± 0.231a	17.066 ± 0.485a	13.141 ± 0.341a	15.696 ± 0.353b	8.663 ± 0.464a
10	0	9.976 ± 0.345de	8.488 ± 1.051f	29.190 ± 0.226e	43.833 ± 0.817f	10.883 ± 0.265d	12.365 ± 0.128b	10.698 ± 0.173f	3.637 ± 0.196d
	100	9.870 ± 0.503e	11.800 ± 0.174cd	40.680 ± 0.832d	56.380 ± 2.614d	11.656 ± 0.780cd	8.896 ± 0.222d	13.571 ± 0.149d	7.036 ± 0.344b
	200	12.301 ± 0.086b	10.215 ± 0.186e	42.523 ± 0.599d	51.283 ± 5.323e	17.643 ± 0.663a	12.421 ± 0.362b	14.421 ± 0.265c	6.545 ± 0.056c
20	0	10.435 ± 0.132de	10.108 ± 0.190e	39.895 ± 2.159d	70.497 ± 1.813b	12.556 ± 0.805c	13.316 ± 0.477a	12.624 ± 0.408e	8.362 ± 0.128a
	100	12.233 ± 0.175b	12.846 ± 0.397b	49.190 ± 1.485c	57.293 ± 0.395d	16.660 ± 0.294a	11.358 ± 0.174c	10.238 ± 0.204g	8.767 ± 0.371a
	200	10.552 ± 0.397d	12.158 ± 0.397bc	50.104 ± 0.226c	53.240 ± 0.450de	17.205 ± 0.582a	13.591 ± 0.224a	14.519 ± 0.098c	6.676 ± 0.098bc

Each value is the mean of three replicates. Means followed by same small letters in the same column are not significantly different at $P \leq 0.05$.

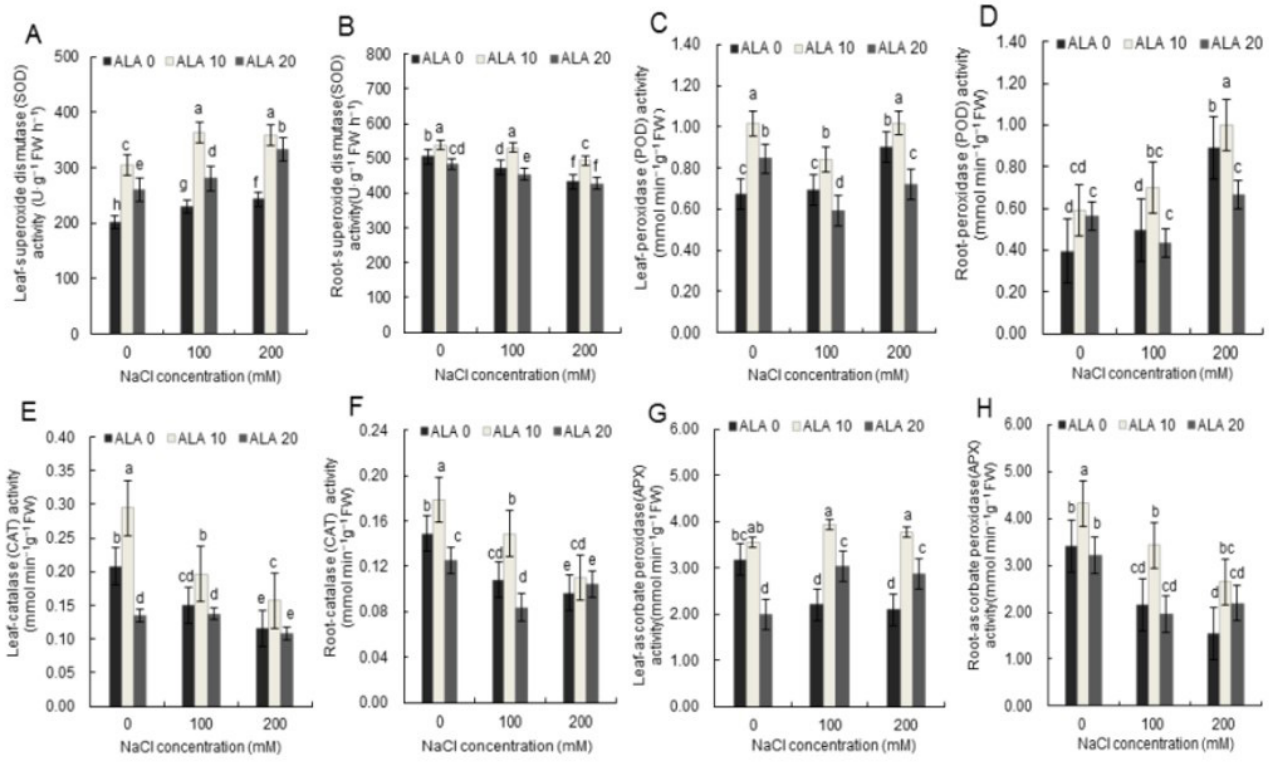

Figure 1. Effects of 5-aminolevulinic acid on the antioxidant enzyme activities in *S. miltiorrhiza* under different salinity stresses: A Leaf-superoxide dismutase (SOD); B Root-superoxide dismutase (SOD); C Leaf-peroxidase (POD); D Root-peroxidase (POD) activity; E Leaf-catalase (CAT); F Root-catalase (CAT); and G Leaf-ascorbate peroxidase (APX); H Root-ascorbate peroxidase (APX). Values are the means of three independent replications ± SE.

increase of salt concentration from 100 to 200 mM (Figure 1EF), however, these trends were not significant in APX activities (Figure 1GH). However, the impact of salinity on POD activities was significant only under higher concentrations of salinity (200 mM), which significantly increased POD activity (Figure 1CD), in both leaves and roots tissues of *S. miltiorrhiza*. SOD activities were significantly enhanced in leaves with the salinity increased from 100 to 200 mM, however, the trends in roots were opposite (Figure 1AB). ALA (10 mg L^{-1}) could significantly regulated different antioxidant enzyme activities in *S. miltiorrhiza* tissues under different salt treatments. For instance, compared with 200 mM salinity stress alone, 10 mg L^{-1} of ALA significantly upregulated POD activity in leaves and roots of *S. miltiorrhiza* by 13.11% and 12.49%, respectively. Similarly, under 200 mM salinity treatment alone, 10 mg L^{-1}ALA application significantly upregulated CAT by 36.42% and 14.13%, and APX by 79.22% and 72.22% in the leaves and roots of *S. miltiorrhiza*, respectively. Noticeably, higher dosage of ALA concentration (20 mg L^{-1}) significantly depressed the activities of antioxidant enzymes in leaf tissues under salinity stresses (100 or 200 mM). It was obvious that an appropriate concentration of ALA (10 mg L^{-1}) could promote antioxidant enzyme activities under salinity condition, but its higher concentration i.e. 20 mg L^{-1}ALA, had a negative effect on antioxidant enzyme activities under salinity.

3.5 Expression profiles of key genes

Higher level (200 mM) of salinity in the growth media significantly inhibited the expression of genes regulating antioxidant enzyme i.e. *CSD1* and key genes associated with secondary metabolites (*DXS1*, *C4H*, *GGPPS*, *HMGR*). In contrast, ALA upregulated the expression of genes associated with stress response (*MYB36*) in *S. miltiorrhiza* root tissues (Figure 2). Meanwhile, 10 mg L^{-1} ALA significantly upregulated most of the studied genes in *S. miltiorrhiza* except *HMGR*, *PAL* and *MYB39*. For instance, *FSD1* and *MSD2* genes were significantly upregulated by 211.24% and 224.89%, respectively, but *HMGR* was significantly downregulated by 11.95% in *S. miltiorrhiza* roots, as compared with the control. The analysis of gene expression data showed that 10 mg L^{-1} ALA was relatively more effective in promoting the expression of key genes under 200 mM salinity stress, particularly the expression of genes such as *FSD1*, *GGPPS*, *HMGR* and *PAL* was increased by 249.31%, 255.65%, 345.05% and 304.81% in the roots of *S. miltiorrhiza*, respectively. We concluded that ALA (10 mg L^{-1}) protected *S. miltiorrhiza* plants from salt-induced toxicity by decreasing oxidative stress and by modulating the expression of ROS scavenging and secondary metabolites and stress-related genes in *S. miltiorrhiza*.

4 Discussion

4.1 ALA mediated modulation of secondary metabolites and stress responsive genes

Secondary metabolites have important adaptive and defense roles in plant interactions with the environment, especially for abiotic stresses (Akula & Ravishankar, 2011). Tanshinone and Rosmarinic acid are the main secondary metabolites of *S.*

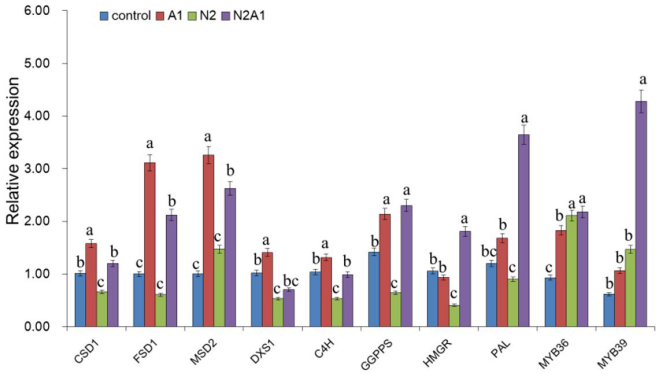

Figure 2. Effects of 5-aminolevulinic acid on the expressions of key secondary metabolites genes in *S. miltiorrhiza* under different salinity stresses. Relative expression levels of these genes were examined in the tested plants by qRT-PCR analysis. Control; A1, treatment with 10 mg L^{-1} ALA alone; N2, treated with NaCl (200 mM) alone; and N2A1, treatment with both NaCl (200 mM) and 10 mg L^{-1} ALA. Means followed by the same letters were not significantly different at $P \leq 0.05$.

miltiorrhiza, and could limit salinity induced intercellular ROS production (Gao et al., 2014; Zhang et al., 2014). ALA strongly stimulated the expression levels of *DXS*, *GGPPS*, *HMGR* and other key genes in tanshinone metabolic pathway (Kai et al., 2011). The other related secondary metabolic pathway gens, like *C4H*, *PAL*, *4CL* were involved in the rosmarinic acid metabolic pathway in previous studies (Song & Wang, 2009; Huang et al., 2008). In addition, it has been reported that exogenous growth regulators can modify the expression of key genes in secondary metabolic pathways,for example, methyl jasmonate can induce the expression of *C4H*, *PAL*, etc in *S. miltiorrhiza* (Xiao et al., 2009). In this study, exogenous ALA could significantly induced some key genes in metabolic pathways, such as *DXS1*,*C4H* and *GGPPS* (Figure 2). We found that the gene expressions of *DXS1*, *C4H*, *GGPPS* and *HMGR* were significantly down-regulated under salt condition, indicating the negative effects of salt stress on *S. miltiorrhiza* root tissue. Similarly, ALA significantly up-regulated the expression of *C4H*, *GGPPS*, *HMGR* and *PAL* genes under salt condition. Interestingly, the expression of *PA*L gene was significantly up-regulated after ALA and salt treatment, indicating that ALA has a positive regulatory effect on *PAL*. However, ALA alone or salt alone had no significant effect on *PAL*. These results suggest that ALA can affect the metabolic pathway of *S. miltiorrhiza* to adapt to salt conditions.

MYB transcription factor is one of the largest members of the plant transcription factor family. It plays an important role in the regulation of plant secondary metabolism, response of hormones and environmental factors, and organ morphogenesis. It can bind to cis-acting elements upstream of stress resistance gene and specifically regulate the expression of the gene in plants, which plays an important role in abiotic stress breeding such as drought resistance, salt tolerance and temperature tolerance (Li et al., 2019b). According to Zhang et al. (2020), R2R3-MYB transcription factor *AtMYB49* activates cuticle deposition and antioxidant defense, contributing to salt tolerance of *Arabidopsis thaliana*. Similarly Wu et al. (2019) verified that *ZmMYB3R* could increase the activities of CAT, POD and SOD, enhance the

sensitivity to ABA, and significantly enhance the regulation of stomatal opening, and enhance the tolerance to drought and salt stress. Similarly, it has reported overexpressed *SmMYB36* could regulate metabolic pathways and promote the accumulation of metabolites (Ding et al., 2017). Recently, Wang et al. (2020) found that *MYB36* regulates *MYB39* to stimulate root development in *Arabidopsis thaliana*. In this study, we found that exogenous ALA and salt could independently up-regulate the expression of *MYB36*, while applying ALA under salt conditions had no significant effect on the expression of *MYB36*, but stimulated the significant up-regulation of *MYB39* in *S. miltiorrhiza* root tissues (Figure 2). There may be some related regulatory relationships between *MYB36* and *MYB39* in *S. miltiorrhiza* root tissues under abiotic stress, which warrants further investigations.

4.2 ALA enhances salinity tolerance by alleviating oxidative damages in S. miltiorrhiza

Salt stress decreases the accumulation and translocation of key mineral ions such as N, K, etc. aggravates osmotic pressure and ionic toxicity (Ahanger & Agarwal, 2017; Ahanger et al., 2019a). The delicate balance of ROS disturbed, when plants are exposed to adverse conditions such as salinity, as they accelerate intra and intercellular ROS levels, leading to ROS burst and injury to membranous structures such as proteins and nucleic acids (Sharma et al., 2012; Ahanger et al., 2019b). To detoxify unregulated ROS, plants modify antioxidant enzymes and their transcript levels, which has been observed in present study (Figure 1). Among these, SOD is a major enzyme, which can effectively decrease oxidative stress by eliminating excessive ROS in plant tissues (Han et al., 2020). For instance, SOD enzyme, as a key enzyme in plant antioxidant system, it converts highly reactive OH^- radical and superoxide (O^-) to less toxic H_2O_2. Lu et al. (2020) found that soybean SOD gene may play a positive role in response to alkaline stress. Han et al. (2020) suggested that *SmSODs* had different responses to cold, salt, drought, heavy metals and plant hormones in *S. miltiorrhiza*. In this study, *S. miltiorrhiza* root tissue under the application of 10 mg L^{-1} ALA significantly increased SOD activity under salt stress (Figure 1B). *CSD1*, *FSD1* and *MSD2* genes expression were also up-regulated (Figure 2). These results indicated that ALA application play a complementary role in the maintenance of total SOD activity and the detoxification of ROS in *S. miltiorrhiza* under stress conditions.

It is established that 1-glutamic acid is catalyzed into l-Glutamyl-tRNA and l-Glutamic acid-1-semialdehyde under the catalysis of enzymes glutamyl-tRNA synthetase and glutamyl-tRNA reductase, severally. Whereafter, under the catalysis of enzyme l-glutamate1-semialdehyde aminotransferase, l-Glutamic acid-1-semialdehyde converted to ALA (Rhaman et al., 2021). And ALA has been found as a ROS scavenges in plants. For example, ALA significantly reduced H_2O_2 content in rice and protected chlorophyll from salt injury by modifying antioxidant enzymes (Nunkaew et al., 2014). Similar positive effect of ALA spraying on cucumber leaves has been observed under salt stressed environments (Zhen et al., 2012). In this study, ALA protected *S. miltiorrhiza* plants from salt injury by adjusting antioxidant enzymes. For example, 10 mg L^{-1} ALA increased the activities of CAT and APX enzymes under high-salt (200 mM) conditions (Figure 1EFGH). In addition, except for ^-OH in leaves, the content of other ROS significantly decreased, indicating that ALA could effectively improve the activity of some antioxidant enzymes to overcome oxidative damage (Table 4). In addition, we found that 10 mg L^{-1} of ALA as an optimum concentration, while higher ALA concentration could increase ROS production and exacerbate oxidative damage in plants as suggested by Rhaman et al. (2021).

4.3 The key regulatory mechanisms of ALA in S. miltiorrhiza under the salinity stress

It is well-known that salt stress degrades photosynthetic pigments, reduces CO_2 fixation, and damage chloroplast ultrastructure and photosynthetic enzymes, impeding photosynthetic systems. (Rhaman et al., 2021). Exogenous ALA at an appropriate concentration can increase chlorophyll content and activated the antioxidant enzymes to cope with abiotic stress in rice and cucumber. (Nunkaew et al., 2014; Wu et al., 2018). The exogenous ALA can also decrease the MDA content, and ROS production in rapeseed, cucumber and peach under salt stress (Naeem et al., 2011; Zhen et al., 2012; Ye et al., 2016). In addition, ALA application in salt-stressed cucumber plant up-regulated the expressions of CAT, APX, Mg-chelatase, and protochlorophyllide oxidoreductase (POR) genes (Zhen et al., 2012; Wu et al., 2018). In conclusion, to promote plant growth and development, ALA application can alleviate salt stress in plants by promoting photosynthetic system, improving antioxidant enzyme system, reducing ROS content and inducing key stress related metabolic genes.

We found that salinity significantly inhibited growth and biomass accumulation of *S. miltiorrhiza* (Table 1). No significant variations in plant height, fresh weight of leaves for root length, fresh/dry weights of roots of *S. miltiorrhiza* under 100 and 200 mM of NaCl, which may be because the plants have experienced maximum growth inhibition within the studied time frame. Salt-induced structural damage to chlorophyll could inhibit carbon assimilation and biomass accumulation in plants. ALA, on the other hand, restored leaf chlorophyll, which was consistent with Naeem et al. (2010) study. This chlorophyll restoration could be associated with positive impact of ALA on chloroplast biosynthesis. Similarly, ALA protected carotenoids in S. *miltiorrhiza* leaves from salt-induced photooxidation by dissipating the excessive excitation energy (Mittler, 2002). The application of ALA could effectively increase SOD enzyme activity. Meanwhile, the expressions of three genes (Figure 2), *CSD1*, *FSD1* and *MSD2* in *S. miltiorrhiza* roots, were significantly down regulated under salinity stress. There are studies which suggest that variation in SOD homologous i.e. *Mn-SOD* and *Cu/Zn-SOD* in sensing and eliminating ROS in plant cells (Jebara et al., 2005; Ueda et al., 2013). SOD enzyme may play complementary roles in maintaining total SOD enzyme activity and ROS in *S. miltiorrhiza* under salinity stress conditions. In addition, the increased oxidative toxicity in *S. miltiorrhiza* under salinity stress possibility arise from the reduced activities of POD, CAT and APX. CAT can eliminate ROS such as H_2O_2 by converting it into H_2O, and reduction in CAT may affect ROS

effectiveness in plants, as detected in *S. miltiorrhiza*. ALA also promoted POD activity, which has an important role in salinity tolerance in the tissues of *S. miltiorrhiza*. Overall, antioxidant data analysis indicated that ALA pretreatment protected *S. miltiorrhiza* against salinity stress by reducing oxidative stress and elevating antioxidant enzyme activities. A pivotal precursor of biosynthesis of metabolites such as tetrapyrrole, ALA can induce plant growth and stresses tolerance even when applied in low concentrations. Our study indicated that ALA could enhance aboveground biomass accumulation, photosynthesis and antioxidant enzyme activity of *S. miltiorrhiza* to reduce salinity injury. ALA had a certain promoting effect on alleviating *S. miltiorrhiza* under salinity stress. However, the mechanism of how ALA improves *S. miltiorrhiza* resistance to salinity stress needs further investigations.

5 Conclusion

Our study indicated that salinity stress can impair growth and biomass production of *S. miltiorrhiza* seedlings via oxidative injury, because the suppressed antioxidant enzyme system is not sufficient enough to confront with the increased ROS under salt stresses, in *S. miltiorrhiza*. ALA restored the growth of salt stressed plants by upregulating antioxidant enzymes and reducing membrane peroxidation. Induction of *CSD1*, *GGPPS* and other stress and secondary metabolic pathway genes indicated a high adaptative response against salinity stress after ALA application. These results suggested that ALA can effectively alleviate salinity stress through promoting antioxidant defense system, reducing ROS generation, and regulating expression profiling of stress responsive genes.

Abbreviations

APX: Ascorbate peroxidase. C4H: Cinnamic acid 4-hydroxylase. CAT: Catalase. CSD: Copper/zinc superoxide dismutase. DXS: 1-deoxy-D-xylulose 5-phosphate synthase. FSD: Fe superoxide dismutase. GGPPS: Geranylgeranyl diphosphate synthase. HMGR: 3-Hydroxy-3-methylglutaryl CoA reductase. MDA: Malondialdehyde. MSD: Manganese Superoxide dismutase. MYB R2R3-MYB: transcription factor. PAL: Phenylalanine ammonia-lyase. POD: Peroxidase. ROS: Reactive oxygen species. SOD: Superoxide dismutase.

Conflict of interest

The authors declare no conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

X.L and L.X conceived the original research plan. X.L, J.J.L and J.M.P conducted the experiment. X.L and J.M.P generated the figures. X.L and J.J.L wrote the article with contributions from all authors. F.I, U.N and L.X revised the manuscript. Z.N.H, J.Y.S and Y.B.Q supervise the experiment. J.Y.S, Y.B.Q and L.X supported the experiment. All authors revised and approved the manuscript.

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