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Exogenous Salicylic Acid Optimizes Photosynthesis, Antioxidant Metabolism, and Gene Expression in Perennial Ryegrass Subjected to Salt Stress

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Abstract: Salicylic acid (SA) is a plant growth regulator that can enhance the abiotic stress tolerance of plants; however, the physiological mechanisms are not yet fully understood. The objective of this study was to examine whether exogenous SA could enhance the salt tolerance of perennial ryegrass (*Loliumperenne* L.; PRG) and investigate the physiological underlying mechanisms. SA was applied to the foliage of PRG at five concentrations (0, 0.25, 0.5, and 1 mM). The SA-treated grass was grown under either control (0 mM NaCl) or salt stress (250 mM NaCl) conditions for 24 d. The SA treatments reduced the leaf electrolyte leakage (EL), malonaldehyde (MDA), and hydrogen peroxide (H_2O_2) content by 36%, 41%, and 40%, respectively, relative to the control under salt stress as measured at 24 d. The SA treatments also alleviated the decline in the leaf photosynthetic rate (Pn), stomatal conductance (gs), nitrate activity (NR), turfgrass quality (TQ) ratings, and chlorophyll (Chl) content under salt stress. In addition, exogenous SA increased the activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (POD) as well as the expression levels of the *Cyt Cu/ZnSOD*, *FeSOD*, *APX*, *CAT,* and *POD* genes under salt stress. The results of this study suggested that the foliar application of SA at 0.25 and 0.5 mM may enhance photosynthesis and antioxidant defense systems and thus improve tolerance to salt stress in perennial ryegrass.

Keywords: salicylic acid; perennial ryegrass; salt tolerance

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1. Introduction

Salinity stress is a major factor limiting plant growth and productivity as potable water supplies for irrigation decrease and secondary water sources with high salinity are used increasingly to mitigate water shortages [1]. Salinity can also lead to ion cytotoxicity due to the replacement of K^+ by Na^+ in biochemical reactions and conformational changes and the dysfunction of proteins [2]. The metabolic imbalances caused by ionic toxicity and osmotic stress, as well as nutritional deficiency under salinity, can disrupt plant photosynthetic function and reduce shoot and root growth [3–5].

The disruption of photosynthetic function can make photosystem II unable to transduce or dissipate the exceeded energy absorbed through the light-harvesting complex [6]. The excess energy may be directed to O_2, resulting in the accumulation of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide anion ($O^{2\bullet-}$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($OH^\bullet$) [7]. Excess ROS can cause damage to proteins, DNA, carbohydrates, and lipids [8]. Plants can activate the enzymatic system, which mainly consists of superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), and ascorbate peroxidases (APX), to cope with the oxidative damage caused by ROS [9].

Briefly, SOD constitutes the first line of defense against ROS by dismutating the $O^{2\bullet-}$ to H_2O_2. H_2O_2 is then regulated by CAT and an array of peroxidases such as POD and APX [9]. In the corresponding cell compartments, the multiple forms of these enzymes coordinate to achieve a balance of the formation and removal of ROS, maintaining H_2O_2 at the levels required for cell signaling [10,11]. Previous studies have shown that salinity stress reduced antioxidant enzyme activity, resulting in lipid peroxidation and cell membrane damage [12].

Plant growth regulators have been used to mitigate salt stress-induced damage to plants. Salicylic acid (SA) is classified as one of the phytohormones. It has an important function in plant defense mechanisms against abiotic stress as a signal molecule [13]. Previous studies have indicated that SA enhances plant salt tolerance by improving the physiological parameters and the activation of antioxidant enzymes [14,15]. Under salinity, exogenous SA at 0.01 mM increased SOD and POD activity and inhibited CAT activity in wheat [9]. SA treatment at 0.1 mM increased CAT, POD, and SOD activities in the salt-affected periwinkle (*Catharanthus roseus* L.) [16]. Syeed et al. (2011) [17] reported that SA treatment at 0.5 mM increased APX activity and decreased SOD activity in salt-tolerant mustard (*Brassica juncea* L.) cultivars under 50 mM NaCl stress. These studies showed that SA application rates ranging from 0.01 to 0.5 mM could improve salt stress tolerance and that SA application can improve antioxidant defense systems and thus salt stress tolerance in various plant species [16–18]. However, the results varied among different studies, and the mechanisms of SA's impact on salt tolerance have not been well documented, especially for turfgrass species.

Perennial ryegrass (*Loliumperenne* L.), a cool-season turfgrass species, is widely used not only as a turfgrass for golf courses, home lawns, and sports fields but also as a forage species with a high yield and good quality [19]. However, perennial ryegrass has medium salt tolerance. Previous studies have shown that salt stress levels (250 mM NaCl) can cause damage to PRG [4]. The utilization of PRG has been limited in regions with high salt content in the soil [4,20]. The objectives of this study were to examine if the application of SA can improve the salt tolerance of PRG and investigate the physiological mechanisms of SA's impact on salt stress tolerance.

2. Materials and Methods

2.1. Plant Culture and Growth Conditions

Seeds of perennial ryegrass (cv. 'Accent') were sown in a plastic container (5 cm in diameter × 10 cm long) filled with perlite (3 mm–6 mm in size) on 22 April 2020. A layer of gauze was used to cover the containers to avoid moisture loss. The containers were placed in a greenhouse with photosynthetic active radiation at 400 $mmol \cdot m^{-2} \cdot s^{-1}$ (at 1400 HR) and temperatures of 24/18 °C (day/night), allowing roots and shoots to establish for 28 days. On 19 May 2020, the grasses were transplanted into reservoirs.

Twenty-eight-day-old seedlings were transplanted into reservoirs filled with 50 L of half-strength Hoagland solution, which had a small pump (1.5 L min^{-1} ventilation) to provide oxygen, and the reservoir was refilled with fresh nutrient solution every week [21,22]. The seedlings were hand-clipped at ≈10 cm height before the initial application of SA. The plants naturally grew under the afore-mentioned conditions for 14 d to allow roots and leaves to recover the ability to uptake water before starting salt and SA treatment.

2.2. Treatments

Perennial ryegrass was subjected to two NaCl levels (0 and 250 mM) and four SA levels (0, 0.25, 0.5, 1 mM). The eight treatments were as follows: (1) nutrient solution (control); (2) 0.25 mM SA; (3) 0.5 mM SA; (4) 1 mM SA; (5) 250 mM NaCl; (6) 250 mM NaCl + 0.25 mM SA; (7) 250 mM NaCl + 0.5 mM SA; and (8) 250 mM NaCl + 1 mM SA. The SA rates were selected based on the previous studies and the high rate (1 mM) was included to test if it could cause negative effects on PRG [23]. The salt stress (250 mM NaCl) was selected based on a previous study by Hu et al. (2012) [4]. There were four replicates for each treatment.

Each of the replicates, consisting of two separate containers (with or without NaCl), was randomly arranged in the greenhouse.

The SA was initially dissolved in 100 µL absolute ethyl alcohol and diluted to concentrations of 0.25, 0.50, and 1.00 mM, respectively, with distilled water. Each treatment was sprayed with a 250 mL SA solution, and the 0 mM SA treatment was sprayed with distilled water. Spraying was carried out in the morning, and the prepared SA solution was evenly sprayed with a CO_2-pressured sprayer at a pressure of 290 kPa on the leaves so that visible water droplets were coagulated on both sides of the leaves. The SA treatments were applied on 16 June and again on 19 June 2020.

Salt treatment began on 22 June 2020. The NaCl was dissolved in a 1/2 strength Hoagland's nutrient solution. The NaCl solution was gradually increased to concentrations of 50, 100, 150, and 250 mM at 24 h intervals, and the final concentration of 250 mM was maintained for 24 d.

2.3. Sample Collection

The turfgrass quality, photosynthetic rate, and stomatal conductance were measured on the day before each of the leaf samples was collected. Leaf samples were collected on 22 June 2020 (before treating with NaCl), 28 June, 4 July, 10 July, and 16 July. A portion of each fresh leaf sample was used to measure the Chl content, EL, and NR activity. The remaining samples for analysis of MDA, H_2O_2, antioxidant enzyme activity, and gene expression were frozen with liquid nitrogen and stored at −80 °C.

2.4. Measurements

Turfgrass quality (TQ) was determined based on the color, density, texture, and uniformity of the turfgrass performance [24]. Turfgrass quality was rated on a visual scale from 1 to 9, with 1 indicating complete senescence; 9 representing dark green, healthy turfgrass; and 6 being of minimally acceptable quality.

Leaf chlorophyll (Chl) content was determined according to the method of Knudson et al. (1977) [25]. In brief, fresh leaf tissue (50 mg) was extracted in 8 mL 95% ethanol for 48 h in the dark, and the extracted solution was analyzed. The chlorophyll a and chlorophyll b were measured with a spectrophotometer (UV-2802S, UNICO Corporation, Spain) by reading the absorbance at 665 and 649 nm. The chlorophyll content was calculated using the formula Chl (mg g^{-1} FW) = $(6.63A_{665} + 18.08A_{649}) \times V/W$, where A = optical density at 665 and 649 nm, V = final volume (mL), and FW = leaf tissue fresh weight (g).

The content of MDA was measured according to Heath and Packer (1968) [26]. Leaf tissues (0.05 g) (W) were homogenized in a solution of 10 mL (V) of 0.1% (*w*:*v*) trichloroacetic acid (TCA). One milliliter of extract was added to a tube containing 4 mL of 20% (*v*/*v*) TCA and 0.5% (*v*/*v*) of thiobarbituric acid (TBA). The homogenate was then incubated in boiling water for 30 min and cooled to room temperature and then centrifuged at 10,000× *g* for 10 min. The absorbance of the supernatant was read at 532 nm (A_{532}) and 600 nm (A_{600}). The absorbance for nonspecific absorption at 600 nm was subtracted from the value at 532 nm. The MDA content was calculated based on the following formula: C_{MDA} (nmol g^{-1}) = $(A_{532} - A_{600}) \cdot V \cdot 10^6 / (155{,}000 \cdot W)$.

For EL analysis, plant tissue (5 mm leaf discs) was transferred to each test tube containing 25 mL of double distilled water (Shi et al., 2006) [27]. The tubes were sealed and then placed on a rotary shaker for 24 h. The electric conductivity (EC_1) of the content of the tubes was determined using an electrical conductivity meter (Leici Instrument Corporation, Shanghai, China). The tubes were sealed and incubated in boiling water for 20 min. The contents of the tubes were cooled at room temperature, followed by a recording of the electric conductivity (EC_2). Membrane damage was calculated using the equation membrane damage (%) = $(EC_1/EC_2) \times 100$.

The content of H_2O_2 was measured according to Patterson et al. (1984) [28]. The absorbance of the titanium peroxide complex was measured with a spectrophotometer

(UV-2802S, UNICO Corporation, Spain) at 415 nm. Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2.

The photosynthetic rate (Pn) and stomatal conductance (gs) were measured in the fully expanded leaves of perennial ryegrass in each treatment using LI-COR 6400 photosynthetic system (LI-COR Inc, Lincoln, NE, USA) (Aftab et al., 2011) [29]. Measurements were taken in the period between 09:00 and 11:00 A.M. All measurements were made at a quantum flux of 680 µmol m^{-2} s^{-1} under red and blue lights. All the measurements were taken at a constant airflow rate of 500 µmol s^{-1}. The ambient CO_2 concentration was 400 µmol mol^{-1} and the temperature was approximately 24 °C.

For determination of antioxidant activities, 0.2 g leaf tissue was ground with liquid nitrogen and extracted with a 1 mL 50 mM phosphate buffer (pH 7.8, including 10 g L^{-1} polyvinylpyrrolidone). The supernatant after centrifugation at 15,000× *g* for 20 min at 4 °C was used for SOD activity determination (Stewart and Bewley, 1980) [30]. The amount of extract that gave 50% inhibition of p-nitro blue tetrazolium chloride reduction was used as one SOD unit.

CAT activity was measured according to Chance, B. and Maehly, A. C. (1955) [31]. The reaction mixer containing 50 mM of phosphate buffer (pH 7.0), 15 mM of H_2O_2, and 100 µL of enzyme extract was mixed in a 3 mL tube. Reaction was started after the addition of the enzyme extract. Within 1 min, the linear decline of absorbance at 240 nm was recorded on a spectrophotometer (Thermo Electron Corporation, CA, USA), and the absorbance change (0.01 units min^{-1}) was used to define CAT activity.

POD activity was determined according to Hammerschmidt et al. (1982) [32]. Leaf tissue (0.2 g) was homogenized in an ice mortar and extracted with a 1 mL 200 mM phosphate buffer (pH 6.0). POD activity was determined by supernatant after centrifugation (5000× *g*, 4 °C, 15 min) using guaiacol as a substrate.

APX activity was estimated by monitoring the decline in absorbance at 240 nm according to Nakano and Asada (1981) [33]. Briefly, the 3 mL reaction mixture contained 100.0 µL enzyme extract, 50 mM phosphate (pH 7.0), 10 mM EDTA-Na_2, 50 mM ascorbic acid, and 10 mM H_2O_2. The absorbance change was measured at 290 nm, and APX activity was expressed as the absorbance change per minute.

Nitrate reductase (NR) activity was measured according to Downs et al. (1993) [34]. The leaf tissue was cut into 5 mm sections. Approximately 200 mg of leaf pieces were placed into tubes containing 2 mL of an incubation substance composed of 0.05 M Tris-HCl pH 7.8 and 0.25 M KNO_3. The tubes were sealed and kept in the dark for 40 min at 37 °C. After incubation, the tissues were treated with a medium containing 1 mL of 1% sulphanilamide in 1 M HCl and 1 mL of 0.01% N-1-napthyl-ethylenediamine hydrochloride (NNEDA). After 15 min with a 40 °C water bath, the optical density was measured at 540 nm with a spectrophotometer (UV-2802S, UNICO Corporation, Shanghai, China). NR activity was expressed as µg g^{-1} h^{-1} FW.

Antioxidant gene expression was analysed using RT-qPCR. The leaf samples of the plant under salt stress that were harvested on 16 July 2020 were used for gene expression analysis. Total RNA was isolated from leaf tissues (about 100 mg of each) using Trizol reagent (Invitrogen, CA, USA) and then treated with RNase-free DNaseI to remove the contaminant DNA (Luo et al., 2011) [35]. The concentration and quality of RNA were, respectively, determined by spectrophotometry (NanoDrop 2000, Thermo Scientific, CA, USA) at 260 and 280 nm and by gel electrophoresis. Reverse transcription of the purified RNA with oligo (dT) primer was performed using the first strand cDNA synthesis kit (Thermo Scientific, CA, USA) according to the manufacturer's instructions. The first cDNA template was then diluted 10-fold and kept at −20 °C for RT-qPCR analysis. The primers of different genes were synthesized based on previous reports and used to amplify the genes in RT-qPCR (Table 1). The *eEF1A(s)* gene was used as the reference gene. The specification of each pair of primers was confirmed by randomly sequencing PCR products and further confirmed by the melting curve analysis using RT-qPCR. The constructing corresponding

plasmid was used to test the amplification efficiency of each pair of primers. Only primers with similar higher amplification efficiency (96%) were used in this experiment.

The transcription levels of genes were analyzed using quantitative real-time PCR with the ABI StepOne PCR System (Applied Biosystems, Foster City, CA, USA). All of the reactions were performed using the TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China). Each 20 µL reaction contains 0.4 µL 0.2 µM of each primer, 10 µL Top Green qPCR SuperMix (TransGen Biotech, Beijing, China), 0.4 µL Passive Reference Dye (50×) (optional), 1 µL of template cDNA, and 7.8 µL ddH_2O. Empty templates (control) were run to verify the level of primer dimer formation and contamination. RT-qPCR parameters were as follows: an initial denaturing step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 50–55 °C for 20 s, and 72 °C for 20 s [35]. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression [36]. The expression of the genes at salt stress without SA was set to 1. There were three technical replicates of each gene per sample for analysis.

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

Gene	Primers Sequences (5′–3′)	Size (bp)	Reference
Cyt Cu/ZnSOD	F GACACMACAAATGGHTGCAT R TCATCBGGATCGGCATGGACAAC	221	Bian and Jiang (2009) [37]
Chl Cu/ZnSOD	F ATGGGTGCATATCDAYAG R GCCAGTCTTCCACCAGCAT	271	Bian and Jiang (2009) [37]
MnSOD	F CAGRGBGCCATCAAGTTCAACG R TACTGCAGGTAGTACGCATG	338	Bian and Jiang (2009) [37]
FeSOD	F TGCACTTGGTGATATTCCACTC R CGAATCTCAGCATCAGGTATCA	297	Bian and Jiang (2009) [37]
POD	F TTCACATTCTGCTCTGCCTG R CCGTGTCTTGTTTCCTCCTG	202	Bian and Jiang (2009) [37]
CAT	F CCTSTCATTGTGMGTTTCTC R GTTAACTCCRAAVCCATCCATATG	292	Bian and Jiang (2009) [37]
pAPX	F CCTGAAAGGTCTGGGTTTGA R TCCTTGGCATAAAGGTCCAC	173	Foito et al. (2009) [38]
eEF1A(s)	F CCGTTTTGTCGAGTTTGGT R AGCAACTGTAACCGAACATAGC	113	Lee et al. (2010) [39]

Chl Cu/ZnSOD: chloroplastic *Cu/ZnSOD*, *Cyt Cu/ZnSOD*: cytosolic *Cu/ZnSOD*, F and R represent forward and reverse.

2.5. Experimental Design and Statistical Analysis

A split plot design with the NaCl treatment as the main plot and the SA treatments as the subplots was used with four replications. The data were subjected to two-way analysis of variance (ANOVA) using SPSS-19 statistical software (SPSS Inc., Chicago, IL, USA). Since salt × SA interactions for all parameters were statistically significant at a 5% probability level for most sampling dates, one-way ANOVA was used for analysis of SA effects under each salt condition. Mean separations were performed using Duncan's multiple range test at $p \leq 0.05$.

3. Results

3.1. Turf Quality (TQ) and Chl Content

The TQ declined during salt stress (Figure 1). The SA treatment increased the TQ under two NaCl conditions at 18 and 24 d. Under salt stress, the SA treatment at 0.25 mM increased the TQ by 26% relative to the 0 mM SA treatment at 24 d. In addition, the SA treatment at 0.5 mM increased the TQ by 23% compared with the 0 mM SA control under salt stress at 24 d. However, SA at 1 mM did not impact the TQ under the 250 mM NaCl treatment.

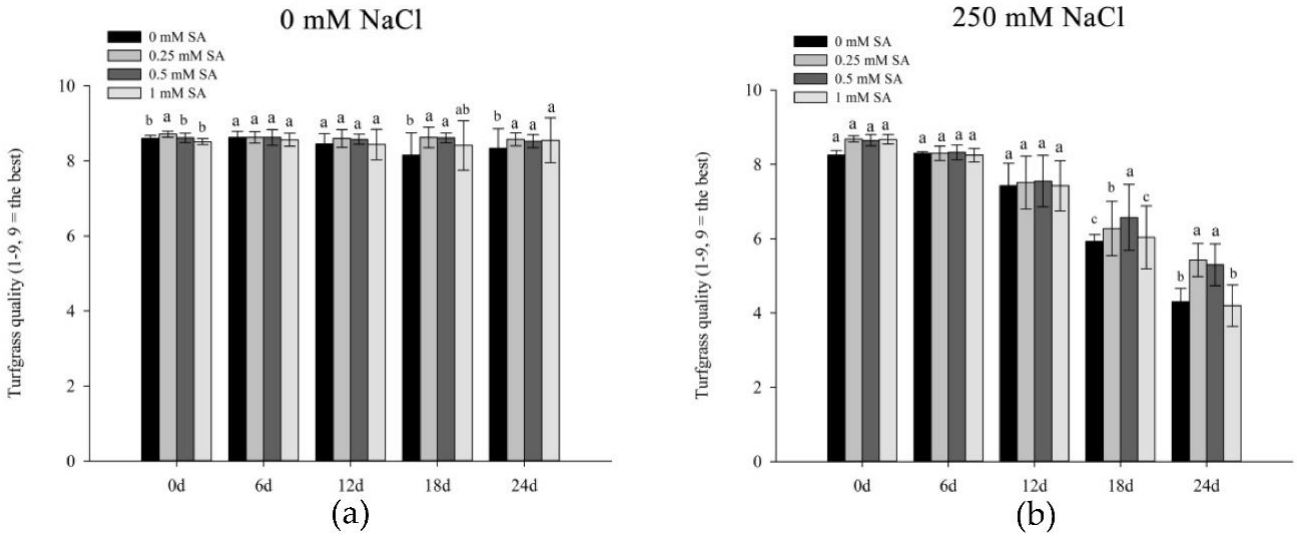

Figure 1. Effect of salicylic acid on turfgrass quality of perennial ryegrass under control (**a**) and 250 mM salt stress (**b**) conditions. The different letters indicate significant differences ($p \leq 0.05$).

Salt stress reduced the Chl content relative to the control at 12, 18, and 24 d (Figure 2). The application of SA at 0.25 mM alleviated the Chl content decline in salt-stressed plants at 6, 12, 18, and 24 d. The SA treatment at 0.25 mM increased the Chl content by 27% relative to the non-SA treatment under salt stress at 24 d. No difference in the Chl content between the 1 mM and 0 mM SA treatments was found under salt stress.

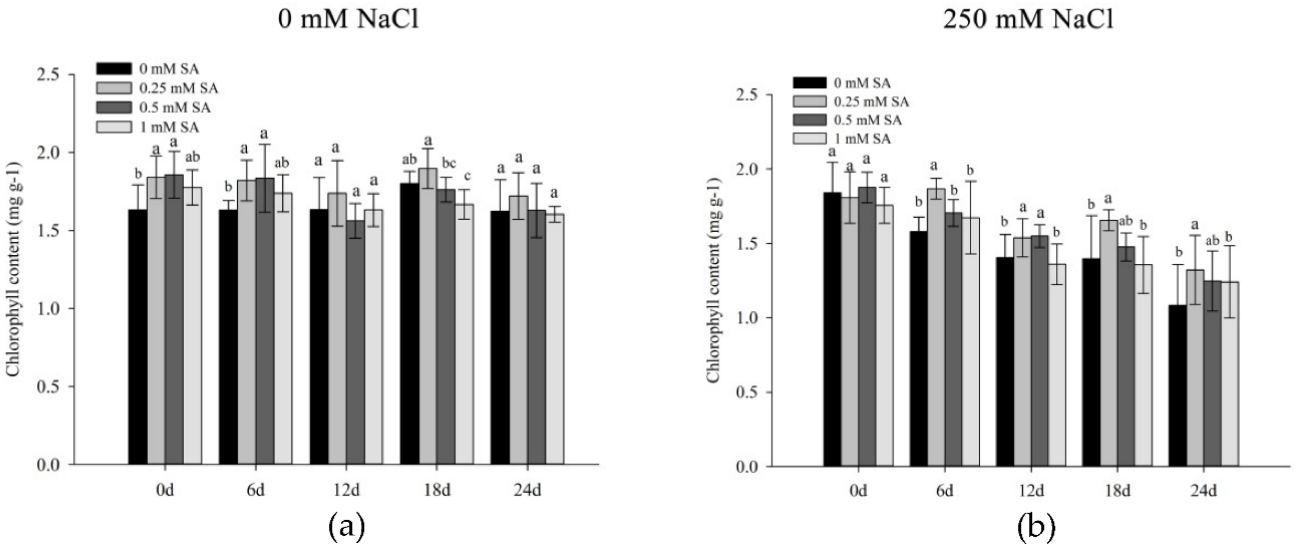

Figure 2. Effect of salicylic acid on chlorophyll content of perennial ryegrass under control (**a**) and 250 mM salt stress (**b**) conditions. The different letters indicate significant differences ($p \leq 0.05$).

3.2. MDA Content, EL, and H_2O_2 Level

Salt stress increased the MDA, EL, and H_2O_2 contents at 12, 18, and 24 d (Table 2). The SA treatments inhibited the salt-induced increases in EL, MDA, and H_2O_2. The application of SA at 0.25 mM reduced the EL of salt-stressed plants at 6, 12, 18, and 24 d. The application of SA at 0.25 mM reduced the EL and MDA contents by 36% and 41%, respectively, compared to the 0 mM SA treatment under salt stress at 24 d. The H_2O_2 content in the plant treated with SA at 0.25 mM was 40% greater than that without SA under salt stress conditions. However, the application of SA at 1 mM had no effect on the EL, MDA, and H_2O_2 contents under either 250 mM NaCl or 0 mM NaCl conditions.

Table 2. Effect of salicylic acid on malondialdehyde (MDA) content, electrolyte leakage, and H_2O_2 levels of perennial ryegrass under two salt conditions.

NaCl (mM)	SA (mM)	Days of NaCl Treatment				
		0	6	12	18	24
		Electrolyte leakage (%)				
0	0	12 a [x]	13 a	22 ba	18 a	15 a
	0.25	12 a	13 a	17 b	22 a	14 a
	0.5	11 a	12 a	16 b	20 a	12 a
	1	15 a	13 a	25 a	19 a	17 a
250	0	12 a	27 a	47 a	57 a	70 a
	0.25	12 a	14 b	31 b	39 b	45 b
	0.5	11 a	16 b	35 b	43 b	58 ab
	1	14 a	16 b	43 b	60 a	70 a
	0 mM NaCl	13 x	13 y	20 y	20 y	14 y
	250 mM NaCl	12 x	18 x	39 x	49 x	61 x
		MDA content (nmol g^{-1})				
0	0	37.0 a	43.3 a	47.2 a	46.7 a	53.3 a
	0.25	38.5 a	43.2 a	44.0 a	39.1 a	43.9 a
	0.5	35.2 a	45.0 a	44.6 a	45.5 a	50.7 a
	1	38.3 a	46.8 a	44.7 a	43.6 a	47.3 a
250	0	36.8 a	49.8 a	63.6 a	72.8 a	105.4 a
	0.25	38.1 a	44.3 a	46.7 b	57.0 b	62.4 b
	0.5	35.5 a	46.0 a	46.0 b	55.9 b	70.1 ab
	1	37.9 a	55.7 a	55.0 ab	67.5 ab	100.1 a
	0 mM NaCl	37.3 x	44.6 x	45.1 y	43.7 y	48.8 y
	250 mM NaCl	37.0 x	49.0 x	52.8 x	63.3 x	84.8 x
		H_2O_2 level (μmol g^{-1})				
0	0	1.0 ab	0.9 a	1.3 a	1.0 a	1.3 a
	0.25	0.8 b	0.8 a	1.1 ab	0.7 a	0.7 a
	0.5	0.8 b	0.8 a	1.1 b	0.8 a	1.1 a
	1	1.2 a	1.0 a	0.8 c	1.0 a	1.4 a
250	0	1.1 a	1.5 ab	2.1 a	3.2 a	3.9 ab
	0.25	0.7 a	0.9 b	1.6 a	2.3 a	2.0 b
	0.5	0.9 a	1.1 ab	1.9 a	2.6 a	2.3 ab
	1	1.1 a	1.7 a	2.1 a	3.7 a	4.1 a
	0 mM NaCl	1.0 x	0.9 y	1.1 y	0.9 y	1.1 y
	250 mM NaCl	0.9 x	1.3 x	1.9 x	2.9 x	3.1 x

[x] Means with the same letters within a column for each treatment are not significantly different at $p \leq 0.05$.

3.3. Photosynthetic Rate (Pn) and Stomatal Conductance (gs)

The Pn and gs were reduced when the grass was subjected to salt stress (Table 3). Under salt conditions, the application of SA at 0.25 and 0.5 mM increased the Pn at 18 and 24 d. The SA treatments at 0.25 and 0.5 mM improved the gs under salt stress at 12, 18, and 24 d. Under non-salt conditions, the application of SA also increased the gs. At 24 d, the SA application at 0.25 mM increased the Pn and gs by 104% and 67%, respectively, compared to the control (0 mM SA) under salt stress. Nevertheless, SA at 1 mM did not impact the Pn and gs under either salt stress or non-salt stress conditions.

Table 3. Effect of salicylic acid on photosynthetic rate (Pn) and stomatal conductance (gs) of perennial ryegrass under two salt conditions.

NaCl (mM)	SA (mM)	Days of NaCl Treatment				
		0	**6**	**12**	**18**	**24**
		Pn (μmol CO_2 m^{-2} s^{-1})				
0	0	11.8 a [x]	14 a	12.3 a	8.5 b	11.9 a
	0.25	11.6 a	13.8 a	13.2 a	11.1 a	13.7 a
	0.5	11.2 a	13.1 a	10.7 a	11.9 a	11.2 a
	1	10.0 b	8.5 b	6.8 b	8.1 b	7.1 b
250	0	11.2 a	4.4 ab	6.5 a	2.7 b	2.7 b
	0.25	11.6 a	4.8 ab	7.9 a	6.1 a	5.5 a
	0.5	10.7 a	5.7 a	6.1 a	6.0 a	5.7 a
	1	8.3 b	3.2 b	5.3 a	2.6 b	3.4 b
	0 mM NaCl	11.1 x	12.3 x	10.8 x	9.9 x	11.0 x
	250 mM NaCl	10.4 x	4.5 y	6.4 y	4.4 y	5.5 y
		gs (mol H_2O m^{-2} s^{-1})				
0	0	0.26 b	0.27 b	0.27 a	0.27 b	0.28 b
	0.25	0.31 a	0.32 a	0.28 a	0.32 a	0.33 a
	0.5	0.27 b	0.30 ab	0.26 a	0.28 ab	0.31 ab
	1	0.22 c	0.22 c	0.18 b	0.20 c	0.18 c
250	0	0.25 b	0.16 bc	0.14 b	0.09 b	0.06 c
	0.25	0.30 a	0.21 a	0.16 a	0.12 a	0.10 a
	0.5	0.27 ab	0.18 ab	0.15 a	0.11 a	0.09 b
	1	0.24 b	0.14 c	0.13 b	0.08 b	0.06 c
	0 mM NaCl	0.26 x	0.28 x	0.25 x	0.27 x	0.28 x
	250 mM NaCl	0.27 x	0.17 y	0.14 y	0.10 y	0.08 y

[x] Means with the same letters within a column for each treatment are not significantly different at $p \leq 0.05$.

3.4. Antioxidant Enzyme Activity and Gene Expression

Salt stress increased SOD activity as measured at 6 and 18 d (Table 4). The SA treatments at 0.25 and 0.5 mM increased SOD activity by 92% and 95%, respectively, compared to the control under salt stress at 24 d. Under normal conditions, the application of SA at 0.25 mM increased SOD activity relative to the control at 6 and 24 d. There was no difference in SOD activity between the 1 mM SA and 0 mM SA treatments under salt stress.

The application of SA significantly upregulated the expression of *CytCu/ZnSOD* under salt stress (Figure 3a). When exposed to 0.25 or 0.5 mM of SA, the expression level of *CytCu/ZnSOD* increased 1.6-fold. The SA treatment at 1 mM caused the downregulation of *CytCu/ZnSOD* gene expression compared to the control. The SA treatment at 0.5 mM caused the downregulation of *ChlCu/ZnSOD* expression relative to the control (Figure 3b). No difference was observed in the *ChlCu/ZnSOD* expression among the 0, 0.25, and 1 mM SA treatments. The SA treatment at 0.25 mM induced the upregulation of *FeSOD* expression (Figure 3c). The downregulation of *MnSOD* was observed in the grass treated with SA at 0.25 and 1 mM SA compared to the control (Figure 3d). The SA treatment at 0.5 mM did not impact *MnSOD* expression. Salt stress increased APX activity as measured at 6 d (Table 5). The application of SA at 0.25 mM increased APX activity by 55% and 84%, respectively, compared to the control under salt stress at 18 and 24 d. The SA treatments at 0.25 and 0.5 mM SA induced the upregulation of APX expression (Figure 4a). Focusing on the general tendency of the gene expression level, we found that the gene expression pattern of the non-stressed samples did not show significant variation compared with the salt-treated samples, so the data were not shown in this section.

Table 4. Effect of salicylic acid on SOD and CAT activity of perennial ryegrass under two salt conditions.

NaCl (mM)	SA (mM)	Days of NaCl Treatment				
		0	6	12	18	24
		SOD (units g^{-1})				
0	0	876 a [x]	888 b	978 a	1023 a	870 b
	0.25	951 a	1026 a	995 a	1068 a	1091 a
	0.5	869 a	1037 a	1119 a	1032 a	995 ab
	1	877 a	1025 a	1010 a	987 a	1093 a
250	0	880 a	1066 a	889 a	925 a	582 b
	0.25	916 a	1244 a	1068 a	1151 a	1115 a
	0.5	885 a	1197 a	999 a	1136 a	1134 a
	1	905 a	1112 a	1082 a	1096 a	640 b
	0 mM NaCl	893 x	994 y	1025 x	1028 x	1012 x
	250 mM NaCl	897 x	1155 x	1010 x	1076 x	958 x
		CAT (units min^{-1} g^{-1})				
0	0	68.5 b	74.0 ab	47.7 b	61.8 c	65.6 c
	0.25	82.5 ab	75.0 ab	66.8 a	81.2 a	84.1 a
	0.5	87.0 a	78.6 a	67.4 a	74.2 ab	79.5 ab
	1	81.3 ab	72.6 b	59.1 ab	64.3 bc	76.1 b
250	0	82.2 a	70.4 a	58.1 ab	50.3 ab	62.6 a
	0.25	79.8 a	78.0 a	62.9 a	63.5 a	40.3 b
	0.5	81.4 a	72.3 a	56.9 ab	62.0 ab	40.8 b
	1	80.3 a	67.0 a	49.8 b	43.3 b	38.7 b
	0 mM NaCl	79.8 x	75.1 x	60.2 x	70.4 x	76.3 x
	250 mM NaCl	80.9 x	71.9 x	57.0 x	54.8 y	51.6 y

[x] Means with the same letters within a column for each treatment are not significantly different at $p \leq 0.05$.

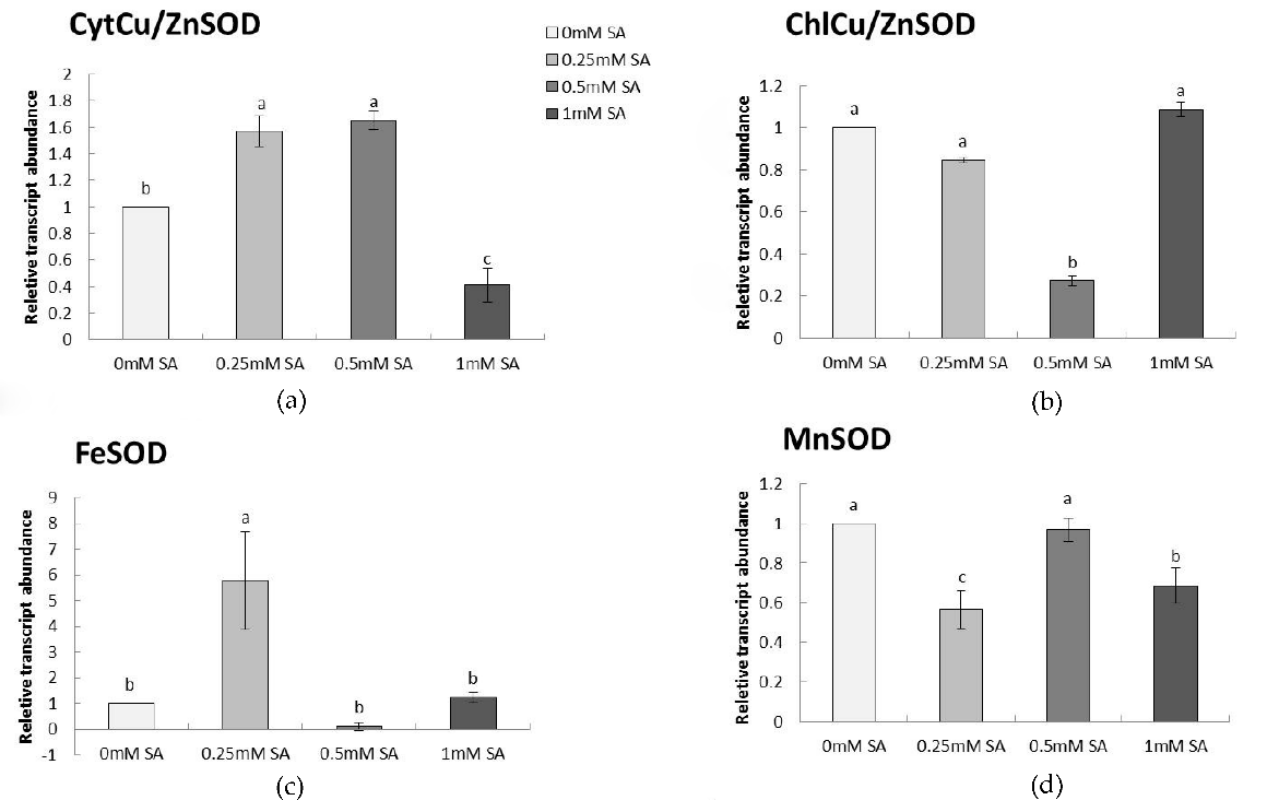

Figure 3. Effect of SA treatment on the relative abundance of *CytCu/ZnSOD* (**a**), *ChlCu/ZnSOD* (**b**), *FeSOD* (**c**) and *MnSOD* (**d**) transcripts determined through RT-qPCR in perennial ryegrass under 250 mM NaCl stress. The values are the means ± SD (n = 4). The different letters indicate significant differences between various SA treatments ($p \leq 0.05$).

Table 5. Effect of salicylic acid on APX and POD activity of perennial ryegrass under two salt conditions.

NaCl (mM)	SA (mM)	Days of NaCl treatment				
		0	6	12	18	24
		APX (units min^{-1} g^{-1})				
0	0	4.4 b [x]	4.3 a	5.3 a	4.9 bc	4.9 a
	0.25	5.0 ab	5.2 a	5.7 a	6.0 ab	6.3 a
	0.5	5.5 a	5.2 a	5.5 a	6.6 a	5.3 a
	1	4.9 ab	4.5 a	5.1 a	4.3 c	5.5 a
250	0	5.4 a	5.3 a	5.5 ab	4.4 bc	3.8 bc
	0.25	5.4 a	5.5 a	6.9 a	6.8 a	7.0 a
	0.5	5.2 ab	5.3 a	6.2 a	5.7 ab	6.4 ab
	1	4.3 b	5.1 a	4.7 b	3.0 c	2.6 c
	0 mM NaCl	5.0 x	4.8 y	5.4 x	5.4 x	5.5 x
	250 mM NaCl	5.1 x	5.3 x	5.8 x	5.0 x	5.5 x
		POD (units min^{-1} g^{-1})				
0	0	1.3 a	1.5 a	1.4 a	1.5 a	1.5 a
	0.25	1.4 a	1.6 a	1.5 a	1.6 a	1.6 a
	0.5	1.4 a	1.5 a	1.4 a	1.7 a	1.6 a
	1	1.4 a	1.4 a	1.3 a	1.5 a	1.4 a
250	0	1.4 a	1.4 a	1.3 b	1.2 c	1.2 a
	0.25	1.5 a	1.4 a	1.6 a	1.6 a	1.4 a
	0.5	1.4 a	1.5 a	1.5 ab	1.5 ab	1.3 a
	1	1.3 a	1.5 a	1.3 b	1.2 bc	1.1 a
	0 mM NaCl	1.4 x	1.5 x	1.4 x	1.6 x	1.5 x
	250 mM NaCl	1.4 x	1.5 x	1.4 x	1.4 y	1.2 y

[x] Means with the same letters within a column for each treatment are not significantly different at $p \leq 0.05$.

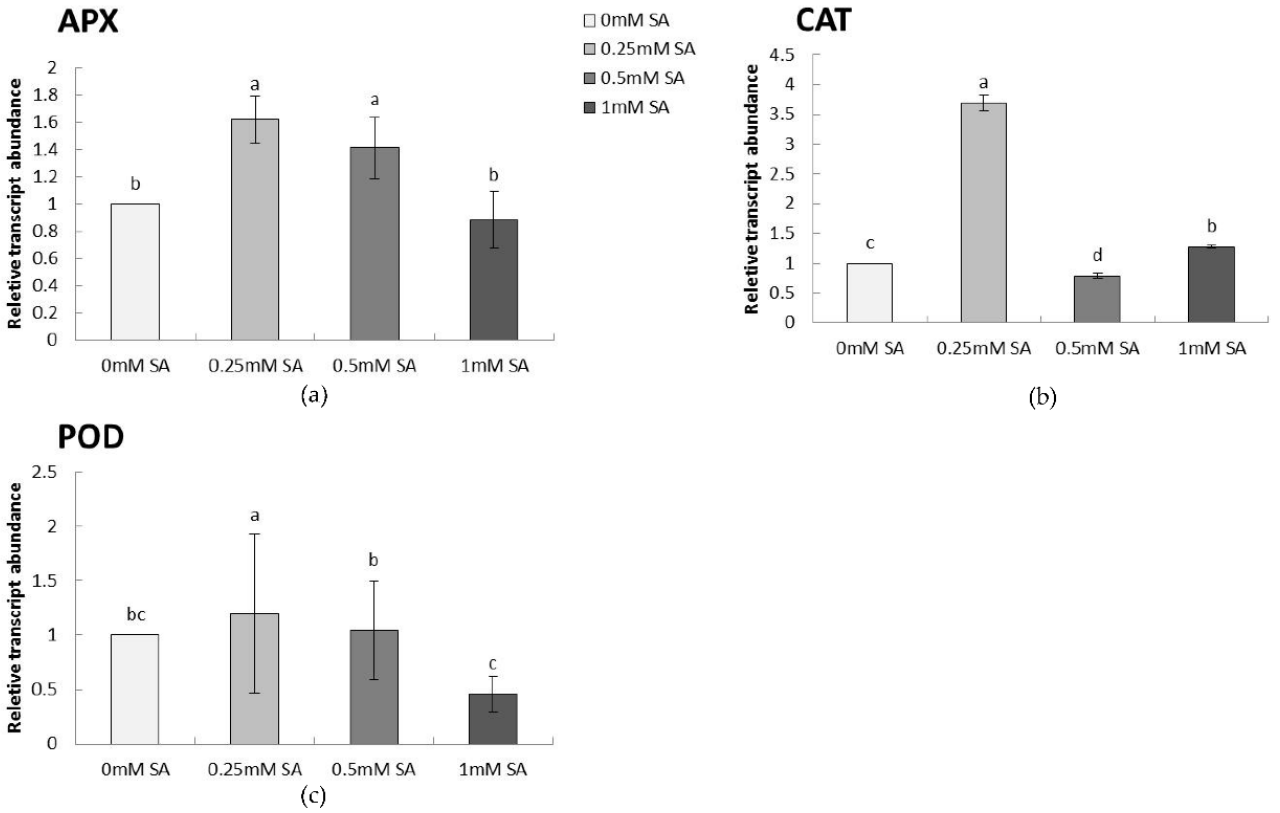

Figure 4. Effect of SA treatment on the relative abundance of *APX* (**a**), *CAT* (**b**) and *POD* (**c**) transcripts determined through RT-qPCR in perennial ryegrass under 250 mM NaCl stress. The values are the means ± SD (n = 4). The different letters indicate significant differences between various SA treatments ($p \leq 0.05$).

Salt stress reduced CAT activity at 18 and 24 d (Table 4). The application of SA did not consistently impact CAT activity under salt stress conditions. Under normal conditions, the application of SA at 0.25 and 0.5 mM increased CAT activity relative to the control at 12, 18, and 24 d. The SA treatment at 0.25 mM increased the *CAT* gene expression level 3.5-fold compared to the control (Figure 4b). The SA treatment at 1 mM also increased *CAT* expression relative to the control.

Salt stress reduced POD activity as observed at 18 and 24 d (Table 5). The application of SA at 0.25 mM increased POD activity by 23% and 33% at 12 and 18 d, respectively, compared to the control. Under non-salt conditions, the SA treatment did not change POD activity relative to the control. The SA treatment at 0.25 mM increased the *POD* gene expression level 1.2-fold compared to the control (Figure 4c). The SA treatments at 0.5 and 1 mM did not impact *POD* gene expression.

3.5. Nitrate Reductase Activity

The NR activity declined as the plant was subjected to salt stress (Figure 5). Under salt stress, the application of SA at 0.25 and 0.5 mM improved NR activity as measured at 6, 12, 18, and 24 d. The SA treatments at 0.25 and 0.5 mM increased NR activity by 29% and 22%, respectively, compared to the control under salt stress at 24 d. However, the SA at 1 mM did not impact NR activity under salt stress.

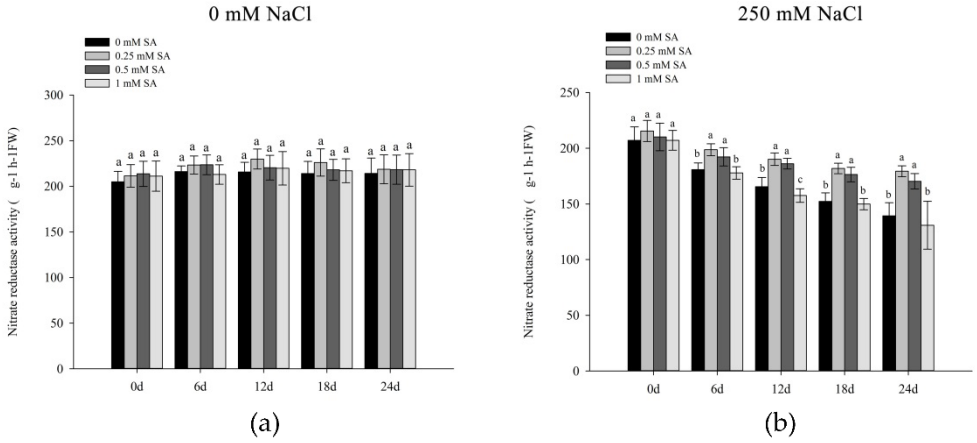

Figure 5. Effect of salicylic acid on nitrate reductase activity (μg g^{-1} h^{-1} FW) of perennial ryegrass under control (**a**) and 250 mM salt stress (**b**) conditions. The different letters indicate significant differences ($p \leq 0.05$).

4. Discussion

The results of this study indicated that salt stress caused oxidative damage to PRG. Exogenous SA at 0.25 and 0.5 mM lowered the EL, H_2O_2, and MDA content of salt-stressed PRG. The SA treatment increased the TQ and Chl contents under salt stress. Our results are consistent with a previous study by Alsahli et al. (2019), which showed that SA alleviated the MDA and H_2O_2 accumulation relative to the non-SA treatment under saline stress [40]. The MDA content and EL have been used to assess oxidative membrane damage caused by salt stress [41]. Under salt stress, plants with a lower level of MDA could indicate less lipid peroxidation and better cell membrane integrity [42]. The results of this study suggest that the foliar application of SA at 0.25 and 0.5 mM can improve salt stress tolerance in PRG. The SA at 1 mM did not improve salt stress tolerance, possibly because high-rate SA can cause oxidative stress. Rao et al. (1997) [43] noted that exogenous SA at 1 to 5 mM increased hydrogen peroxide 3-fold and caused oxidative stress in Arabidopsis plants.

Salt stress decreased the Chl content, Pn, and gs in this study. The application of SA increased the Chl content, Pn, and gs under salt stress, particularly at the 0.25 mM level. The results of this study are consistent with those of Tari et al. (2002) [44] on tomatoes (*Solanum lycopersicum* L.) and Shi et al. (2006) and Yildirim et al. (2008) [27,45]

on cucumbers (*Cucumis sativus* L.), which showed that the application of SA increased the leaf Chl content. Gurmani et al. (2019) and Mahmoud et al. (2021) [46,47] found that the application of SA induced an increase in the Chl content of cucumbers and valencia sweet oranges (*Citrus sinensis*) under salt stress. Aftab et al. (2011) [29] found that the SA-induced alleviation of the Chl content decline was attributed to stimulated Chl biosynthesis and retarded Chl degradation. The increased photosynthetic pigments induced by SA, including chlorophyll a and b, can improve the photosynthetic rate under salinity stress [23]. These results suggested that SA increases in the Chl content of salt-stressed PRG could improve photosynthetic efficiency.

In the present study, we found that SA at 0.25 mM enhanced the Pn and gs relative to the non-SA treatment under salt stress. The SA-induced increase in the gs could be associated with a higher Pn under salt stress [29]. Hamani et al. (2020) [48] found that the foliar application of SA increased the Pn and improved the antioxidant enzymes of cotton under salt stress. When plants are subjected to salt stress, the closure of stomata induced the reduction of CO_2 in chloroplasts and thus decreased the Pn [49]. The results of this study are in agreement with earlier studies on pistachios (*Pistaciavera* L.), mung beans (*Vignaradiata* L.), and mustard that indicated that the enhancement in the Pn by SA was correlated with an increased gs under stress conditions [17,50]. The SA treatment could play a specific role in dealing with the salt stress-induced senescence in leaves [16]. The results of this study suggest that SA applications could improve the Pn associated with elevated gs under salt stress.

The results of this study showed that SOD activity increased in stressed plants relative to non-salted stress plants at 6 d. The SA treatments further increased the SOD activity of PRG under salt stress. However, the application of SA decreased CAT activity under salt stress. This is in general agreement with the results of Li et al. (2014) [6], who proved that SA increased SOD activity but suppressed CAT activity. A previous study indicated that ROS accumulation caused by short-term salt stress could trigger an increase in SOD activity [4]. Idrees et al. (2011) [16] found that a foliar spray of SA increased the activity of SOD in salt-stressed periwinkle. Similar results were observed by Aftab et al. (2011) [29]. Mutlu et al. (2009) [18] reported that SA treatments improved SOD activity but inhibited CAT activity in salt-tolerant wheat cultivars. SA could function on SA-binding protein, which is highly homologous with CAT, thus decreasing CAT activity [51]. Salt stress caused the excess accumulation of toxic ROS in cells. The level of salt stress tolerance is often correlated with various antioxidant metabolites and enzyme activity [52]. These results indicated that SA treatment could enhance SOD activity and thus improve the salt stress tolerance of perennial ryegrass.

The results of this study also indicated that the application of SA increased APX and POD activity under salt stress conditions. This is supported by Syeed et al. (2011) [17], who reported that SA-treated mustard had greater APX activity than the control under salt stress. The efficient destruction of H_2O_2 required the induction of APX [53]. A previous study showed that the enhancement of POD activity by exogenous SA application alleviated salt-induced oxidative damage in sunflowers [54]. Li et al. (2014) [6] demonstrated that SA enhanced the POD activity of Artemisia annua L. when plants were subjected to salinity. The APX and POD are both important enzymes to scavenge H_2O_2 in oxidative-stressed plants. These results suggest that exogenous SA could facilitate the detoxifying of H_2O_2 by enhancing APX and POD activities under salt stress, and APX and POD may have a more important role than CAT in H_2O_2 scavenging.

In the present study, SA treatments promoted the expression level of antioxidant genes including *Cyt Cu/Zn SOD*, *Chl Cu/Zn SOD*, *MnSOD*, *FeSOD*, *CAT*, *APX*, and *POD*. The application of SA at 0.25 mM induced the upregulated expression of *FeSOD*, *Cyt Cu/ZnSOD*, *APX*, *CAT*, and *POD* at 24 d under salt stress. The four different genes *Cyt Cu/Zn SOD*, *Chl Cu/Zn SOD*, *MnSOD*, and *FeSOD* that encode SOD are specifically directed to chloroplasts, mitochondria, peroxisomes, and cytosol. Hu et al. (2012) [4] found that enzymes encoded by *MnSOD* and *FeSOD* were more important in removing ROS than other gene-encoded

enzymes in salt-stressed perennial ryegrass. However, our results suggested that the changes in *Cyt Cu/Zn SOD* and *FeSOD* were more coincident with SOD activity. Our results support the findings of Luo et al. (2011) [35] which showed that *Cyt Cu/Zn SOD* and *FeSOD* could have relatively higher contributions to total SOD activity than other genes induced by SA treatment under salt stress. Our results suggest that the SA-induced expression of the *APX* and *POD* transcripts was related to their activity. Hu et al. (2012) [4] reported that the induced expression of peroxisome *APX* transcripts was higher for salt-tolerant perennial ryegrass cultivars than salt-sensitive ones. In the present study, the SA-induced upregulation of the *CAT* gene did not coincide with CAT activity. Hu et al. (2012) [4] reported that the *POD* transcripts decreased when the POD isoenzyme activities increased in perennial ryegrass under salt stress. These results suggest that the transcript does not always correlate with the corresponding changes in antioxidant enzyme activities. The SA-induced regulations of the antioxidant system can be at both the transcriptional and translational levels in perennial ryegrass under salt stress.

The results of this study indicate that salt stress reduced NR activity and SA treatments increased NR activity under salt stress. A previous study indicated that SA improved the NR activity of lemongrass under water stress [16]. The enhancement of NR activity by SA treatment could protect the plasma membrane from stress-mediated damage. The integrated membrane could increase the uptake of nitrate, thus influencing the synthesis of NR [55]. The results of this study suggest that SA treatment could improve NR activity under salt stress.

5. Conclusions

The results of this study showed that salt stress inhibited the growth of perennial ryegrass and caused oxidative damage. Exogenous SA treatments at 0.25 mM and 0.5 mM were effective in ameliorating the adverse effects of salinity stress on perennial ryegrass, but a high rate of SA (1 mM) did not. The expression of antioxidant genes and efficient activation of antioxidant enzymes induced by SA contributed to the improved salt tolerance of perennial ryegrass. The enhancement of the antioxidant capacities and stomatal conductance due to the SA application protected the photosynthetic system, integrity of the membrane, and N metabolism under salt stress. The results of this study suggest that exogenous SA at 0.25 and 0.5 mM could improve plant antioxidant metabolism and photosynthetic function and alleviate salt stress injury in perennial ryegrass.

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Abbreviations

APX, ascorbate peroxidase; ASA-GSH, ascorbate-glutathione; CAT, catalase; Cd, cadmium; Chl, chlorophyll; *ChlCu/ZnSOD*, chloroplastic copper/zinc SOD; *CytCu/ZnSOD*, cytosolic copper/zinc SOD; EL, electrolyte leakage; FW, fresh weight; GP, glutathioneperoxidase; gs, stomatal conductance; GSH, ascorbate-glutathione; H_2O_2, hydrogen peroxide; MDA, malonaldehyde; NR, nitrate reductase; O_2^-, superoxide; PAR, photosynthetically active radiation; Pn, net photosynthetic rate; POD, peroxidase; PRG, perennial ryegrass; ROS, reactive oxygen species; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TBA, thiobarbituric acid; TQ, turfgrass quality.

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