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Salicylic acid delays leaf rolling by inducing antioxidant enzymes and modulating osmoprotectant content in *Ctenanthe setosa* under osmotic stress

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Abstract: The effects of salicylic acid (SA) on leaf rolling under osmotic stress in *Ctenanthe setosa* (Roscoe) Eichler were evaluated. The leaves were pretreated with SA, paclobutrazol (PAC), and antioxidant enzyme inhibitors for 15 h and then kept in polyethylene glycol (PEG) for 4 h to promote osmotic stress. The degree of leaf rolling was measured every hour during the 4-h PEG treatment. Antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase, and guaiacol peroxidase) and NADPH oxidase (NOX) activities, endogenous SA contents, hydrogen peroxide, proline, and soluble sugar were determined immediately after PEG treatment. SA treatment retarded leaf rolling and induced antioxidant enzyme activities under osmotic stress. Antioxidant enzyme inhibitors and PAC treatments caused reductions in all antioxidant enzyme activities and accelerated leaf rolling. The PAC treatment also decreased endogenous SA, hydrogen peroxide, proline, and soluble sugar contents and NOX activity. In conclusion, the delay in leaf rolling resulting from SA application was related to the antioxidant enzyme activities and also to osmoprotectants. These were supported by the increase in leaf rolling degree due to PAC, which led to a decrease in proline and sugar contents and antioxidant enzyme activities due to a decrease in hydrogen peroxide content followed by a reduction in NOX activity.

Key words: Antioxidant enzymes, *Ctenanthe setosa*, leaf rolling, osmotic stress, salicylic acid

1. Introduction

Leaf rolling is a typical response to drought in numerous plant species such as rice, maize, wheat, and sorghum. However, it also results from other abiotic stresses factors such as salt, temperature, heavy metals, and UV radiation (1). Leaf rolling represents a dynamic behavioral response in plants whereby the normal sunlight orientation of the 2 leaf surfaces may be reversed (2). In higher plants, leaf rolling is caused by bulliform and hypodermis cells (3). The loss of water causes the cells to shrink and the leaves to roll. On the other hand, when the cells become turgid, the leaves maintain a flat position (4). Leaf rolling is an effective protection mechanism for some plants against drought stress (4,5). Leaf rolling was detected as a parameter for determination of drought tolerance in 10 maize cultivars subjected to 34 days of drought stress under greenhouse conditions (6). In another study, Abd Allah (7) studied the genetics of leaf rolling, some root and shoot characters, and their relation with drought resistance characters in 10 rice cultivars under drought stress. The results indicated that the F3 lines had better leaf rolling and, consequently, better drought resistance than their corresponding resistant parents.

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Leaf rolling is a hydronastic mechanism that reduces light interception, transpiration, and leaf dehydration. Leaf rolling might play a similar role in osmotic adjustment to maintain internal plant water status (8). Delayed leaf rolling indicates the ability to sustain turgor despite drought stress, for instance through increased water uptake or osmotic adjustment. In addition, leaf rolling provides protection from the effects of excess radiation (4). It is suggested that plants with rolled leaves have a certain resistance to drought and high temperature (4). Plants exposed to drought stress generate reactive oxygen species (ROS), including superoxide anion radicals (O_2^-), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (9). ROS may react with proteins, lipids, or deoxyribonucleic acid, causing oxidative damage and devastating the normal functions of cells (10). The ROS in plants are scavenged by a variety of antioxidant enzymes (11). Upon exposure to abiotic stresses, plants activate their antioxidant enzymes, which then start quenching ROS and protecting the cell (12). Changes in antioxidant enzyme activities during leaf rolling were detected in *Ctenanthe setosa* under drought stress (13,14). In these studies, exposure to drought stress enhanced the activities

of antioxidant enzymes catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), dehydroascorbate reductase, and monodehydroascorbate reductase; however, superoxide dismutase (SOD) activity did not change significantly during leaf rolling. On the other hand, ROS, especially nitric oxide and H_2O_2, can be regulatory signal molecules for antioxidant enzyme induction under stress conditions (15). Accumulation of H_2O_2 parallel to an increase in leaf rolling in *C. setosa* under drought stress was determined (13,16). Therefore, by inducing antioxidant systems, H_2O_2 can be an important regulator molecule for leaf rolling. It is well documented that H_2O_2 plays a central role in responses to both abiotic and biotic stresses in plants. This molecule seems to be a master hormone that controls a variety of stress responses and physiological adjustments including the ROS/hormonal homeostasis in the cell (17). Depending on the site of generation and the interaction with specific hormonal compounds, such as salicylic acid (SA), ethylene, auxins, abscisic acid (ABA), and others, H_2O_2 is able to act in a specific manner (18). SA is an important signaling molecule that has role in a series of plant physiological processes including photosynthesis and plant defense responses. It has been found that plants treated with SA generally exhibit better resistance to drought stress (19). Exogenous SA treatment induces the antioxidant enzyme system in plants under chilling and osmotic stress conditions (20,21).

Moderate leaf rolling increases photosynthesis under mild water stress (22); however, leaf rolling may also cause decreases in photosynthesis and yield under mild water stress due to reduced light absorption by the photosynthetic system (23). In the present study we claim that a delay in leaf rolling may be beneficial for plants under mild water stress. In addition, delayed leaf rolling associated with drought resistance in rice cultivars subjected to drought stress was reported (24). Moreover, SA-mediated delays in leaf rolling as a result of water loss prevention and the induction of antioxidant enzymes has been reported in mature maize plants after 17-day drought stress under a rainout shelter in the field (25) and in *C. setosa* subjected to 38-day severe water stress by withholding water in a growth chamber (26). However, it is still unclear how SA is able to control the delay in leaf rolling. Furthermore, the leaves of some plants accumulate soluble sugars during rolling for osmotic adjustment. For example, reducing and soluble sugars are accumulated as the degree of leaf rolling increases in *C. setosa* (27). Proline is also an important solute related to the adaptation to low water potential by leaf rolling. Similar to soluble sugars, its concentration is higher in the rolled leaves of *C. setosa* than in unrolled leaves (27). Hare and Cress (28) have proposed that proline might function as a regulatory signal. It is now well established that a molecular signal system capable of sensing proline levels acts in plant cells to control gene expression (29). Proline signaling interacts with the soluble sugar signaling pathways, with proline exhibiting dual functions as an osmolyte compound and an ROS scavenger. In addition, soluble sugars are important signals in the regulation of plant metabolism and development (30). In plants, soluble sugars have been shown to fulfill a dual role as both metabolites and signaling molecules (31) that may play important roles in the adaptive mechanisms to stress (32). Soluble sugar protection against oxidative stress seems to be partly due to activation of specific ROS scavenging systems and a consequent reduction in oxidative damage (32,33). In addition, SA is reported to induce accumulation of proline in osmotically stressed seedlings (34). An increase in proline content in sunflower plants under drought stress conditions by SA application was also determined (35). Additionally, an increase in soluble sugar content by SA application was reported in grape seedlings (36) and in wheat plants subjected to drought followed by treatment of the seeds with 0.5 M SA before sowing (37). Thus, it is important to learn the extent to which soluble sugars, proline, and antioxidants can intervene in delayed leaf rolling resulting from SA and how SA regulates these signal mechanisms. In order to study these signal mechanisms and their relation to leaf rolling phenomena, a short period of osmotic stress was applied to the detached leaves of *C. setosa*.

2. Materials and methods

2.1. Plant material and growth conditions

Twenty *C. setosa* (Roscoe) Eichler (Marantaceae) plants were vegetatively propagated from their rhizomes and grown in plastic pots containing peat and sand (5:1) in a growth chamber for 6 months under the following conditions: 16 h light and 8 h darkness at 25 °C, photon flux density at the surface of the leaves of 300 μmol m^{-2} s^{-1}, and 70% relative humidity. Each plant was the same age and had at least 15 fully grown leaves of similar size. From these plants, 30 randomly selected leaves were used for each of the experiments. Each experiment was repeated 3 times.

2.2. SA and paclobutrazol treatments

To determine the effect of SA on leaf rolling, the detached leaves of well-watered plants were placed in distilled water for 1 h to eliminate wound stress, and then the cut ends of the petioles were placed in tubes of the following solutions and kept in them for 15 h. There were 2 treatment groups; the first was treated with SA while the other was nontreated (NT). The SA-treated group included the following (prepared with distilled water): 1 μM SA as control, 1 μM SA + 1.5 mM dithiothreitol (DTT; an inhibitor of SOD, APX, GPX, and CAT), and 1 μM SA + 100 μM

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; an inhibitor of GPX inhibitor). The NT group included the following (prepared in dH_2O): distilled water as control, 1.5 mM DTT, and 100 μM DTNB. After the 15-h pretreatments, leaves were transferred to 2.5% polyethylene glycol (PEG 6000) for 4 h to induce osmotic stress.

In order to evaluate the role of SA on leaf rolling, detached leaves of the well-watered plants were treated with distilled water (control), 1 μM SA, 500 μM paclobutrazol (PAC; a biosynthesis inhibitor of SA), and 1 μM SA + 500 μM PAC for 15 h, separately. After these pretreatments, the leaves were transferred to 2.5% polyethylene glycol (PEG 6000) for 4 h. A PAC concentration of 500 μM was used due to an increase in leaf rolling at this concentration. Degree of leaf rolling (%) was measured during the 4 h in PEG. Immediately after the 4-h PEG treatment, antioxidant enzyme and NADPH oxidase (NOX) activities, endogenous SA, proline, soluble sugar, and H_2O_2 contents were determined.

2.3. Determination of leaf rolling degree

Degree of leaf rolling (%) was measured during the 4-h PEG treatment. Degree of leaf rolling (%) was calculated according to the methods of Premachandra et al. (38). The width of the middle portion of the leaves was measured and the degree of leaf rolling was calculated as percentage reduction in leaf width.

2.4. Enzyme activity assays

To determine antioxidant enzyme activities (except in NOX), 0.25 g of frozen leaf tissue was homogenized with a mortar and pestle in 5 mL of 50 mM potassium-phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 0.1% PVP. In the case of the APX assay, 5 mM ascorbic acid (ASC) was added to the buffer. Leaf tissue homogenized in buffer was centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant was decanted into centrifuge tubes and stored at –20 °C. Enzyme activities were measured in the supernatants by the following methods.

GPX (EC 1.11.1.7) activity was measured by monitoring the increase in absorbance at 470 nm in 100 mM potassium-phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 5 mM guaiacol, 15 mM H_2O_2, and 50 μL of enzyme extract (39). GPX activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1} for tetraguaiacol at 470 nm.

APX (EC 1.11.1.11) activity was assayed by the method of Nakano and Asada (40). The rate of decrease in the absorbance of ASC at 290 nm for 3 min (ε = 2.8 mM^{-1} cm^{-1}) was measured in 1 mL of a reaction mixture containing an aliquot of supernatant, 0.25 mM ASC, and 5 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0).

CAT (EC 1.11.1.6) activity was determined from decrease in absorbance at 240 nm for 3 min following the consumption of H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}) (41). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 10 μL of enzyme extract in a 1-mL volume.

SOD (EC 1.15.1.1) activity was determined using the nitroblue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (42). For the activity assay, 50 mM potassium-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 2 μM riboflavin, 13 mM L-methionine, and 75 μM NBT was used. Samples were then exposed to 200 μmol photons m^{-2} s^{-1} for 10 min. Absorbance at 560 nm was then measured (Thermo Nicolet Evolution 100, UK). The SOD activity was expressed in U, with 1 unit corresponding to the amount needed to cause a 50% inhibition in the absorbance of controls performed for each individual assay.

NOX; EC 1.6.3.1 was extracted from 0.25 g leaf tissue at 4 °C with 1.0% PVP in 100 mM potassium-phosphate buffer (pH 7.0) including 0.1 mM EDTA and 0.1% Triton. The extract was centrifuged at 15,000 × *g* for 20 min at 4 °C. The supernatant was used for the enzymatic assay. NOX was assayed according to the method of Cakmak and Marschner (43). The reaction mixture contained 0.05 M NADPH, 100 mM potassium-phosphate buffer (pH 7.0), and 0.1 mM EDTA. The reaction was initiated by adding the enzyme extract, and the oxidation of NADPH was recorded by spectrophotometer at 340 nm.

2.5. Determination of H_2O_2 content

H_2O_2 content was determined according to the method of Velikova et al. (44). Leaves (250 mg) were homogenized in 3 mL of 5% trichloroacetic acid with 0.1 g of activated charcoal at 0 °C. The homogenate was centrifuged at 12,000 × *g* for 15 min. To a 0.5-mL aliquot of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 0.75 mL of 1 M KI were added. The absorbance of the supernatant was measured at 390 nm.

2.6. Determination of proline and soluble sugar contents

Proline content was determined by the ninhydrin method (45). Dry leaf samples (0.1 g) were homogenized in 10 mL of 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate (1 mL) was mixed with equal volumes of glacial acetic acid and ninhydrin reagent and then incubated for 1 h at 100 °C. The reaction was stopped by placing the test tubes in an ice bath. The samples were rigorously mixed with 3 mL of toluene. The toluene phase was read at 520 nm on a UV-VIS spectrophotometer (Thermo Nicolet Evolution 100, UK). The proline (Merck, Germany) concentration was determined using a standard curve. Free proline content was expressed as μg g^{-1} DW.

Total soluble sugar content was determined by phenol-sulfuric acid method (46). Dry leaves (0.1 g) were extracted with 5 mL of 80% ethanol by boiling the samples in glass tubes in a 95 °C water bath for 10 min. After extraction,

the tubes were centrifuged at 2500 rpm for 5 min, and the supernatants of the extractions were used for sugar analysis. To 900 µL of distilled water 100 µL of the sample was added, and the mixture was shaken vigorously. To 1 mL of sample, 1 mL of 5% phenol and 5 mL of H_2SO_4 were added, and the mixture was stirred. After cooling at room temperature for 15 min, the absorbance of the sample was recorded at 490 nm.

2.7. Determination of SA content

SA was extracted from plant tissue according to the method of Kadioglu et al. (26). Leaves (0.5 g) were homogenized in 3 mL of 90% methanol by homogenizer (IKA Labortechnik, Ultraturrax T25, Staufen, Germany) and centrifuged at 12,000 × *g* for 15 min. Supernatant was collected. The pellet was homogenized in 3 mL of 90% methanol and centrifuged again, and then the supernatants were combined. Supernatants were evaporated by rotary evaporator (Heidolph, Laborota 4000, Germany) at 68 °C for 5 min. The residue was dissolved in 2.5 mL of 5% trichloroacetic acid and centrifuged at 12,000 × *g* for 10 min. Next, 5 mL of a mixture of ethyl acetate, cyclopentane, and isopropanol (100:99:1, v/v/v) was added to supernatant. The supernatant was evaporated at 90 °C for 10 min. The residue was dissolved in 2.5 mL of 20% methanol, filtered by a 45-µm syringe filter, and injected (100 µL) into a high-performance liquid chromatography (HPLC) system. The HPLC system consisted of an LC 20 AT/Prominence (Shimadzu, Japan) equipped with a quaternary HPLC pump, micro vacuum degasser, thermostated column compartment, refractive detector, standard micro fluorescence detector, and preparative autosampler. SA analysis was performed on a Lichrosorb SI-60 (250 mm × 4.0 mm i.d., 5-µm particle size; Teknorama, Barcelona, Spain) column containing methanol and water (50:50, v/v) in a mobile phase operating at 22 °C with a flow rate of 1 mL min^{-1}. The measurements were performed using a Shimadzu RF10-AXL fluorescence detector (excitation/emission detection at 313/405 nm). SA was identified by comparing retention times to those of authentic standards under the analysis conditions stated above. Standard solutions of SA (Sigma, Germany), each containing an adequate concentration in mobile phase, were injected into the column. Calibration curves for SA were made with standard solutions and were later used for assessing the concentrations corresponding to the different peaks in the chromatograms. The areas of compound peaks were quantified by Shimadzu LC Solution Software.

2.8. Statistical analysis

All experiments were repeated 3 times with 30 leaves. For each experiment, the means from 3 repetitions were calculated. Variance analysis of mean values was performed by Duncan multiple comparison test (1- and 2-way ANOVA), and significance level was determined at the 5% level ($P < 0.05$). Relationships between variables were determined using Pearson's correlation coefficient test. All statistical analyses were done with SPSS (standard released version 16.0 for Windows; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Leaf rolling degree

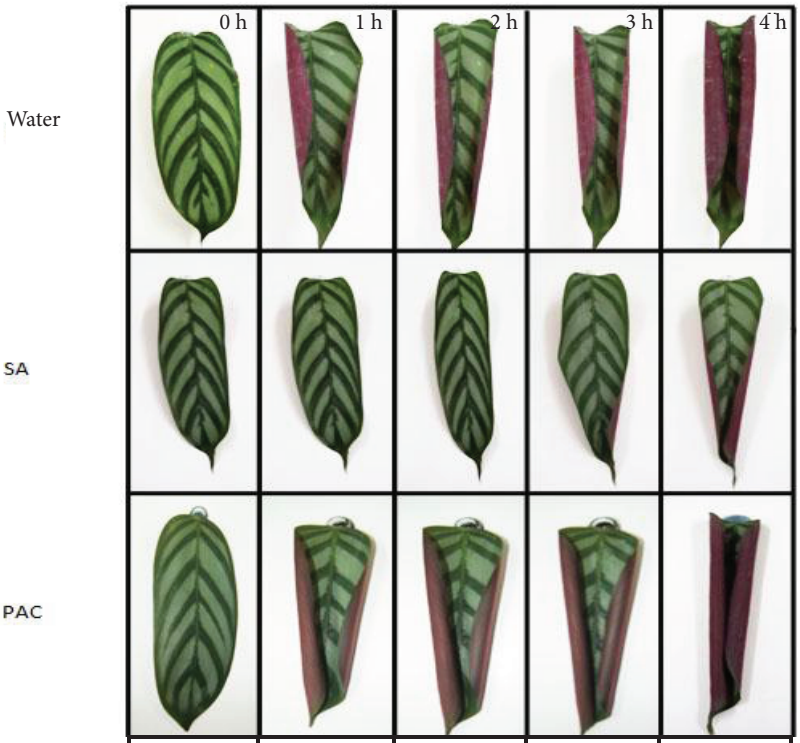
Exogenous SA application delayed leaf rolling (Figure 1). The antioxidant enzyme inhibitor treatments increased leaf rolling compared to SA application (Figure 2). At the end of a 1-h osmotic stress application, leaf rolling degrees in the NT group were 25.6%, 48.9%, 54.5%, and 42.3% for the control (H_2O), PAC, DTT, and DTNB, respectively. However, leaf rolling degrees in the SA-treated group were 0%, 17.8%, 32.4%, and 38.3% for SA, SA + PAC, SA + DTT, and SA + DTNB at the end of a 1-h osmotic stress application, respectively. In addition, leaf rolling degrees in the control (H_2O), PAC, DTT, and DTNB treatments of NT were 69.1%, 71.4%, 72.3%, and 72.4% at the end of a 4-h osmotic stress application, respectively (Figure 2). Furthermore, the leaf rolling degrees for SA, SA + PAC, SA + DTT, and SA + DTNB were 25%, 65.4%, 70.5%, and 72%, respectively (Figure 2). In addition, a significant negative correlation between SA application and leaf rolling degree under osmotic stress treatment (Table) was observed ($r = –0.912$, $P < 0.01$). This implies a close linkage between SA and leaf rolling.

3.2. Enzyme activities

SA treatment increased GPX activity. In the SA-treated control, GPX activity was higher than the NT control (H_2O) under osmotic stress. The GPX activity in the SA-treated control was 1.2-fold higher than in the NT control (Figure 3a). In contrast, GPX activities diminished when PAC was applied. The lowest GPX activities were measured in DTT in both NT and SA (Figure 3a). The GPX activities were 96.8 and 95.7 U g^{-1} DW in DTT and SA + DTT treatments, respectively.

SA treatment increased CAT activity in comparison with the NT control after 4 h of osmotic stress (Figure 3b). The highest activity was determined in the SA control leaves under osmotic stress (6.2 U g^{-1} DW). Activity in the SA-treated control was 1.1-fold higher than in the NT control. However, antioxidant enzyme inhibitors decreased CAT activity under osmotic stress. CAT activities in the SA control were 1.2-, 1.9-, and 2.0-fold higher than in the SA + PAC, SA + DTT, and SA + DTNB groups. The lowest CAT activity was measured in the DTT treatment of NT (2.1 U g^{-1} DW). However, there was no statistical difference between SA + PAC and PAC treatments (Figure 3b).

APX activity increased more in treated groups than in the NT control. The APX activity in the SA control was



Treatments	0 h	1 h	2 h	3 h	4 h
	Leaf rolling degrees (%)				
Water	0	26 ± 0.4	40 ± 1.1	63 ± 0.7	69 ± 0.8
SA	0	0	0	18 ± 0.5	25 ± 2.1
PAC	0	49 ± 1.6	62 ± 0.8	62 ±0.8	77 ± 0.3

Figure 1. Visual leaf rolling and degrees of leaf rolling during 4-h PEG treatment following dH_2O, SA, and PAC applications.

1.2-fold higher compared to the NT control. On the other hand, PAC and antioxidant enzyme inhibitors decreased APX activity according to the control groups (SA and NT). Activities in SA + PAC, SA + DTT, and SA + DTNB were 1.7-, 2.0-, and 1.7-fold lower than in the SA control. On the other hand, APX activity in the NT control was 2.0-, 1.7-, and 1.3-fold higher than in PAC, DTT, and DTNB treated groups, respectively. The lowest activity was observed in the PAC treatment of NT (34.2 U g^{-1} DW) (Figure 3c).

SOD activity in the SA control was higher than in the NT control (Figure 3d). SOD activity in the SA control was 1.2-fold higher than in the NT control. However, PAC and antioxidant enzyme inhibitors caused decreases in SOD activity (Figure 3d). The activities in PAC, DTT, and DTNB treatments were 1.9-, 1.5-, and 1.5-fold lower than in the NT control (H_2O), respectively. Similarly, SOD activities in SA + PAC and in SA + DTT and SA + DTNB were 2.2- and 2-fold lower than SA control, respectively.

Treatment with exogenous SA caused a significant enhancement in NOX activity compared to control, PAC, and SA + PAC treatments (Figure 4a). The NOX activity after SA treatment was 2.3- and 3.5-fold higher than in the control and PAC, respectively. In addition, the lowest NOX activity (0.65 U g^{-1} DW) was detected after PAC application. Moreover, there was no considerable difference between SA + PAC and PAC treatments (Figure 4a).

To further illuminate the relationships among NOX, antioxidant enzyme activities, and degree of leaf rolling, a correlation analysis was performed. Significant ($P < 0.01$) negative correlations between all enzyme activities and leaf rolling were determined (Table). The correlation coefficient between GPX and the leaf rolling was –0.884. In addition, the correlation coefficient between CAT and the leaf rolling was –0.937. On the other hand, positive and significant correlations between endogenous SA content and all enzyme activities were determined (Table). SA content was significantly ($P < 0.01$) related to SOD ($r = 0.825$), GPX ($r = 0.880$), APX ($r = 0.934$), CAT ($r = 0.875$), and NOX ($r = 0.877$).

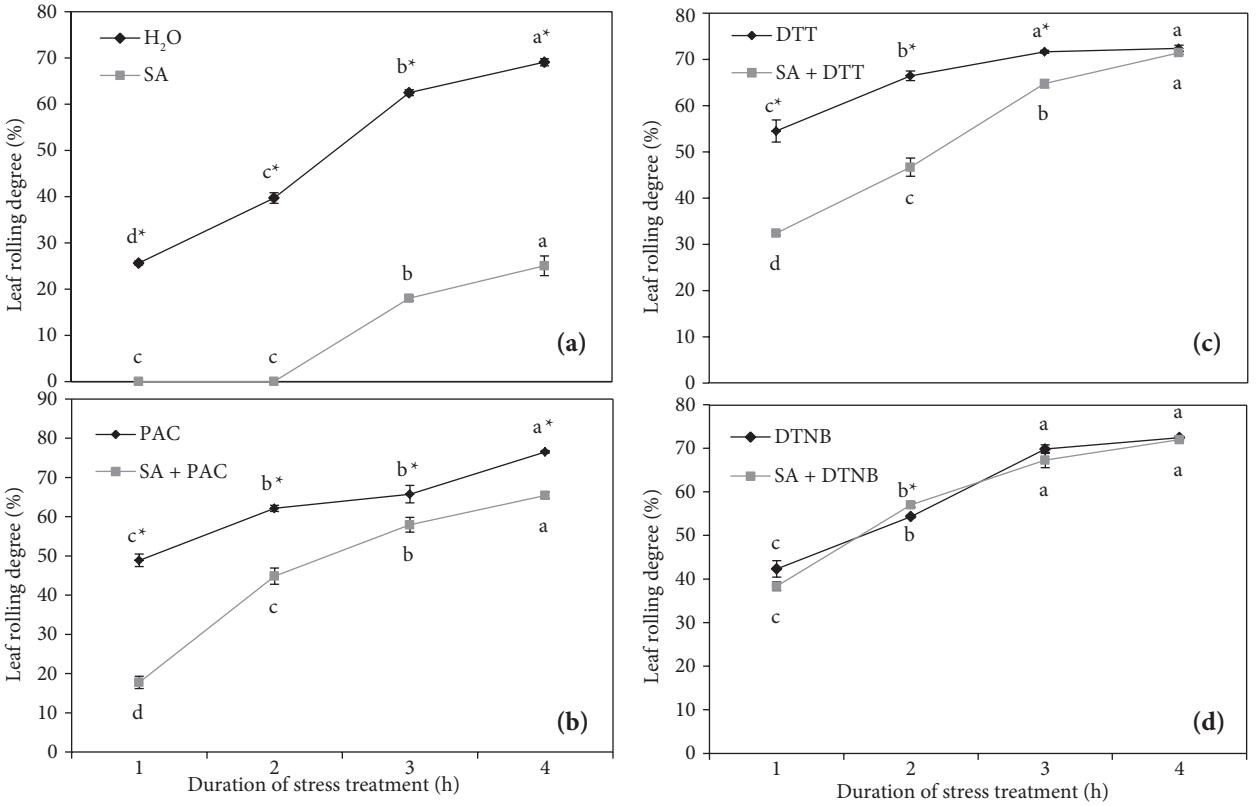


Figure 2. Leaf rolling degrees (%) during 4-h PEG treatment after dH_2O, SA, PAC, DTT, and DTNB applications. The same lowercase letters are not significantly different from each other ($P < 0.05$) during 4 h of the same treatments. Asterisks show significant differences between treatments in the same hours. Bars are standard deviations.

Table. Linear correlations among the descriptive parameters of physiological state and leaf rolling, SA, and H_2O_2 contents.

	SA	H_2O_2	Proline	Sugar	SOD	APX	CAT	GPX	NOX
Leaf rolling	–0.912**	–0.748**	–0.919**	–0.976**	–0.773**	–0.856**	–0.937**	–0.884**	–0.961**
SA		0.846**	0.968**	0.942**	0.825**	0.934**	0.875**	0.880**	0.877**
H_2O_2			0.914**	0.851**	0.988**	0.975**	0.876**	0.940**	0.851**

The correlation coefficient (r) and significant differences are given; **: $P < 0.01$.

3.3. Endogenous H_2O_2 content

H_2O_2 content was enhanced significantly by exogenous SA treatment compared to the control, SA + PAC, and PAC under osmotic stress. H_2O_2 content in the SA-treated leaves was 1.2-, 1.7-, and 1.6-fold higher than in the control, PAC, and SA + PAC, respectively. The lowest quantity was determined in PAC treatment (16.3 μM g^{-1} DW) in comparison with the other treatments. There was no significant difference between PAC and SA + PAC treatments (Figure 4b).

To study the relationship between endogenous H_2O_2 and SA contents in detail, correlation analysis was performed (Table). A positive and significant correlation was determined between H_2O_2 and SA contents ($r = 0.846$, $P < 0.01$).

3.4. Soluble sugar and proline content

Exogenous SA treatment gave rise to an increase in soluble sugar content in comparison to the control, PAC, and SA + PAC (Figure 5a). Sugar content in the SA treatment was 1.5-, 1.9-, and 1.6-fold higher than in the control, PAC, and SA + PAC treatments, respectively. The lowest soluble sugar amount measured was 11.3 mg g^{-1} DW in PAC treatment (Figure 5a).

Under osmotic stress, the proline content was significantly increased by exogenous SA treatment. On the other hand, PAC and SA + PAC caused decreases in proline content in comparison to the control (Figure 5b). Proline content in SA was 1.4-, 2.2-, and 1.6-fold higher than in the control, PAC, and SA + PAC, respectively. The

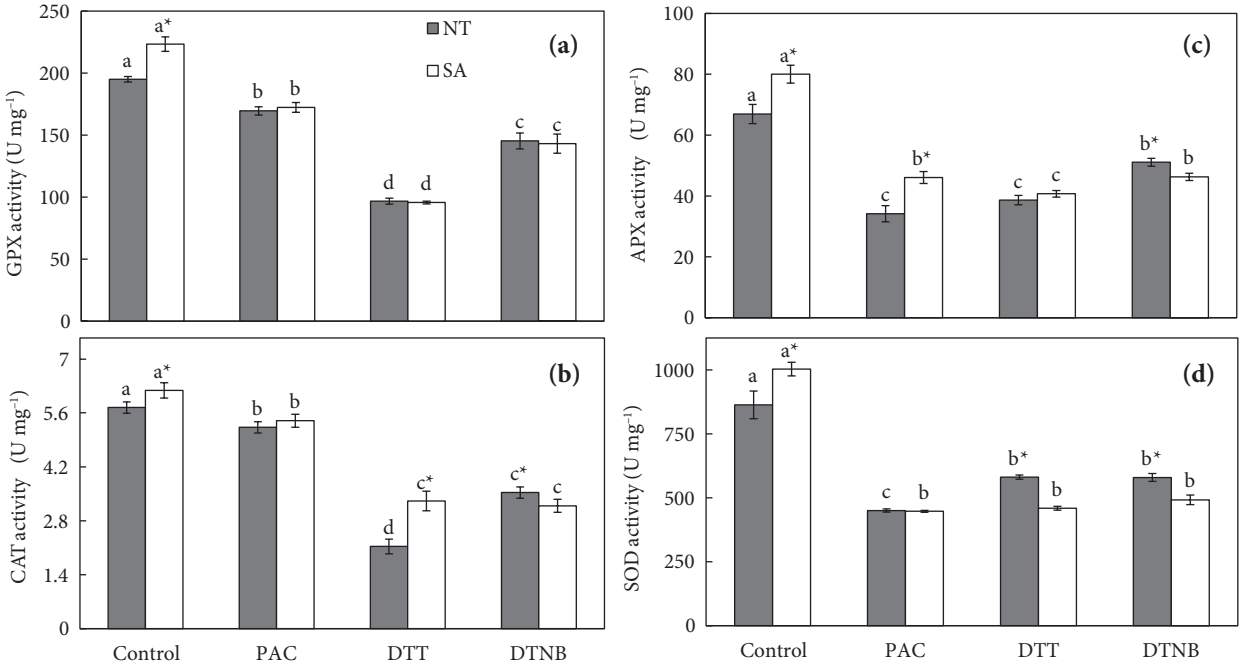


Figure 3. Antioxidant enzyme activities after 4-h PEG treatment following dH_2O (control), SA (control), PAC, DTT, and DTNB applications. The same lowercase letters are not significantly different from each other ($P < 0.05$) in each column among different treatments. Asterisks indicate significant differences ($P < 0.05$) in each column for each treatment. Bars are standard deviations.

lowest proline content was measured in the PAC treatment (30.3 mg g^{-1} DW) (Figure 5b).

Endogenous SA content and the osmoprotectants (proline and soluble sugar) were well correlated (Table). Significant and positive correlations were determined between soluble sugars and SA content (r = 0.942) and proline and SA content (r = 0.968). Furthermore, the relationship between leaf rolling and osmoprotectants was studied. Negative and significant ($P < 0.01$) correlations were determined between soluble sugar content and leaf rolling (r = –0.976) and proline content and leaf rolling (r = –0.919).

3.5. SA content

The exogenous SA treatment caused a significant increase in SA content in comparison to other treatments under osmotic stress. Endogenous SA content in the SA-treated leaves was 1.1-, 1.2- and 1.1-fold higher than in control, PAC, and SA + PAC treatments. On the other hand, minimum SA content was measured after PAC treatment among all groups (Figure 6). The SA content in the PAC treatment was 37 ng g^{-1} FW.

4. Discussion

The exposure of plants to certain environmental stresses often leads to the generation of ROS (9). Increasing ROS levels cause oxidative damage to cell components such as lipids, proteins, and nucleic acids (47). When plants are exposed to stress, antioxidant systems become active and begin to scavenge ROS. Antioxidant defense systems play vital roles in helping plants tolerate stressful conditions. The induction of antioxidant enzymes (SOD, CAT, and APX) by SA treatment under drought (25,48), salt (49), and UV radiation (50) stresses is known. In the present study, we investigated the activity of certain enzymes (SOD, APX, CAT, and GPX) to determine whether SA may have a role in the regulation of antioxidant defense in plants under osmotic stress. In our study, SA delayed leaf rolling and induced antioxidant enzymes under osmotic stress in comparison with the control group. When antioxidant enzyme inhibitors DTT (51) and DTNB (52) were applied to detached leaves, leaf rolling formed faster than in the control group under drought stress. This suggests that SA may delay leaf rolling by activating antioxidant enzymes. It is well known that antioxidant enzymes in plant cells play a major role in the preservation of membrane integrity. Therefore, water loss can be reduced and leaf rolling can be retarded through maintenance of leaf hydration by SA application. On the other hand, H_2O_2 may be an important molecule for controlling leaf rolling in *C. setosa*. It was determined that exogenous H_2O_2 treatment caused leaf rolling formation in *C. setosa* leaves kept in a series of

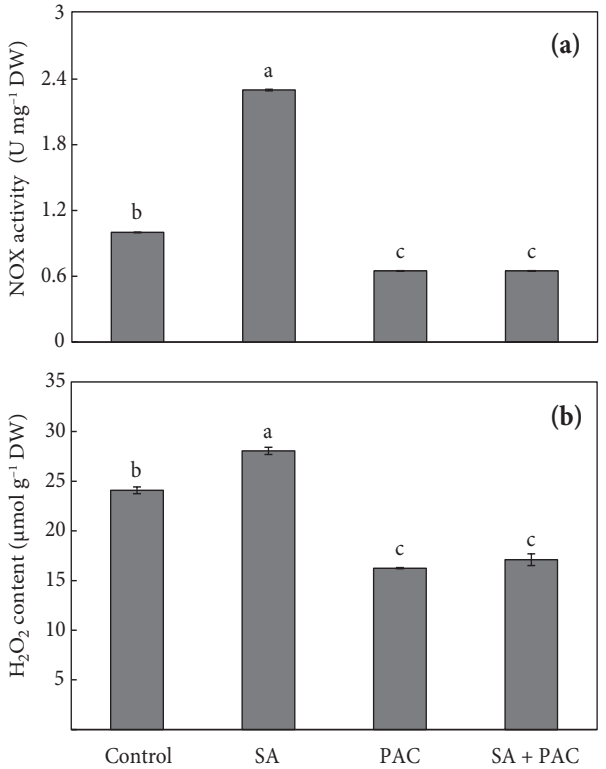


Figure 4. Endogenous H_2O_2 content and NOX activity after 4-h PEG treatment following dH_2O (control), SA, PAC, and SA + PAC applications. The same lowercase letters are not significantly different from each other ($P < 0.05$) in each column among different treatments. Bars are standard deviations.

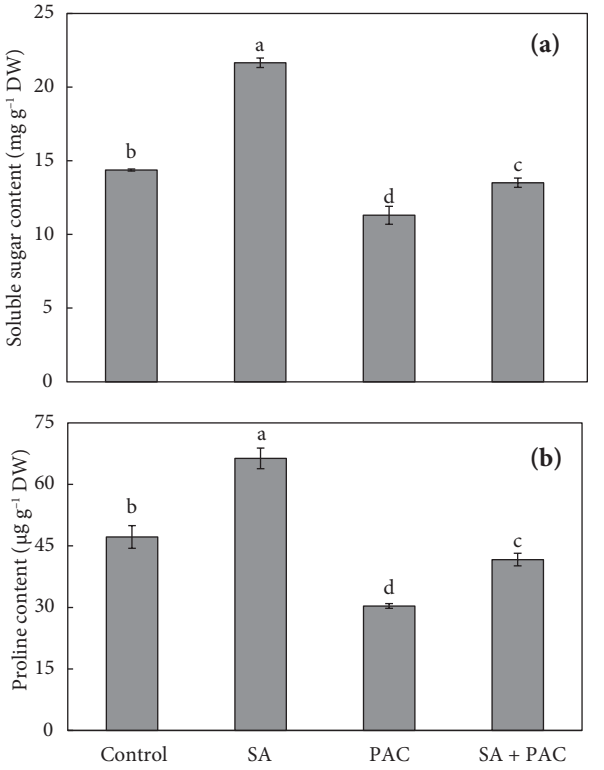


Figure 5. Soluble sugar and proline content after 4-h PEG treatment following dH_2O (control), SA, PAC, and SA + PAC applications. The same lowercase letters are not significantly different from each other ($P < 0.05$) in each column among different treatments. Bars are standard deviations.

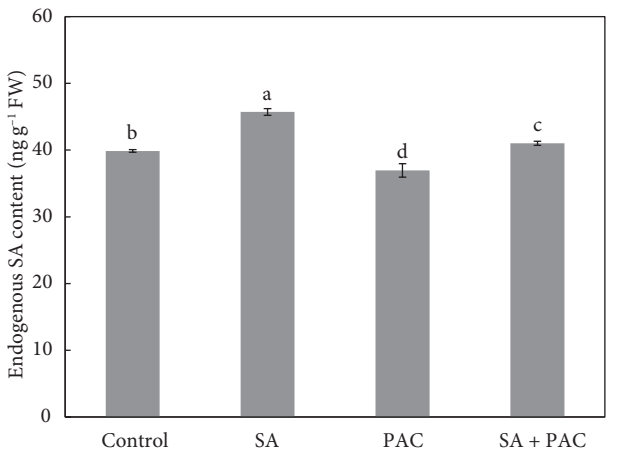


Figure 6. Endogenous SA content after 4-h PEG treatment following dH_2O (control), SA, PAC, and SA + PAC applications. The same lowercase letters are not significantly different from each other ($P < 0.05$) in each column among different treatments. Bars are standard deviations.

H_2O_2 solutions (0–300 mM) prepared with distilled water for 120 min (data not given). Leaves in 50 mM H_2O_2 began to roll after 30 min. Leaf rolling degrees at 120 min were 60%, 72%, 74%, and 80% at 50, 100, 200, and 300 mM concentrations, respectively. In addition, accumulation of H_2O_2 paralleled an increase in leaf rolling in *C. setosa* under drought stress (13,16). Therefore, in this study, NOX activity and endogenous H_2O_2 content were measured to test whether SA-stimulated increases in antioxidant enzyme activity were H_2O_2-mediated. Significant increases in NOX activity and endogenous H_2O_2 content were determined after SA treatment. On the other hand, it was previously determined that SA-induced increases in H_2O_2 content resulted from an increase in NOX activity (53,54). It was clear that treatment of leaves with SA caused an increase in H_2O_2, which may act as a signal for the induction of an observed increase in antioxidant enzyme activities. Positive and significant correlations between H_2O_2 content and antioxidant enzyme activities support this idea. To test the role of endogenous SA on leaf rolling, PAC, a SA biosynthesis inhibitor that affects benzoic acid 2-hydroxylase (BA2H) (55), was applied to the leaves. It has been suggested that the conversion of benzoic acid to SA occurs via inducible BA2H. In tobacco, increases in BA2H activity paralleled or preceded SA accumulation induced by *Tobacco mosaic virus* infection, UV exposure, or treatment

with benzoic acid or H_2O_2 (56). Decreases in antioxidant enzyme activities after PAC application were determined for the first time in the present study. On the other hand, various studies indicated that PAC induced antioxidant enzyme activities (57,58). To explain these decreases in the enzyme activities resulting from PAC treatment in the present study, endogenous SA and H_2O_2 contents and NOX activity were measured after PAC treatment. PAC treatment inhibited NOX activity and decreased H_2O_2 and SA contents. Similar to the present study, Zhou and Zhong (59) added different concentrations of exogenous SA (0, 50, and 100 µM) and PAC (50 and 100 µM) to *Taxus chinensis* cell cultures to manipulate the endogenous SA level. This led to a lower level of internal SA content. Endogenous SA accumulation in 100 µM PAC was 30% lower than it was without the addition of inhibitor. It is well known that the generation of ROS, and especially H_2O_2, is a signal of the activation of plant defense mechanisms under abiotic stresses (60,61). In these cases, H_2O_2 causes alterations in the activity of several antioxidant enzymes. Therefore, the decrease in endogenous H_2O_2 content via a decrease in NOX activity by PAC resulted in reductions in enzyme activities in the present study. In accordance with the above results, decreases in enzyme activity may be due to low H_2O_2 content.

In the present study, interactions between SA and the accumulation of compatible solutes (proline and sugar) and delay in leaf rolling were investigated in addition to the antioxidant system. The increase in solute accumulation under osmotic stress after SA treatment indicated that a delay in leaf rolling could be related to osmotic adjustments made through proline and sugar accumulation. It was previously reported that a notable increase in the degree of leaf rolling, proline, and reducing sugar contents was determined after exposing *C. setosa* plants to a severe water deficit (62). In addition, Clifford et al. (63) reported that *Ziziphus mauritiana* made osmotic adjustments by accumulating soluble sugar and proline under drought stress. Furthermore, the decrease in solute concentration after PAC treatment in the present study suggested that endogenous SA content might be effective in the rise of solute concentrations. In other words, SA induces the accumulation of solutes and this may result in the suspension of leaf rolling because the leaf rolling phenomenon depends on leaf water content. Loss of water from the hypodermis cells is followed by the rolling of leaves inward. In the present study, accumulation of osmoprotectants by SA provided the leaf with the osmotic adjustment needed to save its own water content; thus, leaf rolling was delayed. In addition, the positive and significant correlations among H_2O_2, soluble sugar, and proline contents may indicate that soluble sugars and proline are also related to ROS scavenging during leaf rolling.

In conclusion, SA may have a H_2O_2-mediated regulatory role in leaf rolling through the induction of antioxidant enzymes. The induced antioxidant enzymes reduce oxidative stress damage resulting from excess accumulation of H_2O_2 and then delay leaf rolling. On the other hand, increases in compatible solute concentrations following SA treatment facilitate water uptake by osmoregulation. Therefore, stress is alleviated. The reduction in the effects of stress delays leaf rolling. Therefore, this delay might be related to compatible solutes as well as the antioxidant system. Compatible solute accumulation may enable leaves to take in water and scavenge ROS. In addition, the inhibitory effect of PAC on antioxidant enzyme activities and osmolyte accumulation has been shown here for the first time. However, whether PAC inhibits antioxidants and osmolyte accumulation directly or through an H_2O_2-like molecule is unknown. Therefore, further studies are necessary to explain this mechanism.

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