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Exogenous Salicylic Acid Alleviates Growth Inhibition and Oxidative Stress Induced by Hypoxia Stress in *Malus robusta* Rehd

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Abstract Salicylic acid (SA) as a signal molecule mediates many biotic and environmental stress-induced physiologic responses in plants. In this study we investigated the role of SA in regulating growth and oxidative stress in *Malus robusta* Rehd under both normoxic and hypoxic conditions. Hypoxia stress inhibited plant growth and dramatically reduced biomass. Addition of SA significantly alleviated the plant growth inhibition. The amounts of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) significantly increased in leaves of the plants exposed to hypoxia stress and resulted in oxidative stress, which was indicated by accumulated concentration of malondialdehyde (MDA) and electrolyte leakage. Addition of SA significantly decreased the level of O_2^-, electrolyte leakage, and lipid peroxidation and enhanced the activities of superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) under hypoxia stress. As important antioxidants, ascorbate (AsA) and glutathione (GSH) contents in the plant leaves were slightly increased by SA treatment compared to hypoxia stress treatment alone. It was concluded that SA could alleviate the detrimental effects of hypoxia stress on plant growth and of oxidative stress by enhancing the antioxidant defense system in leaves of *M. robusta* Rehd.

Keywords Salicylic acid · Hypoxia stress · *M. robusta* Rehd · Oxidative stress · Antioxidant enzymes · Antioxidants

Introduction

Higher plants require oxygen to maintain growth and metabolism. A well-oxygenated root-zone environment is essential for a healthy root system, but frequently plants experience root-zone hypoxia mainly due to waterlogged soil, soil compaction, or poor drainage (Chérif and others 1997; Drew 1997; Geigenberger 2003; Garnczarska and Bednarski 2004). Hypoxia stress is considered one of the main environmental factors limiting plant growth and yield worldwide, especially in higher-rainfall regions. Oxygen deficiency in the root zone has drastic effects on plant growth, development, and survival (Drew 1983; Kato-Noguchi 2002). Hypoxia stress has been found to inhibit growth of roots and shoots because it affects many physiologic processes of the plant, including chlorophyll biosynthesis, reactive oxygen species (ROS) metabolism, and anaerobic metabolism (Chérif and others 1997; Geigenberger 2003; Matsui and Tsuchiya 2006; Fan and others 2008).

Oxidative stress is one of the most important factors that cause damage to plants exposed to many abiotic stresses (Horváth and others 2007; Ma and others 2008). Excessive generation of ROS is an integral part of many stress situations, including hypoxia. Numerous studies have shown that hypoxia stress triggers formation of ROS and induces

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oxidative stress in plants (Drew 1997; Geigenberger 2003; Garnczarska and Bednarski 2004; Narayanan and others 2005). These ROS include superoxide radicals (O_2^-), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2), which are produced during electron transport activities in the cell membrane as well as by a number of metabolic pathways (Mittler and others 2004; Verma and Mishra 2005). ROS accumulation induces oxidative processes such as membrane lipid peroxidation, protein oxidation, and DNA and RNA damage, resulting in cell damage and eventually cell death (Foyer and others 1994; Blokhina and others 2003). To scavenge ROS and protect against oxidative stress, plants have evolved an efficient antioxidant defense system composed of both antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), glutathione reductase (GR), and ascorbate peroxidase (APX), and antioxidants such as ascorbate (AsA), glutathione (GSH), flavonoids, and the lipid-soluble compounds such as the carotenoids and tocopherols (Foyer and others 1997; Garnczarska and Bednarski 2004; Molassiotis and others 2006). Among the enzymes, SOD is involved in O_2^- scavenging and CAT, APX, and GR are involved in H_2O_2 scavenging (Bowler and others 1992). In addition, among the antioxidants, GSH and AsA not only act as substrate for GR and APX, respectively, but are directly involved in ROS scavenging (Foyer and others 1997; Hernandez and others 2001; Ma and others 2008). Of the antioxidant enzymes, SOD and CAT play important roles in the formation and degradation of H_2O_2, respectively, and APX and GR are involved in AsA regeneration (Noctor and Foyer 1998; Hernandez and others 2001). Results have proved that the antioxidant system is a significant responsive mechanism of plants to overcome oxidative stress (Bowler and others 1992; Garnczarska and Bednarski 2004; Fan and others 2008). Thus, an understanding of the interplay between enzymatic and nonenzymatic ROS scavenging machinery is crucial for identifying key components involved in oxidative stress defense and manipulating tolerance of plants to hypoxia.

Salicylic acid (SA) is a phytohormone with ubiquitous distribution in plants; it plays an important role in the regulation of plant growth and development (Malamy and others 1990; Durner and others 1997; Horváth and others 2007). Recent studies indicate that it also participates in the signaling of abiotic stresses. There is strong evidence that SA mediates the oxidative burst that precedes the hypersensitive response and the development of systemic acquired resistance. Previous studies have shown that SA conferred plant resistance to various abiotic stresses such as heat acclimation (Dat and others 1998), chilling tolerance (Kang and Saltveit 2002), salinity stress (Gunes and others 2007), and cadmium toxicity (Krantev and others 2008). All these studies demonstrated that the majority of SA-regulated abiotic stresses in plants are involved in antioxidant responses, thus indicating that protection of plants from oxidative damage by SA is associated with an enhanced antioxidant system (Dat and others 1998; Horváth and others 2007).

In view of all aforementioned reports, there is little information on the effects of exogenously applied SA on hypoxia-induced oxidative stress, although more studies have shown that exogenous application of SA increased plant resistance to various abiotic stresses. Apple (*Malus*) is one of the most economically important fruits worldwide. *M. robusta* Rehd, which originates in China, has high salinity tolerance and nematode resistance so it is widely used as rootstock for commercial apple production in China. However, it is sensitive to waterlogged soil. Our previous studies also showed that *M. robusta* Rehd is intolerant of hypoxia stress (Bai and others 2008).

The objective of this work was to study the influence of SA on the growth and the antioxidant system of *M. robusta* Rehd grown under normoxic and hypoxia conditions, and to find whether hypoxia tolerance could be induced by endogenous SA, thus supplying information on the possible involvement of oxidative stress in the mechanism of damage by hypoxia stress.

Materials and Methods

Plant Materials and Treatments

The present study was conducted at Northwest A&F University, Yangling (34°20′N, 108°24′E), China. Seeds of *M. robusta* Rehd were stratified at 0−4°C for 50 days. Then three germinated seeds were planted in their own plastic pot (12 cm × 12 cm) filled with sand. The plastic pots were then placed in a greenhouse under natural light and temperature conditions. When reaching the two true-leaf stage, the seedlings were watered with half-strength Hoagland's nutrient solution (Hoagland and Arnon 1950) every other day. The pH of the nutrient solution was adjusted to 6.5 ± 0.1 by adding diluted NaOH or HCl. Thirty days later, similarly sized seedlings (15 leaves, about 5 cm in height) were transferred into plastic tubs (52 cm × 37 cm × 15 cm) containing 20 L half-strength nutrient solution. The plastic tubs were wrapped with black plastic to prevent exposure of roots to light and then placed in the growth chamber at day/night temperatures of 20−25°C/15–20°C and under a 14-h photoperiod at a photon flux density of 160 μmol m^{-2} s^{-1} provided by sodium lamps. The solution was continuously aerated with an air pump (20 min aeration/h). Seedlings were cultivated for 10 days to allow plants to adapt to the growth chamber conditions. After 10 days of precultivation seedlings were randomly

divided into three groups: (1) control (normoxic): the seedlings were cultured in half-strength Hoagland nutrient solution and the solution was continuously aerated with an air pump and maintained at dissolved oxygen (DO) concentrations of 8.0−8.5 mg L^{-1} by a DO controller (FC-680, Corporation of Super, Shanghai China). (2) Hypoxia: aeration was by N_2 gas and maintained the DO at 1.5−2.0 mg L^{-1} by another DO controller; the leaves of seedlings were sprayed with distilled water. (3) Hypoxia + SA (0.5 mM). Each treatment contained three replicates with 30 plants in each replicate for a total of 90 plants per treatment. All experiments were carried out in a completely randomized block design. At 0, 5, 10, 15, and 20 days after treatment, leaves were collected and immediately frozen in liquid nitrogen and then stored at −70°C until use.

Growth Measurements

After 20 days of treatments, shoot and root lengths were measured by a ruler and the number of leaves was manually counted on ten plants per treatment, and then each plant was divided into shoots and roots. Dry weights of shoots and roots were measured by drying at 70°C for at least 72 h.

Electrolyte Leakage and Lipid Peroxidation Assays

Electrolyte leakage (EL) was determined following the method described by Dionisio-Sese and Tobita (1998). Ten leaf discs (5 mm in diameter) were placed in test tubes containing 10 ml distilled water. The tubes were incubated in a water bath at room temperature for 2 h and the initial electrical conductivity of the medium (EC_1) was analyzed using an electrical conductivity analyzer (DDS-307, Shanghai Precision Scientific Instrument Co., Ltd, China). The samples were autoclaved at 100°C for 20 min to release all electrolytes, cooled to 25°C, and then the final electrical conductivity (EC_2) was measured. The EL was calculated using the formula: $EL = (EC_1/EC_2) \times 100$.

Lipid peroxidation was estimated as the content of all 2-thiobarbituric acid reactive substances and expressed as equivalents of malondialdehyde (MDA), as described by Heath and Packer (1968).

O_2^- and H_2O_2 Assays

The amount of O_2^- was determined following Doke's method (1983) by measuring the capacity to reduce nitroblue tetrazolium in the dark at room temperature. The light absorbance was measured at 530 nm. The absorbance of the end-product was measured at 530 nm. O_2^- formation was expressed as ΔA_{530}/g FW of the sample.

H_2O_2 content was determined according to a method described by Patterson and others (1984). Frozen leaves were homogenized in cold acetone at the ratio of 1 g sample:2 ml ice-cold acetone. Titanium reagent (2% $TiSO_4$) was added to a known volume of extract supernatant to give a Ti concentration of 2%. The Ti–H_2O_2 complex, together with unreacted Ti, was then precipitated by adding 0.2 ml of 17 M ammonia solution to each milliliter of extract. The precipitate was washed five times with ice-cold acetone by resuspension, drained, and dissolved in 2 M H_2SO_4 (3 ml). The absorbance of the solution was measured at 410 nm against blanks, which had been prepared similarly but without plant tissue.

Enzyme Extractions and Assays

SOD, POD, and CAT Activities

Frozen leaves (0.5 g) were homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 3 mM 2-mercaptoethanol, and 2% (w/v) polyvinylpolypyrrolidone in a chilled mortar and pestle. The homogenate was centrifuged at 16,000 *g* for 30 min at 4°C and the supernatant was used for the following enzyme assays.

SOD activity was assayed by the nitroblue tetrazolium (NBT) method (Dhindsa and others 1981).The reaction mixture (3 ml) contained 50 mM Na–phosphate buffer (pH 7.3), 13 mM methionine, 75 mM NBT, 0.1 mM EDTA, 4 mM riboflavin, and enzyme extract (0.2 ml). The reaction was started by the addition of riboflavin, and the glass test tubes were shaken and placed under fluorescent lamps (160 μmol m^{-2} s^{-1}). The reaction was allowed to proceed for 5 min and was then stopped by switching off the light. The absorbance was measured at 560 nm. Blanks and controls were run in the same manner but without illumination and enzyme, respectively. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT reduction under assay conditions.

POD and CAT activities were assayed following the method of Chance and Maehly (1955) with some modification. The POD reaction solution (3 ml) contained 50 mM phosphate buffer (pH 7.8), 25 mM guaiacol, 200 mM H_2O_2, and 0.5 ml enzyme extract. Changes in absorbance of the reaction solution at 470 nm were determined every 30 s. One unit of POD activity was defined as an absorbance change of 0.01 units/min.

The CAT reaction solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 200 mM H_2O_2, and 50 μl enzyme extract. The reaction was initiated by adding the enzyme extract. Changes in absorbance of the reaction solution at 240 nm were read every 30 s. One unit of CAT

activity was defined as an absorbance change of 0.01 units/min.

Protein was estimated by the method of Bradford (1976), using bovine serum albumin as a standard. All the antioxidant enzyme activities were expressed as units (U) mg^{-1} protein.

APX and GR Activities

APX and GR activities were assayed using the method described by Ma and Cheng (2003). Leaves (0.5 g) were extracted with 8 ml of 50 mM phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 0.1% triton X-100, and 2% PVP-3000. The homogenates were centrifuged at 16,000 *g* for 20 min at 4°C and the supernatants were collected for assay of enzyme activities. One unit of APX activity was the amount of APX that catalyzes the oxidation of 1 mmol ascorbate/min. One unit of GR activity was defined as the reduction of 1 mmol glutathione (GSSG)/min.

AsA and GSH Contents Assays

Samples (0.5 g) were homogenized in 8 ml of 6% (w/v) trichloroacetic acid precooled on ice. The extract was then centrifuged at 12,000 *g* for 20 min. The AsA content was determined according to a method of Ma and Cheng (2003). GSH was assayed according to a method described by Griffith (1980). Nonprotein thiols were extracted by homogenizing the samples in 5% (w/v) sulfosalicylic acid on ice and then centrifuged at 10,000 *g* for 20 min. The supernatants were collected and used for analysis. GSH content was determined in neutralized samples after reduction of oxidized GSSG with yeast-GR, 5,5′-dithio-bis-nitrobenzoic acid (DTNB) and NADPH by a spectrophotometer at 412 nm.

Statistical Analysis

Statistical analyses were carried out by analysis of variance (ANOVA) using SPSS v11 for Windows (SPSS, Inc., Chicago, IL). Results are given as mean ± standard error (SE). Differences between treatments were separated by the least significant difference (LSD) test at $p < 0.05$.

Results

Growth Parameters

Growth inhibition ($p < 0.05$) was detected in response to hypoxia stress and was indicated by reductions in plant shoot and root lengths and leaf number by 76.67, 83.31, and 83.96%, respectively, compared with the control (Table 1). However, this inhibition was significantly alleviated by addition of 0.5 mM SA ($p < 0.05$). Shoot and root dry masses of *M. robusta* Rehd were decreased ($p < 0.05$) by hypoxia stress by 59.47 and 52.23%, respectively, compared with the control (Table 2). Moreover, the ratios of roots to shoots were also affected, but the differences were not statistically significant. Under hypoxia stress conditions, exogenous SA application resulted in significant increases in the dry weight of the plants, with an average increase in the dry weight of shoots and roots of 14.56 and 23.76%, respectively.

Table 1 Effects of exogenous SA application on lengths of shoots and roots and leaf number of *M. robusta* Rehd grown in normoxic or hypoxic nutrient solution for 20 days

Treatment	Shoot length (cm)		Root length (cm)		Leaf number (No./plant)	
	M ± SE	%	M ± SE	%	M ± SE	%
Control	8.21 ± 0.80 a	100	25.22 ± 4 .28 a	100	21.20 ± 2.39 a	100
Hypoxia	6.29 ± 0.54 b	76.67	20.25 ± 2.37 b	80.31	20.25 ± 2.37 b	83.96
Hypoxia + SA	7.33 ± 1.03 ab	89.30	22.00 ± 2.49 ab	87.26	22.00 ± 2.49 ab	90.57

Values are mean ± SE, $n = 10$. Data with different letters in the same column indicate a significant difference at $p < 0.05$ by LSD test

Table 2 Effects of exogenous SA application on shoot and root dry weights and the root/shoot ratio of *M. robusta* Rehd grown in normoxic or hypoxic nutrient solution for 20 days

Treatment	Shoot dry weight (g $plant^{-1}$)		Root dry weight (g $plant^{-1}$)		Root/shoot	
	M ± SE	%	M ± SE	%	M ± SE	%
Control	1.21 ± 0.08 a	100	0.43 ± 0.02 a	100	0.36 ± 0.03 a	100
Hypoxia	0.72 ± 0.10 b	59.47	0.23 ± 0.05 b	52.23	0.32 ± 0.02 a	88.89
Hypoxia + SA	0.84 ± 0.04 ab	68.13	0.27 ± 0.01 ab	64.64	0.34 ± 0.03 a	95.08

Values are mean ± SE, $n = 10$. Data with different letters in the same column indicate a significant difference at $p < 0.05$ by LSD test

Membrane Damage

Damage to the membranes was investigated by monitoring MDA content and electrolyte leakage. MDA content and electrolyte leakage increased significantly in leaves of plants when exposed to hypoxia stress compared with the controls (Fig. 1a, b). Exogenously applied SA reduced the accumulation of MDA, but the change was not as great as that for electrolyte leakage.

Free Radicals Production

Compared with the control, hypoxia stress quickly increased leaf O_2^- and H_2O_2 content during all treatments ($p < 0.05$). H_2O_2 concentration in leaves increased after 0, 5, 10, and 15 days of the treatment (Fig. 1c). Exposure to hypoxia also resulted in an increase in the O_2^- content compared with the control (Fig. 1d). Under hypoxia stress, exogenous SA significantly suppressed O_2^- production, whereas the level of H_2O_2 increased slightly by SA treatments under hypoxia stress conditions.

Activities of Antioxidant Enzymes

The activities of antioxidant enzymes involved in scavenging ROS changed significantly during hypoxia treatment. SOD, POD, and CAT activities in the leaves increased significantly during the first 15 days ($p < 0.05$) and decreased gradually with prolonged hypoxia stress. The combination of hypoxia stress and SA produced much higher SOD and POD activities than in the control (Fig. 2a, b). However, unlike SOD and POD, SA application had less effect on CAT activity (Fig. 2c). Immediately after hypoxia stress, APX and GR activities increased sharply, peaked at 5 days, and then decreased. However, APX and GR activities were higher than in the control throughout the treatment (Fig. 2d, e). APX activity was considerably promoted by treatments of SA under hypoxia stress ($p < 0.05$), and the highest APX activity was observed under the combination of hypoxia stress and SA at 15 days. However, SA application had little effect on GR activity.

AsA and GSH Concentrations

When the plants were exposed to hypoxia stress, the content of AsA and GSH of the leaves increased, reached the highest point at 10 and 5 days, respectively, and then declined with increasing duration of treatment (Fig. 3a, b). Under hypoxia stress, exogenous SA application slightly enhanced AsA and GSH content, but the differences were not statistically significant.

Discussion

Oxygen deficiency in the root zone has drastic effects on plant growth, development, and survival (Drew 1983; Kato-Noguchi 2002). There have been numerous studies reporting that root-zone hypoxia inhibits plant growth and reduces dry matter accumulation. Sojka and others (1975) found an 83% decrease in leaf area of wheat after 25 days of root hypoxia. The root biomass, shoot biomass, and total plant biomass of plants were reduced by 65.9, 24.6, and 30.2%, respectively, under root-zone hypoxia (Shi and others 2007). In the present study, similar results were observed: almost all the measured growth parameters were significantly reduced in plants under hypoxia stress when compared to the control. However, exogenous SA

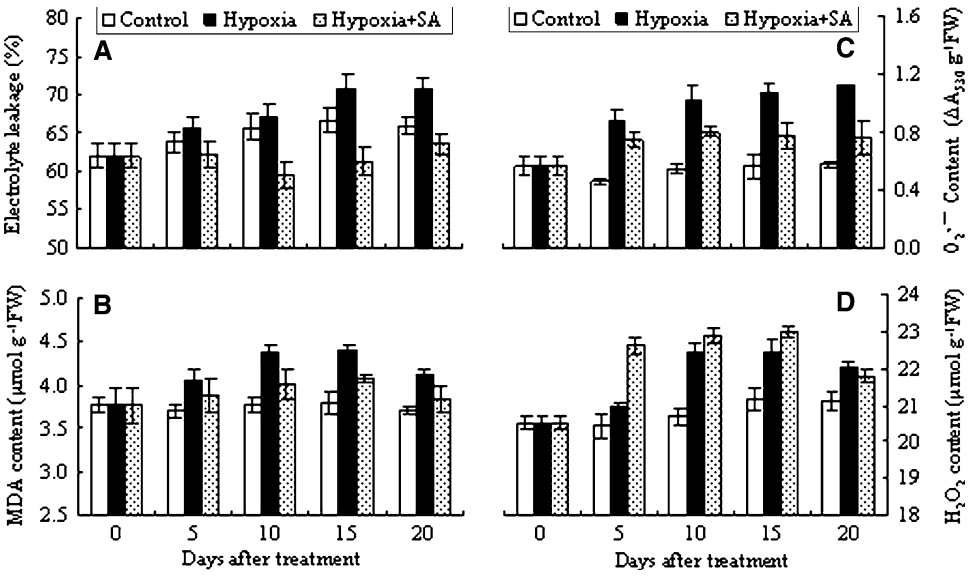

Fig. 1 Effect of exogenous SA application on electrolyte leakage (**a**) and MDA (**b**), O_2^- (**c**), and H_2O_2 (**d**) content of *M. robusta* Rehd grown in normoxic or hypoxic nutrient solution. Each value is the mean of three independent experiments, and the vertical bars indicate standard error (SE) ($n = 3$)

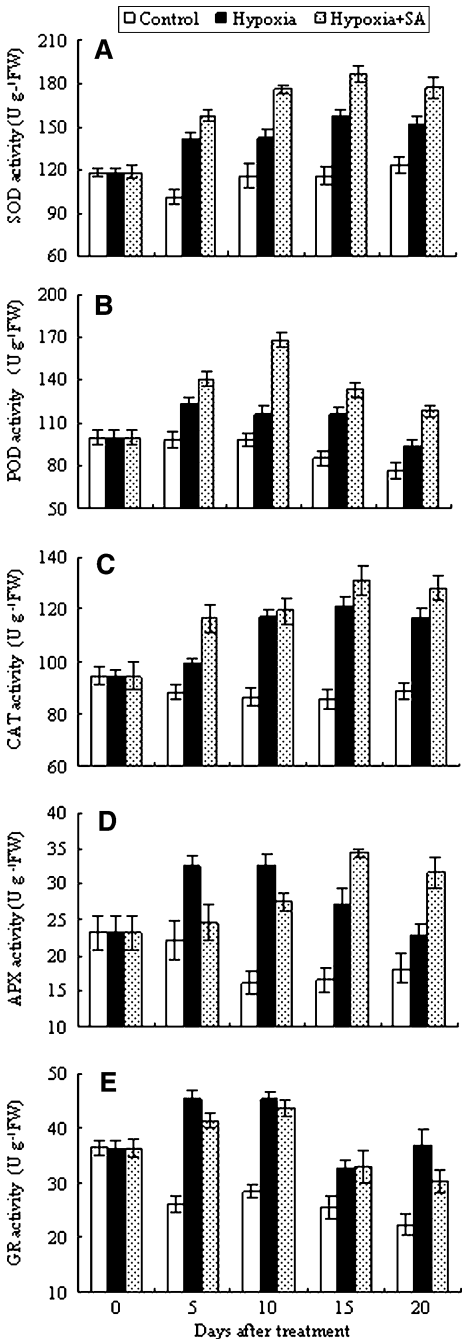

Fig. 2 Effect of exogenous SA application on SOD (**a**), POD (**b**), CAT (**c**), APX (**d**), and GR (**e**) activity of *M. robusta* Rehd grown in normoxic or hypoxic nutrient solution. Each value is the mean of three independent experiments, and the vertical bars indicate standard error (SE) ($n = 3$)

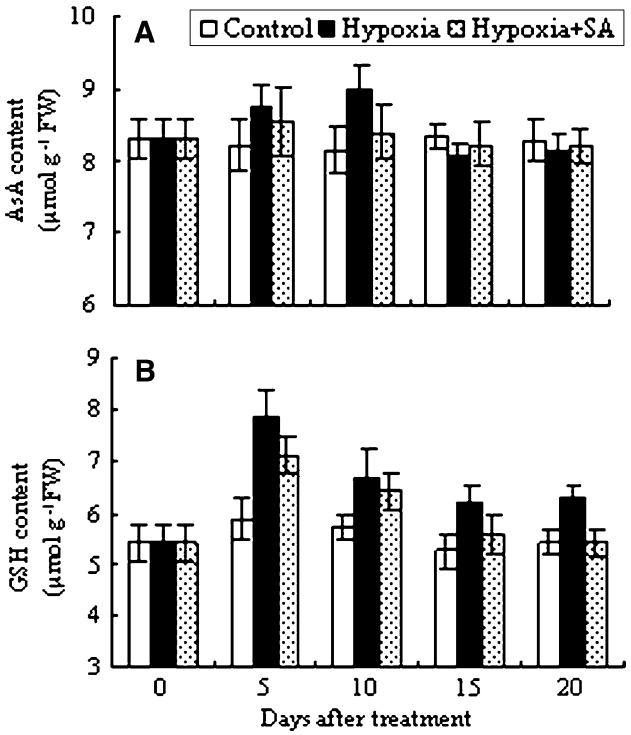

Fig. 3 Effect of exogenous SA application on AsA (**a**) and GSH (**b**) contents of *M. robusta* Rehd grown in normoxic or hypoxic nutrient solution. Each value is the mean of three independent experiments, and the vertical bars indicate standard error (SE) ($n = 3$)

treatments alleviated the inhibitory effects of hypoxia stress on growth (Tables 1 and 2). This agrees with the findings that SA-treated maize plants had higher dry mass compared with that of untreated seedlings under salinity stress (Gunes and others 2007). SA treatment was also shown to have an ameliorating effect on seed germination and seedling growth under lead or mercury stress in two rice plants (Mishra and Choudhuri 1997). Pretreatment of barley seedlings with SA prevented the lipid peroxidation caused by Cd and increased the fresh weight of shoots and roots (Metwally and others 2003).

Enhanced formation of ROS under stress conditions induced protective responses and cellular damage (Blokhina and others 2003). In the present study we found that hypoxia stress induced the generation of O_2^- and H_2O_2 in the leaves of the plants (Fig. 1c, d). These results agreed with previous reports by Zhang and others (1994) for wheat, Garnczarska and Bednarski (2004) for lupine, and Narayanan and others (2005) for sea buckthorn. Addition of SA suppressed O_2^- production under hypoxia stress; this has also been observed in plants under oxidative stress as shown in a study of Shi and Zhu (2008). The increased concentrations of O_2^- and H_2O_2 led to lipid peroxidation, causing membrane damage and leakage of electrolytes (Sairam and Srivastava 2002; Ma and others 2008). It is well documented that MDA is a product of cell membrane lipid peroxidation (Bailly and others 1996; Shah and others 2001), and its content in vivo can indicate the extent of oxidative stress in plants and cell membrane homeostasis. The present results showed that hypoxia stress caused a dramatic increase in MDA content and electrolyte leakage

in leaves of *M. robusta* Rehd indicating the occurrence of oxidative stress (Fig. 1a, b). However, exogenously applied SA reduced the accumulation of MDA and electrolyte leakage under hypoxia stress, indicating an alleviation of oxidative stress due to hypoxia. In a few experiments, SA was also found to be an effective preventative agent against these compounds. Pretreatment of cucumber and maize leaves by spraying with SA can protect them from oxidative stress induced by manganese and salinity (Gunes and others 2007; Shi and Zhu 2008).

The improvement of stress tolerance is often related to the enhancement of activities of antioxidant systems in plants. It has been suggested that an increased level of ROS triggers the upregulation of the activity of antioxidant enzymes, which in turn protected plants from oxidative stress (Davey and others 2000; Ma and others 2008). SOD, POD, and CAT are the most important detoxifying enzymes and work together with APX and GR of the ascorbate-glutathione cycle to promote scavenging of ROS (Hernandez and others 2001; Parida and others 2004; Ma and others 2008). SOD is responsible for the scavenging of toxic O_2^- in different cell organelles (Fridovich 1986). H_2O_2 might be detoxified by CAT, POD, and the ascorbate–glutathione cycle (Parida and others 2004; Molassiotis and others 2006). In our study, the activities of SOD, POD, CAT, APX, and GR in leaves under hypoxia stress have also been shown to increase and then decreased with the duration of hypoxia stress (Fig. 2a–e). These results suggest that the modulation of SOD, POD, CAT, APX, and GR in response to stress is limited beyond which they would be damaged by stress. At the same time, this implies that leaves have been damaged by hypoxia stress.

SA plays important roles in abiotic stress tolerance, and considerable interest has been focused on SA because of increased antioxidant enzymes which induce a protective effect on plants under stress (Malamy and others 1990; Horváth and others 2007). Studies showed that SA increased resistance of maize, cucumber, and rice to chilling (Kang and Saltveit 2002), wheat to salinity (Shakirova and others 2003), and cucumber to manganese (Shi and Zhu 2008). In this study, the activities of SOD, POD, GR, and APX significantly increased in SA-treated plants compared to those in hypoxia-stressed plants. These data suggest that endogenous SA plays an important antioxidant role in protecting *M. robusta* Rehd from oxidative stress.

AsA and GSH are two important antioxidants. A high level of endogenous AsA is essential in maintaining the antioxidant capacity that protects plants from oxidative stresses (Foyer and others 2001; Ma and Cheng 2003). GSH is a well-known antioxidant that plays a prominent role in the defense against ROS (Noctor and Foyer 1998; Foyer and others 2001; Ma and others 2008). In this study, hypoxia caused an increase in AsA concentration during the early hypoxia period, but as the time under hypoxia progressed, AsA concentration gradually decreased to reach a lower level compared with that of the control (Fig. 3a, b). This behavior is representative of a process of regulation that reduces ascorbate and restores the antioxidant potential of the plants. In the long term, this regulatory mechanism is probably unable to impair oxidative damage. A similar response was observed by Badiani and others (1993) in soybean leaves exposed to high levels of CO_2. GSH concentrations increased in the early hypoxia period and then decreased, but they remained at a higher level than that of the control. Exogenous SA elevated the levels of AsA and GSH under hypoxia stress, thus promoting the cycle of ascorbate–glutathione, which made it clear that SA improved the hypoxia stress tolerance of *M. robusta* Rehd by promoting the accumulation of AsA and GSH. However, the direct relationship and the regulating mechanism of SA to ROS metabolism remain to be further explored.

In conclusion, the application of 0.5 mM exogenous SA partially alleviated growth inhibition, delayed membrane lipid peroxidation, and reduced hypoxia injury in *M. robusta* Rehd under hypoxia stress. The role of SA in alleviating hypoxia injury of *M. robusta* Rehd may be attributed to its ability to induce the antioxidant system. This study showed an interesting effect of SA in the stress response that should be important not only for a basic understanding of the role of the hormone but also for potential use of the chemical in agriculture.

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