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Full Length Research Paper

Effects of salicylic acid on monoterpene production and antioxidant systems in *Houttuynia cordata*

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Salicylic acid (SA) plays important roles in plant defense responses. However, little is available about its effects on monoterpene responses. Therefore, monoterpene contents and antioxidant systems were measured three days after foliar application of SA with different concentrations in *Houttuynia cordata*. SA at low concentrations (≤ 1 mM) suitably enhanced the efficiency of antioxidant system, however, at high concentrations (10 and/or 100 mM), a strong accumulation of peroxidase, catalase and malondialdehyde suggested the induction of oxidative stress. High concentrations of SA resulted in a marked accumulation of total monoterpenes, which might be attributed to the oxidative burst. A significant increase in total monoterpene contents was also observed at 0.01 mM SA, which might be favorable for the induction of monoterpenes because of less oxidative damage to the plants. Total monoterpene contents showed a decline in the treatments of 0.1 and 1 mM SA. However, not all of monoterpene individuals were significantly induced and/or suppressed in the corresponding treatments. This implied that different monoterpenes might be differentially mediated at different concentrations of SA.

Key words: *Houttuynia cordata,* monoterpenes, oxidative stress, salicylic acid.

INTRODUCTION

Houttuynia cordata Thunb. a single species of the genus *Houttuynia* in Saururaceae, is an aromatic wild herb native to some Asian countries, such as China (Wu et al., 2005a), Thailand (Nuengchamnong et al., 2009), India (Chakraborti et al., 2006), Vietnam (Ogle et al., 2003) and Korea (Kim et al., 2001). It is perennial and widely distributed in ravines, stream sides, forests, wet meadows, slopes, thicket and field margins, trailsides, roadsides or ditch banks in these regions (Wu et al., 2005b). Its plants are used as a kind of traditional Chinese medicine for hundreds of years and exhibit a variety of pharmacological activities like anti-cancer, anti-oxidative, anti-inflammatory, anti-mutagenic, anti-bacterial and anti-virus (Han et al., 2009; Hayashi et al., 1995; Lau et al., 2008). It therefore has been identified as one of the most potential wild plant resources by the Chinese State Health Department (Wu et al., 2005c). Monoterpenes are one of the major effective components of *H. cordata* (Hayashi et al., 1995).

The plants produce essential oils rich in monoterpenes, such as β-myrcene, α-pinene, β-phellandrene, β-pinene, β-ocimene, D-limonene and γ-terpinene (Chen et al., 2008). Monoterpenes possess a lot of important pharmacology activities, for example, limonene and perillyl alcohol have chemopreventive activity against cancer (Chan et al., 2006; Crowell, 1997; Gould, 1995). Moreover, monoterpenes generally have fragrance and other biological activities. Some monoterpenes are used

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Abbreviations: CAT, Catalase; **H_2O_2,** hydrogen peroxide; **MDA,** malondialdehyde; **NBT,** nitroblue tetrazolium; **$\cdot O_2^-$,** superoxide radicals; **POD,** peroxidase; **SA,** salicylic acid; **SAR,** systemic acquired resistance; **SOD,** superoxide dismutase; **ROS,** reactive oxygen species; **EDTA,** ethylene diamine tetraacetic acid; **UV-Vis,** ultraviolet/visible; **FW,** fresh weight; **TCA,** trichloroacetic acid; **GC-MS,** gas chromatography-mass spectrometry; **SE,** standard errors.

as important flavor agents and are added to foods, drinks, perfumes, cosmetics and tobacco (Aharoni et al., 2005; Verlet, 1993). Some monoterpenes such as 1, 8-cineole, pinene and carvacrol have been considered as important biopesticides (Choi et al., 2006; Prates et al., 1998). Monoterpenes therefore are widely used in medicine, industry and agriculture. Monoterpenes, a kind of terpenoids secondary metabolites, are thought to have essential ecological roles and could be induced by biotic and abiotic stresses, such as pathogen attack, wounding, ozone and high temperature (Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010). Some signal transduction pathways are generally involved in the regulation of monoterpene biosynthesis in response to these stresses (Zhao et al., 2006). It has been suggested that signal molecules could be employed directly or indirectly for the production of plant secondary metabolites (Zhao et al., 2005).

The signal components involved in monoterpene biosynthesis therefore might be served as potential allelochemicals for induction of monoterpenes. SA is a phytohormone with roles in signal transduction of a wide range of defense responses including the biosynthesis of some secondary metabolites (Hayat et al., 2010; Pieterse and van Loon, 1999). For example, alkaloids such as vincristine and vinblastine in periwinkle (Idrees et al., 2010), scopolamine and hyoscyamine in *Brugmansia candida* (Pitta-Alvarez et al., 2000) and pilocarpine in jaborandi (Avancini et al., 2003) could be induced by SA. Exogenous application of SA significantly stimulates the production of anthraquinones (Bulgakov et al., 2002) and glucosinolates (Kiddle et al., 1994).

A survey of literature indicates that SA could affect antioxidant enzyme activities and then cause a moderate increase in the content of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) (Ali et al., 2006; Chen et al., 1993; Harfouche et al., 2008; Mahdavian et al., 2007), which acts as a second messenger in regulating plant defense responses (Dempsey and Klessig, 1994; Hayat et al., 2010; Jaspers and Kangasjärvi, 2010). In recent years, effects of SA on terpenoids secondary metabolism also have received attentions gradually. Ali et al. (2006) and Shabani et al. (2009) showed that SA induced the accumulation of triterpenoids, ginsenosides in ginseng and glycyrrhizin in licorice, respectively. Production of sesquiterpenoids, such as bilobalide in ginkgo (Kang et al., 2006), crepidiaside, deoxylactucin and sonchuside in chicory (Malarz et al., 2007), and artemisinin in *Artemisia annua* (Aftab et al., 2010; Pu et al., 2009) could also be stimulated by SA. Although there are only little information about monoterpenes, SA accumulates diterpenes including ginkgolides (Kang et al., 2006) and taxol (Miao et al., 2000; Wang et al., 2007) which share the common non-mevalonate pathway in plastids with monoterpenes (Lichtenthaler, 1999), and monoterpene biosynthesis is suppressed in SA-deficient transgenic *Arabidopsis* plants (Munné-Bosch et al., 2007). In addition, a recent study showed that methyl salicylate, SA derivatives, transitorily increases monoterpene emission rates in a woody plant, *Quercus ilex* (Peñuelas et al., 2007). All of these suggest that SA might be used as a potential enhancer to improve monoterpene production. Therefore, keeping the importance of monoterpenes and *H. cordata* with enormous commercial value, we employed SA as an elicitor to study whether foliar application of SA with different concentrations could alter the yield of monoterpenes and assess whether oxidative stress is involved in the induction.

MATERIALS AND METHODS

Plant materials and growth conditions

H. cordata line w01-100 was used in the experiment (Chen et al., 2008; Wu et al., 2003). Stoloniferous rhizomes were transplanted into individual earthen pots (50 cm diameter) containing a mixture of soil, sand and perlite (2: 2: 1) and the pots were kept in an greenhouse at Ya'an (latitude 29°59′ 08″N, longitude102°58′ 56″E and altitude 595 m) maintained at 25 ± 4°C with relative humidity of 70%. Water was uniformly added to each pot until field moisture capacity for the uniformity in growth. After leaf emergence, plants were limited to ten plants per plot. Plants were conducted to foliar application of SA when five leaves appeared.

SA application and sample preparation

Plants were sprayed with 0.01, 0.1, 1, 10 or 100 mM SA dissolved in distilled water by using an atomizer onto the leaves until it ran off. The control plants were sprayed with distilled water. Each treatment consisted of three replicates. After three days, the third fully expanded leaves were collected randomly from uniform plants in replicate plots, kept in deep freezer at -70°C and used for determinations of malondialdehyde (MDA) contents, antioxidative enzyme activities, H_2O_2 concentrations, and monoterpene contents.

Antioxidative enzyme activities

Leaves (0.5 g) were ground in liquid nitrogen and homogenized with 10 ml 50 mM (4°C) sodium phosphate buffer (pH 7.8) for superoxide dismutase (SOD), 50 mM sodium phosphate buffer (pH 6.0) for peroxidase (POD) and 200 mM sodium phosphate buffer (pH 7.0) for catalase (CAT), respectively. The homogenate was centrifuged at 4°C (15,000 *g* × 5 min) and the supernatant was used for assays. The whole extraction procedure was carried out. SOD activity was measured according to its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich, 1971). Briefly, the reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM L-methionine, 75 µM NBT, 10 µM ethylene diamine tetraacetic acid (EDTA) -Na_2, 2 µM riboflavin, and 0.05 ml enzyme extract. Absorbance was recorded on Ultraviolet/Visible (UV-Vis) spectrophotometer (UV-2450, Shimazu Company, Kyoto, Japan). One activity unit (U) was defined as the amount of SOD to inhibit the reduction of NBT by 50%. SOD activity was calculated in terms of U g^{-1} fresh weight (FW). POD activity was determined based on guaiacol oxidation (Hassan et al., 2005). Briefly, the reaction mixture contained 50 mM sodium phosphate buffer (pH 6.0), 5 mM guaiacol, 10 mM H_2O_2, and 0.05 mL enzyme extract.

Absorbance change due to guaiacol oxidation was measured at

470 nm by using the UV-Vis spectrophotometer. One U of activity was calculated by the variety absorbance of 0.01 min^{-1}. POD activity was expressed as U g^{-1} FW. CAT activity was measured by monitoring the destruction of H_2O_2 (Rout and Shaw, 2001). Briefly, the reaction mixture consisted of 200 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2, 0.05 ml enzyme extract. Decrease in the absorbance due to decomposition of H_2O_2 was recorded at 240 nm on the UV-Vis spectrophotometer. One U of activity was defined as the variety of 0.01 absorbance min^{-1}. CAT activity was calculated in terms of U g^{-1} FW.

H_2O_2 and MDA contents

H_2O_2 content was determined as titanium complex (Brennan and Frenkel, 1977). Briefly, leaves (0.5 g) were homogenized in 10 ml cold (4°C) acetone. The homogenate was centrifuged (15,000 *g* × 5 min) at 4°C. The supernatant (1 ml) was mixed with 0.1 ml titanium reagent (5% titanic tetrachloride in concentrated hydrochloric acid, v/v), followed by the addition of 0.2 ml concentrated ammonia to precipitate the peroxide-titanium complex. The mixture was then centrifuged (15,000 *g* × 5 min). The complex was washed with acetone repeatedly and then solubilized in 5 ml of 2 M sulphuric acid. The intensity of yellow color of supernatant was measured at 415 nm by using the UV-Vis spectrophotometer. H_2O_2 concentration in the supernatant was calculated by comparing its absorbance to a standard calibration curve representing H_2O_2-titanium complex from 0 to 1 mM. H_2O_2 content in plants was expressed as µmol g^{-1} FW. MDA content was measured as described by Heath and Packer (1968). Briefly, leaves (0.5 g) were homogenized in 10% (w/v) cold (4°C) trichloroacetic acid (TCA) and centrifuged (15,000 *g* × 5 min). 3.0 ml of the supernatant was mixed with 3.0 ml TBA (0.5%) in 10% (w/v) TCA and heated at 100°C for 10 min. After cooling, the mixture was centrifuged (15,000 *g* × 5 min) and the supernatant was determined at 532 nm with UV-Vis spectrophotometer. The values were corrected for non-specific absorbance by subtracting the absorbance at 600 nm. MDA content was calculated by using extinction coefficient of 155 mM^{-1} cm^{-1}.

Monoterpene contents

Leaves (50 g FW) were subjected to hydrodistillation for 4 h to extract essential oils by using a Clevenger apparatus (Clevenger, 1928). Ethyl acetate was used as solvent. The ethyl acetate layers were settled to the constant volume of 10 ml by using ethyl acetate. The extracts were dehydrated by passing through anhydrous sodium sulphate and stored in a clean glass vial sealed with Parafilm at 4°C in the dark until used for gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS analysis was performed on an Agilent 6890 series GC system using a fused silica capillary column (HP-5MS: 30 × 0.25 mm, film thickness 0.25 µm), coupled to an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Palo Alto, CA,USA). For all analyses, the injector port was maintained at 250°C, carrier gas was helium, the column flow was held constant at 1 ml min^{-1}, injection volume of each sample was 1 µl, split ratio was 1: 50, interface temperatures was 280°C and the MS operated at 70 eV. The temperature program was as follows: an initial temperature of 60°C (2 min hold) was increased to 110°C at 10°C min^{-1}, and held at 110°C for 4 min followed by a 10°C min^{-1} until 220°C (5 min hold). The constituents of essential oils were identified by the use of a combination of mass spectrum database search (NIST 2.0 Database) and relative retention indices as well as comparison of patterns of mass spectra with those of authentic references reported (Chen et al., 2008). The relative content of individual monoterpene was expressed as percent peak area relative to total peak area.

Statistical analysis

Values are presented as means ± standard errors (SE). Difference significance was identified statistically by Duncan's multiple range tests ($P < 0.05$ or 0.1). All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Antioxidative enzyme activities

The activities of antioxidative enzymes, SOD, POD and CAT in the leaves of *H. cordata* were significantly affected by SA treatments with different concentrations (Figure 1). However, different responses were observed among these enzymes. In detail, SOD activity was not induced by SA applications and was significantly suppressed in the treatments of high concentrations of SA (10 and 100 mM) as compared to the control plants. Treatments with SA resulted in an increase in POD activity, but a burst increase was only observed at the highest SA concentration (100 mM). CAT activity showed no significant increase at low concentrations of SA (≤ 1 mM). However, it was strongly stimulated by high concentrations of SA. These, therefore suggest that the concentrations of SA are critical for the inhibition of SOD and the induction of POD and CAT.

MDA and H_2O_2contents

H_2O_2 content was measured to evaluate whether application of SA could improve the generation of ROS, which are generally used as signaling molecules to finely regulate SA-induced defense responses (Asghari and Aghdam, 2010). As compared to the control plants, increased H_2O_2 was not detected in all the treatments with SA after three days (Figure 2a). However, although no significant change of MDA contents was also observed at low concentrations of SA, application of SA at high concentrations showed a marked increase in MDA content (Figure 2b). Increased MDA indicate that phospholipid membrane suffered oxidative damage from excess SA because it was an indicator of lipid peroxidation. Therefore, although H_2O_2 accumulation was absent in the leaves of *H. cordata* after three days, this result incorporated with the strong induction of POD and CAT suggest that SA at high concentrations may lead to oxidative stress.

Monoterpene contents

Total monoterpene contents in the leaves of *H. cordata* were significantly influenced by SA applications (Table 1). Total monoterpenes showed a significant increase in its content in the treatment of 0.01 mM SA as compared to the control. However, application of 0.1 and 1 mM SA

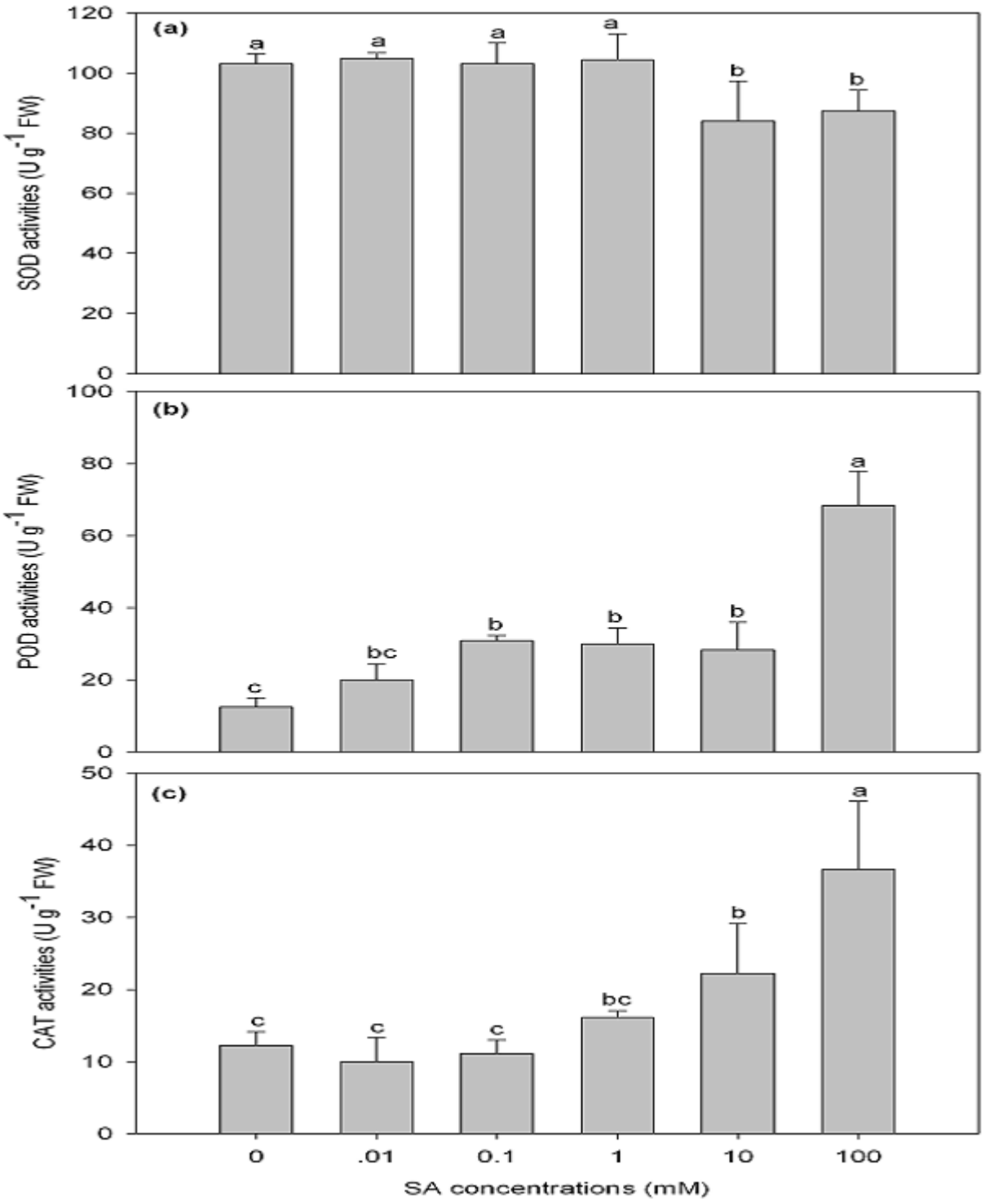

Figure 1. Effects of different SA treatments on antioxidative enzyme activities in *H. cordata* leave; **a**, SOD; **b**,POD; **c**, CAT. Values are means ± SE (n=3). Bars carrying different letters are significantly different at $P < 0.05$.

significantly suppressed monoterpene production. Interestingly, accumulation of total monoterpenes was again induced by higher concentrations of SA, and the contents showed a stronger increase than that at 0.01 mM SA. The results imply that differential regulation mechanism might be involved in monoterpene biosynthesis induced by different concentrations of SA. Different monoterpenes got different effects in the

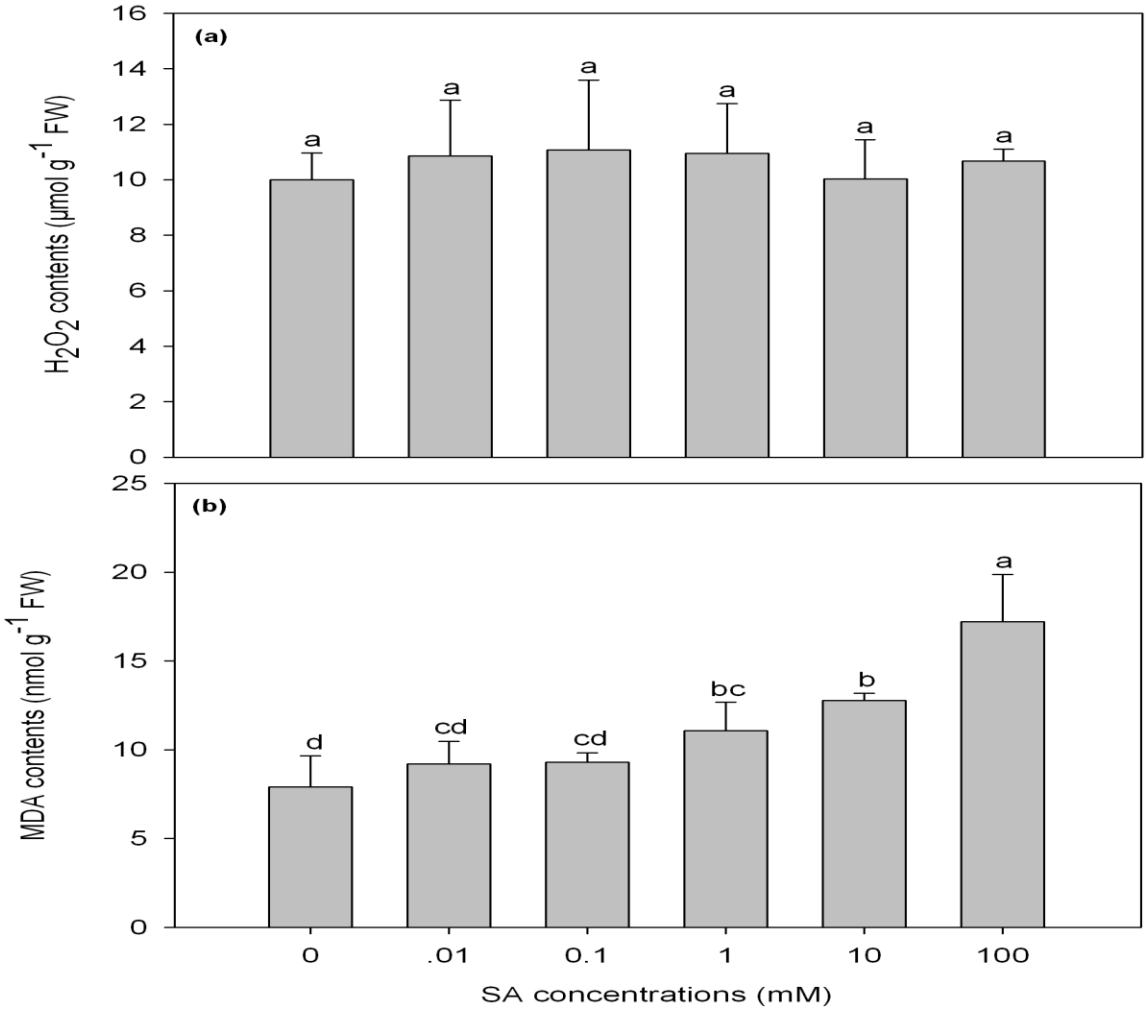

Figure 2. Effects of different SA treatments on H_2O_2 and MDA contents in *H. cordata* leaves; **a,** H_2O_2 and; **b**, MDA. Values are means ± SE (n=3). Bars carrying different letters are significantly different at $P < 0.05$.

treatments with SA (Table 1). In detail, only the contents of β-myrcene, D-limonene, β-ocimene, γ-terpinene and terpinolene were significantly increased at 0.01 mM SA. The biosynthesis of β-phellandrene, α-terpinene, p-cymene, terpinolene, 4-terpineol and geraniol was not significantly suppressed by the application of 0.1 and 1 mM SA. High concentrations of SA could not significantly improve the production of camphene, p-cymene, α-terpineol and geraniol. The results suggest that the biosynthesis of these monoterpenes might be differentially mediated in the treatments of SA with different concentrations.

DISCUSSION

Exogenous application of SA at low concentrations might suitably enhance the efficiency of antioxidant system in *H. cordata*. Although SOD and CAT activities were not elevated in the treatments with SA at low concentrations in this study, moderately increased POD activity suggest that plants were endowed with a state of antioxidant capacity. It has been demonstrated that SA plays an important role in the induction of systemic acquired resistance (SAR) in plants (Hayat et al., 2010). The corresponding mechanism is that SA as a long distance mediator could move freely among the cells, tissues and organs (Klessig and Malamy, 1994; Shulaev et al., 1995), stimulate the activities of one or more antioxidative enzymes such as SOD, POD and CAT and then improve plant tolerance to overcome the oxidative stresses caused by various biotic and abiotic stresses (Dong et al., 2010; Idrees et al., 2010). For example, by improving antioxidative enzyme activities, SA effectively alleviated the negative effects induced by metal (Ahmad et al., 2011; Chen et al., 2007; Kazemi et al., 2010; Panda and Patra, 2007; Wang et al., 2009; Zhou et al., 2009), salinity (Idrees et al., 2010), virus (Radwan et al., 2006; Radwan

Table 1. Effects of different SA treatments on the contents of monoterpenes (%) in *H. cordata* leaves.

Compound	Control	SA 1	SA 2	SA 3	SA 4	SA 5
α-Thujene	0.29 c	0.30 c	0.26 d	0.28 cd	0.36 b	0.41 a
α-Pinene	1.66 c	1.67 c	1.47 d	1.47 d	1.94 b	2.17 a
Camphene	0.17 ab	0.15 bc	0.15 bc	0.14 c	0.17 a	0.17 ab
β-Phellandrene	11.34 cd	11.56 c	10.11 d	10.47 cd	13.70 b	16.90 a
β-Pinene	1.88 c	1.92 c	1.65 d	1.67 d	2.25 b	2.54 a
β-Myrcene	10.15 c	10.82 b	9.67 cd	9.49 d	11.40 a	11.08 ab
α-Terpinene	1.30 bc	1.52 ab	1.13 c	1.16 c	1.57 ab	1.68 a
p-Cymene	0.18 ab	0.15 ab	0.11 b	0.13 ab	0.24 a	0.18 ab
D-Limonene	0.42 c	0.45 b	0.37 d	0.38 d	0.50 a	0.53 a
β-Ocimene	3.79 d	4.41 c	3.39 e	3.41 e	4.86 b	5.31 a
γ-Terpinene	2.26 b	2.57 a	1.92 c	1.93 c	2.67 a	2.81 a
Terpinolene	0.51 b	0.59 a	0.45 b	0.45 b	0.61 a	0.63 a
3-Carene	0.18 b	0.18 b	0.16 c	0.15 c	0.20 b	0.22 a
4-Terpineol	3.82 bc	4.17 ab	3.27 c	3.27 c	4.25 ab	4.46 a
α-Terpineol	0.19 a	0.20 a	0.16 b	0.15 b	0.21 a	0.20 a
Geraniol	1.10 a	1.01 bc	1.13 a	1.04 ab	1.14 a	0.93 c
Total monoterpenes	39.25 d	41.67 c	35.40 e	35.59 e	46.05 b	50.22 a

Data indicates mean ± SE (n=3). The data followed by different letters in the same lines are significantly different according to Duncan's multiple range test at P <0.1. Control, SA1, SA 2, SA 3, SA 4 and SA 5 represent 0, 0.01, 0.1, 1, 10 and 100 mM SA, respectively.

et al., 2007), insects (Molinari and Loffredo, 2006; Urbanek Krajnc et al., 2011), herbicides (Ananieva et al., 2004), drought (Singh and Usha, 2003) and temperature (He et al., 2005; Janda et al., 1999). However, there is a decline in these enzyme activities especially CAT; is observed in some other plants (Ali et al., 2006; Asghari and Aghdam, 2010; Choudhury and Panda, 2004; Hayat et al., 2010; Molinari and Loffredo, 2006). An alternative assumption is that SA inhibits antioxidative enzymes by bonding, thus gradually increasing the generation of ROS such as H_2O_2, which act as second messengers that induce the expression of defense-related genes associated with SAR (Chen et al., 1993; Dempsey and Klessig, 1994). Recent studies indicate that the inhibition of enzymes and the induction of ROS are transient or temporary; approximately one day after exogenous application of SA (Janda et al., 1999; Pu et al., 2009).

In this study, an increase of H_2O_2 content and a significant decrease of CAT activity were not observed three day after application of SA at low concentrations. Therefore, in further studies determination of ROS and enzyme activities at early stages is suggested to assess whether low concentrations SA could result in temporary reduction of these enzyme activities and increased H_2O_2 content in *H. cordata*.

However, high concentrations of SA itself might cause a high level of oxidative stress in *H. cordata*. A strong accumulation of MDA in the treatments with SA at high concentrations indicates the induction of oxidative stress in plants. Although H_2O_2 content was increased three days later, this result suggest that excess generation of ROS was more or less induced at early stages (Dai et al., 1997; Heath and Packer, 1968). In addition, both POD and CAT are responsible for quenching H_2O_2, and they are usually activated when excessive H_2O_2 is generated under various stress, protecting plants against oxidative damage (Apel and Hirt, 2004; Nayyar and Chander, 2004). Thus, as compared to the other treatments, markedly elevated H_2O_2-scavenging enzymes at high concentrations of SA also implied excess accumulation of H_2O_2 particularly at early stages. Moreover, the suppression of SOD activity might lead to accumulation of superoxide radicals ($^{\cdot}O_2^-$) (Alscher et al., 1997; Bowler et al., 1992). Further studies therefore are needed to determine whether oxidative burst is transiently induced after excess application of SA in *H. cordata*. The burst accumulation of total monterpenes at high concentrations of SA might be attributed to SA-induced oxidative stress. As observed in this study, 0.1 and 1 mM showed adverse effects on monoterpene biosynthesis.

Surprisingly, the treatments with SA at higher concentrations (10 and 100 mM) markedly promoted monoterpene production. Incorporated with the changes of MDA contents and antioxidative enzyme activities, we assumed that oxidative burst might partially contribute to monoterpene accumulation. It is well known that ROS as signal components are involved in activation of monoterpene biosynthetic enzymes and oxidative burst could induce monoterpene biosynthesis (Zhao et al., 2005; Zhao et al., 2006; Zhao and Sakai, 2003). Certainly, application of SA combined with antioxidants in *H. cordata* is to be used to support this hypothesis in

further works.

A suitable concentration of SA could significantly promote monoterpene production, but not lead to oxidative stress in *H*. *cordata*. In the study, 0.01 mM SA was favorable for the induction of monoterpenes, and this lower concentration of SA should not cause oxidative damage according to the moderate production of MDA. However, higher concentrations of SA resulted in either the suppression of biosynthesis (0.1 and 1 mM) or strong oxidative stress (10 and 100 mM). Similar responses are observed in many aspects of plant growth and development, for example, 0.01 mM SA showed a significant increase in dry matter, leaf number, root length, pigment contents, chlorophyll contents, net photosynthetic rates and root nodule number, while higher concentrations of SA resulted in an inhibitory effect (Fariduddin et al., 2003; Gutiérrez-Coronado et al., 1998; Hayat et al., 2005).

The suitable SA concentration for phenolic compound accumulation was 6.25 to 22.5 mg L^{-1} in *Salvia miltiorrhiza* cell culture (Dong et al., 2010), while higher concentrations of SA proved to be inhibitory. 20 mg L^{-1} was proved to be the optimal SA concentration with less damage to *Taxus cells* and marked production of taxol (Wang et al., 2007). Therefore, these suggest that a critical concentration of SA might be important for its beneficial effects.

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