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Full Length Research Paper

Salicylic acid-induced physiological responses and monoterpene accumulation in *Houttuynia cordata* Thunb

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Houttuynia cordata **Thunb. is a medicinal herb rich in monoterpenes. The roles of salicylic acid (SA) in monoterpene accumulation and physiological responses of *H. cordata* are still limited in knowledge, hampering its application in the field. After foliar application of 0.01 mM SA, monoterpene contents, antioxidative enzyme activities, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents were measured. The maximum of total monoterpene concentrations was observed 8 days after treatment. A continuous increase of superoxide dismutase and peroxidases activities after 4 and 2 days, respectively, suggested the induction of plant tolerance. H_2O_2 content did not showed an increase in SA-treated plants at the early stage, while a significant decline in MDA content was observed as compared to the controls. Moreover, although H_2O_2 was markedly induced at the later stage, MDA content was not increased. These implied that SA application led to lower lipid peroxidation. The study suggested that the application of SA might have little adverse effects on growth and development, and harvest for 8 days after SA application is critical to quality assurance of monoterpenes in *H. cordata*.**

Key words: Antioxidative system, *Houttuynia cordata*, monoterpenes, salicylic acid.

INTRODUCTION

Houttuynia cordata Thunb. is one of the most potential medicinal and edible wild plant resources (Wu et al., 2005). It is an aromatic herb and in Chinese is known as 'Yuxingcao', which means 'producing unique fishy smell'. It is a wild vegetable and has been used as a kind of traditional Chinese medicine for hundreds of years. Monoterpenes produced as secondary metabolites are its key components due to their various pharmacological activities such as anti-cancer, anti-oxidative, anti-inflammatory, anti-bacterial and anti-viral (Gould, 1995; Lau et al., 2008; Lu et al., 2006). In addition, monoterpenes usually process unique fragrances and are widely added to foods, drinks, perfumes, cosmetics and tobacco (Aharoni et al., 2005; Verlet, 1993). In recent years, farmers are trying to raise the yield of *H. cordata* by its commercial cultivation. Undoubtedly, the content of monoterpenes is a very important index of its quality. It has been demonstrated that signal molecules are very potential elicitors for induction of plant secondary metabolites (Zhao et al., 2005). These metabolites generally have essential defense roles in response to various stress. Thus, biotic and abiotic stress could stimulate accumulation of these defense metabolites (Holopainen and Gershenzon, 2010). Some signal compounds as messengers are involved in the regulation of the defense responses. For example, calcium G-proteins and jasmonate signalling pathways mediate yeast elicitor-induced β-thujaplicin biosynthesis (Zhao and Sakai, 2003). Recent years, the applications of signal components as elicitors have evolved an effective strategy for the production of target secondary metabolites in plant cell cultures. However, it is still uncommon for commercial application (Zhao et al., 2005).

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

It therefore suggested that application of elicitors *in vivo* is an easy and direct channel to promote the yield of plant secondary metabolites in the field conditions. Salicylic acid (SA), a signal molecule has been found to generate metabolic and physiological responses. It can affect anti-oxidative enzyme activities and mediate production of reactive oxygen species (ROS) (Dempsey and Klessig, 1994; Larkindale and Knight, 2002). In addition to growth and development, the SA signalling pathway plays a key role in plant defense responses (Hayat et al., 2010). It has been used as a potent enhancer of some secondary metabolites in previous studies, for example, exogenous application of SA improves the production of alkaloids (Idrees et al., 2010; Pitta-Alvarez et al., 2000), anthraquinones (Bulgakov et al., 2002) and glucosinolates (Kiddle et al., 1994). Recently, some studies indicate that SA signalling pathway is involved in biosynthesis of terpenoids including triterpenoids (Shabani et al., 2009), diterpenoids (Wang et al., 2007), and sesquiterpenoids (Aftab et al., 2010).

Our preliminary study still showed that lower concentrations of SA could increase monoterpene contents in *H. cordata*. However, there is limited knowledge of the regulation of SA-induced monoterpene accumulation and physiological responses. This hampers the use of SA on a practical level for production of *H. cordata* in the field. In the present study, the primary aim of this work was to determine the optimal harvest period and determine whether the SA application resulted in oxidative damage to the plants. After application of SA at lower concentrations in *H. cordata*, monoterpene contents, antioxidative enzyme activities, H_2O_2 and MDA contents were dynamically measured.

MATERIALS AND METHODS

Plant growth and SA application

H. cordata line w01-100 was planted in earthen pots (75 cm diameter) containing a mixture of soil, sand and perlite (2: 2: 1) and the pots were kept in the field, Ya'an (latitude 29° 59′ 08″N, longitude102° 58′ 56″E, and altitude 595 m). Plants were watered uniformly to maintain optimum water regime in each plot for the uniformity in growth. After leaf emergence, plants were limited to 20 plants per plot. When five leaves appeared, plants were conducted to foliar application of 0.01 mM SA. The control plants were sprayed with distilled water. Each treatment consisted of three replicates.

The third fully expanded leaves were collected randomly from uniform plants in replicate plots 1, 2, 4, 8 and 12 d after treatments. Samples were enclosed with self-sealed bags, placed in liquid nitrogen, and then stored at -70°C for determinations as follows.

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 the trapping solvent. The ethyl acetate layers were brought to a constant volume of 10 ml with 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 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 m × 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). Identification of components was based on the comparison of their mass spectra and retention indices with those of authentic compounds and by computer matching with NIST 2.0 and Wiley libraries as well as by comparison of the mass spectral data with those reported in the authentic references (Chen et al., 2008). The relative content of individual monoterpene was expressed as percent peak area relative to total peak area.

Measurement of 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; EC 1.15.1.1), 50 mM sodium phosphate buffer (pH 6.0) for peroxidase (POD; EC 1.11.1.7) and 200 mM sodium phosphate buffer (pH 7.0) for catalase (CAT; EC 1.11.1.6), 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 at 4°C. SOD activity was measured according to its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich, 1971). The reaction mixture contained of 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, 0.05 ml enzyme extract. Absorbance was recorded on UV-Vis spectrophotometer (UV-2450, Shimazu Co, 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). CAT activity was measured by monitoring the destruction of H_2O_2 (Rout and Shaw, 2001). 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. POD activity was determined based on guaiacol oxidation (Hassan et al., 2005). The reaction mixture contained of 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 using the UV-Vis spectrophotometer. One U of activity was calculated by the change in absorbance of 0.01 min^{-1}. POD activity was expressed as U g^{-1} FW.

Determination of 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

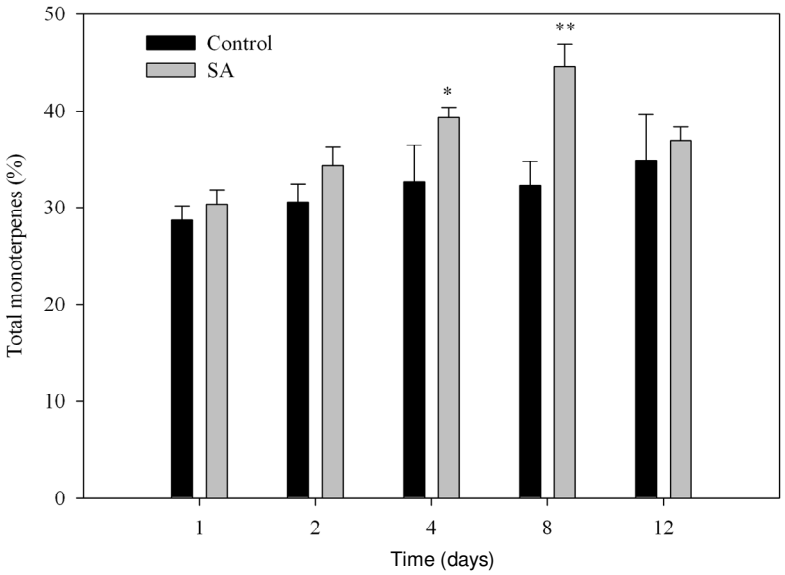

Figure 1. Changes in total monoterpene content in the leaves of *H. cordata* after 0.01 mM SA treatment. Measurements were made 1 to12 days after the foliar application of SA. The results are expressed as percentage of total essential oils by measuring the area under the peaks from GC-MS analysis. Each value represents the mean ± SE (n = 3). The asterisk indicates a statistically significant difference between the data from the SA-treated plants and respective controls (*$P < 0.05$, **$P < 0.01$).

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 an extinction coefficient of 155 mM^{-1} cm^{-1}.

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

Monoterpene accumulation after SA treatment

Production of total monoterpenes in the leaves after foliar application of SA is shown in Figure 1. Individual monoterpenes (≥0.2%), α-thujene, α-pinene, β-phellandrene, β-pinene, β-myrcene, α-terpinene, p-cymene, D-limonene, β-ocimene, γ-terpinene, terpinolene and 4-terpineol were included in calculation of total monoterpene contents (Table 1). The result showed that exogenous SA significantly promoted monoterpene accumulation as compared to the controls and the maximum of total monoterpene concentrations was observed 8 days after SA application.

Changes of antioxidative enzyme activities after SA application

The changes in the activities of SOD, CAT and POD in the leaves were observed in Figure 2. The activities of SOD and POD showed a significant and continuous increase 4 and 2 d after SA application as compared to the controls, respectively. However, CAT activity was only slightly increased during the period of experiment. This suggested that SA induced differential responses of antioxidative enzyme activities.

Changes of H_2O_2 and MDA contents after SA application

The changes of H_2O_2 contents in the leaves after application of SA were showed in Figure 3. The results

Table 1. Monoterpene profile of *H. cordata* after SA application.

Compounds	RI	Time (days)				
		1	2	4	8	16
α-Thujene	461	0.24±0.01	0.29±0.02	0.30±0.01	0.35±0.03	0.27±0.01
α-Pinene	472	1.38±0.08	1.48±0.10	1.57±.03	1.81±0.12	1.44±0.07
β-Phellandrene	533	8.44±0.87	9.59±0.38	9.67±0.09	12.61±0.36	10.56±0.43
β-Pinene	539	1.55±0.07	1.71±0.13	1.90±0.08	2.09±0.14	1.64±0.07
β-Myrcene	557	8.20±0.30	9.15±0.52	12.08±0.61	13.18±0.73	10.48±0.34
α-Terpinene	600	1.37±0.11	1.46±0.13	1.69±0.01	1.59±0.12	1.14±0.04
p-Cymene	612	0.22±0.01	0.24±0.04	0.27±0.04	0.28±0.10	0.18±0.02
D-Limonene	619	0.39±0.03	0.41±0.03	0.49±0.02	0.51±0.04	0.40±0.02
β-Ocimene	630	1.82±0.21	3.12±0.16	4.07±0.30	4.57±0.34	4.97±0.12
γ-Terpinene	666	2.23±0.08	2.38±0.26	2.69±0.13	2.62±0.28	1.96±0.11
Terpinolene	714	0.53±0.02	0.57±0.06	0.63±0.03	0.63±0.08	0.47±0.03
4-Terpineol	883	3.93±0.16	3.89±0.34	4.00±0.33	4.32±0.33	3.47±0.15

Notes: All values are the average of three repeats. Data indicates mean ± SE (n = 3).

indicate that H_2O_2 was not induced at the early stage after SA treatment while a strong increase in its contents was observed at the later stage as compared to the control plants. This implied that ROS was generated 8 days after SA application. The changes of MDA contents in the leaves were showed in Figure 4. Although there was no significant difference in H_2O_2 contents between the SA-treated and control plants, MDA content showed a significant decrease at the early stage after SA treatment.

In addition, at the later stage, although H_2O_2 was markedly induced, a significant increase was not observed as compared to the controls. MDA content is an indicator of lipid peroxidation. These results suggest that SA application resulted in inhibition of oxidative stress.

DISCUSSION

Optimum harvest period is critical to quality assurance of monoterpenes in *H. cordata*. It is well known that the accumulation of plant defense metabolites is due to the activation of corresponding enzymes (Zhao et al., 2006). It is suggested that monoterpene biosynthesis uses signal transduction and transcription factors that bind to *cis*-acting elements in promoter regions of monoterpene biosynthetic genes (Zhao et al., 2005). When stresses or signal molecules were removed, the rate of monoterpene biosynthesis would be decreased. In the present study, 8 d after exogenous application of 0.01 mM SA would be the optimal harvest time according to the highest values of total monoterpene contents in the leaves of *H. cordata*. Similar results were observed in production of other secondary metabolites induced by exogenous SA by others (Dong et al., 2010; Kang et al., 2006; Pu et al., 2009; Wang et al., 2007). Exogenous application of SA in this study induced plant tolerance by improving the antioxidant defense system. Previous studies have indicated that SA application could ameliorate the severe oxidative stress generated by environmental stresses such as drought (Singh and Usha, 2003) and heat (Larkindale and Knight, 2002), heavy metals (Kazemi et al., 2010; Panda and Patra, 2007), salinity (Idrees et al., 2010), insects (Molinari and Loffredo, 2006) and herbicides (Ananieva et al., 2004). The alternative mechanism is that SA improves antioxidative enzyme activities (Hayat et al., 2010; Idrees et al., 2010; Larkindale and Knight, 2002).

In the present study, although CAT activity showed a little response, markedly enhanced SOD and POD activities suggested the acquirement of ROS-scavenging capacity. It is suggested that SA induces systemic acquired resistance (SAR) in *H. cordata.* Exogenous treatment with 0.01 mM SA could protect *H. cordata* against oxidative damage. In the present study, excess H_2O_2 was produced in the leaves of SA-treated plants as compared to the controls at the later stages. Alternatively, SA not only induced antioxidative enzyme activities but also improved the accumulation of monoterpenes, which have greater antioxidant capacity because of their conjugated double bond system (Tepe et al., 2004; Vickers et al., 2009). It has been demonstrated that monoterpenes as antioxidant agents play an important defense role against oxidative stress induced by various environmental stresses such as heat (Loreto et al., 1998). These might partly contribute to the low MDA content at the later stages. Therefore, the results suggested that 0.01 mM SA application might not result in the adverse effects on growth and development and it could be used as a potential elicitor to improve mononterpene production in *H. cordata*.

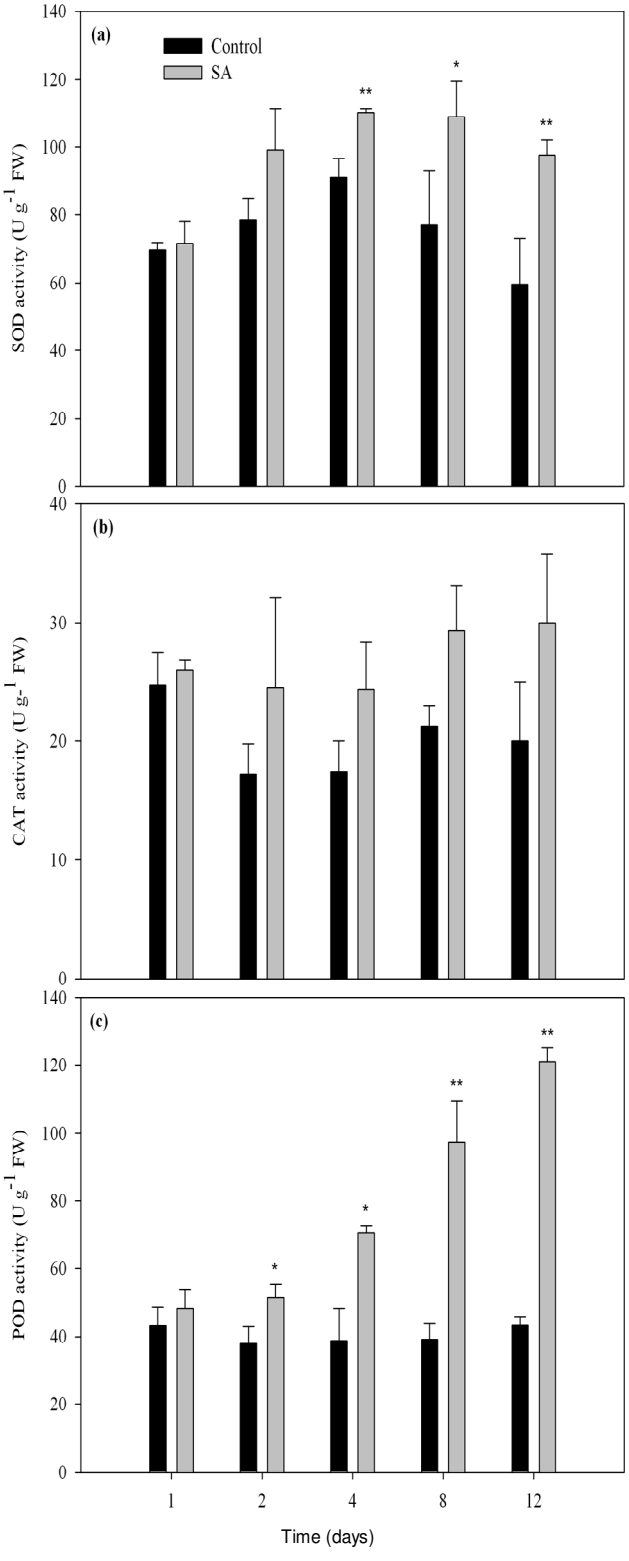

Figure 2. Changes in antioxidative enzyme activities in the leaves of *H. cordata* after 0.01 mM SA treatment. (a) - SOD, (b) - CAT, (c) - POD. Measurements were made 1 to 12 days after the foliar application of SA. Each value represents the mean ± SE (n = 3). The asterisk indicates a statistically significant difference between the data from the SA-treated plants and respective controls (**P < 0.05, **P < 0.01*).

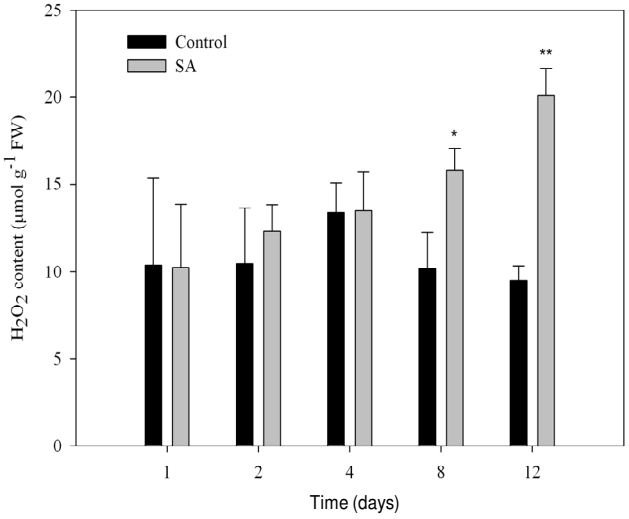

Figure 3. Changes in H_2O_2 contents in the leaves of *H. cordata* after 0.01 mM SA treatment. Measurements were made 1 to 12 days after the foliar application of SA. Each value represents the mean ± SE (n = 3). The asterisk indicates a statistically significant difference between the data from the SA-treated plants and respective controls *(*P < 0.05, **P < 0.01*).

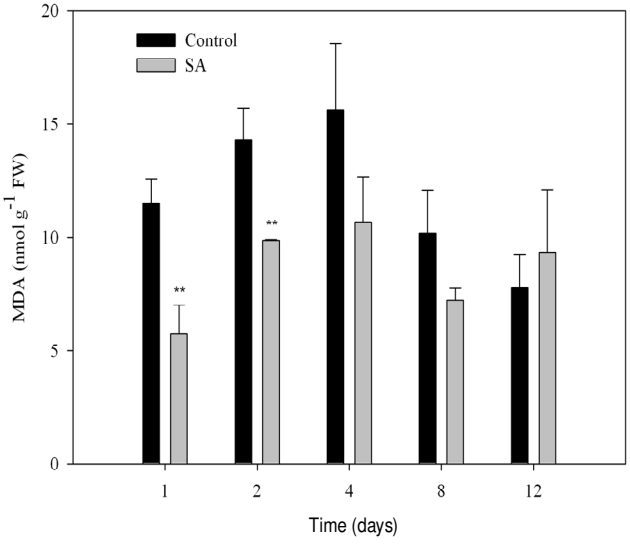

Figure 4. Changes in malondialdehyde (MDA) contents in the leaves of *H. cordata* after 0.01 mM SA treatment. Measurements were made 1 to12 days after the foliar application of SA. Each value represents the mean ± SE (n = 3). The asterisk indicates a statistically significant difference between the data from the SA-treated plants and respective controls *(*P < 0.05, **P < 0.01*).

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