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BRIEF COMMUNICATION

Effects of salicylic acid pre-treatment on cadmium and/or UV-B stress in soybean seedlings

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Abstract

The present study examined the effect of salicylic acid (SA) pre-treatment on soybean seedlings exposed to cadmium and/or UV-B stress. Dry mass, pigment content, net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) were decreased by the Cd and/or UV-B stress. SA alleviated the adverse effects of Cd and/or UV-B on growth, pigment content, P_N, and gs, but did not mitigate the inhibitory effect of Cd/UV-B on E, or that of Cd on chlorophyll fluorescence parameters. Cd and/or UV-B induced oxidative stress and increased lipid peroxidation that was significantly decreased by SA pre-treatment. The Cd and/or UV-B increased superoxide dismutase (SOD) activity, decreased peroxidase (POD) activity, and catalase (CAT) activity was mostly unaltered. SA might act as one of the potential antioxidants as well as a stabilizer of membrane integrity to improve plant resistance to the Cd and/or UV-B stress.

Additional key words: antioxidants, chlorophyll, fluorescence, *Glycine max*, net photosynthetic rate, oxidative stress, stomatal conductance, transpiration rate.

Cadmium is a highly toxic heavy metal that enters the environment *via* several sources. It is significant pollutant because of its high toxicity and water solubility (Pinto *et al*. 2004). Many papers illustrate toxic effects of Cd on plant metabolism, such as a decrease in the photosynthetic rate (Hasan *et al*. 2011), an inhibition of chlorophyll biosynthesis (Tao *et al*. 2012), an enhancement of oxidative stress (Sharma and Dietz 2009), an induction of lipid peroxidation (Belkhadi *et al*. 2010), and a promotion or inhibition of antioxidant enzyme activities (Liu *et al*. 2011).

The effects of increased UV-B radiation (280 - 320 nm) at the earth's surface on plant growth have been investigated extensively. Increases in solar UV-B have raised concerns about the damaging impact of UV-B radiation on crop plants (Caldwell *et al*. 2007). Enhanced UV-B radiation has been found to depress plant growth, decrease the content of chlorophyll, inhibit photosynthesis, and alter the antioxidant system (Tao *et al*. 2012).

Salicylic acid (SA) is considered to be an important signalling molecule, which plays an important role in regulating a number of physiological processes and plant resistances to stresses (Saruhan *et al*. 2012). Many reports have illustrated that SA can ameliorate the injurious effects of heavy metals on plants (Moussa and El-Gamal 2010, Liu *et al*. 2012). Plants accumulate large amounts of SA when exposed to UV radiation (Bandurska and Cieślak 2013). The exogenous application of salicylic acid alleviates the damaging effects induced by UV-B radiation in Kentucky bluegrass (Ervin *et al*. 2004). Further, SA stimulates photosynthetic machinery by increasing the content of chlorophyll in UV-stressed

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Abbreviations: CAT - catalase; Chl - chlorophyll; E - transpiration rate; F_v/F_m - variable to maximum chlorophyll fluorescence ratio (potential photochemical efficiency of photosystem II); F_v/F_0 - variable to basal fluorescence ratio (potential activity of photosystem II); g_s - stomatal conductance; MDA - malondialdehyde; P_N - net photosynthetic rate; POD - peroxidase; SOD - superoxide dismutase; WUE - water use efficiency
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plants (Mahdavian *et al*. 2008). SA has been shown to accumulate in plants in response to oxidative stress and to be directly involved in signalling various antioxidant responses (Larkindale and Knight 2002). However, the mechanism of SA-induced resistance is still unclear.

Under natural conditions, plants are often exposed to several stress factors simultaneously. Generally, the enhanced UV-B radiation and Cd pollution can occur together. The previous studies found that the UV-B irradiation and Cd are able to stimulate the formation of ROS and to cause oxidative stress (Ambasht and Agrawal 2003, Zhang *et al*. 2010). However, no work has been done on the ameliorative role of exogenous SA on the damaging effects induced by the combination of Cd and UV-B. Hence, the present study investigated the possible role of salicylic acid in alleviating Cd and/or UV-B radiation damage. Plant growth, photosynthetic parameters, chlorophyll fluorescence, antioxidant enzymes, and lipid peroxidation were assessed to provide essential information on Cd and/or UV-B radiation toxicity.

Soybean (*Glycine max* L. cv. Liaoxing 1) seedlings were grown from seeds obtained from the Shenyang Agricultural University. Each pot was filled with *Vermiculite*, and seedlings (two per pot) were watered to full saturation on alternate days with Hoagland solution. They grew in a greenhouse at day/night temperatures of 25/20 °C, a 16-h photoperiod, irradiance of 600 μmol m^{-2} s^{-1} (photosynthetically active radiation), and relative humidity of 75 %. After 15 d, the seedlings were divided into two groups. The leaves of one half of the seedlings were sprayed (a hand sprayer) with 0.5 mM SA firstly dissolved in absolute ethanol, and then added drop wise to water (ethanol:water, 1:1000, v/v). Preliminary studies had shown that this concentration was optimal for enhancing the Cd/UV-B tolerance of seedlings. A control group of seedlings was sprayed with ethanol:water (1:1000, v/v). The control and SA treatment solution contained 0.1 % (v/v) *Tween 20*. The volume of the spray was 30 cm^3 per pot. After 24 h, the seedlings were subjected to four treatments: *1*) control, *2*) increased Cd^{2+} content (Cd), *3*) increased UV-B irradiation (UV-B), and *4*) increased Cd^{2+} and increased UV-B irradiation (Cd+UV). Cadmium ($CdCl_2 . 2.5\ H_2O$) was dissolved in the Hoagland solution and applied to the pots to reach final concentration of 40 mg(Cd) kg^{-1}(*Vermiculite*). Supplemental UV-B radiation (280 - 320 nm) was provided by 40 J s^{-1} UV-B fluorescent tubes (Electric Light Source Research Institute, Beijing, China) according to Li *et al*. (2012). Seedlings were irradiated 6 h per day, from 10:00 to 16:00. All parameters were measured at 6 d after the beginning of each treatment. Each treatment was carried out in triplicate.

The height and root length of ten seedlings were measured. Shoot and root dry masses were obtained by drying the samples at 80 °C to a constant mass. Total chlorophyll (Chl) and carotenoid (Car) content was determined as described by Agrawal and Rathore (2007). Net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) were measured as described Li *et al*. (2012). Chlorophyll *a* fluorescence parameters of intact leaves (one leaf per plant, three plants per replicate) were measured using *Li-6400-40LCF* (*LI-COR*, Lincoln, NE, USA). The value of F_v/F_m, reflecting the maximal quantum yield of photosystem (PS) II photochemistry, was calculated according to the formula $F_v/F_m = (F_m - F_0)/F_m$, where F_v is variable fluorescence, F_0 is basal fluorescence, F_m is maximal fluorescence (for details see Strasser and Srivastava 1995). F_v/F_0 reflected the potential activity of PS II. The content of flavonoids was determined according to the method of Lois *et al*. (1994). Malondialdehyde (MDA) content was estimated according to the method of Islam *et al*. (2008). Fresh samples were homogenized in extraction buffer (0.1 M PBS, pH 6.8). The homogenate was centrifuged at 12 000 *g* and 4 °C for 15 min and the supernatant was used for enzyme assay. SOD activity was determined following the method of Beyer and Fridovich (1987), POD and CAT activities according to Chance and Maehly (1955). One unit of SOD was defined as the amount of enzyme that inhibits the reduction of nitroblue tetrazolium by 50 %. One unit of POD was defined as the amount of enzyme which produces 1.0 absorbance change at 470 nm per min. One unit of CAT was defined as the amount of the enzyme causing the decomposition of 1 μmol of H_2O_2 per min. All data were subjected to the one-way analysis of variance (*ANOVA*) and LSD multiple comparison test ($P < 0.05$ for significance of differences) using the *SPSS v. 13* statistical package (*SPSS*, Chicago, IL, USA).

Under Cd and UV-B stresses separately, no significant differences were observed for seedling height, whereas a decline was found in shoot dry mass (Table 1). Cd+UV significantly decreased seedling height but had no effect on shoot dry mass. Cd and Cd+UV significantly decreased root length and root dry mass, whereas UV-B alone had no effect. SA pre-treatment did not result in significant differences in seedling height or shoot dry mass, but increased root length and root dry mass under all the treatments, with the exception of root length under UV-B treatment. No significant difference was observed for plant growth parameters and biomass production between the SA pre-treatment group and the control.

The content of Chl *a*, Chl *b*, and Cars were decreased under Cd, UV-B, and Cd+UV (except Chl *b* content under UV-B) (Table 1). SA pre-treatment did not affect the pigment content in the controls but alleviated negative effects of Cd and UV-B. The flavonoid content increased in the UV-B and Cd+UV-treated plants and this increase was reduced by the SA pre-treatment. No significant difference in flavonoid content was observed between the SA pre-treatment and the control.

All the stresses decreased P_N, g_s, and E but did not affect WUE (Table 1). The SA pre-treatment significantly increased P_N and g_s in all treated plants but had no effect on E and WUE (except E in Cd+UV-treated plants). The

Table 1. Growth characteristics, content of Chl *a*, Chl *b*, Car, and flavonoids, net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E), water use efficiency (WUE), the potential photochemical efficiency of PS II (F_v/F_m), the potential activity of PS II (F_v/F_0), activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), and malondialdehyde (MDA) content of *Glycine max* seedlings pre-treated with SA or not and treated with Cd, UV-B, and Cd+UV. Means ± SD, $n = 3$. Data followed by the same letter are not significantly different ($P > 0.05$).

Parameters	SA	Control	Cd	UV-B	Cd+UV
Seedling height [cm]	- SA	23.19 ± 2.59ab	22.10 ± 2.47bc	21.17 ± 3.08bcd	20.16 ± 1.59cd
	+SA	25.42 ± 2.08a	22.25 ± 0.50b	24.07 ± 1.41cd	18.80 ± 2.17d
Root length [cm]	- SA	15.75 ± 2.09a	12.88 ± 1.62bc	14.20 ± 1.32abc	11.98 ± 1.87c
	+SA	16.08 ± 1.74a	15.20 ± 1.3a	15.33 ± 0.58ab	13.75 ± 1.5abc
Shoot dry mass [g plant^{-1}]	- SA	1.33 ± 0.21ab	0.89 ± 0.13d	0.97 ± 0.17d	1.10 ± 0.11bcd
	+SA	1.42 ± 0.11a	0.98 ± 0.14d	1.15 ± 0.17cd	1.27 ± 0.12abc
Root dry mass [g plant^{-1}]	- SA	0.32 ± 0.04ab	0.24 ± 0.04c	0.31 ± 0.03b	0.23 ± 0.04c
	+SA	0.36 ± 0.05ab	0.31 ± 0.03b	0.38 ± 0.03a	0.31 ± 0.04b
Chl *a* content [mg g^{-1}(f.m.)]	- SA	3.14 ± 0.09ab	2.31 ± 0.07e	2.89 ± 0.09c	2.15 ± 0.04f
	+SA	3.12 ± 0.03ab	3.24 ± 0.07a	3.12 ± 0.07b	2.46 ± 0.08d
Chl *b* content [mg g^{-1}(f.m.)]	- SA	0.93 ± 0.01bc	0.72 ± 0.03de	0.91 ± 0.01c	0.68 ± 0.01e
	+SA	0.96 ± 0.03b	0.96 ± 0.04b	1.07 ± 0.09a	0.75 ± 0.03d
Car content [mg g^{-1}(f.m.)]	- SA	0.54 ± 0.048a	0.40 ± 0.01d	0.49 ± 0.02b	0.44 ± 0.03cd
	+SA	0.55 ± 0.01a	0.53 ± 0.02a	0.47 ± 0.00bc	0.49 ± 0.08b
Flavonoid content [A_{334} g^{-1}(f.m.)]	- SA	0.49 ± 0.02cd	0.51 ± 0.03cd	0.58 ± 0.07ab	0.59 ± 0.03a
	+SA	0.52 ± 0.06bc	0.45 ± 0.02d	0.51 ± 0.03c	0.50 ± 0.02cd
P_N [μmol(CO_2) m^{-2} s^{-1}]	- SA	13.51 ± 0.15a	9.13 ± 0.37e	10.19 ± 0.62d	9.08 ± 0.89e
	+SA	13.96 ± 0.32a	10.36 ± 0.68cd	11.16 ± 0.62bc	11.90 ± 0.72b
g_s [mol m^{-2} s^{-1}]	- SA	0.11 ± 0.00a	0.06 ± 0.00d	0.07 ± 0.00d	0.06 ± 0.01d
	+SA	0.11 ± 0.00a	0.08 ± 0.00c	0.09 ± 0.00b	0.09 ± 0.01b
E [mmol(H_2O) m^{-2}s^{-1}]	- SA	2.59 ± 0.15a	1.65 ± 0.16de	1.93 ± 0.14bc	1.61 ± 0.14e
	+SA	2.53 ± 0.15a	1.71 ± 0.11cde	1.92 ± 0.11bcd	2.13 ± 0.14b
WUE (P_N/E)	- SA	5.21 ± 0.33b	5.53 ± 0.46ab	5.28 ± 0.29b	5.63 ± 0.59ab
	+SA	5.15 ± 0.55b	6.06 ± 0.35a	5.80 ± 0.52ab	5.59 ± 0.20ab
F_v/F_m	- SA	0.81 ± 0.00a	0.80 ± 0.00b	0.81 ± 0.00a	0.80 ± 0.00ab
	+SA	0.81 ± 0.01a	0.80 ± 0.01b	0.81 ± 0.01a	0.81 ± 0.00ab
F_v/F_0	- SA	4.39 ± 0.14a	3.97 ± 0.13c	4.23 ± 0.13ab	4.13 ± 0.13bc
	+SA	4.37 ± 0.24ab	3.94 ± 0.19c	4.25 ± 0.25ab	4.17 ± 0.08bc
SOD [U mg^{-1}(protein)]	- SA	194.40 ± 28.8a	90.09 ± 21.9c	138.65 ± 6.33b	89.93 ± 11.3c
	+SA	201.72 ± 6.86a	96.23 ± 9.56c	156.15 ± 8.63b	149.49 ± 13.8b
POD [U mg^{-1}(protein)]	- SA	38.54 ± 3.32d	49.27 ± 3.70b	45.50 ± 4.58cd	46.88 ± 3.76cd
	+SA	40.64 ± 2.40d	63.37 ± 2.99a	44.24 ± 3.85d	66.08 ± 4.17a
CAT [U mg^{-1}(protein)]	- SA	1.43 ± 0.42cd	1.44 ± 0.09cd	1.21 ± 0.04	1.37 ± 0.07d
	+SA	1.64 ± 0.06b	1.65 ± 0.09b	1.55 ± 0.08bc	1.95 ± 0.11a
MDA content [nmol g^{-1}(f.m.)]	- SA	18.68 ± 0.32c	25.34 ± 0.81a	22.01 ± 0.56b	24.92 ± 0.50a
	+SA	19.46 ± 0.57c	21.69 ± 1.33b	18.60 ± 0.30c	22.34 ± 0.66b

Cd treatment decreased F_v/F_m, whereas the UV-B and Cd+UV treatments showed no significant effects. The Cd and Cd+UV treatments decreased F_v/F_0 but the UV-B treatment did not affect F_v/F_0. The SA pre-treatment did not affect chlorophyll fluorescence parameters in the control as well as after the stress treatments.

Exposures to Cd, UV-B, and Cd+UV significantly decreased SOD activity compared to the control (Table 1). The SA pre-treatment significantly increased the SOD activity only in the Cd+UV-treated plants. POD activity increased under the Cd treatment but showed no significant change under UV-B and Cd+UV. The SA pre-treatment further increased the POD activity in the Cd and Cd+UV-treated plants. CAT activity significantly decreased under the UV-B treatment, but exhibited no significant change under the Cd and Cd+UV. However, the SA pre-treatment increased the CAT activities in the Cd, UV-B, and Cd+UV-stressed plants. The SA pre-treatment alone did not affect the SOD and POD activities but increased the CAT activity. The content of MDA markedly increased in the Cd, UV-B, and Cd+UV-treated plants in comparison to the control. The SA pre-treatment counteracted the stress-induced increase in MDA. There was no significant difference in MDA

between the SA pre-treatment and the control.

Exposure to Cd and/or UV-B resulted in decreased shoot and root dry masses of soybean plants. The damaging effect induced by Cd+UV was greater than that of the Cd or UV-B treatment alone. The growth inhibition was found to be associated with a stress-induced decrease in P_N. Earlier studies have demonstrated that SA can ameliorate the injurious effects of abiotic stresses on crops (Nazar *et al*. 2011, Bandurska and Cieślak 2013). In the present study, we indeed observed an ameliorative impact of the SA pre-treatment on the root dry mass of the soybean seedlings under the Cd and/or UV-B stresses. This could have been due to the fact that SA is known to reduce the accumulation and uptake of Cd (Belkhadi *et al*. 2010).

Cd and/or UV-B significantly decreased Chl content primarily because Cd or UV-B inhibits its synthesis and increases its degradation (Sharma and Dietz 2009). The reduction in the Chl content under Cd+UV was higher than under Cd or UV-B alone. Belkhadi *et al*. (2010) found that SA pre-soaking counteracted Chl destruction, and the foliar application of SA proved to be equally fruitful in increasing the pigment content (Hayat *et al*. 2005). Similarly, our results indicate that exogenous SA contributed to the increase in the Car content under the Cd and Cd+UV stresses. However, the mechanism of Car responses to SA may be different according to the concentration applied and species, *etc*. Flavonoids have effective radical scavenging capacities, and can contribute directly to enhancing protection against UV-B radiation (Karioti *et al*. 2008). In the present study, the flavonoid content significantly increased under the UV and Cd+UV treatments, whereas the SA pre-treatment counteracted this increase.

Our study revealed that the Cd and/or UV-B stress significantly reduced P_N with a concomitant decrease in the pigment content, g_s, and E. Several studies also found that a UV-B or Cd treatment decreased photosynthesis (Wang *et al*. 2008, Moussa and El-Gamal 2010). F_v/F_m and F_v/F_0 were also significantly decreased by Cd and the latter ratio also by Cd+UV. Similar results have been obtained in other plants (Ekmekçi *et al*. 2008). Therefore, it is possible to suppose negative effects of Cd and Cd+UV on the photochemical reactions in which electron transport is blocked (Tyystjärvi 2008). SA pre-treatment enhances P_N, E, g_s, and WUE in several plants (Fariduddin *et al*. 2003). Our results indicate that the SA pre-treatment contributed to the increase in P_N in the stressed plants, and this increase in P_N was attributed to the fact that SA induced the increase in g_s. In the present study, we did not observe any beneficial effect of the SA pre-treatment on photosystem efficiency.

Elevated MDA content is regarded as a sensitive indicator of oxidative stress in plants exposed to different stresses including Cd and UV-B (Wang *et al*. 2008). SA alleviates the Cd or UV-B-induced lipid peroxidation (Moussa and El-Gamal 2010, Bandurska and Cieślak 2013). Our results also demonstrate that the lipid peroxidation was boosted by Cd and/or UV-B, and that the SA pre-treatment decreased it which confirms the role of SA against oxidative damage.

Cd and/or UV-B influence antioxidative enzyme activities in dependence on species, exposure time, and dose (Prasad *et al*. 2005, Bočová *et al*. 2012). For example, Cd increases SOD and APX activities (Mobin and Khan 2007), whereas decreases CAT activity (Mishra *et al*. 2006). Prasad *et al*. (2005) observed that Cd+UV-B enhanced SOD and CAT activities. In the present study, Cd promoted the POD activity but decreased the SOD activity, whereas UV-B decreased the SOD and CAT activities. Only the SOD activity was decreased by Cd+UV. Several reports show that SA can induce an antioxidant activity under multiple stresses (Mutlu *et al*. 2009, Saruhan *et al*. 2012). A decline in activities of CAT, POX, and SOD was observed in plants treated with SA (Choudhury and Panda 2004). Our data show that exogenous SA resulted in an increase in the POD and CAT activities in the Cd-treated plants, and an increase in the CAT activity in the UV-treated plants. Additionally, we found that the SA pre-treatment promoted the activities of all three enzymes in the Cd+UV-treated plants.

The SA-induced alleviation of the negative effects of Cd and/or UV-B may have resulted from various factors as elucidated below. The SA pre-treatment alleviated Cd and/or UV-B damages in the seedlings through an increase of pigment and flavonoid content, and an improvement of photosynthetic capacity. Results from this study also show that SA enhanced the antioxidant enzymes in leaves subjected to the Cd and/or UV-B stresses, thus suppressing stress-induced oxidative damage and enhancing tolerance. Thus, the adverse effects of Cd and/or UV-B could be ammeliorated by foliar spray of SA.

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