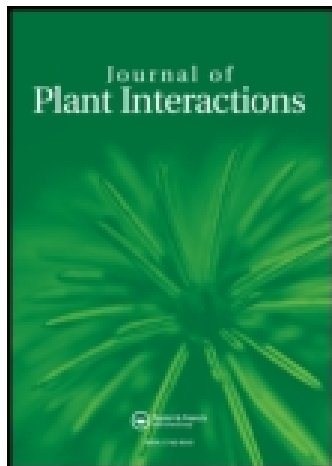


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## RESEARCH ARTICLE

### Salicylic acid increases tolerance to oxidative stress induced by hydrogen peroxide accumulation in leaves of cadmium-exposed flax (*Linum usitatissimum* L.)

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The aim of this study is to investigate the impacts of exogenous salicylic acid (SA) pretreatments on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) accumulation, protein oxidation, and H<sub>2</sub>O<sub>2</sub>-scavenging enzymes in leaves of Cd-treated flax seedlings. Cd-enhanced H<sub>2</sub>O<sub>2</sub> levels were related to increased activities of guaiacol peroxidase (POX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11), and were independent of changes in catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1) activities. In control flax seedlings, exogenous SA pretreatments inhibited the activity of CAT, resulted in an enhanced production of H<sub>2</sub>O<sub>2</sub> suggesting that SA requires H<sub>2</sub>O<sub>2</sub> to initiate an oxidative stress. However, although leaves of Cd-free flax seedlings pretreated with SA accumulated in vivo H<sub>2</sub>O<sub>2</sub> by 1.2-fold compared with leaves of Cd-only exposed ones; the damage to growth and proteins after the exposure to Cd was significantly less, indicating that SA can regulate the Cd-induced oxidative stress. Moreover, the Cd-treated seedlings primed with SA exhibited a higher level of total antioxidant capacities and increased activities of H<sub>2</sub>O<sub>2</sub>-detoxifying enzymes.

**Keywords:** cadmium; salicylic acid; hydrogen peroxide; antioxidant enzymes

#### 1. Introduction

Flax (*Linum usitatissimum* L.), one of the most important fiber crops in the world, is generally exposed to cadmium (Cd) pollution, notably affecting its growth performance. Cd is not a nutrient element for animals and plants with no biological function, but known to cause a range of negative effects on plants (Hayat et al. 2012; Xu et al. 2013; Zhang et al. 2013). At the leaf level, Cd stress causes the chlorosis, inhibits photosynthesis, and damages the structure and function of photosystem II (Zhang et al. 2013). Cd also changes hormonal status, disturbs mineral nutrition and water balance, and affects membrane structure and permeability (Xu et al. 2013). At the cellular level, Cd stress can cause the dislocation of nutrients resulting in deficiency effects in plant cell. Within the cell, Cd stress may generate oxidative stress (Belkadhî et al. 2013) and triggers indirectly the production of reactive oxygen species (ROS) including the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Mittler et al. 2004). At the same time, plants develop many defensive mechanisms in response to Cd stress for their survival. A first barrier against Cd stress is retention of Cd in the cell wall because they can somewhat restrict the Cd into cell (Douchiche et al. 2010). Since ROS can damage nucleic acids, proteins, lipids and amino acids, they must be scavenged by ROS detoxification mechanisms (Mittler et al. 2004; Djebali et al. 2008). These include the antioxidants (e.g. glutathione and

ascorbate) and enzymes, such as catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), and superoxide dismutase (SOD, EC 1.15.1.1). Thus, Cd tolerance is also associated with the activation of antioxidant defense system (Rodríguez-Serrano et al. 2009; Gallego et al. 2012).

To better characterize the enhanced response of plants to Cd-mediated oxidative stress, Gallego et al. (2012) established that exogenous salicylic acid (SA) application notably reduces the oxidative damage of Cd in plant cells. Recent studies demonstrated that it also participates in the signaling of abiotic stresses such as Cd stress (Hayat et al. 2012; Noriega et al. 2012). Exogenous SA was also found to enhance the efficiency of antioxidant defense system in plants and their tolerance to oxidative stress (Shi et al. 2009). Under Cd stress, several studies showed that SA-induced priming enhanced numerous antioxidant enzyme activities, thereby lowering the amounts of ROS inside cells (Shi et al. 2009; Zhang & Chen 2011).

Among ROS species, H<sub>2</sub>O<sub>2</sub> is also an important signaling molecule which is produced by the chloroplast and involved in the response of the plant to different types of environmental stressors (Pérez-Ruiz et al. 2006). But when it accumulates at a high level, it becomes toxic because it can be converted to highly reactive hydroxyl radicals. Although increasing evidence indicates that SA

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may cause oxidative stress to plants through the accumulation of  $H_2O_2$  (Chao et al. 2010), the preliminary treatment of plants with low concentrations of SA might have stress-induced adaptation effect, causing enhanced tolerance toward most kinds of abiotic stresses due primarily to enhanced antioxidative capacity (Janda et al. 2003).

In the present study, the relative relationships between SA pretreatments,  $H_2O_2$ , and  $H_2O_2$ -metabolizing enzymes have been studied in leaves of Cd-treated flax plantlets.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Flax seeds (cv. Viking), were soaked for 8 h in SA solutions (250 and 1000  $\mu M$ ) or water (0  $\mu M$  SA) as previously shown in Belkadi et al. (2010). After that they were germinated for four days at 25°C in the dark, uniform plantlets were transferred to a continuously aerated nutrient solution (pH 5.5) containing 1 mM  $MgSO_4$ , 2.5 mM Ca ( $NO_3$ )<sub>2</sub>, 1 mM  $KH_2PO_4$ , 2 mM  $KNO_3$ , 2 mM  $NH_4Cl$ , 50 mM EDTA-Fe-K, 30 mM  $H_3BO_3$ , 10 mM  $MnSO_4$ , 1 mM  $ZnSO_4$ , 1 mM  $CuSO_4$ , and 30 mM  $(NH_4)_6Mo_7O_{24}$ . The nutrient solution was buffered with HCl/KOH and changed twice per week. After growing for 2 days, plantlets were subjected during 10 days to 50 or 100  $\mu M$   $CdCl_2$ . Plantlets were grown in a growth chamber at a day/night cycle of 16 h/8 h, at 23°C/18°C, respectively, a relative humidity close to 75% and a light intensity of 200  $\mu mol$  photons  $m^{-2} s^{-1}$ .

### 2.2. Plant growth parameters

The shoot-to-root ratio is one measure to assess the overall growth of control and Cd-treated plants. Healthy shoot system is a key to healthy plant. Any changes from control shoot to root ratio would be an indication of a change in the overall health of plants. The shoot-to-root ratio can be calculated for each treatment as follows: Fresh weight for shoots/Fresh weight for roots = shoot/root ratio.

### 2.3. Chlorophyll and shoot water contents

The soil plant analysis development (SPAD) chlorophyll content in the leaf attached to their parent plant was measured by using Minolta chlorophyll meter (SPAD-502; Konica Minolta Sensing Inc, Japan) whereas the water content (WC) of stems and leaves was estimated accordingly: Fresh weight (FW) was recorded using an analytical balance, after which optical properties were measured and samples were dried at 60°C in an oven, until constant weight (dry weight, DW) was reached. WC was calculated as follows:  $FW - DW / FW$  ( $g g^{-1}$  FW).

### 2.4. $H_2O_2$ and carbonyl group contents

Root samples were homogenized in 0.2 N perchloric acid (pH 7.5). The flax  $H_2O_2$  content in roots was

determined as described by O'Kane et al. (1996). Protein-bound carbonyl content was determined by using the dinitrophenylhydrazine (DNPH) method, according to Reznick and Packer (1994).

### 2.5. Antioxidant enzyme assays

For enzyme assays, frozen leaf samples were ground to a fine powder with liquid nitrogen and extracted with ice-cold 50 mM phosphate buffer (pH 7.0). The extracts were centrifuged at 4°C for 30 min at 20,000  $\times g$  and the resulting supernatants; henceforth referred to as crude extracts, was collected and used for protein content assay and enzyme activities. Protein content was determined according to Bradford (1976) with bovine serum albumin as standard.

CAT activity was determined by following the consumption of  $H_2O_2$  (extinction coefficient 39.4  $mM^{-1} cm^{-1}$ ) at 240 nm for 30 s (Aebi 1984). POX activity was measured according to (Fielding & Hall 1978). APX activity was determined following the procedure described by (Nakano & Asada 1981). Total SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971).

### 2.6. 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and ferric-reducing antioxidant power (FRAP) assays

For antioxidant potential analysis, extracts were prepared following Ferreres et al. (2007). Antioxidant potential of the samples was determined spectrophotometrically by the FRAP assay developed by Benzie and Strain (1996) and by monitoring the disappearance of radical DPPH according to the method of Brand-Williams et al. (1995).

### 2.7. Statistical analysis

All presented data are the mean values of three independent experiments with five replicates. Statistical calculations were performed with SPSS-17 statistical software. Mean difference comparison among different treatments was done by analysis of variance and Tukey's (HSD) test at a 0.05 probability level.

## 3. Results

### 3.1. Growth, hydration, and SPAD values

Ten days after the addition of Cd to the nutrient medium, flax plantlets exhibited reduced shoot growth as indicated by decrease in shoot/root ratio (Figure 1a). Moreover, in comparison with the control, the SPAD chlorophyll values of the leaves showed approximately 24% drop in 100  $\mu M$  Cd- treated plants (Figure 1b). However, no alteration of stem and leaf water contents was detected (Figure 2).

In contrast, presoaking with SA for 8 h led to an increase in the shoot/root ratio in dose-dependent manner (Figure 1a). In the presence of Cd, 8-h SA pretreatment

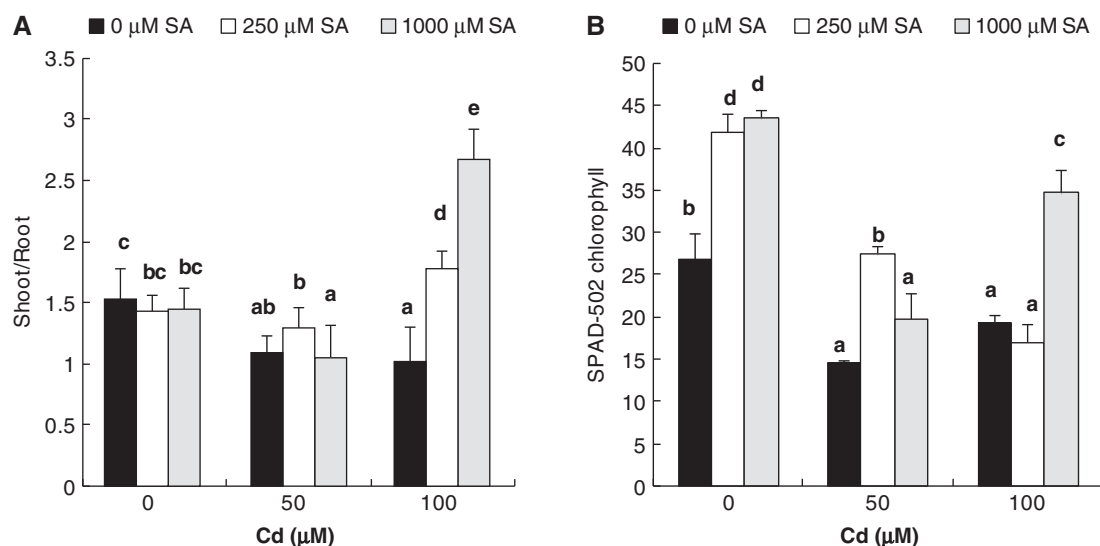


Figure 1. The effects of 8-h pretreatment with SA on shoot/root ratio (A) and SPAD chlorophyll value (B) in flax plantlets after 10 days of exposure to Cd stress. Values are the means of five replicates experiments  $\pm$  SE. Bars with different letters are statistically different at ( $P \leq 0.05$ ).

(250 or 1000  $\mu\text{M}$ ) significantly increased the chlorophyll contents and also neutralized the harmful effect of the metal, completely at its lower and higher levels (Figure 1b). As a result, already after treatments with Cd, the whole shoot system showed healthy appearance and was taller and more voluminous (result not shown). However, no further amelioration in shoot hydration has been noticed in 8-h SA pretreated plantlets (Figure 2).

### 3.2. Hydrogen peroxide, total soluble protein contents, and protein carbonylation

In leaves, Cd treatments showed a significant increase of  $\text{H}_2\text{O}_2$  content compared with control (Figure 3a). It increased by about 100% over control at 100  $\mu\text{M}$  Cd. However, upon the 8-h SA pretreatment (250  $\mu\text{M}$ ), there was a slight significant decrease in the  $\text{H}_2\text{O}_2$  content in flax leaves compared to those with Cd only (Figure 3a).

The Cd-associated damages to proteins were investigated by comparing total protein and carbonyl groups contents (Figure 3b, c). The accumulation of total soluble protein content was increased in comparison with control plantlets. In addition, 8-h SA pretreated flax plantlets exhibited a significant induction of leaf total protein content. Thus, at the highest metal concentration, the major result was at 1000  $\mu\text{M}$  SA; a nearly 103% increase was noticed compared to unpretreated plantlets (Figure 3b). In parallel, relative to the control, Cd-treated flax plantlets with 100  $\mu\text{M}$  exhibited a slightly higher rate of protein oxidation (Figure 3c). However, it was observed that 8-h SA pretreatments (250 or 1000  $\mu\text{M}$ ) exhibited a significant reduction of carbonyl groups content with or without Cd treatments (Figure 3c). At 100  $\mu\text{M}$  Cd, these main reductions were by about 30% and 50%, respectively, compared to unpretreated plantlets (Figure 3c).

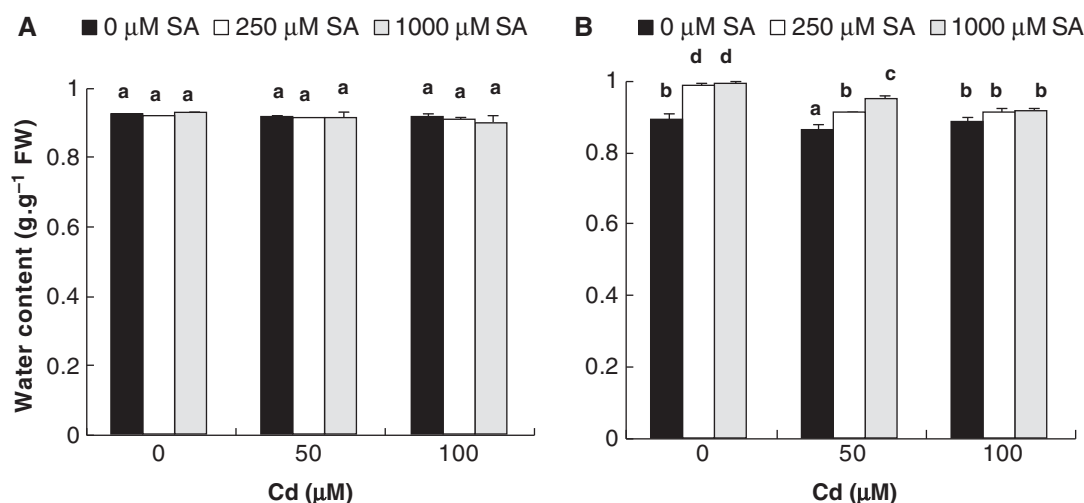


Figure 2. The effects of 8-h pretreatment with SA on WC in stems (A) and leaves (B) of flax plantlets after 10 days of exposure to Cd stress. Values are the means of five replicates experiments  $\pm$  SE. Bars with different letters are statistically different at ( $P \leq 0.05$ ).

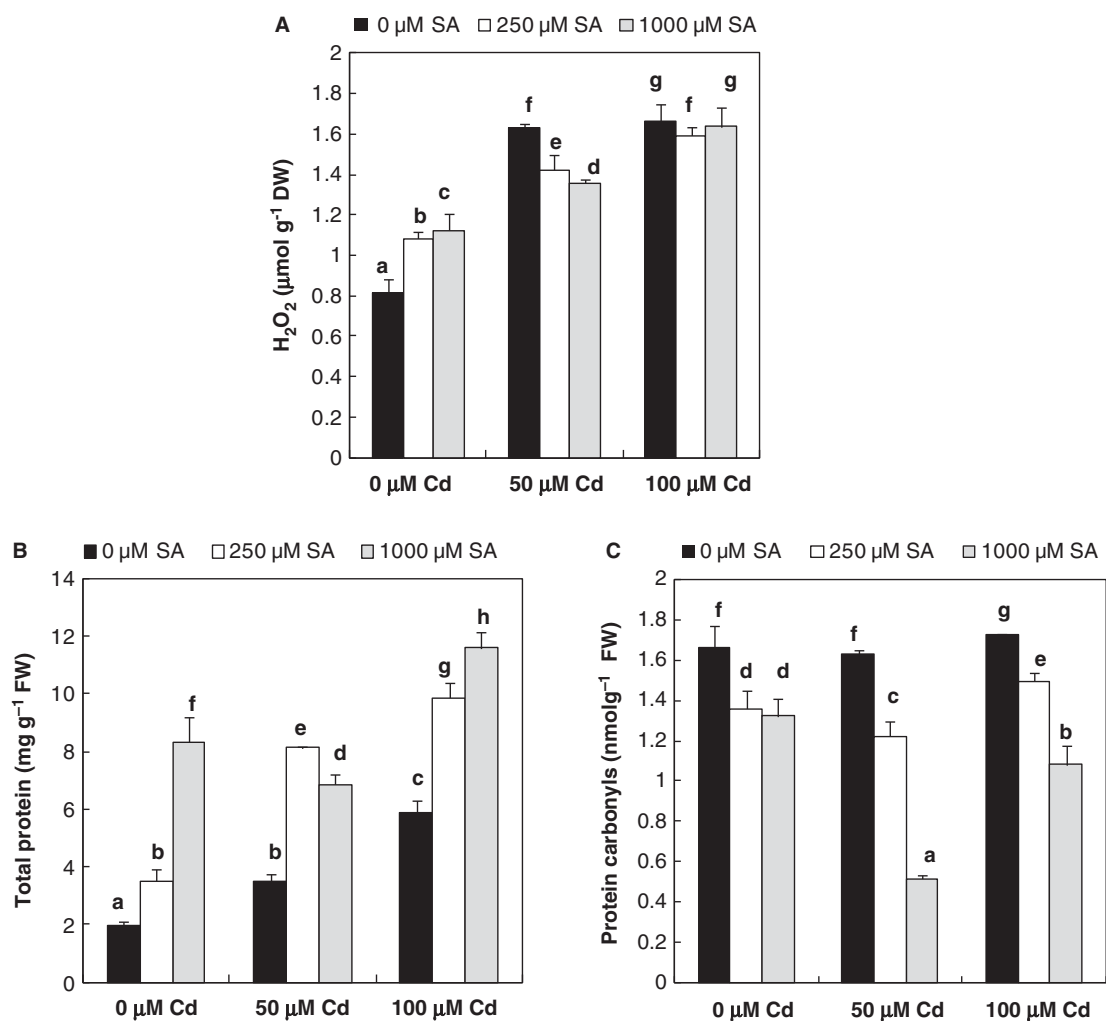


Figure 3. The effects of 8-h pretreatment with SA on hydrogen peroxide content (A), total protein (B), and protein carbonyl (C) contents in leaves of flax plantlets after 10 days of Cd stress. Values are the means of five replicates experiments  $\pm$  SE. Bars with different letters are statistically different at ( $P \leq 0.05$ ).

### 3.3. Catalase, guaiacol peroxidase, ascorbate peroxidase, and superoxide dismutase activities

The effect of Cd on the activities of antioxidative enzymes (Figure 4) was significant in most Cd-contaminated leaves. CAT activity was significantly decreased in Cd-treated flax plantlets, and the effect was similar with the application of 8-h SA pretreatment (250 or 1000  $\mu$ M; Figure 4a). At 50 and 100  $\mu$ M Cd, leaf POX and APX showed a significantly higher activities level than control, particularly at the highest concentration of the contaminant. Significant enhanced response in both enzymes activities was occurred by 8-h SA pretreatment followed by Cd exposure (Figure 4b, c). In contrast, SOD activity was declined by Cd stress (Figure 4d). However, SA (250 or 1000  $\mu$ M) fully suppressed the Cd response of leaf SOD activity (Figure 4d).

### 3.4. FRAP and DPPH-radical scavenging capacities

Under stress conditions, the antioxidant capacities, evaluated by monitoring FRAP and DPPH-radical scavenging activity, may not be sufficient to reduce the injurious effect of oxidative damages to flax leaves

(Figure 5a, b). However, upon both 8-h SA pretreatments increases in the total antioxidant capacities were noticed in contaminated flax leaves, with respect to the untreated plantlets (Figure 5a, b). These SA-induced increases were in a dose-dependent manner. At 100  $\mu$ M Cd, the maximum values have been registered at 1000  $\mu$ M SA for both assays (Figure 5a, b).

## 4. Discussion

Cd is considered as one of the most toxic factors affecting plant growth performance. Thus, the responses of growth, under Cd stress, become the most important phenomena of plant physiology. In this work, we investigate the potential relationship between SA and H<sub>2</sub>O<sub>2</sub> levels and growth performance and carbonyl groups. We also suggest that SA is able of generating H<sub>2</sub>O<sub>2</sub> and imposed oxidative damage to growth and proteins.

Flax roots grown for 10 days with 50 and 100  $\mu$ M CdCl<sub>2</sub> adversely affected the shoot morphological aspects and shoot/root ratio is decreased (Figure 1a). These observations were accompanied by normally



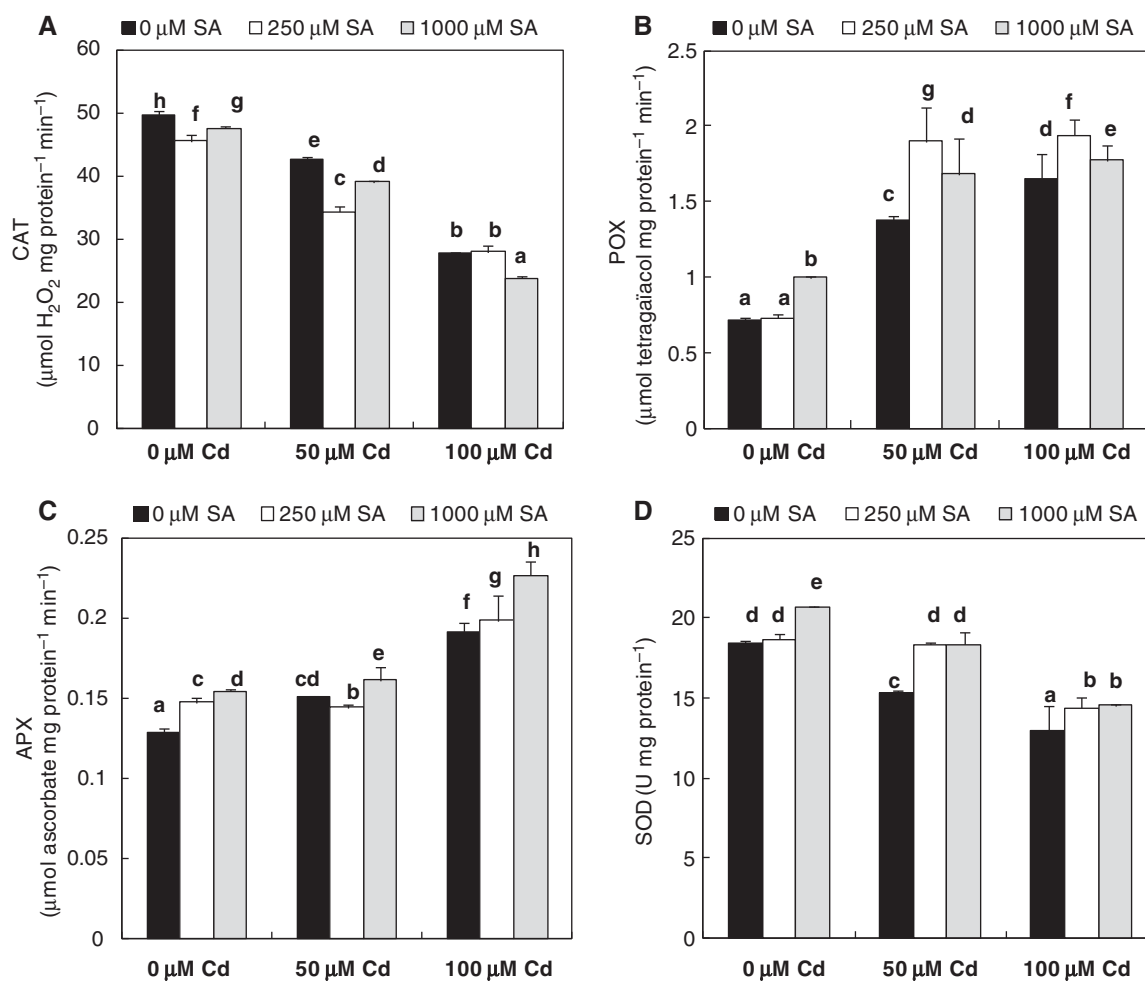


Figure 4. The effects of 8-h pretreatment with SA on the activities of catalase (A) guaiacol peroxidase (B), ascorbate peroxidase (C), and superoxide dismutase (D) in leaves of flax plantlets after 10 days of cadmium (Cd) stress. Values are the means of five replicates experiments  $\pm$  SE. Bars with different letters are statistically different at ( $P \leq 0.05$ ).

hydrated tissues (Figure 2a, b). Both processes could be a consequence of an important adaptive response of Cd-treated plantlets due to the fast inhibition of shoot

growth (which limits plant biomass production) while root growth is better preserved (Figure 1a). It was reported that under Cd stress, growth processes may be

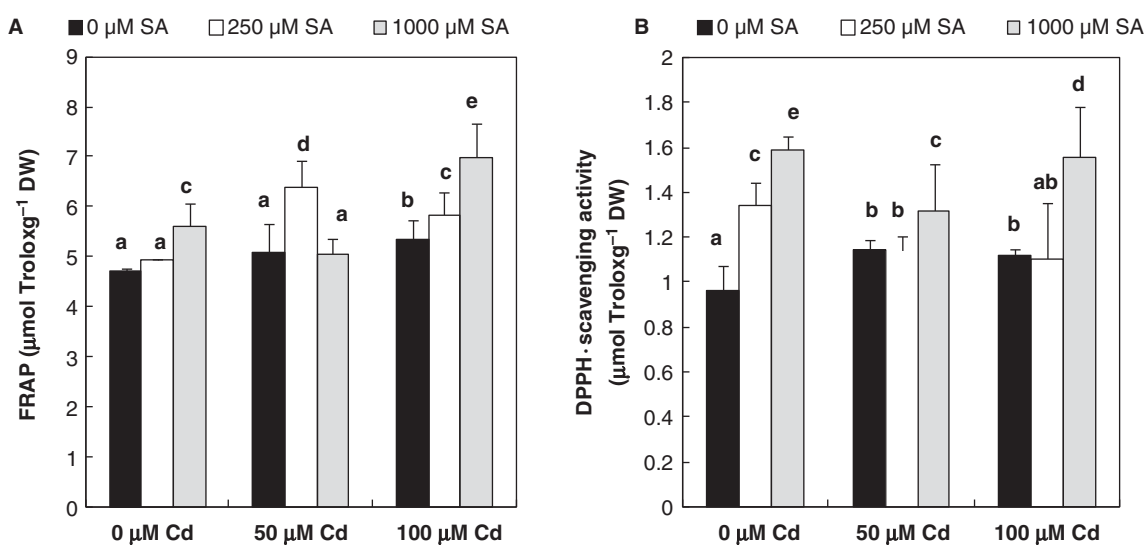


Figure 5. The effects of 8-h pretreatment with SA on FRAP (A) and DPPH-radical scavenging activity (B) in leaves of flax plantlets after 10 days of Cd stress. Values are the means of five replicates experiments  $\pm$  SE. Bars with different letters are statistically different at ( $P \leq 0.05$ ).

regulated by exogenous plant hormone concentrations (Hassan et al. 2008). While low concentrations of salicylic acid (Belkadhi et al. 2013), cytokinin (Prasad 1995), gibberellin (Ghorbanli et al. 2000), auxin (Hu et al. 2013), and brassinosteroid (Hayat et al. 2007) accelerate growth of Cd-treated plants, abscisic acid (Hsu & Kao 2004), and jasmonic acid retard it (Maksymiec 2007). Moreover, in this experiment, the reductions in shoot/root ratio were alleviated by applying SA to Cd-treated flax plantlets (Figure 1a). This may be linked to the SA-increased levels of chlorophylls under Cd stress (Figure 1b).

Chlorophylls represent the central part of the entire metabolism of the green plant system. Therefore, any significant change in their level is likely the reason of large marked effects manifested primarily on the growth. In fact, it is well known that increase of chlorophyll occurs during growth. The results showed that Cd decreased the SPAD chlorophyll values (Figure 1b) which agree with previous reports by Ishimaru et al. (2012) for rice and Hasan et al. (2013) for tomato. SA-increased chlorophyll concentration led to active photosynthetic activity causing growth. Furthermore, Li et al. (1992) established that SA inhibited the activity of ACC synthase enzyme, preventing the formation of ethylene and chlorophyll alteration. Besides, under Cd stress, an increase in cytokinin content in 8-h SA-pretreated plantlets might also be responsible for their growth response. In fact, cytokinins have been shown to influence the activity of expansins (proteins involved in the control of cell expansion; Downes & Crowell 1998). Consequently stimulation of leaf expansion of flax plantlets pretreated with SA might be due to a SA-induced cytokinin synthesis. This assumption is supported by the fact that in this study the inhibitory effect of Cd on leaf growth was lower in plants which were pretreated with SA. Accordingly, Gadallah and El-Enany (1999) reported a decline in the growth inhibitory effect of Cd in plants treated with exogenous cytokinin.

Since Cd-treatment is known to produce oxidative stress, increased oxidation of proteins might be due to this effect (Figure 3b). This is in agreement with previous studies by Djebali et al. (2008). Since SA is a signaling molecule, it is probable that it reprogrammed the gene expression (Foyer & Noctor 2005), leading to *de novo* protein synthesis (Zhang et al. 1998) and a membrane repair mechanism (Belkadhi et al. 2013). The results of *in vivo* measurement of the effect of SA on the levels of protein and carbonyl groups (Figure 3b, c) suggest a more direct effect of this hormone on antioxidative enzyme activities (Rodríguez-Serrano et al. 2009). In accordance with this, we found that 8-h SA pretreatment (250 or 1000  $\mu\text{M}$ ) enhanced the total antioxidant capacities in contaminated leaves (Figure 5a, b). The ability of SA to influence the activity of different antioxidant enzymes is known from the literature. Besides, the increased FRAP and DPPH-radical scavenging activity by injured leaves is also not excluded as contributing to the increase observed in the total protein contents of Cd-treated flax plantlets.

Other process implicated in the prevention of Cd-induced protein damage involves the regulation of the synthesis of antioxidant enzymes. In this study, Cd affected leaf SOD and CAT activities in flax plantlets, with their impact being higher at 100  $\mu\text{M}$  Cd (Figure 4a, d) but, tended to increase APX and POX activities, especially at this concentration (Figure 4b, c) which is similar to the findings of Mishra et al. (2006) in *Bacopa monnieri*. However, our results showed that the 8-h SA pretreatment caused a significant increase in POX, APX, and SOD in Cd-treated plantlets (Figure 4b–d). Similarly, SA is believed to inhibit CAT by the chelation of heme Fe and by causing conformational changes (Ruffer et al. 1995). The enhanced activities of these antioxidative enzymes under Cd stress, seems to be the result of *de novo* synthesis and/or activation of the enzymes, mediated through the transcription-specific genes (Foyer & Noctor 2005). Similarly, different plant species grown under Cd stress and pretreated with SA possessed higher antioxidative enzymatic activity, for instance rice (Panda & Patra 2007) and maize (Krantev et al. 2008).

It was proposed that high levels of endogenous SA can bind and inhibit  $\text{H}_2\text{O}_2$ -removing enzymes, such as CAT and APX (Durner & Klessig 1996). In addition, APX could post-transcriptionally suppressed by SA (Yuan & Lin 2004). In this study, under stress conditions, the observed stimulation of APX, POX, and SOD in leaves pretreated with low concentrations of SA (250 or 1000  $\mu\text{M}$ ) was coupled with a decrease in intracellular  $\text{H}_2\text{O}_2$  (Figure 3a). Thus, the stimulation of these three major  $\text{H}_2\text{O}_2$ -degrading enzymes could cause further inactivation by dose-dependent manner of  $\text{H}_2\text{O}_2$  accumulation. This suggests that SA-mediated activation of  $\text{H}_2\text{O}_2$ -degrading enzymes may further reduce  $\text{H}_2\text{O}_2$  levels. It was found that increased  $\text{H}_2\text{O}_2$  levels also act upstream of exogenous SA to induce endogenous SA (Leon et al. 1995) which leads to SA and  $\text{H}_2\text{O}_2$  to compose a positive response to stress conditions. Thus, SA-pretreated plants may produce less ROS and at the same time enhance their own capacity to scavenge  $\text{H}_2\text{O}_2$ , which result in the less accumulation of ROS under Cd stress (Zhang & Chen 2011). Moreover, SA-induced redox regulation was found to be accompanied by accumulations of phytohormones like ethylene, nitric oxide, and jasmonate (Dat et al. 2003).

## 5. Conclusion

It is concluded that SA priming-induced improvements in Cd-treated flax plantlets growth were associated with chlorophyll synthesis, protein repair mechanisms and greater antioxidant capacities. In injured leaf cells, SA and  $\text{H}_2\text{O}_2$  may probably use a common signaling mechanism to improve flax tolerance to Cd-induced oxidative stress. Our results suggest that these strategies can be of great help in the production of flax seed capable of growing vigorously under polluted conditions.

## Acknowledgments

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