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Treatment with salicylic acid decreases the effect of cadmium on photosynthesis in maize plants

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Summary

The present study investigated the possible mediatory role of salicylic acid (SA) in protecting photosynthesis from cadmium (Cd) toxicity. Seeds of maize (*Zea mays* L., hybrid Norma) were sterilized and divided into two groups. Half of the seeds were presoaked in 500 μM SA solution for only 6 h, after which both groups were allowed to germinate for 3 d and were then grown for 14 d in Hoagland solution at 22/18 °C in a 16/8-h light/dark period and 120 μmol m^{-2} s^{-1} PAR. All seedlings (without H_2O and SA controls) were transferred to Cd-containing solutions (10, 15, and 25 μM) and grown for 14 d. The rate of CO_2 fixation and the activity of ribulose 1,5-bisphosphate carboxylase (RuBPC, EC 4.1.1.39) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) were measured. Changes in the levels of several important parameters associated with oxidative stress, namely H_2O_2 and proline production, lipid peroxidation, electrolyte leakage, and the activities of antioxidative enzymes (superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), and guaiacol peroxidase (POD, EC 1.11.1.7)) were measured. Exposure of the plants to Cd caused a gradual decrease in the shoot and root dry weight accumulation, with the effect being most pronounced at 25 μM Cd. Seed pretreatment with SA alleviated the negative effect of Cd on plant growth parameters. The same tendency was observed for the chlorophyll level. The rate of CO_2 fixation was lower in Cd-treated plants, and the inhibition was partially overcome in SA-pretreated plants. A drop in the activities of RuBPC and PEPC was observed for Cd-treated plants. Pretreatment with SA

Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; CAT, catalase; DTT, dithiothreitol; MDA, malondialdehyde; NBT, nitroblue tetrazolium; PEP, phosphoenol pyruvate; PEPC, phosphoenol pyruvate carboxylase; POD, guaiacol peroxidase; PS2, photosystem 2; RuBP, ribulose 1, 5-bisphosphate; RuBPC, ribulose 1, 5-bisphosphate carboxylase; RWC, relative water content; SA, salicylic acid; SOD, superoxide dismutase; TCA, trichloroacetic acid

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alleviated the inhibitory effect of Cd on enzyme activity. Proline production and the rates of lipid peroxidation and electrolyte leakage increased in Cd-treated plants, whereas the values of these parameters were much lower in SA-pretreated plants. Treatment of plants with Cd decreased APX activity, but more than doubled SOD activity. Pretreatment with SA caused an increase in both APX and SOD activity, but caused a strong reduction in CAT activity. The data suggest that SA may protect cells against oxidative damage and photosynthesis against Cd toxicity.

Introduction

Cadmium (Cd) is a highly toxic trace element that enters the environment mainly from industrial processes and phosphate fertilizers. It can reach high levels in agricultural soils and is easily assimilated by plants. When taken up in excess by plants, it induces various visible symptoms of phytotoxicity, e.g. leaf roll, chlorosis, growth reduction in root and shoot, browning of root tips, and eventually death (Kahle, 1993). A number of toxic effects of Cd on metabolism have been reported, such as decreased uptake of nutrients (Sandalio et al., 2001), changes in nitrogen metabolism (Boussama et al., 1999), interaction with the water balance of the plant, and inhibition of stomatal opening (Barcelo and Poschenrieder, 1990). Cd^{2+} ions are known to cause alterations in the functionality of membranes by affecting the lipid composition (Quariti et al., 1997) and certain enzymatic activities associated with membranes, such as H^+-ATPase (Fodor et al., 1995). Net photosynthesis is also sensitive to Cd because it directly affects chlorophyll biosynthesis (Stobort et al., 1985; Padmaja et al., 1990; Gadallah, 1995) and the proper development of the chloroplast ultrastructure (Stoyanova and Merakchiiska-Nikolova, 1992; Stoyanova and Tchakalova, 1997). Early studies indicated that Cd ions affect the oxidizing side of photosystem 2 (PS2) and lead to the uncoupling of electron transport in the chloroplasts (Mohanty and Mohanty, 1988; Atal et al., 1993). With regard to the site and mechanism of inhibition, it is generally accepted that the water-oxidizing system of PS2 is affected by Cd by replacing the Mn^{2+} ions, thereby inhibiting the reaction of PS2 (Baszynski et al., 1980). The negative effects of Cd can also be observed in the carboxylating phase of photosynthesis. The main targets of the influence of Cd are two key enzymes of CO_2 fixation, ribulose 1,5-bisphosphate carboxylase (RuBPC) and phosphoenol pyruvate carboxylase (PEPC). It has been shown that Cd^{2+} ions lower the activity of RuBPC and damage its structure by substituting for Mg^{2+} ions, which are important cofactors of carboxylation reactions, and may also shift RuBPC activity towards oxygenation reactions (Siedlecka et al., 1998). Stiborova (1988) and Malik et al. (1992) demonstrated that Cd caused an irreversible dissociation of the large and small subunits of RuBPC, thus leading to total inhibition of the enzyme.

Evidence has been reported suggesting that Cd toxicity takes the form of oxidative stress, caused by the stimulation of free oxygen radical production (Sanita di Toppi and Gabrielli, 1999) and by the modified activity of various antioxidant enzymes (Hegedűs et al., 2001). Under severe stress conditions, however, the antioxidant capacity may not be sufficient to minimize the harmful effect of oxidative injury. Survival under stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals, and induce biochemical changes that adjust the metabolism accordingly. Therefore, the search for signal molecules that mediate the stress tolerance is an important step towards a better understanding of how plants adjust to an adverse environment.

Salicylic acid (SA) is a potent signaling molecule in plants and is involved in eliciting specific responses to biotic and abiotic stresses. It has been shown that SA provides protection against low-temperature stress in maize (Janda et al., 1999) and winter wheat plants (Tasgin et al., 2003), induces thermotolerence in mustard seedlings (Chen et al., 1997; Dat et al., 1998a, b), and modifies plant responses to salt and osmotic stresses (Borsani et al., 2001), ozone or UV light (Sharma et al., 1996), drought (Senaratna et al., 2000), herbicides (Ananieva et al., 2004), and pathogens (Malamy et al., 1990; Durner et al., 1997). Further, SA is also known to be involved in plant protection against heavy metals. SA pretreatment alleviated Pb- and Hg-induced membrane damage in rice (Mishra and Chudhuri, 1999) and Cd toxicity in barley (Metwally et al., 2003) and maize plants (Pál et al., 2002). SA has been shown to accumulate in plants in response to various oxidizing stresses, such as H_2O_2 (Leon et al., 1995), ozone (Sharma et al., 1996), and heat (Dat et al.,

1998a), and it has been suggested that it is directly involved in signaling various antioxidant responses (Larkindale and Knight, 2002).

This study was undertaken to determine the physiological and biochemical changes in maize plants treated with SA during Cd-induced stress, to investigate whether this plant regulator is involved in the induction of defense responses, and to test the hypothesis that the ability of SA to protect photosynthesis against Cd stress is mediated by its effect on the antioxidant defense system.

Materials and methods

Plant growth and treatment with Cd

Seeds of maize (*Zea mays* L., hybrid Norma) obtained from the Agricultural Research Institute, Martonvásár, Hungary, were sterilized and divided into two groups. One half of the seeds were soaked in 500 μM SA solution for 6 h, while the other half were soaked in water (control). Both groups were then allowed to germinate on moist filter paper in the dark. Three-day-old, dark-grown seedlings were placed in polyethylene pots filled with 0.6 L modified Hoagland solution (0.3125 mM KNO_3, 0.45 mM $Ca(NO_3)_2$, 0.0625 mM KH_2PO_4, 0.125 mM $MgSO_4 \cdot 7H_2O$, 11.92 μM HBO_3, 4.57 μM $MnCl_2 \cdot 4H_2O$, 0.191 μM $ZnSO_4 \cdot 7H_2O$, 0.08 μM $CuSO_4 \cdot 5H_2O$, 0.024 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 15.02 μM $FeSO_4 \cdot 7H_2O$, and 23.04 μM $Na_2EDTA \cdot 5H_2O$). The nutrient solution was aerated twice a day, and changed three times a week. $CdCl_2$ was added at concentrations of 10, 15, and 25 μM. The plants were grown in a growth chamber with a day/night cycle of 16/8 h, at 22/18 °C, respectively, relative humidity between 50% and 60%, and 120 $\mu mol\, m^{-2}\, s^{-1}$ PAR. After 14 d of growth, i.e. 3 d after soaking, the plants were harvested for analysis.

$^{14}CO_2$ fixation

Photosynthetic rates were measured using leaf slices as described by Popova et al. (1987). Briefly, 1 g of leaf blade tissue was cut perpendicular to the veins into 1-mm slices, which were incubated in 5 mL buffer in a 25 mL Erlenmeyer flask at 25 °C for 5 min at 120 $W\, m^{-2}$ irradiance. The buffer contained 0.33 M sorbitol, 0.05 M HEPES-NaOH, 0.002 M KNO_3, 0.002 M EDTA, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, 0.0005 M K_2HPO_4, 0.02 M NaCl, and 0.2 M Na-isoascorbate, pH 7.6. At the end of the preincubation period, 20 mM $NaHCO_3$ containing 40 μCi $NaH^{14}CO_3$ (14.3 $\mu Ci\, \mu M^{-1}$) was added to each sample. After allowing the plants to fix $^{14}CO_2$ for 10 min, the reaction was stopped by adding boiling 80% ethanol. The tissues were subsequently extracted eight times with boiling ethanol of the same concentration. The combined extracts were brought to dryness *in vacuo* at 40 °C and were dissolved in 10 mL distilled water. An aliquot was measured into 5 mL of scintillation fluid for radioactivity assay using a Packard Tri-Carb liquid scintillation counter.

Enzyme extraction and assays

Leaf tissue without the major veins was ground in a mortar on ice at a ratio of 1 g fresh mass to 5 mL cold extraction medium containing 0.33 M sorbitol, 0.05 M HEPES-NaOH, 2 mM KNO_3, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM K_2HPO_4, 0.02 M NaCl, and 0.2 M Na-isoascorbate, pH 7.6. The homogenate was quickly filtered through four layers of cheesecloth and centrifuged at 20,000*g* for 15 min, and the supernatant was used directly for enzyme assay.

RuBPC and PEPC activities were assayed from the activated crude preparation by following the incorporation of $NaH^{14}CO_3$ into acid stable products (Popova et al., 1988). The assay mixture for RuBPC contained, in 50 mM HEPES-NaOH (pH 8.0), the following: 20 μmol $MgCl_2$, 1 μmol dithiothreitol (DTT), 20 μmol $NaHCO_3$ (containing 1.48 MBq, specific radioactivity 0.38 $MBq\, \mu mol^{-1}$), and the enzyme extract equivalent to 0.3–0.4 mg protein. Reactions were initiated at 25 ± 1 °C, by the addition of 2 μmol RuBP, and stopped after 1 min reaction time with 6 M HCl. The assay mixture for PEPC activity contained 20 μmol $MgCl_2$, 0.4 μmol NADH, 20 μmol $NaHCO_3$ (containing 1.48 MBq, specific radioactivity 0.38 $MBq\, \mu mol^{-1}$), 1 μmol DTT, and enzyme extract equivalent to 0.3–0.4 mg protein in 50 mM HEPES-NaOH (pH 8.0). The reaction volume was 1 mL. Reactions were initiated at 30 ± 1 °C by addition of 3 μmol PEP. The reaction time was 1 min. The amount of fixed $^{14}CO_2$ was measured in a liquid scintillation spectrometer.

Determination of stress markers

The levels of some non-specific stress markers were measured. Fresh material (about 0.150 g) was homogenized in 0.1% cold trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000*g* for 25 min. The supernatant obtained was used for the determination of both hydrogen peroxide and lipid peroxidation levels.

Endogenous hydrogen peroxide was measured spectrophotometrically ($\lambda = 390$ nm) by reaction with 1 M KI. The results were calculated using a standard curve prepared with fresh hydrogen peroxide solutions (Jessup et al., 1994).

The level of lipid peroxidation was measured by the method of Heath and Packer (1968) with slight modifications. Leaf material weighing 0.2–0.3 g was homogenized in 3 mL 0.1% TCA and centrifuged at 15,000*g* for 30 min at 4 °C, after which 0.5 mL buffer and 1 mL reagent (0.5% thiobarbituric acid (TBA) in 20% TCA, w/v) were added to a 0.5 mL aliquot of the supernatant. As a blank, 0.5 mL 0.1% TCA+0.5 mL buffer and 1 mL reagent were used. The test tubes were heated at 95 °C for 30 min and then quickly cooled in an ice bath. After cooling and centrifugation to give a clear supernatant, the absorbance of the supernatant at 532 nm was read and the value for the non-specific absorption at 600 nm was subtracted. The level of malondialdehyde (MDA) was estimated using an extinction coefficient of 155 $mM^{-1}\, cm^{-1}$.

The degree of membrane integrity was also assessed by the leakage of electrolytes from the whole above-ground part of three plants with approximately similar sizes. The leaf segments were washed, blotted dry, weighed, and put in stopper vials filled with the exact volume of bidistilled, deionized water. The vials were then incubated for 20 h in the dark with continuous shaking. The amount of electrolyte leakage was measured conductometrically.

Proline concentration was determined spectrophotometrically at 520 nm after Bates et al. (1973).

Antioxidative enzyme assay

Superoxide dismutase (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), taking into account the comments of Beyer and Fridovich (1987). Leaf samples were homogenized in 4 volumes (w/v) of an ice-cold buffer containing 0.1 M Tris–HCl (pH 7.8), 0.1 mM EDTA, and 0.05% Triton X-100. The homogenates were filtered through four layers of cheesecloth and centrifuged at 4 °C for 30 min at 15,000*g*. The crude extracts were dialyzed for 24 h against a half-strength extraction buffer without Triton X-100, centrifuged for 20 min at 15,000*g*, and the supernatants were used for SOD assay. The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.053 mM NBT, 10 mM methionine, 0.0053 mM riboflavin, and an appropriate aliquot of enzyme extract. The reaction was started by switching on the light and was allowed to run for 7 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition in the reduction of NBT as monitored at 560 nm.

Ascorbate peroxidase (APX) activity was determined in the soluble fraction and in the chloroplast membrane fraction in 2 mL reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate (extinction coefficient 2.8 mM cm^{-1}), 0.1 mM H_2O_2, and leaf extract causing a linear decrease in absorbance at 290 nm for 20 s (Nakano and Asada, 1981).

The crude extract for guaiacol peroxidase (POD) measurements was isolated according to Polle et al. (1994). Maize leaves (0.2 g) were homogenized with 3 mL 100 mM K_2HPO_4 (pH 7.0) containing 1 mM EDTA and 1% (w/v) polyvinyl pyrrolidone (PVP-40) at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 20 min. The activity was measured by following the change in absorption at 470 nm due to guaiacol oxidation in a reaction solution (3 mL final volume) composed of 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM H_2O_2, and 50 μL of crude extract.

Catalase (CAT) activity was determined by following the consumption of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm for 30 s (Aeby, 1984). The assay mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2, and 50 μl leaf extract in a 3 mL volume.

Soluble protein content was determined by the method of Bradford (1976), while chlorophyll was extracted by acetone and measured spectrophotometrically according to Arnon (1949).

Determination of Cd content

Around 1 g of dry root and leaf material was wet digested in a H_2SO_4:HNO_3 mixture (1:5 v/v) for 24 h, after which it was treated with HNO_3:$HClO_4$ mixture (5:1 v/v). The Cd concentration in the digest was measured using an atomic absorption spectrophotometer (Perkin-Elmer, Germany).

Salicylic acid extraction and analytical procedure

The SA content was measured according to Meuwly and Métraux (1993). Leaf tissue (1.5 g) was ground in liquid nitrogen with a mortar and pestle, in the presence of 1 g quartz sand. The tissue powder was transferred to a centrifugation tube and mixed with 2 mL of 70% methanol containing 250 ng *ortho*-anisic acid (oANI) (used as internal standard) and 25 μg *para*-hydroxy-benzoic acid (pHBA) (used as extraction carrier). The extract was centrifuged at 10,000*g* for 20 min. The pellet was resuspended in 2 mL 90% methanol, vortexed, and centrifuged as above.

The methanol content was evaporated from 2 mL of the mixed supernatants at room temperature under vacuum. A volume of 1 mL of 5% (w/v) TCA was added to the residual aqueous phase and the mixture was centrifuged at 15,000*g* for 10 min. The supernatant was gently partitioned twice against 3 mL of a 1:1 (v/v) mixture of ethyl acetate/cyclohexane. The upper organic layers contained the free phenolic portion. The aqueous phases containing the methanol-soluble bound phenolics were acid hydrolyzed. In all, 250 ng oANI, 25 μg pHBA, and 1.3 mL 8 N HCl were added to the aqueous phase and incubated for 60 min at 80 °C before partitioning twice as above. Just prior to the HPLC analysis, the organic phases were evaporated to dryness under vacuum and resuspended in 1 mL of the HPLC initial mobile phase.

SA was quantified fluorimetrically (W474 scanning fluorescence detector, Waters, USA), with excitation at 305 nm and emission at 407 nm.

Statistics

Student's *t*-tests were applied to determine the significance of the results between different treatments.

Results

Effect of SA on growth response to Cd toxicity

When Cd was present in the nutrient solution, maize plants exhibited reduced root and shoot growth. Shoot fresh weight accumulation decreased proportionally with increasing Cd concentration and 25 μM Cd caused 76% reduction in the values of this

parameter. For root dry weight, the reduction was 18%. Shoot and root length were also reduced in Cd-treated variants (Table 1). Maize plants treated with Cd concentrations of 25 μM or higher showed leaf and root dissolution and chlorosis, and their survival rate deteriorated greatly. These symptoms were less pronounced in Cd-treated plants pretreated with SA for 6 h.

The levels of chlorophyll also indicated the toxic nature of Cd in the plant system. The chlorophyll content showed a reduction of approximately 20% and 50% in treated plants, 15 and 25 μM Cd, respectively. Pretreatment with SA before exposure to Cd, however, led to 17% and 41% restoration of the chlorophyll levels (Table 1).

No significant changes were observed in the level of leaf-soluble protein, with the exception of variants treated with 25 μM Cd, where a decrease of 10% was measured. SA pretreatment had a non-significant effect on the protein level after Cd exposure (Table 1).

Cd treatment did not induce a water deficit, as RWC was only slightly affected. In the control plants, RWC was about 94–95%. The reduction in this parameter was only approximately 5% for the treated plants (data not shown).

Effect of cadmium on the free and bound salicylic acid contents in leaves and on root cadmium accumulation

In the present work, it was found that control leaves initially contained little SA in either free or bound form. Treatment with 10 and 15 μM Cd increased the level of free SA by 26% and 50%, respectively. Increase in the bound SA was more pronounced, and 25 μM Cd caused a more than 10-fold increase compared with the control. The total content of free and bound SA was significantly higher in all Cd-treated variants (Table 2).

The Cd content of dry seeds was very low 0.237 ng (kg DW)$^{-1}$, while that in the root tissue of control plants (no Cd in the growth medium) was 27.2 ng (kg DW)$^{-1}$, increasing by 13 and 18 times in samples from plants treated with 10 and 25 μM Cd, respectively. SA pretreatment reduced the Cd accumulation in the roots of plants treated with 15 and 25 μM Cd (Table 2).

Effect of SA and Cd on CO_2 fixation, PEPC, and RuBPC activities

The inhibition of maize plants' growth was accompanied by a decrease in the rate of photosynthetic CO_2 fixation. Data for this parameter are given both for fresh weight and for chlorophyll content, because Cd treatment caused a reduction in the level of chlorophyll. When the seeds were presoaked in 500 μM SA for 6 h, the rate of CO_2 fixation fully recovered (Fig. 1A and B).

Table 2. Effect of Cd and SA on the accumulation of free and bound SA in leaves of maize and on root Cd accumulation

Treatment	Free SA ng (g FW)$^{-1}$	Bound SA ng (g FW)$^{-1}$	Cd content in roots ng (g FW)$^{-1}$
Control	272.2	217.0	27.23
10 μM Cd	343.7	325.3	368.05
15 μM Cd	387.3	399.5	417.76
25 μM Cd	217.5	2778.7	497.33
SA (500 μM)	311.3	381.0	35.79
10 μM Cd+SA	278.7	2581.7	428.74
15 μM Cd+SA	248.5	1090.0	375.29
25 μM Cd+SA	163.0	1601.5	427.6

Dry seed Cd content was measured to be 0.237 ng (kg DW)$^{-1}$. Details of treatment and growth are as in Table 1. Each value corresponds to a typical experiment among at least three replicates.

Table 1. Effect of Cd and SA on some physiological parameters of maize plants

Treatment	Shoot FW (g plant^{-1})	Root DW (g plant^{-1})	Shoot length (s m)	Root length (s m)	Chlorophyll mg (g FW)$^{-1}$	Protein mg (g FW)$^{-1}$
Control	0.68±0.04	0.017±0.001	26.3±1.8	25.4±3.8	2.46±0.17	11.84±1.31
10 μM Cd	0.38±0.03	0.02±0.004	19.9±2.1	20±2.7	2.27±0.11	11.03±0.96
15 μM Cd	0.24±0.05	0.017±0.003	18.7±3.3	18.9±3.0	2.01±0.09	11.28±1.53
25 μM Cd	0.16±0.05	0.014±0.002	17.2±3.0	15.9±2.8	1.29±0.20	10.64±1.05
SA (500 μM)	0.41±0.06	0.019±0.005	24.3±2.6	21.4±2.6	2.17±0.11	11.31±1.54
10 μM Cd+SA	0.49±0.04	0.019±0.004	19.9±1.6	18.6±2.8	2.24±0.21	10.87±1.25
15 μM Cd+SA	0.56±0.07	0.015±0.004	20.5±2.6	18.2±2.7	2.27±0.19	12.13±1.59
25 μM Cd+SA	0.42±0.05	0.017±0.002	18±2.0	18.6±1.8	1.72±0.08	11.09±1.25

Dry seeds were soaked in 500 μM SA or water for 6 h, and were germinated on moist filter paper for 3 d. Then, they were transferred to hydroponic medium and were grown for 14 d without or with Cd in the medium.
The data of root and shoot length, root dry weight, shoot fresh weight, chlorophyll and protein contents are means±s.e. from three experiments.

The activities of both carboxylating enzymes (RuBPC and PEPC) were also affected by Cd treatment. PEPC activity was reduced only after exposure to 25 μM Cd, while RuBPC activity showed a strong reduction at all Cd concentrations. Pretreatment of maize plants with SA before exposure to Cd alleviated the inhibitory effect of Cd and led to a nearly 2-fold increase in PEPC activity compared with untreated plants. SA was observed to have a very strong protective effect on RuBPC activity (Fig. 1C and D).

Effect of SA and Cd on hydrogen peroxide level, electrolyte leakage, lipid peroxidation, and proline level

No major changes were observed in the hydrogen peroxide level in Cd-treated plants or those pretreated with SA before exposure to Cd (Fig. 2A).

Because Cd is known to induce oxidative stress, damage to the membranes was investigated by monitoring MDA content and electrolyte leakage. Relative to the control, Cd-treated maize plants exhibited a higher rate of lipid peroxidation. SA alone caused a decrease in the MDA level. Presoaking the seeds with SA before the application of Cd decreased the level of MDA; the effect was more pronounced in plants treated with 25 μM Cd (Fig. 2B).

Electrolyte leakage was also affected by pretreatment with SA, but the extent of change was not so great as for change in the MDA level (Fig. 2C).

Concentrations of the stress metabolite proline increased upon Cd exposure. The most prominent effect was at 25 μM Cd; a nearly 2-fold increase compared with the control was observed. SA pretreatment counteracted the Cd-induced increase in proline levels (Fig. 2D).

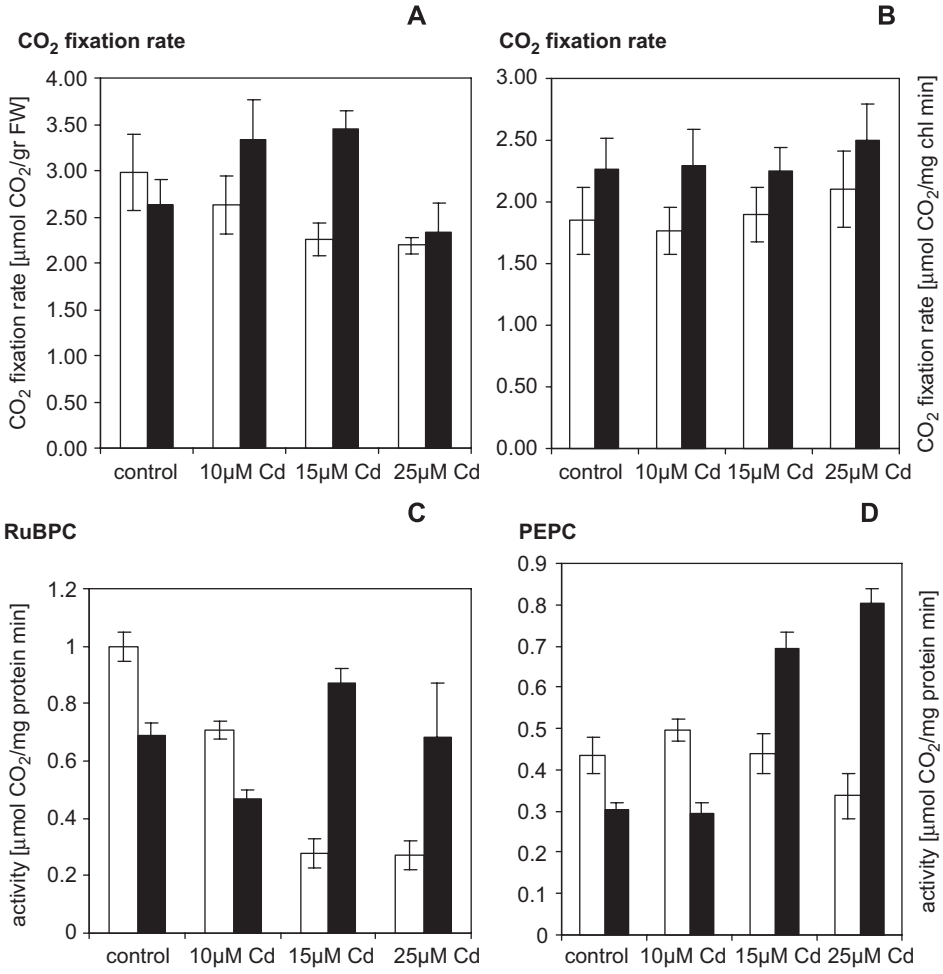

Figure 1. (A) Photosynthetic CO_2 fixation rate in terms of FW base or (B) chlorophyll content and (C) activities of the carboxylating enzymes RuBPC and (D) PEPC in maize plants treated with Cd. Dry seeds were soaked in 500 μM SA (black bars) or water (white bars) for 6 h and were germinated for 3 d on moist filter paper. They were grown for 14 d in the hydroponic medium with or without Cd (control). Data are the means of three independent experiments ± s.e.

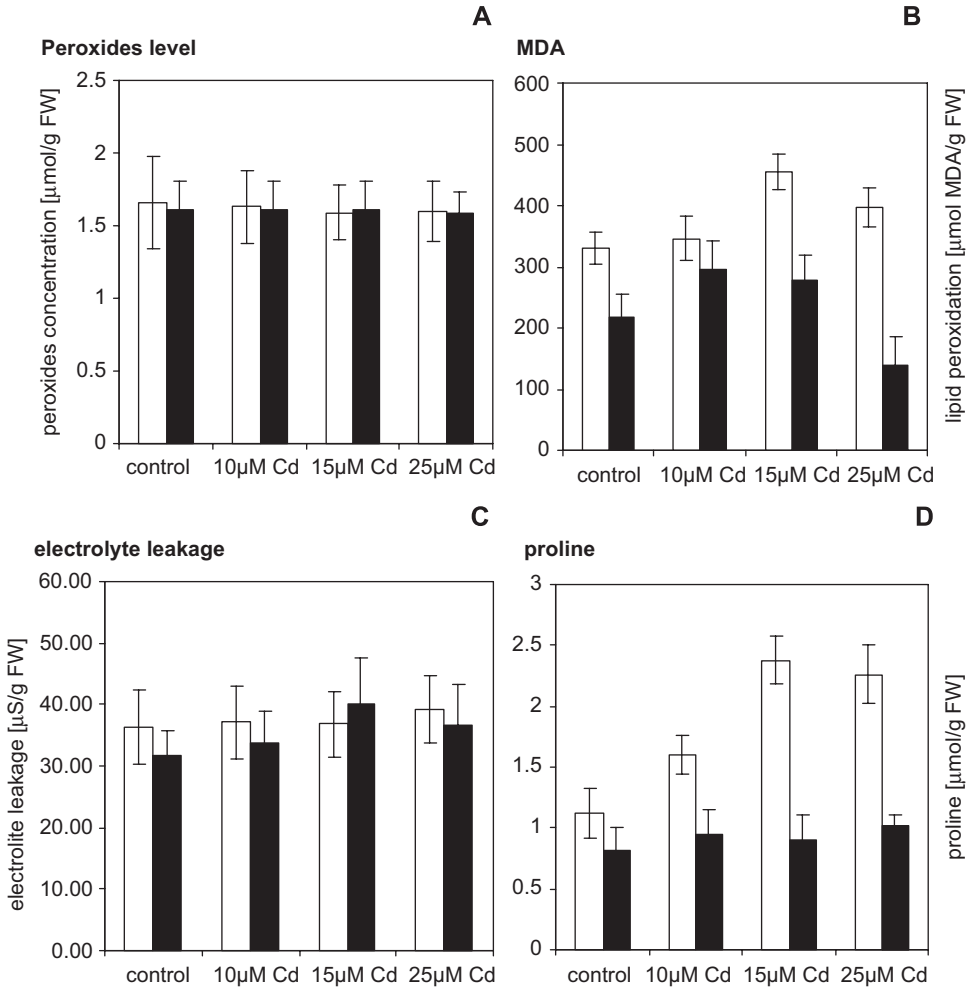

Figure 2. Effect of Cd and SA (A) on hydrogen peroxide production, (B) lipid peroxidation, (C) electrolyte leakage, and (D) proline accumulation in 14-d-old maize plants. For variants and treatments, see Fig. 1. Data are the means of three independent experiments ± s.e.

Effect of SA and Cd on SOD, APX, CAT, and POD activities

The presence of Cd in the nutrient solution (10, 15, and 25 μM Cd) led to disturbances in the activity of the antioxidative enzymes (Fig. 3). The SOD activity, for instance, increased at all Cd concentrations, with the highest increase (2-fold increase) in plants treated with 25 μM Cd (Fig. 3A).

CAT activity was not affected by Cd treatment but dropped to approximately 50% in SA-pretreated plants (Fig. 3B).

In contrast to SOD, APX activity was suppressed by all Cd concentrations. After exposure to 25 μM Cd, the APX activity dropped more than 2-fold (Fig. 3C).

POD activity showed a relatively small difference in activity in all the treatments when compared with SOD and APX. Cd treatment caused an increase of 14% and 12% upon exposure to 10 and 15 μM Cd, respectively (Fig. 3D).

SA pretreatment caused a significant increase in SOD activity upon Cd exposure and alleviated the inhibitory effect of Cd on APX activity. Pretreatment with SA reduced the activity of CAT. The SA-mediated changes in POD activity were much smaller and resembled those observed for Cd treatment alone.

Discussion

The present study was performed to analyze the mechanisms of the beneficial effect of SA on maize plants exposed to toxic Cd concentrations.

It has been shown that presoaking maize seeds for 6 h with 500 μM SA before exposure to Cd has a protective effect on photosynthesis (Fig. 1A and B) and diminishes the oxidative damage caused by Cd (Fig. 2A–D).

Maize plants grown for 14 d with 10, 15, and 25 μM $CdCl_2$ exhibited a significant inhibition of

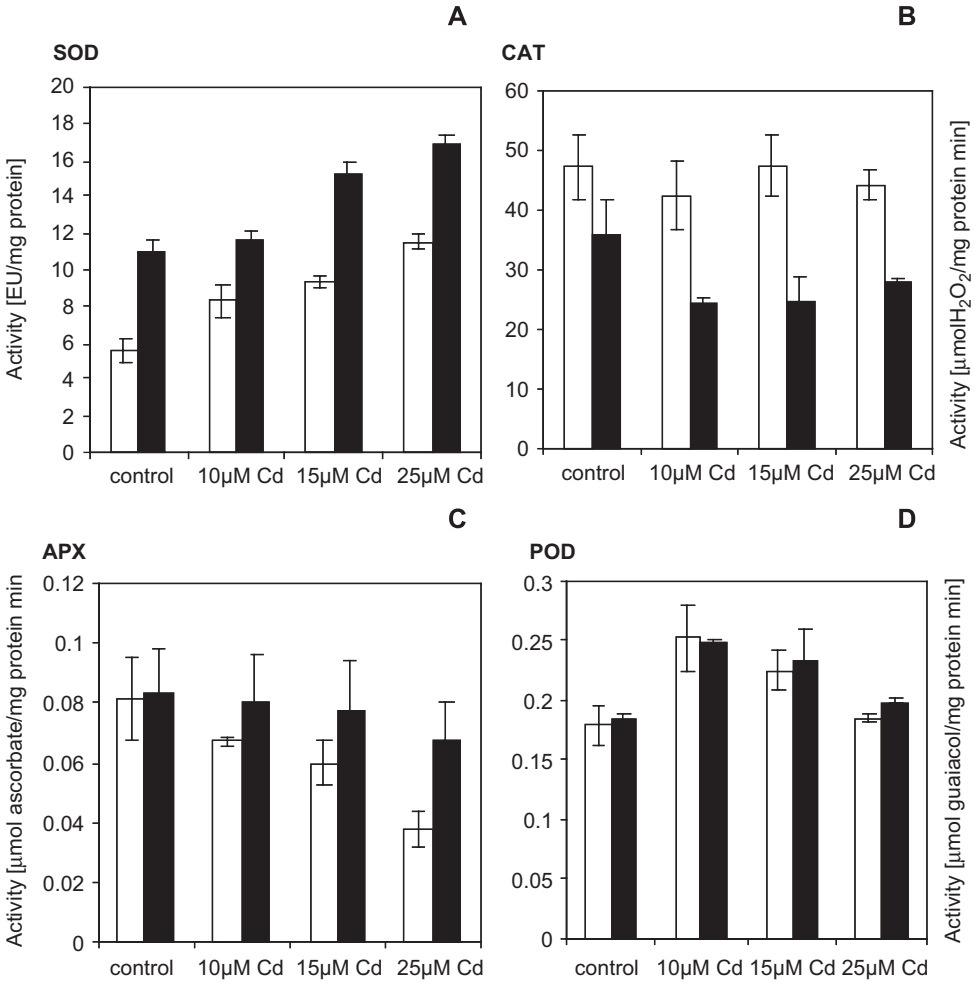

Figure 3. Activity of (A) SOD, (B) CAT, (C) APX, and (D) POD in the leaves of 14-d-old maize plants subjected to Cd. For variants and treatments, see Fig. 1. Values are means of three independent experiments for SOD, APX, and POD and two for CAT, with three to five determinations from each experiment.

growth, as measured by shoot fresh weight and root dry weight accumulation and shoot and root length (Table 1). After 14 d of treatment, plants grown on 25 μM Cd showed visible toxicity symptoms and the survival rate declined greatly. In this model system, the maize plants were unable to tolerate Cd concentrations higher than 25 μM. This can be attributed to the fact that the plants were exposed to Cd at a very early stage of development. In general, it can be said that the sensitivity of a given plant species to heavy metal toxicity depends on its concentration, treatment duration, plant species, age at treatment, and plant organ examined.

The data showed that the chlorophyll content was reduced in Cd-treated plants (Table 1).

The rate of photosynthesis decreased, and 25 μM Cd caused over 26% inhibition in the rate of CO_2 assimilation (Fig. 1A). The activity of both carboxylating enzymes, PEPC and RuBPC, also decreased with rising Cd concentrations, with the effect being very pronounced for RuBPC (over 3-fold) (Fig. 1C and D).

The MDA content in Cd-treated maize plants was observed to be greater than that in the control grown in standard nutrient solution. This showed that Cd toxicity in maize plants was linked to lipid peroxidation. Similar data have been reported for many plant species, including bean (Chaoui et al., 1997), pea (Lozano-Rodriguez et al., 1997), sunflower (Gallego et al., 1996), and maize (Pál et al., 2005).

These severe alterations in the chlorophyll level, chloroplast structure, photochemistry and carboxylating enzyme activities, and the high extent of lipid peroxidation are ultimately responsible for the destruction of photosynthesis caused by Cd.

The results indicated that, although SA has a beneficial effect on photosynthesis in the case of Cd stress, the compound itself stressed the plants (Table 1 and Fig. 1). This has also been

demonstrated in earlier investigations. It was shown that the long-term treatment (7 d) of barley seedlings with 500 μM and 1 mM SA reduced root and seedling growth, chlorophyll and protein contents, and the rate of CO_2 assimilation. The effect of SA depended on the time of treatment duration: no changes were observed in these parameters when barley seedlings were treated with SA for 2 h; the inhibition appeared 6 h after the start of treatment (Pancheva et al., 1996). However, the pretreatment of barley seedlings with 500 μM SA for 6 h before exposure to paraquat led to protection of photosynthesis (Ananieva et al., 2002) and diminished the oxidative damage caused by paraquat (Ananieva et al., 2004). Similar data were presented by Janda et al. (1999) for maize plants experiencing cold stress. These data confirm the suggestion that SA plays different roles based on its endogenous levels in a particular plant species under specific developmental and environmental conditions.

In the present study, it was found that control leaves of maize plants contained both free and bound SA forms. Cd treatment caused an accumulation of free and conjugated SA, with a higher increase in the bound form (Table 2). A similar effect of Cd on SA accumulation was reported by Pál et al. (2005) for maize plants.

The Cd content of dry seeds and root tissue was low in the absence of Cd in the growth medium, and strongly increased after treatment with Cd. SA pretreatment led to a non-significant decrease in the root level of Cd in plants treated with 15 and 25 μM Cd (Table 2). The results exclude the possibility that the formation of stable SA–Cd complexes lowered the Cd toxicity after SA pretreatment. Cd–SA complex formation in the hydroponic solution could not be the cause of the beneficial effect of SA because the exposure to Cd started 3 d after the 6-h SA soaking of the seeds. Another reason why the formation of such a complex can be ruled out is that pretreatment with SA only reduced the level of root Cd accumulation to a very low extent (Table 2).

Proline is known to accumulate in plants under various stress conditions including drought, salt, hypoxia, and UV radiation. Proline accumulation thus appeared to be a suitable indicator of heavy metal stress. The observed decrease in the level of proline in SA-pretreated seeds indicated partial relief from Cd stress (Fig. 2D). In addition to this, it was found that SA pretreatment decreased MDA accumulation and the level of electrolyte leakage caused by Cd, indicating its involvement in protection against oxidative damage. These data are in agreement with those reported by Metwally et al. (2003).

A variety of abiotic stresses, including heavy metals, cause molecular damage to plant cells either directly or indirectly through a burst of active oxygen species (AOS) (Cuipers et al., 2002; Zhang et al., 2005). These oxygen species (O^-, OH^-, H_2O_2) can convert fatty acids to toxic lipid peroxides, which destroy biological membranes. Although Cd does not generate AOS directly, like other heavy metals such as Cu and Fe, it generates oxidative stress via interference with the antioxidant defense system (Somashekaraiah et al., 1992; Sanita di Toppi and Gabrielli, 1999). Among the H_2O_2-destroying enzymes, it was the POD activity that was stimulated by Cd (Fig. 3D). This result is in good agreement with the increased rate of lipid peroxidation (Fig. 2B) and with other observations (Shaw, 1995). POD, which participates in ignin biosynthesis, might build up a physical barrier against toxic heavy metals. Earlier data in the literature on the CAT response in leaves exposed to Cd stress are contradictory, since both enzyme activation (Lee et al., 1976) and inhibition (Gallego et al., 1996) have been described. In the present experiments, Cd did not induce changes in CAT activity (Fig. 3B), while the activity of APX decreased (Fig. 3C). Summarizing the results, it can be concluded that, among the H_2O_2-eliminating enzymes, POD responds to Cd stress. The absence of changes in CAT activity suggests a different role of CAT in the oxidative stress induced by heavy metals. SOD is an important AOS-scavenging antioxidant enzyme. By catalyzing the detoxification of O_2^- (superoxide radical) to O_2, SOD blocks O_2^--driven cell damage. The results showed that Cd treatment increased SOD activity, indicating the activation of the antioxidative system.

At first glance, it may appear surprising that Cd, which is not a transient metal, is able to cause oxidative stress. However, Cd binds to thiol groups and thereby inactivates thiol-containing enzymes. This could be the reason for the observed inhibition of APX activity, as it is known that the enzyme is sensitive to thiol reagents.

The physiological role of SA is usually considered to be as a signal molecule, altering the antioxidative system by inhibiting CAT and stimulating peroxidases (Rao et al., 1997). The observed decrease in the levels of MDA, proline, and the rate of electrolyte leakage in maize plants pretreated with SA is confirmation of the protective role of SA against Cd stress. The high activity of SOD in SA-pretreated plants suggests that hydrogen radicals were accumulated as a result of Cd treatment.

The data suggest that endogenous SA plays an important antioxidant role in protecting maize

plants from oxidative stress. SA is a direct scavenger of hydroxyl radicals and iron-chelating compounds, as well as generates them via the Fenton reaction (Dinis et al., 1994; Halliwell et al., 1995). Data have been presented suggesting a salicylate–iron complex, with SOD activity catalyzing the dismutation of superoxide radicals (Jay et al., 1999). Therefore, high levels of SA in maize plants may act directly as a preformed antioxidant to scavenge AOS and/or indirectly modify the redox balance by activating antioxidant responses, as suggested by Yang et al. (2004) for rice plants.

Rao and Davis (1999) proposed two different mechanisms to explain the role of SA in ozone-induced cell death in *Arabidopsis*. In some cases SA potentiates the activation of antioxidant defense responses to minimize the oxidative stress induced by ozone, while in other cases high levels of SA led to the activation of oxidative bursts and cell death. These examples demonstrate that SA is an important component in modifying stress responses and may play a pro- or antioxidative role depending on its endogenous level (Yang et al., 2004).

An important question in this study was how this short-term treatment with SA (presoaking of seeds for 6 h) affected certain physiological processes, such as plant growth, photosynthesis, and the antioxidant defense system. The beneficial effect of SA during the earlier growth period may help plants to avoid cumulative damage upon exposure to Cd. Alternatively, SA could be involved in the expression of specific proteins or defense-related enzymes.

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