SALICYLIC ACID DECREASES Cd TOXICITY IN MAIZE PLANTS

A. Krantev, R. Yordanova, L. Popova*

Institute of Plant Physiology "Acad. M. Popov", Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 21, 1113 Sofia, Bulgaria

Summary. The present study is focused on the possible mediatory role of salicylic acid (SA) in photosynthesis under cadmium (Cd) toxicity. Maize seeds were sterilized and divided into two groups. One half of the seeds was presoaked in 500 μM SA solution for 6 h and then both groups were allowed to germinate for 3 days. Plants were grown for 12 days in Hoagland solution at 22/18 °C under 16/8-h light/ dark periods and 120 μmol. $m^{-2}s^{-1}$ PAR. All seedlings (without H_2O and SA controls) were transferred to Cd-containing solutions (10, 15, 25 μM) and grown for 12 days. Exposure of plants to Cd caused a gradual decrease in chlorophyll content, the effect being expressed to a higher extent at 25 μM Cd. Pretreatment of seeds with SA alleviated the Cd negative effect on this parameter. 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 in Cd-treated plants. Pretreatment with SA alleviated the inhibitory effect of Cd on the studied enzymatic activities. Proline production and the rate of lipid peroxidation in Cd-treated plants were much lower in SA-pretreated plants.

Key words: Cadmium; Photosynthesis; Salicylic acid; *Zea mays* L.

***Abbreviations**:* MDA - malondialdehide; PEPC - phosphoenol pyruvate carboxylase; RuBPC - ribulose 1,5-bisphosphate carboxylase; RWC - relative water content; SA - salicylic acid.

*Corresponding author, e-mail: lpopova@obzor.bio21.bas.bg

INTRODUCTION

Cadmium (Cd^{2+}) is one of the most toxic air, water and soil pollutants which penetrates into environment mainly through industrial processes and phosphate fertilizers. It can reach high levels in agricultural soils and is easily assimilated by plants. In most environmental conditions Cd enters from roots, and they are the first site which experiences the negative influence of Cd. It has been suggested that growth inhibition of root by Cd is due to a direct effect on the nucleus or to interaction with hormones (Barcelo and Poschenrider, 1990). In the aerial part of plants, growth reduction appears to be a consequence of inhibited photosynthesis, chlorophyll metabolism, electron transport activity, and stomata functionality.

Certain heavy metals like Cu and Fe can be toxic by their participation in redox reactions producing hydroxyl radicals which are extremely toxic to living cells (Stochs and Bagchi, 1995). By contrast, Cd^{2+} is unable to participate in Fenton-type reactions, but indirectly produces reactive oxygen species (Sandalio and Dalurzo, 2001). Cadmium damages nucleoli, alters RNA synthesis, reduces the absorption of nitrate and its transport, interacts with water balance and damages the photosynthetic apparatus (Di Toppi and Gabrielli, 1998).

Survival under stressful conditions depends on plant's ability to perceive the stimulus, to generate and transmit signals, and to induce biochemical changes that accordingly adjust metabolism. Therefore, the search for signal molecules that mediate stress tolerance is an important step to better understanding plant acclimation to adverse environment.

Salicylic acid (SA) is an endogenous growth regulator with many physiological functions. SA has been identified as an important signaling element involved in establishing local and systematic disease resistance responses of plants after pathogen attack (Alvarez, 2000); it mediates some positive acclimation responses to abiotic stress factors, such as heavy metals, herbicides, low temperatures and salinity (Metwally A. et al., 2003; Ananieva et al., 2002; Janda et al., 1999). SA pretreatment alleviates Cd toxicity in barley (Metwally et al., 2003) and maize plants (Pal et al., 2002).

The present study investigated possible mediatory role of salicylic acid (SA) in photosynthesis under cadmium (Cd) toxicity. Changes in some parameters associated with oxidative stress, namely proline production, lipid peroxidation, CO_2 fixation and the activity of the carboxilating enzymes RuBCase and PEPCase, were assessed since they are known to be mostly affected by Cd treatment.

MATERIALS AND METHODS

Growth and treatment of seedlings

Maize (*Zea mays L.*) seeds were presoaked for 6h, either in 500 μM SA or in water as a control. After that they were germinated in the dark for 3 days, and placed on polyethylene pots with modified Hoagland solution for 2 weeks (0.3125 mM KNO_3, 0.45mM $Ca(NO_3)_2$, 0.0625 mM KH_2PO_4, 0.125 mM $MgSO_4$ x $7H_2O$, 11.92 μM HBO_3, 4,57 μM $MnCl_2$ x $4H_2O$, 0.191 μM $ZnSO_4$ x $7H_2O$, 0.08 μM $CuSO_4$ x $5H_2O$, 0.024 μM $(NH_4)_6Mo_7O_{24}$ x $4H_2O$, 15.02 μM $FeSO_4$ x $7H_2O$ and 23.04 μM Na_2EDTA x $5H_2O$). Four cadmium concentrations were tested, and plants belonged to 8 experimental groups: 1) control plants on nutrient solution without Cd^{2+}; 2) presoaked in water and treated with 10μM Cd^{2+}; 3) presoaked in water and treated with 15μM Cd^{2+}; 4) presoaked in water and treated with 25μM Cd^{2+}; 5) presoaked in SA without Cd^{2+}; 6) presoaked in SA and treated with 10μM Cd^{2+}; 7) presoaked in SA and treated with 15μM Cd^{2+}; 8) presoaked in SA and treated with 25μM Cd^{2+}.

Enzyme extraction and assays

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. Slices were incubated in 5 mL buffer in a 25 mL Erlenmeyer flask at 25 ºC for 5 min at 120 W m^{-2} light intensity. Buffer contained: 0.33 M sorbitrol, 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 μM $NaHCO_3$ containing 40 μCi $NaH^{14}CO_3$ (14.3 μCi/ μM) was added to each sample. They all were allowed to fix $^{14}CO_2$ for 10 min. The reaction was stopped by adding boiling 80% ethanol. Tissues were subsequently extracted eight times with boiling ethanol of the same concentration. Combined extracts were brought to dryness in *vacuo* at 40 ºC and were dissolved in 10 mL distilled water. An aliquot was measured in 5 mL of scintillation fluid for radioactivity assay using Packard Tri-Carb liquid scintillation counter.

RuBPC (E.C. 4.1.1. 39) and PEPC (EC 4.1.1.31) 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 50 mM HEPES-NaOH (pH 8.0), 20 μmol $MgCl_2$, 1 μmol ditiothreitol (DTT), 20 μmol $NaHCO_3$ (containing 1.48 MBq, specific radioactivity 0.38 MBq μmol^{-1}), and enzyme extract equivalent to 0.3-0.4 mg protein. Reactions, at 25±1 ºC, were initiated 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 50 mM HEPES-NaOH (pH 8.0): 20 μmol $MgCl_2$, 0.4 μmol NADH, 20 μmol $NaHCO_3$ (containing 1.48 MBq,

specific radioactivity 0.38 MBq mol^{-1}), 1 μmol DTT, and enzyme extract equivalent to 0.3-0.4 mg protein. The reaction volume was 1 mL. Reactions, at 30±1 °C, were initiated by the addition of 3 μmol PEP. Reaction time was 1 min. The amount of fixed $^{14}CO_2$ was measured using a liquid scintillation spectrometer.

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

Level of lipid peroxidation was measured according to the method of Heath and Packer (1968) with slight modifications. A 0.2- 0.3 g leaf material was homogenized in 3 mL 0.1% TCA and centrifuged at 15, 000 g for 30 min at 4 °C. To 0.5 mL aliquot of the supernatant 0.5 mL buffer and 1 mL reagent (0.5% thiobarbituric acid, TBA in 20% TCA, w/v) were added. The blank contained 0.5 mL 0.1% TCA + 0.5 mL buffer and 1 mL reagent. 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 was read at 532 nm and the value for the non-specific absorbtion at 600 nm was subtracted. Malondialdehyde level (MDA) was estimated by using the mM extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Chlorophyll was extracted by acetone and measured spectrophotometrically according to Arnon (1949).

RESULTS

CO_2 fixation, PEPC and RuBPC activities

In a previous study we have shown that treatment of maize plants with Cd caused a reduction in root and shoot growth. Dry weight accumulation, both shoot and root lengths were reduced in the treated plants (Krantev et al., 2007).

Here we demonstrated that growth inhibition of maize plants was accompanied by a significant decrease in the rate of photosynthetic CO_2 fixation expressed on both fresh weight and chlorophyll content bases because Cd treatment caused a reduction in chlorophyll level.

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 RuDPC activity exhibited a strong reduction at all Cd concentrations applied. Pretreatment of maize plants with SA before exposure to Cd alleviated the inhibitory effect of Cd and led to nearly two-fold increase in PEPC activity compared to untreated plants. A very strong protective effect of SA was observed on RuBPC activity (Fig. 1).

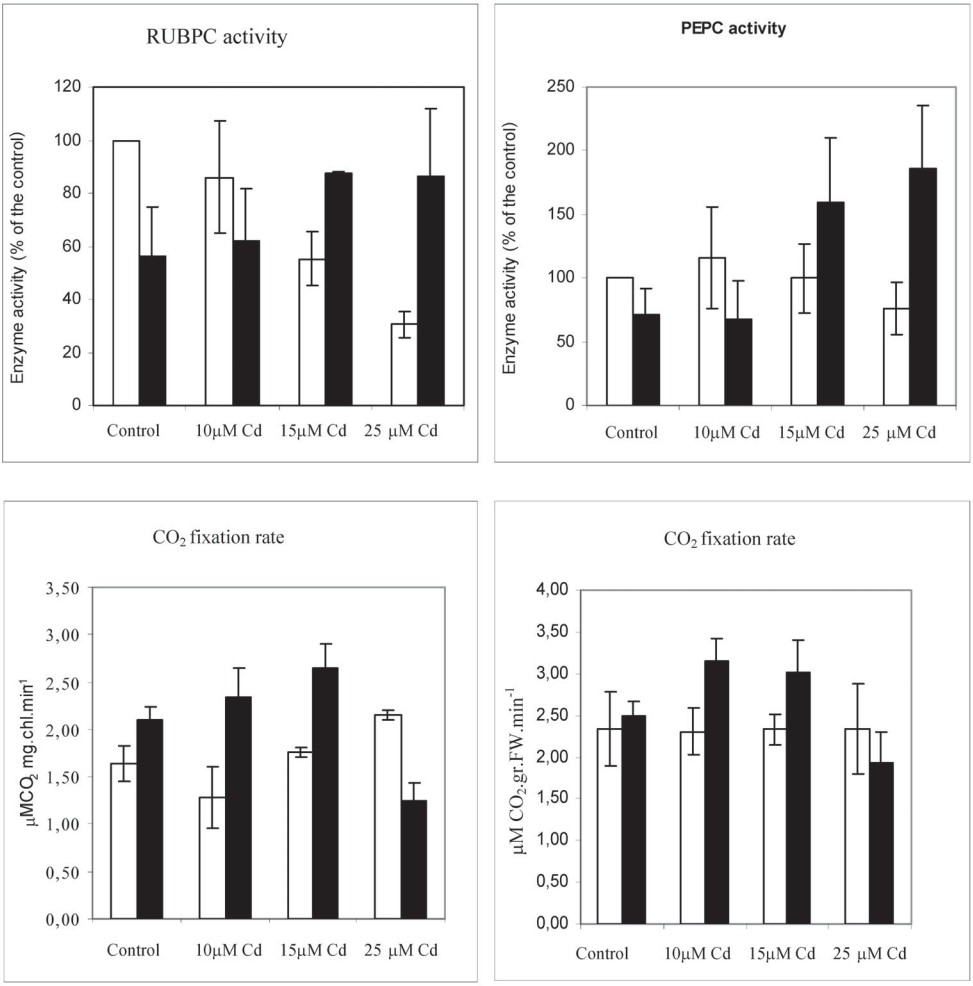

Figure 1. Photosynthetic CO_2 fixation rate and activities of carboxylating enzymes (RuBPC and PEPC) in maize plants treated with Cd or pretreated with SA before exposure to Cd. Dry seeds were soaked in 500 μM SA (black bars) or water (white bars) for 6 h and were germinated for three days in moist filter paper. They were grown for 14 days in hydroponic medium without Cd or with Cd (in the respective concentrations). Data are means ± SD, (n=3) from three experiments.

Effect of SA on lipid peroxidation, chlorophyll content and proline level

Since Cd is known to induce oxidative stress we studied membranes damage by monitoring of MDA content.

Cadmium-treated maize plants with SA before Cd application decreased MDA, the effect being more pronounced in 25 μM Cd-treated plants.

The concentration of the stress metabolite proline increased upon Cd exposure. The most prominent effect was observed at 25 μM Cd (a nearly two-fold rise compared to the control). SA pretreatment counteracted the Cd-induced increase in proline levels. Chlorophyll content decreased slightly with increasing cadmium concentration, i.e. it was higher in control and 10, 15 μM Cd^{2+}- treated plants for H_2O-presoaked group of plants. Chlorophyll measured in plants at 25 μM Cd^{2+} whose seeds were presoaked with SA was higher than in the group under the same stress conditions, but without preliminary seed treatment with SA.

Exposure of maize plants to Cd^{2+} led to a slight decrease in leaf RWC. The values of this parameter for the control plants were 95-97% and 92-93% for H_2O presoaked and SA presoaked plants, respectively. The reduction in RWC was approximately 6% and 11% for H_2O presoaked and SA presoaked plants, respectively in the variants treated with 10 μM and 15 μM Cd^{2+} (Table 1).

DISCUSSION

The rate of photosynthesis decreased and 25 μM Cd caused inhibition of CO_2 assimilation. The activity of both carboxylating enzymes (PEPC and RuBPC) also decreased at higher Cd concentrations, the effect being strongly expressed on RuBPC (over 3-fold) (Fig. 1).

Proline accumulation appeared to be a suitable indicator for heavy metal stress. This compound accumulates in plants under different stress conditions including drought, salt, hypoxia, UV radiation. The observed decrease in proline level in plants grown from SA-pretreated seeds indicated partial recovery from Cd stress (Table 1). In addition, it was demonstrated that SA pretreatment decreased MDA accumulation and electrolyte leakage caused by Cd, which confirms the role of this compound against oxidative damage. Our data are in agreement with those reported by Metwally

Table 1. Effect of Cd and SA on relative water content (RWC), chlorophyll content, lipid peroxidation, and proline accumulation.

	RWC %	Chlorophyll a+b μg chl/gFW	MDA μmol/gFW	Proline μmol/gFW
Control	95,7	1,7	329,98	1,12
10μM Cd	91,5	1,7	346,15	1,6
15μM Cd	91,82	1,6	455,57	2,38
25μM Cd	90,01	0,9	396,04	2,26
Control+SA	92,68	1,57	216,77	0,82
10μM Cd+SA	81,385	1,11	295,91	0,94
15μM Cd+SA	82,9	1,10	278,02	0,91
25μM Cd+SA	88,285	1,37	140,04	1,02

and co-workers (2003). Cadmium toxicity leads to water balance distruction that is manifested by the RWC decrease which is in agreement with data published elsewhere (Costa end Morel, 1994). Our results showed also that chlorophyll content was reduced in Cd-treated plants (Table 1). These severe alterations in chlorophyll level, the high extent of lipid peroxidation, the decrease in RuBPC and PEPC activity and CO_2 fixation rates are some of the factors responsible for damages in photosynthetic process.

It has been demonstrated that presoaking of maize seeds for 6 h with 500 μM SA before exposure to Cd has a protective effect on photosynthesis and diminishes the oxidative damage caused by Cd. An important issue arising from this study was how the short-term treatment with SA affected several physiological processes, such as plant growth, photosynthesis and antioxidant defense system. We assume that the beneficial effect of SA during an earlier growth period could be related to avoidance of cumulative damage upon exposure to cadmium. Alternatively, SA could be involved in the expression of specific proteins or defense-related enzymes.

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