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Changes of photosynthetic activities of maize (*Zea mays* L.) seedlings in response to cadmium stress

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Abstract

Maize (*Zea mays* L.) seedlings were grown in nutrient solution culture containing 0, 5, and 20 μM cadmium (Cd) and the effects on various aspects of photosynthesis were investigated after 24, 48, 96 and 168 h of Cd treatments. Photosynthetic rate (P_N) decreased after 48 h of 20 μM Cd and 96 h of 5μM Cd addition, respectively. Chl *a* and total Chl content in leaves declined under 48 h of Cd exposure. Chl *b* content decreased on extending the period of Cd exposure to 96 h. The maximum quantum efficiency and potential photosynthetic capacity of PSII, indicated by F_v/F_m and F_v/F_o, respectively, were depressed after 96 h onset of Cd exposure. After 48 h of 5μM Cd and 24 h of 20 μM Cd treatments, the activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.39) and phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) in the leaves started to decrease, respectively. We found that the limitation of photosynthetic capacity in Cd stressed maize leaves was associated with Cd toxicity on the light and the dark stages. However, Cd stress initially reduced the activities of Rubisco and PEPC and subsequently affected the PSII electron transfer, suggesting that the Calvin cycle reactions in maize plants are the primary target of the Cd toxic effect rather than PSII.

Additional key words: cadmium; chlorophyll fluorescence; maize; photosynthetic rate; phospho*enol*pyruvate carboxylase; ribulose-1, 5-bisphosphate carboxylase/oxygenase.

Introduction

Cadmium (Cd) is one of the key heavy metal pollutants toxic for humans, animals and plants. It enters agricultural soils mainly from industrial processes, phosphate fertilizers and atmospheric deposition, after which it is transferred to the food chain (Wagner 1993).

The presence of excessive amount of Cd results in retardation of plant growth and inhibition of diverse metabolic processes such as photosynthesis, respiration and nitrate assimilation (Sanità di Toppi and Gabbrielli 1999, Kučera 2008). A marked decrease of photosynthetic rate (P_N) for different plant species under exposure to Cd stress has been demonstrated (Baszyński *et al.* 1980, Baryla *et al.* 2001, Burzyński and Żurek 2007, Küpper *et al.* 2007, Krantev *et al.* 2008).

Cd stress affects photosynthesis in various ways. It inhibits the synthesis of chlorophylls (Chl) (Padmaja *et al.* 1990, Burzyński and Kłobus 2004, Azevedo *et al.* 2005, Shukla *et al.* 2008) and their stable binding to proteins (Horváth *et al.* 1996), thereby decreasing the accumulation of pigment-lipoprotein complexes, particularly photosystem (PS) I (Krupa *et al.* 1987, Sárvári 2005). Ahmed and Tajmir-Riahi (1993) reported that Cd interacted with the protein subunits of light harvesting complex II. *In vitro* studies with isolated chloroplasts showed that PSII is extremely sensitive to Cd (Li and Miles 1975, Chugh and Sawhney 1999). The maximum quantum efficiency of PSII as shown by ratio of variable fluorescence to maximum fluorescence (F_v/F_m) decreased under Cd stress, indicating inhibition of electron transfer reactions in PSII (Sigfridsson *et al.* 2004, Azevedo *et al.* 2005, Küpper *et al.* 2007). The target sites of Cd action have been suggested at both the donor and the acceptor

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Abbreviations: Cd – cadmium; Chl – chlorophyll; DM – dry mass; F_0 – minimal fluorescence in dark adapted state; F_m – maximal fluorescence in dark adapted state; F_v – variable fluorescence; F_v/F_m – maximal quantum efficiency of PSII; PEPC – phospho*enol*pyruvate carboxylase; P_N – net photosynthetic rate; PS – photosystem; ROS – reactive oxygen species; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase.
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sides of PSII (Sigfridsson *et al.* 2004). The degradation of chloroplast structure caused by Cd stress are often observed in plants (Baszyński *et al.* 1980, Barcelo *et al.* 1988, Horváth *et al.* 1996, Baryla *et al.* 2001), which may be associated with a significant accumulation of reactive oxygen species (ROS) in plants resulting from Cd-induced oxidative stress (Dietz *et al.* 1999, Schützendübel *et al.* 2001, Romero-Puertas *et al.* 2004, Kučera 2008). However, *in vitro* studies indicate that CO_2 fixation is inhibited by Cd without any significant effect on photochemical reactions in isolated protoplasts and chloroplasts (Weigel 1985a,b), which implies that Cd limits P_N by affecting the dark phase of the photosynthetic process. It has been suggested that the site of Cd action locate either at the carboxylation step and/or the regenerative phase of Calvin cycle (Weigel 1985a,b). Disturbances in the activities of Rubisco and other enzymes of the dark phase of photosynthesis were observed in Cd-stressed plants (Malik *et al.* 1992, Krupa and Moniak 1998, Burzyński and Żurek 2007). In addition, Cd toxicity decreases stomatal conductance to CO_2 (Baryla *et al.* 2001) and number of open stomata (Barcelo *et al.* 1988), which would lower CO_2 uptake and the intercellular CO_2 concentration for assimilation.

Küpper *et al.* (2007) suggested that Cd inhibited the photosynthetic light reactions more than Calvin cycle in the metal hyperaccumulator *Thlaspi caerulescens*. Vassilev *et al.* (2004) found that leaf gas exchange, Chl content and electron transport activity decreased, but lipid peroxidation status of thylakoids did not alter when barley plants were exposed to 42 mg kg^{-1} Cd treatment in sand culture for 10 days. Baszyński *et al.* (1980) noted that the decline in Chl content preceded that of CO_2 fixation and proposed this to be the primary cause of diminished photosynthetic activity by Cd stress. However, some researchers revealed that Cd stress initially destroyed the photosynthetic carbon reduction cycle and subsequently influenced the photosynthetic electron transport (Burzyński and Kłobus 2004, Burzyński and Żurek 2007). In most of the studies, Cd treatments were set long enough to cause substantial metabolic changes in plants. In such experiments, the effects caused by relatively long exposure to Cd might reflect a general failure of the plant photosynthetic activities. Little is known about the responses of photosynthetic activities of plants during the short-term Cd stress. And thus Cd influences on P_N have been intensively studied, but the primary sites of Cd action on P_N remain unclear.

In this study, maize (*Zea mays* L.) seedlings were grown in nutrient solution culture containing 0, 5, and 20 μM Cd with relatively short exposure periods (24 h to 168 h). The time-related changes of P_N, Chl level, Chl fluorescence parameters, and activities of the enzymes in Calvin cycle such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.39) and phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) were investigated. The purpose was to examine the primary responses of photosynthetic activities in maize plants at the early stages of Cd stress.

Materials and methods

Plants: Maize crop (cv. Nongda 108) seeds of similar size were surface-sterilised by soaking in 10 % H_2O_2 for 30 min and germinated on the filter paper at 25 °C for 48 h after washing thoroughly in deionized water. Initially, the germinated seeds were sown in quartz sands. The uniform 7 day-seedlings were transplanted to nutrient solution containing the following chemicals [mM]: $Ca(NO_3)_2$ 2.0, K_2SO_4 0.75, KH_2PO_4 0.25, KCl 0.1, $MgSO_4$ 0.6, FeEDTA 4.0×10^{-3}, H_3BO_3 1.0×10^{-3}, $MnSO_4$ 1.0×10^{-3}, $ZnSO_4$ 1.0×10^{-3}, $CuSO_4$ 1.0×10^{-4}, $(NH_4)_6Mo_2O_4$ 5.0×10^{-6}. Plants were grown in an environmentally-controlled growth chamber, where the temperature of day/night was 26/22 °C and daily irradiance was set at approximately 500–600 μmol m^{-2} s^{-1} for 14 h, the relative humidity was about 70 %.

Cd treatment: When the transplanted plants had grown in normal nutrient solution for one week, three levels 0, 5, and 20 μM Cd as $CdCl_2$ were added into nutrient solution.

Cd analysis: The plant samples were rinsed using distilled water, killed at 105 °C for 30 min, dried at 65–70 °C for 2 days and weighed. Dry plant materials were digested in HNO_3-$HClO_4$ and the extracts were analyzed for Cd using inductively coupled plasma atomic emission spectrometry (*ICPS-1000IV, Shimadzu*, Kyoto, Japan).

Chl: Leaf samples collected after 24, 48, 96 and 168 h of Cd exposure, respectively, were extracted with 80 % acetone in the dark for 48 h at 25 °C until leaves were blanched. Absorbances of the clear extract at 652.0, 665.2, and 750.0 nm were recorded and concentrations of Chl *a*, *b*, and *a*+*b* were computed as described by Porra *et al.* (1989) .

P_N of the fully expanded leaves in plants exposed to Cd stress for 24, 48, 96 and 168 h, respectively, was measured with a portable infrared gas analysis system (*LCA-4, ADC Bio Scientific Ltd.*, Herts, England). During the measurement the intact plants were removed out from the growth chamber. The relative air humidity was about 65 %, the leaf temperature ranged from 26 to 30 °C and the ambient CO_2 concentration was 320 –380 μmol mol^{-1}.

Chl fluorescence parameters were recorded in parallel to gas exchange measurements in the same leaf, using a direct portable fluorometer (*PEA, Hansatech Ltd.*,

Kings Lynn, Norfolk, England). Leaves were acclimated to dark for 20 min before measurements were taken. The time of measuring was 5 s , and irradiance was set at 75 % of maximum irradiance (>3000 μmol m^{-2} s^{-1}). Minimal (F_o), maximum (F_m), and variable ($F_v = F_m–F_o$) fluorescence in the dark adapted state, F_v/F_o ratio, and maximum quantum efficiency of PSII (F_v/F_m) were recorded. The ratio of F_v/F_o was used to assess potential photosynthetic capacity of PSII (Roháček 2002).

Rubisco and PEPC activities: Fresh leaves were sampled after 24, 48, 96 and 168 h of Cd exposure, respectively, and immediately frozen in liquid nitrogen and stored at –80 °C until the analysis. 0.5 g fresh leaves were ground with a pestle in an ice-cold mortar with 2 ml buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM DTT, 0.5 mM EDTA and 10 % (v/v) glycerol. The homogenate was centrifuged at 4 °C for 20 min at 15 000 × *g*, and the supernatant was used for the determination of Rubisco and PEPC activities.

The Rubisco activity was determined spectrophotometrically at 340 nm by monitoring NADH oxidation at 30 °C. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 40 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.2 mM NADH, 4 mM ATP, 0.2 mM EDTA, 5 mM DTT, 1 unit of glyceraldehyde 3-phosphodehydrogenase and 1unit of 3-phosphoglycerate kinase. The reaction was initiated after the addition of 0.1 ml enzyme extract mixed with 0.2 mM ribulose-1,5-bisphosphate. The Rubisco activity was expressed as μmol CO_2 fixed at one min per 1mg of protein.

The assay mixture for the PEPC activity contained 20 mM $MgCl_2$, 0.4 mM NADH, 20 mM $NaHCO_3$, 1 mM DTT, and 0.1 ml enzyme extract in 50 mM Tris-HCl (pH 8.0). The reaction was initiated with 4 mM phospho-*enol*pyruvate and lasted for 2 min. The rate of NADH consumption was determined by the absorbance change at 340 nm. The PEPC activity was expressed as μmol NADH oxidized in one min per mg of protein in the enzyme extraction.

Estimation of protein was done according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis: The experiment was set up in a completely randomised factorial design (3 Cd treatments × 4 replicates). All data were subjected to analysis of variance (*ANOVA*). Means were compared using the least significant difference (*LSD*) test at the 5 % probability level.

Results

Plant growth: When maize plants were exposed to 20 and 5 μM Cd for three and four days, respectively, the visual symptoms of Cd toxicity such as wilting was first observed in old leaves. The symptoms such as marginal chlorosis and necrosis were subsequently visible. Injury became more serious with the increasing Cd concentration and the Cd exposure duration.

After 96 h of 5 and 20 μM Cd stress, the dry mass (DM) of shoots has decreased. At 168 h, 20 μM Cd caused more reduction of the shoot DM than 5 μM Cd treatment. The effects of 5 and 20 μM Cd addition on the root DM during the treatment period of 96 h were not significant. Only 20 μM Cd exposure for 168 h reduced the root DM (Fig. 1).

Cd concentration in plants: The Cd concentration in roots and shoots increased significantly with the increasing Cd concentrations in growth media and with the time of Cd stress (Fig. 2).

Leaf Chl concentration: Cd stress reduced Chl content in leaves (Table 1). At 48 h onset of Cd exposure, the contents of Chl *a* and total Chl were found to decrease without significant changes in Chl *a* /Chl *b* ratio. On extending the period of exposure to 96 h, Chl *b* concentration declined. Chl *a* showed more sensitivity to Cd toxicity than did Chl *b*.

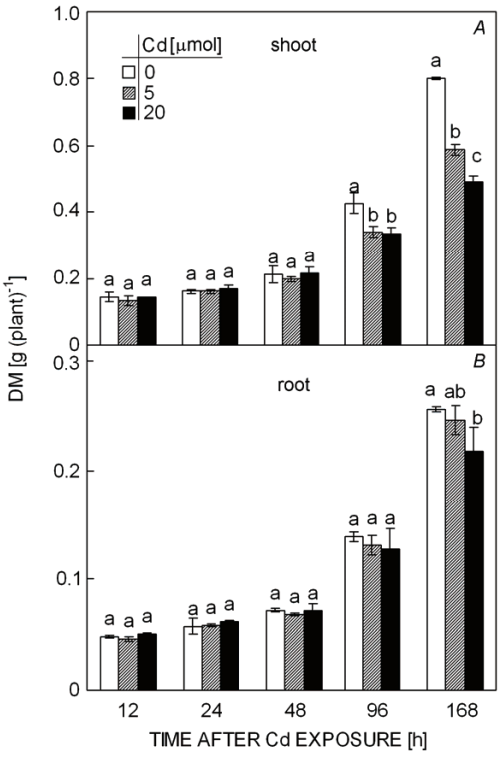

Fig. 1. Dry mass (DM) of maize plants as affected by Cd stress. *A*: shoot, *B*: root. Means±SD, *n*=4. The column of different Cd treatments with the same time of Cd exposure marked by the same letter are not significantly different at p=0.05.

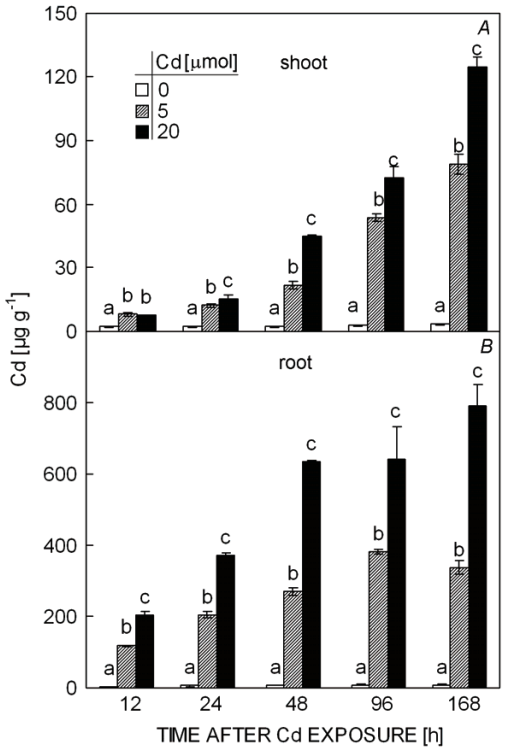

Fig. 2. Cd content in maize plants as affected by Cd stress. *A*: shoot, *B*: root. Means±SD, *n*=4. The column of different Cd treatments with the same time of Cd exposure flanked by the same letter are not significantly different at p=0.05 level.

***P*$_N$**: As shown in Table 1, when maize plants were grown with the 20 μM Cd treatment for 48 h, P_N began to decrease. While P_N decreased after 96 h of 5 μM Cd addition. P_N was inhibited earlier at higher concentration of Cd stress.

Chl fluorescence parameters: In presence of Cd for 96 h, there was a decrease in F_v without effect upon F_o. After Cd stress for 168 h, F_o increased and F_v showed less change. The ratio F_v/F_m declined significantly with 96 h and 168 h of Cd exposure compared with no Cd addition, indicating that the potential maximal quantum yield of PSII was inhibited by Cd stress. The ratio of F_v/F_o under 96 h and 168 h of 5 to 20 μM Cd stress was also below the normal condition, implying the potential photosynthetic capacity of PSII was reduced by Cd toxicity. However, Cd exposure for 24 and 48 h did not change F_v/F_m and F_v/F_o ratios (Table 1).

The activities of Rubisco and PEPC: Cd exerted a deleterious effect on the activities of Rubisco and PEPC (Table 1). After 24 h of treatment, the activities of Rubisco and PEPC in 20 μM Cd-stressed plants showed decreases. After 48 and 96 h of 5μM Cd treatment, the activities of Rubisco and PEPC in the leaves began to lower, respectively.

Discussion

It was found that Cd toxicity caused a notable reduction in P_N in different plant species (Baszyński *et al.* 1980, Baryla *et al.* 2001, Burzyński and Żurek 2007, Küpper *et al.* 2007, Krantev *et al.* 2008). We also observed that a significant decrease of P_N occurred in maize seedling subjected to 20 μM Cd for 48 h and to 5μM Cd for 96 h. Higher concentration of Cd caused P_N inhibition earlier. Our research suggested that the destruction of photosynthesis caused by Cd was associated with the alterations in the Chl level, photochemistry and carboxylating enzyme activities in maize plants.

Cd inhibits Chl biosynthesis through δ-aminolevulinic acid dehydratase (Myśliwa-Kurdziel and Strzałka 2002), photoactive protochlorophyllide (Pchlide) and NADPH/ Pchlide oxidoreductase ratio (Schoefs and Bertrand 2005) because of Cd's interference with the sulfhydryl site (Prasad and Strzałka 1999). In addition, the decreases in the ion contents of magnesium, iron, and manganese (Azevedo *et al.* 2005) were found in the Cd-stressed plants and these minerals are essential in Chl synthesis and/or photosynthetic electron transport. Regarding Cd toxicity to PSII activities in plants, some researchers suggested that Cd binds in sites on both the acceptor and the donor side of PSII (Sigfridsson *et al.* 2004). On the donor side, the presence of Cd^{2+} exchanges, with high affinity in a slow reaction, for the Ca^{2+} cofactor in the Ca/Mn cluster that constitutes the oxygen-evolving center (Sigfridsson *et al.*, 2004, Faller *et al.*, 2005), which results in inhibition of photosynthetic oxygen evolution. Cd also inhibits electron transfer from redox-active tyrosine residues D1-161. On the acceptor side of PSII, Cd^{2+} binds to, or closes to, the Q_B binding site and not in the vicinity of Q_A (Sigfridsson *et al.* 2004). Cd's damage to PSII is found to be strongly dependent on the irradiance conditions (Küpper *et al.* 2002, 2006). In low irradiance including a dark phase, the inhibition of PSII under Cd stress is largely attributed to the function impairment of light harvesting antenna as Mg^{2+} ion in the Chl molecules of the light harvesting complex II is substituted by Cd^{2+}. In high irradiance, Cd direct damage to the PSII reaction centre occurs instead (Küpper *et al.* 2002).

We showed a decrease in the Calvin cycle activity as indicated by the reduction in Rubisco and PEPC activities in the Cd-exposed maize plants. Moreover, the inhibitory influences of Cd stress on the activities of Rubisco and PEPC occurred earlier than on the PSII electron transfer. Di Cagno *et al.* (2001) noted that F_v/F_m ratio did not alter in sunflower plants subjected to Cd treatments for 15 days, but Rubisco activity was reduced, indicating that

Table 1. Effects of Cd on P_N, Chl level, Chl fluorescence parameters, and the activities of Rubisco and PEPC in leaves of maize plants The data of different Cd treatments with the same time of Cd exposure marked by the same letter are not significantly different at p=0.05.

Cd [μM]	Time after Cd exposure [h]	Chl [mg g^{-1} (FM)]			P_N [μmol m^{-2} s^{-1}]	F_o	F_m	F_v/F_m	F_v/F_o	Enzyme activities [μmol min^{-1} mg^{-1} (protein)]	
		Chl *a*	Chl *b*	Total Chl						Rubisco	PEPC
0	24	1.57[a]	0.79[a]	2.35[a]	11.08[a]	422[a]	1825[a]	0.769[a]	3.373[a]	4.6[a]	52.9[a]
5		1.25[a]	0.63[a]	1.88[a]	10.96[a]	379[a]	1802[a]	0.789[a]	3.755[a]	2.8[ab]	41.3[ab]
20		1.44[a]	0.55[a]	1.98[a]	9.83[a]	366[a]	1742[a]	0.789[a]	3.754[a]	2.3[b]	27.4[b]
0	48	1.86[a]	0.59[a]	2.45[a]	12.51[a]	415[a]	2066[a]	0.798[a]	3.975[a]	2.5[a]	64.0[a]
5		1.34[b]	0.60[a]	1.94[b]	8.99[ab]	402[a]	1992[a]	0.796[a]	3.961[a]	1.9[b]	59.2[a]
20		1.18[b]	0.74[a]	1.92[b]	8.28[a]	405[a]	1932[a]	0.789[a]	3.772[a]	1.8b	36.7[b]
0	96	1.70[a]	0.64[a]	2.34[a]	12.18[a]	426[a]	2208[a]	0.807[a]	4.187[a]	2.4[a]	55.6[a]
5		1.26[b]	0.46[b]	1.72[b]	8.61[b]	411	1808[b]	0.772[b]	3.405[b]	1.0[b]	35.9[b]
20		1.15[b]	0.42[b]	1.57[b]	8.36[b]	416[a]	1800[b]	0.769[b]	3.337[b]	0.9[b]	38.4[b]
0	168	1.58[a]	0.58[a]	2.16[a]	13.00[a]	417[a]	1988[a]	0.789[a]	3.764[a]	4.2[a]	67.1[a]
5		1.17[b]	0.43[b]	1.60[b]	8.78[b]	440[a]	1886[a]	0.766[b]	3.290[b]	3.2[b]	33.5[b]
20		1.00[b]	0.36[b]	1.35[b]	5.69[c]	465[a]	1879[a]	0.749[b]	3.042[b]	2.5[b]	41.9[b]

the photosynthetic process is mainly altered by this factor. PEPC in maize leaves was also found to be inactivated by Cd (Iglesias and Andreo 1984, Vojtechova and Leblova 1991). It has been suggested that Cd^{2+} ions lower the activity of Rubisco and damage the structure of Rubisco by substituting for Mg^{2+} ions and may also shift Rubisco activity towards oxygenation reactions (Siedlecka *et al.* 1998). Malik *et al.* (1992) demonstrated that Cd caused an irreversible dissociation of the large and small subunits of Rubisco, which lead to total inhibition of the enzyme. Hajduch *et al.* (2001) observed drastic reductions and fragmentation of Rubisco in Cd-stressed rice leaves.

Pietrini *et al.* (2003) suggested that loss of Rubisco activity under Cd stress could not be fully explained by the decrease of Rubisco content. Cd stress leads to the accumulation of ROS in the plant cells because it reduces glutathione level and inhibits antioxidative enzymes, such as glutathione reductase, catalase, and ascorbate peroxidase (Dietz *et al.* 1999, Schützendübel *et al.* 2001, Romero-Puertas *et al.* 2004, Kučera 2008). Ortega-Villasante *et al.* (2005) observed that oxidative stress occurred on alfalfa plants with 30 μM of Cd treatment just after 6–24 h. The enhanced ROS levels, an early response in Cd stressed plants, are active to oxidize thiol groups in Rubisco protein (Liu *et al.* 2008). Enhanced oxidation of Rubisco protein under Cd stress is correlated with the reduction of photosynthetic activity, indicating that oxidative damage to Rubisco is an important factor lowering the photosynthetic activity in Cd-treated plants. Krupa *et al.* (1993) found that during short term exposure of 10 to 50 μM Cd in the early stages of growth, the Calvin cycle reactions in bean plants are more likely than PSII to be the primary target of the Cd toxic influence. The reduced demand for ATP and NADPH upon Calvin cycle inhibition results in down-regulations of the PSII photochemistry and the yield of linear electron transport. Other researchers also found that Cd stress initially destroyed the photosynthetic carbon reduction cycle and subsequently influenced the photosynthetic electron transport in cucumber plants (Burzyński and Kłobus 2004, Burzyński and Żurek 2007).

In conclusion, Cd-stress resulted in P_N decrease in maize plants, which might be associated with Cd toxicity on the light and the dark stages of photosynthetic activity. The inhibitory effects of Cd stress on the activities of Rubisco and PEPC occurred earlier than on the PSII electron transfer, suggesting that the Calvin cycle rections in maize plants are more likely than PSII electron transfer to be the primary target of the Cd toxic effect.

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