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Effect of Cadmium on Sunflower Growth, Leaf Pigment and Photosynthetic Enzymes

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Abstract: The characteristics of photosynthetic metabolism, during treatment with cadmium, in two stories of sunflower seedlings were investigated. After 7 days of application of the metal, chlorosis and necrosis appeared especially in the first story. These stories approved a high decrease in the amount of Fe and Mg, this decrease was more important in the first story. Pigments were analyzed in the two stories and a significant decrease was recorded. However, no changes observed in the maximal efficiency of PSII photochemistry (Fv/Fm). Furthermore, the majority of carboxylation enzymes showed a decrease of their activity. Results of the present study suggested that the photosynthetic apparatus remained functional after Cd exposition and showed a tolerance for this metal despite effects on growth, mineral nutrition and enzymes activities.

Key words: Chlorophyll fluorescence, *Helianthus annuus*, Cd^{2+} toxicity

INTRODUCTION

Cadmium is considered as major environmental pollutant and its phytotoxicity is well established[1]. The most studies on Cd in plants concern, essentially, uptake[2-3] and translocation mechanism[4-6]. Cadmium toxicity is correlated with disturbances in uptake and distribution of macro and micronutrients in plants[7]. This toxicity is also, related with the increase of lipid peroxydation and alterations in antioxidant systems[8-10].

Photosynthesis is too sensitive to Cd. Chlorophyll being one of the targets[8,11-12] as well as enzymes involved in CO_2 fixation[13-14].

The aims of this work were to characterize the response of *Helianthus annuus* seedlings grown in the presence of two Cd concentrations and to have more information about effects of this metal on photosynthesis in two different leaf stories.

MATERIALS AND METHODS

Plant material and growth conditions: Sunflower seeds (*Helianthus annuus* L. Cv. Airelle) were disinfected with 10% H_2O_2 for 20 min and then washed with distilled water and germinated in the dark for 4 days over two sheets of filter paper moistened with distilled water at 25°C. Seedlings were transferred to full nutrient solution containing; 2 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 30 μM H_3BO_4, 50 μM Fe-K-EDTA,10 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$, 0.03 mM $(NH_4)_6Mo_7O_{24}$, pH 5.7. Plants were grown in a growth chamber (16 h light/ 8 h dark) under mercury lamps, providing a light intensity of 150 μmol m^{-2} s^{-1}, day/ night temperature of 25-20°C and 65 (±5) % relative humidity. The hydroponic cultures were continuously aerated and renewed every three days to minimize pH shift and nutrient depletion. After 10 d, treatments were performed, for 7 d, by adding 0 (control), 50 μM or 100 μM $Cd(NO_3)_2$. The samples designated for plant growth were dried after at least 48 h desiccation at 70°C. The dry weight was used as a measure of growth. Fresh plant material for enzyme determinations was stored in liquid nitrogen until use.

Nutrient analysis and cadmium accumulation: Nutrients content and cadmium in the two stories of leaves were determined on dried material[15]. The samples were dissolved in HNO_3 (65%). Mg, Fe and Cd were analyzed by atomic absorption spectrophotometry (spectrophotometer Perkin Elmer-model 2380).

Leaf pigment analysis: Pigment analysis was performed by HPLC[16]. Leaves of known weight were frozen in liquid nitrogen and stored at -80°C until use. Leaves were ground in cold 100% HPLC-grade acetone (1/10, w/v). The mixture was centrifuged at 500 g for 5 min and thus filtered through 0.2 μm Minisart filter and immediately analyzed. The HPLC pigment separation was performed at room temperature with a ODS column (HP ODS Hypersil: C8, 5 μm particle size, 250x4 mm Ø). The pigments were eluted using 100% solvent (acetonitrile/ methanol, 75/25, v/v).

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The column was allowed to re-equilibrate with the solvent for 10 min prior the next injection. The flow rate was 1 ml min^{-1}. The pigments were detected by their absorbance at 445 nm. To determine the pigment content, known amounts of pure standard were injected into the HPLC system and equation, correlating peak area to pigment concentration, was formulated.

Chlorophyll fluorescence: Chlorophyll fluorescence of photo-system II (PSII) was measured with a Plant Efficiency Analyser (PEA, Hansatech instruments Ltd, P02. 002). The measurements were done at room temperature (25°C). Plants were first adapted for 30 min in total darkness. The maximal fluorescence (Fm) was determined at the beginning of each measurement using a saturating pulse (3000 µmol m^{-2} s^{-1}). The initial fluorescence (Fo) and the maximal fluorescence were determined after dark acclimation. The variable fluorescence (Fv) was taken from the formula: Fv=Fm-Fo and the parameter used were Fv/Fm and 1/Fo-1/Fm.

Enzyme extraction: Samples of leaves were ground at 4°C in a mortar with an extraction buffer containing: 100 mM Tris-HCl, pH 7.5, 25% (W/V) polyvinylpyrrolidone (PVP), 3 mM dithioerythriol (DTT), 1 mM EDTA, 0.05% trition X-100, 5 mM $MgCl_2$, 2 mM $MnCl_2$. The homogenate was centrifuged for 5 min at 15000 g at 4°C. The supernatant was stored at -80°C until use to determine Phosphoenolpyruvate carboxylase (PEPC) and Rubisco activities[17].

For the MDH activity, leaves were ground in a mortar at 4°C with an extraction buffer containing: 20 mM Tris-HCl and 5 mM ascorbic acid. The homogenate was centrifuged for 20 min at 25000g at 4°C. The supernatant was stored at -80°C until use.

Enzyme activities: Phosphoenolpyruvate carboxylase, Rubisco and MDH activities were determined spectrophotometrically (spectrophotometer Shimadzu UV/visible). PEPC and Rubisco were determined by monitoring NADH oxidation at 340 nm, at 30°C in 2 cm^3 reaction mixture. The reaction mixture for Rubisco[18] contained: 100 mM Tris-HCl, pH 8; 40 mM $NaHCO_3$; 10 mM $MgCl_2$; 0.2 mM NADH; 4 mM ATP; 0.2 mM EDTA; 5 mM DTT; 0.2 mM ribulose-1, 5-bisphosphate (RuBP); 1 UE glyceraldehyde 3-phosphodehydrogenase (G3PDH); 1 UE 3-phosphoglycerate kinase (3 PGK). The Rubisco activity was measured after addition of enzyme extract at 30°C. For PEPC[19] the reaction mixture contained: 100 mM Tris-HCl, pH 7.2; 2 mM $NaHCO_3$; 0.2 mM NADH; 6.6 mM $MgCl_2$; 1.6 mM NaF; 1.9 mM DTT; 4 mM phosphoenolpyruvate (PEP); 2 UE dehydrogenase (MDH). Assays of PEPC were initiated by the addition of enzyme extract. Enzymes activities are expressed as µmoles CO_2 (Rubisco) or HCO_3^- (PEPC) degraded per minute in relation to total proteins. The reaction mixture for MDH contained: 0.1 M sodium pyrophosphate, pH 9; 33 mM L-malate Na; 2.5 mM NAD. The reaction was initiated by the addition of enzyme extract and the activity was determined by following the NAD reduction at 340 nm[20].

Total soluble proteins were determined with bovine serum albumin (BSA) as a standard[21].

Statistical analysis: The experiment was repeated at least twice. The significance of differences between control and treatment was determined using the student's t-test at 0.05 level of probability.

RESULTS

Growth, Fe, Mg and Cd concentration in leaf tissues: After 7 days of treatment, cadmium affected dramatically leaves of sunflower. Visual symptoms of Cd toxicity were observed at the first story (S_1) and in the second story (S_2) with the two used concentrations of metal . These symptoms were reduction of leaf area and dry weight, chlorosis and necrosis (Table 1).

Cadmium seemed to be more accumulated in the second story (S_2). In fact, 42% of total cadmium was accumulated in S_1 however, almost 58% was accumulated in S_2 with the two treatments (Table 1).

Mg and Fe concentrations in leaves were significantly decreased by Cd treatment (Fig. 1). The greatest reduction is observed at the higher level of Cd addition. However, the first story seemed to be more affected by the treatment. In fact, Fe concentrations were reduced by 38% in S_1 but this reduction was not significant in S_2 at 50 µM Cd. At the high concentration of metal (100 µM) a significant decrease of Fe was observed at the two stories with 72 % and 62 % of reduction in S_1 and S_2 , respectively (Fig. 1C and D). Mg is also reduced in the two stories, but this reduction seemed to be more intense in S_1 (Fig. 1A and B).

Pigment content: Leaf pigment analysis showed a significant difference in the leaf pigment content between the control and treated leaves. Chlorophyll a and b contents were reduced with the two Cd concentrations. But this decrease appeared more intensive in chlorophyll b at S_1. In fact, the ratio chl a:b was increased significantly with the higher Cd concentration. In S_2, this

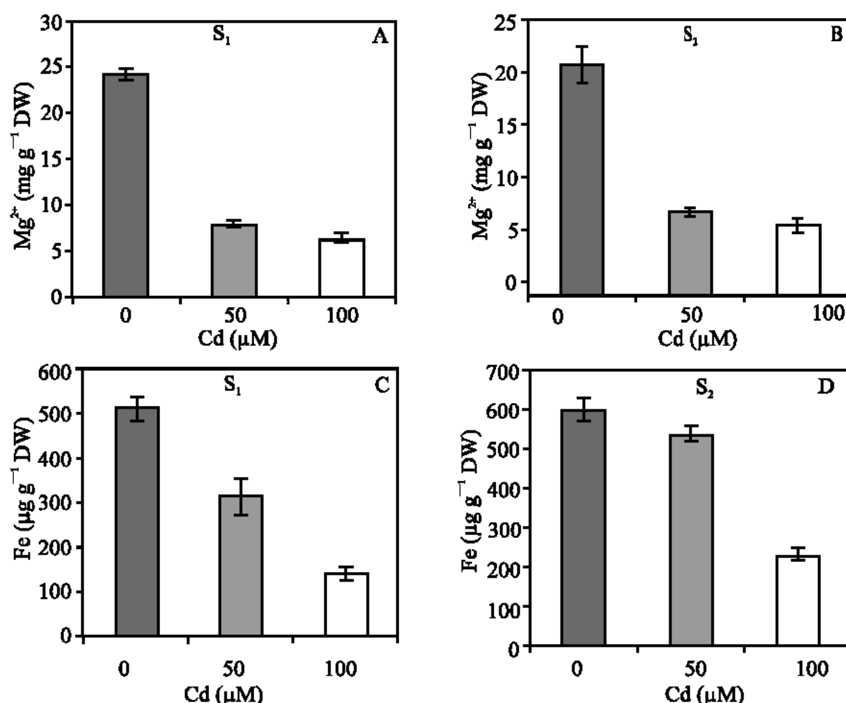

Fig. 1: Effect of cadmium on Mg and Fe content in two leaf stories of 17-day-old sunflower seedlings grown in control medium solution and with two concentrations (50 and 100 μM) added in the medium for 7 days. Data are mean values ± SE of 6 replicates. SE is indicated by bars A and C: first story, S_1, B and D: second story, S_2

Table 1: Dry weight (DW), leaf area and cadmium accumulation in two leaf stories of 17-day-old sunflower plants grown in control nutrient solution (0) and with addition of two concentrations of cadmium for 7 days. Data are mean values ± SE of 6 replicates

	S_1			S_2		
Story treatments	0 (μM)	50 (μM)	100 (μM)	0 (μM)	50 (μM)	100 (μM)
DW (mg)	55.34±0.15	40.42±0.20	32.87±0.20	36.45±0.50	20.08±0.20	15.66±0.10
Leaf area (cm^2)	4.13±0.12	3.61±0.17	2.76±0.15	3.46±0.21	2.58±0.14	1.30±0.12
Cd (μg g^{-1} DW)	-	921.80±90.30	1224.80±28.60	-	1234.90±59.90	1717.90±183.40

Table 2: Evolution of fluorescence parameters measured *in situ* on sunflower leaves of 17 day-old seedlings grown in hydroponic medium in presence of two concentrations of cadmium or without metal addition (0) for 7 days. Data are mean values ± SE of 12 replicates

	S_1			S_2		
Story treatments	0 (μM)	50 (μM)	100 (μM)	0 (μM)	50 (μM)	100 (μM)
Fv/Fm	0.850±0.0011	0.850±0.0013	0.850±0.0014	0.858±0.0007	0.859±0.0016	0.859±0.0016
1/F0-1/Fm	0.002±2 10^{-5}	0.002±1.5 10^{-5}	0.002±1.6 10^{-5}	0.002±1.9 10^{-5}	0.002±4.5 10^{-5}	0.002±5 10^{-5}

ratio was not statistically modified (Fig. 2). β-carotene showed a similar pattern to that of the chlorophylls: In fact, there was a significant reduction of pigment content with the two concentrations and in the two stories. However, this decrease was more severe in S_2 (Fig. 3A and B). The level of xanthophyll was not modified by Cd treatment in S_1 but it was weakly reduced in S_2, only at the high concentration of Cd (Fig. 3C and D).

Chlorophyll fluorescence: The potential photochemical yield after dark adaptation, Fv/Fm, which can be used as a stress indicator[22] was monitored after 7 days of treatment in the two stories. Fv/Fm showed no statistically significant change after treatment with Cd in the two stories and with the two treatments (Table 2).

No statistically significant change was noted in 1/Fo-1/Fm, which can inform in the excitation-energy trapping capacity at the reaction center of PSII[23].

Enzymes activities: Activity of Rubisco (Fig. 4A and B) was significantly decreased in sunflower leaves submitted to Cd treatment in the two stories. This enzyme showed the maximum decline in its activity from 50 μM Cd. On the contrary, PEPC activity (Fig. 4C) was weakly stimulated in

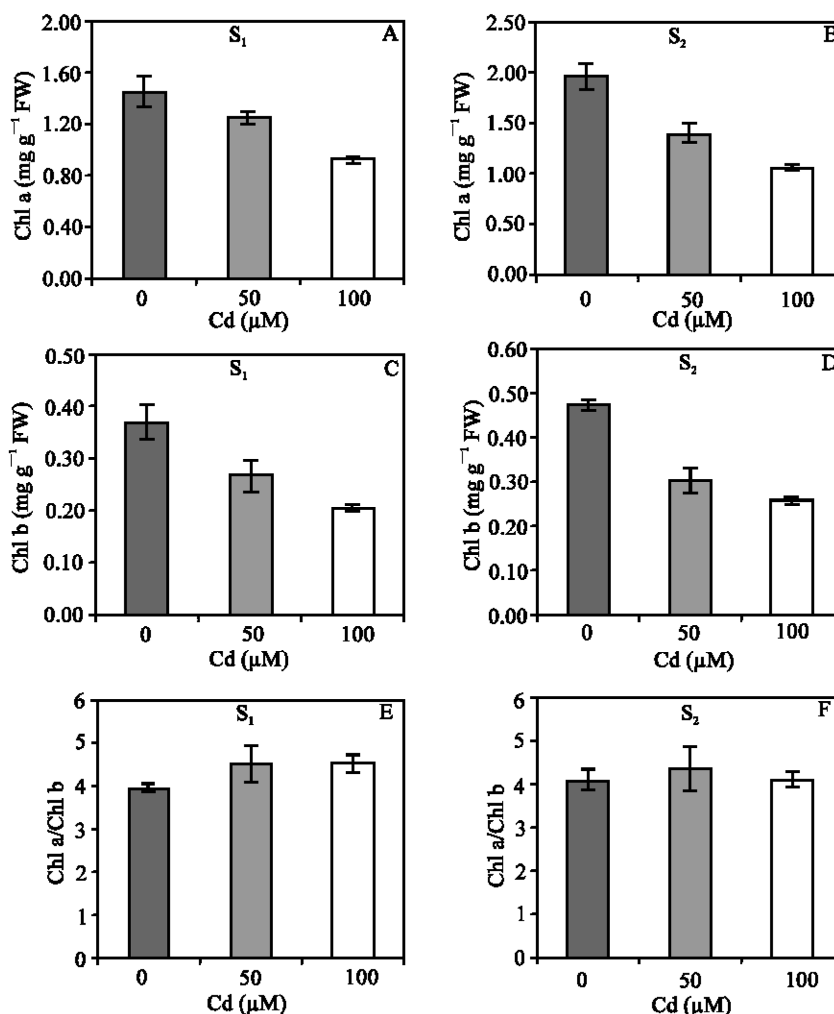

Fig. 2: Effect of cadmium on chlorophylls in two leaf stories of 17-day-old sunflower seedlings grown in absence or in presence of two concentrations of cadmium for 7 days. Data are mean values ± SE of 6 replicates. SE is indicated by bars A and B: chlorophyll a , C and D: chlorophyll b, E and F: chlorophylla/ chlorophyll b

S_1 at 50 μM. In the same story, this activity was not modified at 100 μM Cd. In the second story, this enzyme had a different response to S_1 and its activity tended to be decreased by Cd treatment. This effect was only statistically significant at the high concentration of metal used (Fig. 4D).

Activity of MDH was strongly affected by Cd treatment. In fact, its activity showed a severe decrease after exposure to cadmium. This decrease was more important in the first story (Fig. 4E and F).

DISCUSSION

Exposure to 50 and 100 μM Cd reduced leaf growth of sunflower seedlings. The two leaf stories showed a decrease of their weight and leaf area. Morphological toxicity symptoms were observed, especially, at high Cd concentration in the first story of leaves. Growth reduction due to Cd may result from decreased photosynthesis and impaired mineral nutrition.

Cadmium exposure of *Helianthus annuus* plants led to substantial changes in nutrient composition in the two leaf stories. In fact, leaves of plants grown in the presence of Cd contained less Fe than control leaves. Decrease of this nutrient could be the major origin of necrosis and chlorosis appeared in S_1. Iron deficiency was reported in several plant species treated by cadmium[7,24,25]. Mg, was also reduced by Cd treatment in the leaves. Cd inhibits Mg transport to the shoots of sugar beet and could be the cause of leaf area decrease and loss of chlorophyll[24].

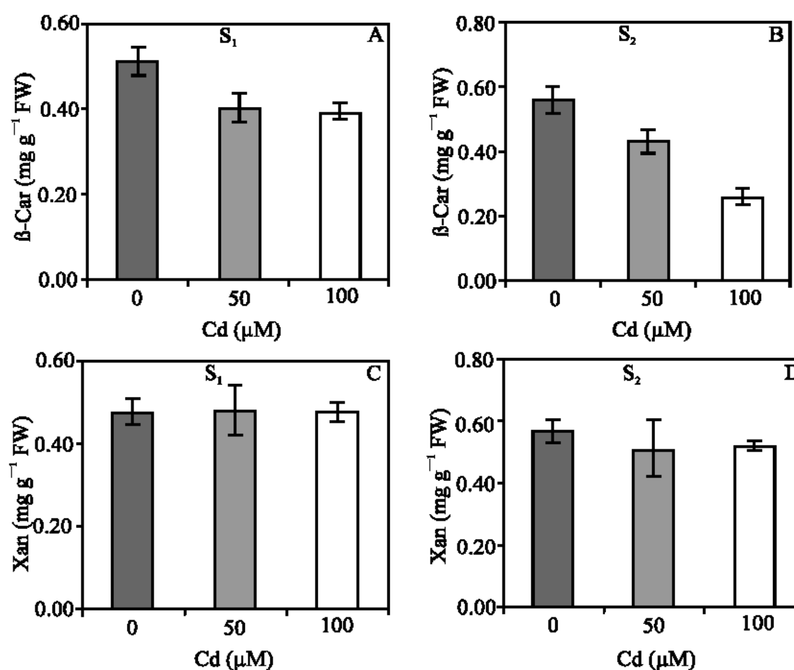

Fig. 3: β-carotene and xanthophyll contents in two leaf stories of 17-old-day sunflower plants grown in control nutrient solution and supplemented with 50 or 100 μM of cadmium for 7 days. Data are mean values ± SE of 6 replicates. SE is indicated by bars A and B: β-carotene, C and D: xanthophyll

Decrease of leaf growth could be due to competition between Cd and essential cations, leading to deficiency of these ions[7].

Cadmium decreased the content of both chlorophylls and β-carotene. This decrease of chlorophyll should be due to inhibition of protochlorophyllide reductase and inhibition of aminolevulinic acid synthesis[11]. Effect of Cd on chlorophyll content has been reported in several works[15,26-30]. However, the percentage decrease in chl b was larger than that for chl a at the higher Cd concentration in the first story. In fact, an increase of chl a:b ratio was recorded. In the second story and in the first one at the low concentration no significant change in this ratio was observed. Furthermore, the reduction of chlorophyll content seemed to be more intensive in S_2 than in S_1 which can be explained by the high accumulation of the metal at this story. It is suggested that Cd inhibits chlorophyll biosynthesis via inhibition of enzymes implicated in this biosynthesis[12,31] more than as a result of nutrients deficiency. In fact, Cd was more accumulated in the second story and its interaction with SH groups could possibly explain the inhibition of protochloropyllide reductase. The same results were obtained for β-carotene, with a high reduction at the second story (S_2). Several reports have shown the decrease of carotene after Cd treatment in several plants[15,28,29,32-36]. Furthermore, Mg^{2+} deficiency caused significantly lower levels in the chlorophyll and carotenoid contents[37]. Cd also produced a decrease in the content of xanthophyll, although this decrease was only observed in S_2 at the high concentration. This pigment seemed to be less sensitive for Cd treatment than chlorophylls and carotene. Thus, degradation of pigments or inhibition of their biosynthesis have been proposed as being responsible for the photosynthesis reduction produced by the metal[8,33].

The maximal potential photochemical efficiency of PSII under dark-adapted conditions (Fv/Fm) and the excitation-energy trapping capacity at the reaction center of PSII (1/Fo-1/Fm) were not significantly affected by cadmium treatment. Similar results were presented by several authors[13,26,28,30,34,35]. These results suggested that Cd don't affect the photochemical efficiency and the capacity of trapping excitation energy by PSII reaction centers.

Some enzymes of the photosynthetic carbon reduction cycle are sensitive to Cd and could also be responsible for the productivity decrease[14]. The most important enzyme, Rubisco, was studied in this work to clarify the mechanism of action of Cd on photosynthesis. Results obtained showed a high inhibition in activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase.

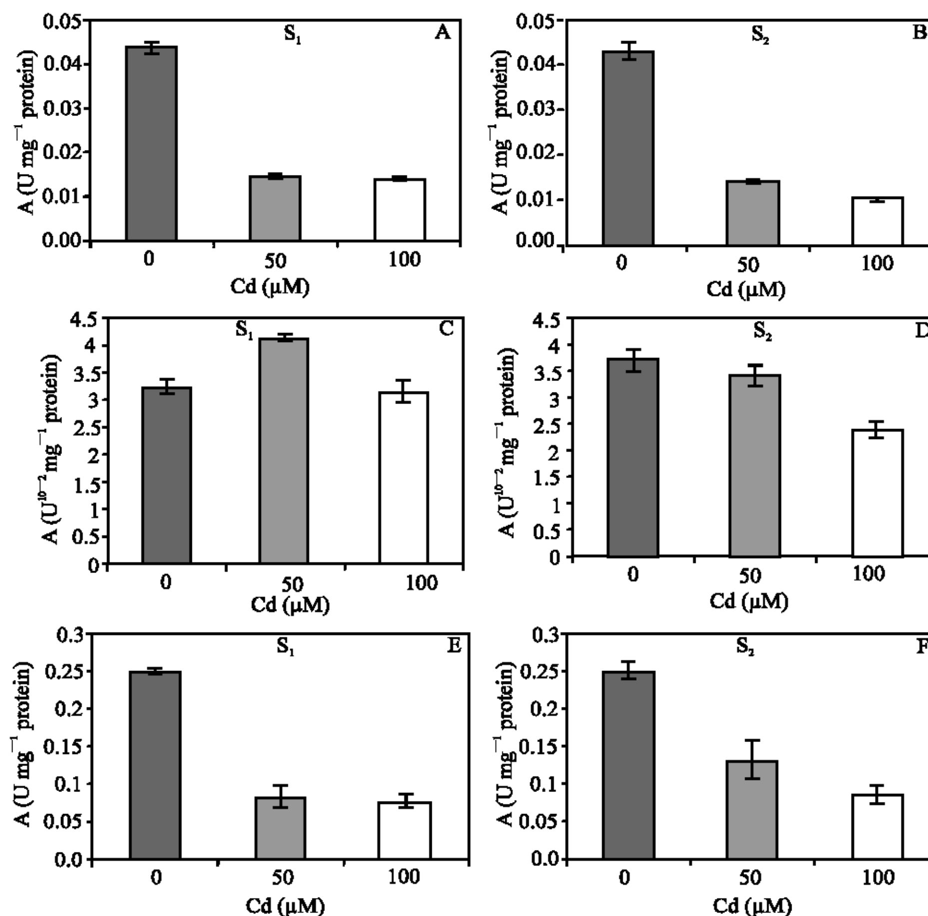

Fig. 4: Activity of some enzymes in two leaf stories of 17-old-day sunflower seedlings grown in control medium solution and added with 50 or 100 µM of cadmium for 7 days. Data are mean values ± SE of 4 replicates. SE is indicated by bars A and B: activity of Rubisco in S_1 and S_2, respectively; C and D: activity of PEPC in S_1 and S_2, respectively; E and F: activity of MDH in S_1 and S_2, respectively

Several authors indicated that the activity of Rubisco decreased, though to different extent, upon the Cd treatment[27-30,38-44]. This enzyme seems to be very sensitive to Cd treatment, in fact it was considerably affected from the little concentration of the metal. However, PEPC was less sensitive to Cd treatment. In S_1, it showed a small increase of its activity at 50 µM and not modified at 100 µM. This little stimulation of PEPC activity could be responsible of malate accumulation. In the second story, its activity was reduced significantly at the high concentration of the metal. However, the role of this enzyme in C_3 plants is uncertain[45]. Suggestions have included a role in recapturing respiratory carbon, in synthesizing four-carbon acids, principally malate and aspartate. MDH, another photosynthetic enzyme, was increasingly depressed with Cd treatment. So it can be suggested that Cd^{2+}exerted a profound deleterious effect on photosynthetic enzymes.

In conclusion, this idea improved that cadmium is a toxic metal for plants. It induces some physiological and metabolic disturbances. Its toxicity seems to be more important in the first story (mature leaves). In fact, a dramatic decrease of pigment and enzyme activities were recorded in this story. This negative effect can induce a strong inhibition of plant growth, specially of the young leaves.

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