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Changes in Activities of Nitrogen Metabolism Enzymes in Cadmium Stressed Marrow Seedlings

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Abstract: Plants of marrow (*Cucurbita pepo* L.) were grown in a controlled environmental chamber and were submitted to different concentrations of cadmium (Cd) applied to NO₃⁻ the nutrient solution at 0, 10, 20, 30, 40 and 50 µM. The activities of primary nitrogen enzymes as well as ammonium ion contents were investigated in both roots and shoots of marrow seedlings. Toxic cadmium ion accumulation in seedlings significantly modified activity of primary nitrogen assimilating enzymes. There were significant decreases in glutamine synthetase (GS; EC 6.3.1.2), glutamate synthase (GOGAT; EC 1.4.1.13), nitrate reductase (NR; EC 1.6.6.1) and nitrite reductase (NiR; EC 1.6.6.4) activities in both root and shoot of the seedlings. In contrast, the same previous Cd concentrations expressed significant increase in glutamate dehydrogenase (GDH; EC 1.4.1.2) activity in both shoot and root of the same seedlings. Corresponding with the increase in glutamate dehydrogenase activity, ammonium ion contents in shoots of cadmium treated seedlings, showed significant increase with the increase in cadmium concentration. However, ammonium ion contents of roots of the same seedlings, exhibited significant increase up to 30 µM Cd only. These results suggest that, in general, treatment with Cd²⁺ affect nitrogen assimilation and metabolism to a great extent in both roots and shoots of marrow seedlings.

Key words: Heavy metal, nitrogen metabolism, enzyme activity, ammonium content

INTRODUCTION

Nowadays the heavy metal pollution of the soil is causing ever greater problems, exacerbated by the fact that the heavy metals accumulated in plants may, either directly or indirectly, find their way into animals and human beings. Cadmium (Cd) is one of the most important metals in terms of food-chain contamination, because it is readily taken up by the cells of different plant species (Gomes-Junior *et al.*, 2006; Liu *et al.*, 2007).

Cadmium has been shown to cause many morphological, physiological, biochemical and structural changes in plants, such as growth inhibition, water imbalance and inhibition of seed germination (Benavides *et al.*, 2005; Mishra *et al.*, 2006). The bases of Cd toxicity are still not completely understood, but might result from its high affinity for sulfhydryls e.g., threefold higher than Cu ions (Schutzendubel and Polle, 2002). Cd binds to sulfhydryl groups of structural proteins and enzymes, leads misfolding, inhibition of activity and/or interference with redox-enzymatic regulation (Hall, 2002). Another important toxicity mechanism is due to the chemical similarity between Cd²⁺ and functionally active ions situated in active sites of enzymes and signaling

components. Thus, Cd²⁺ ions can interfere with homeostatic pathways for essential metal ions (Roth *et al.*, 2006) and the displacing of divalent cations, such as Zn and Fe, from proteins could cause the release of free ions, which might trigger oxidative injuries via free Fe/Cu-catalyzed Fenton reaction (Polle and Schutzendubel, 2003).

The growth and yield of a plant often depend on N supplementation (Mattson *et al.*, 1991; McDonald *et al.*, 1996; Lopez-Cantarero *et al.*, 1997) in order to form amino acids, proteins, nucleic acids and other cell constituents needed for development (Srivasankar and Oaks, 1996). Glutamine synthetase (GS; EC 6.3.1.2) is the central enzyme in ammonium assimilation in plants (Lam *et al.*, 1995, 1996). Glutamine Synthetase (GS) is considered key to NH₄⁺ assimilation and catalyses glutamine synthesis from glutamate, ATP and NH₄⁺. In contrast, the enzyme that catalyzes glutamate synthesis is NADH-glutamate synthase (GOGAT; EC 1.4.1.13) and generally less active than GS (Baker *et al.*, 1998).

Ammonium can be directly incorporated into glutamate by the aminating reaction of glutamate dehydrogenase (NADH-GDH; EC 1.4.1.2). Since GDH reversibly deaminates glutamate to NH₄⁺ and

2-oxoglutarate, the physiological role of GDH *in vivo* remains controversial (Ireland and Lea, 1999). Studies on source-sink relations have shown that GDH is induced in old leaves when nitrogen remobilization is maximal (Srivastava and Singh, 1986; Msclaux *et al.*, 2000). This led to the proposal that the physiological role of GDH is to synthesize glutamate for translocation in senescing leaves (Mifflin and Habash, 2002). However, there is no evidence to discern a reductant or indispensable role of GDH and GOGAT for glutamate synthesis and nitrogen remobilization. In addition, GDH catalyzes the reversible oxidative deamination of glutamate to supply 2-oxoglutarate and ammonium (Aubert *et al.*, 2001).

Plants utilize nitrate, ammonium and dinitrogen (N₂) molecules as external nitrogen sources. Ammonium is the final form of inorganic nitrogen prior to the synthesis of organic nitrogen compounds (Ireland and Lea, 1999). Some studies support the involvement of GDH in the assimilation of ammonium produced in stress conditions like heavy metallic stress such that induced by Cd (Boussama *et al.*, 1999; Dominguez *et al.*, 2003; Kwinta and Kolik, 2006). Generally, those conditions bring about disturbances on the activities of enzymes involved in the ammonium assimilation such as an inhibition of GS and GOGAT (Gouia *et al.*, 2000; Chien *et al.*, 2002; Kwinta and Cal, 2005).

The first stage in NO₃⁻ assimilation is the reduction to NO₂⁻ by nitrate reductase (NR; EC 1.6.6.1), this stage being the most prone to regulation and in turn, being limiting for NO₃⁻ (Srivastava and Oaks, 1996; Huber *et al.*, 1996; Ruiz *et al.*, 1998). The next step in NO₃⁻ assimilation is the conversion of NO₂⁻ to NH₄⁺ by nitrite reductase (NiR; EC 1.6.6.4) (Srivastava and Oaks, 1996; Migge and Becker, 1996). In different plant species (e.g., tomato, maize, pea and barley) Cd alters the activity of different enzymes involved in nitrogen metabolism (Nussbaum *et al.*, 1988; Boussama *et al.*, 1999). At the root level, the reduction of nitrate absorption may be due to transpiration inhibition. Moreover, both the nitrate reductase and nitrite reductase activities in roots and leaves are affected (Chaffei *et al.*, 2004) as well as nitrite transport from roots to shoots (Di Toppi and Gabbriellini, 1999) leading to a reduced nitrate assimilation by the whole plant.

In the present investigation we study the toxic effect of Cd on nitrogen assimilating enzymes and accumulation of ammonium ion in both roots and shoots of marrow plants.

MATERIALS AND METHODS

Seeds of marrow (*Cucurbita pepo* L.) obtained from Agricultural Research Center, Ministry of Agriculture,

Giza. This project was conducted from 2009 to 2010. Healthy seeds were surface sterilized in 10% H₂O₂ for 20 min followed by repeated washing with distilled water and germinated on wet filter paper at 25°C in the dark for 3 days. Subsequently plants were cultivated hydroponically in a growth chamber under controlled conditions at temperature of 28/22°C (day/night) and 16/8 h photoperiod.

After 3 days of growth, Cd was applied in the form of cadmium chloride (CdCl₂) diluted in a 100 μM NO₃⁻ solution at different concentrations (0, 10, 20, 30, 40 and 50 μM Cd²⁺). After 24 h of Cd treatment, the plants were harvested and immediately separated into shoots and roots. The roots were washed with cold distilled water repeatedly and dried with filter paper.

Biochemical measurements

Measurement of NR and NiR and GOGAT activities

Extraction: Known fresh weights of the plant organs (root and shoot) were ground, with a ratio of 1: 10 (w/v), in a mortar at 0°C in 50 mM KH₂PO₄ buffer, pH 7.5, containing 2 mM EDTA, 1.5 % (w/v) soluble casein, 2 mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpyrrolidone (PVP). The homogenate was filtered and then centrifuged at 3,000 g for 5 min, after which time the supernatant was centrifuged at 30,000 g for 20 min. The resulting extract was used to measure enzyme activities (nitrate reductase, nitrite reductase and glutamate synthase). The extraction medium was optimized for the enzymatic activities.

Assay of NR (EC 1.6.6.1): NR assay followed the methodology of Kaiser and Lewis (1984). In a final volume of 2 mL, the reaction mixture contained 100 mM buffer KH₂PO₄, pH 7.5, 100 mM KNO₃, 10 mM cysteine, 2 mM NADH and enzyme extract. For the NR assay, the incubation was carried out at 30°C for 30 min and the reaction was terminated by the addition of 1000 mM zinc acetate. The nitrite formed was colorimetrically determined at 540 nm after azocoupling with sulfanilamide and naphthylethylenediamine dihydrochloride as described by Hageman and Hucklesby (1971). The NR activity was expressed as % in relation to control.

Assay of NiR (EC : 1.6.6.4): NiR activity was determined by the disappearance of NO₂⁻ from the reaction medium (Lillo, 1984). The reaction mixture contained 50 mM buffer KH₂PO₄, pH 7.5, 20 mM KNO₂, 5 mM methylviologen, 300 mM NaHCO₃ and 0.2 mL of enzyme extract. After incubation at 30°C for 30 min, the nitrite content was determined colorimetrically as above (Hageman and Hucklesby, 1971) and expressed as % in relation to control.

Assay of GOGAT (EC 1.4.1.13): GOGAT activity was assayed spectrophotometrically as described by Chen and Cullimore (1988). The assay mixture in a 3 mL final volume, consisted of 40 mM potassium phosphate buffer (pH 7.5), 10 mM L-glutamine, 10 mM 2-oxoglutarate, 0.14 mM NADH and enzyme extract. The control lacked glutamine, NADH and 2-oxoglutarate. Reaction was started with the addition of enzyme. The oxidation of NADH was observed at 340 nm and the activity is expressed as % in relation to control.

Measurement of total GS (EC 6.3.1.2) activity: GS activity in both root and shoot samples was determined according to a modification of the standard biochemical assay (Canovas *et al.*, 1991). Aliquots (200 μ L) of extract were added to 660 μ L of a reaction mix (100 mM Tris-HCl, 20 mM $MgSO_4$, 30 mM glutamate, 6 mM NH_2OH , 6 mM aspartate, 4 mM EDTA and 12 mM ATP, pH 7.6), at 26°C. After 10 min, the reaction was terminated with 860 μ L of stop solution (0.37 M $FeCl_3$, 0.2 M trichloroacetic acid and 0.67 M HCl). After centrifugation for 15 min at 16,000 g, supernatants were used for the colorimetric determination of γ -glutamylhydroxamate at 540 nm after complexation with acidified ferric chloride. Blanks were treated like the samples, but without the addition of ATP. The values were expressed as % in relation to control value.

Measurement of GDH (EC 1.4.1.2) activity: GDH was assayed spectrophotometrically, at 30°C by monitoring the oxidation of NADH at 340 nm essentially as indicated by Singh and Srivastava (1986). The reaction mixture contained 150 μ mol NH_4Cl , 1 μ mol $CaCl_2$, 0.3 μ mol NADH, 20 μ mol 2-oxoglutarate and 100 μ mol Tris buffer (final pH 8.2 and final volume 1 mL). Control without NH_4^+ and without 2-oxoglutarate to correct for endogenous NADH oxidation.

Quantification of ammonium: NH_4^+ was analyzed from an aqueous extract of 0.2 g of dried and ground plant material in 10 mL of distilled water. NH_4^+ was measured using the method of Bewthelot reaction (Hoshida *et al.*, 2000). Equal amount (50 μ L) of plant extracts and phenol reagent (1% phenol, 0.005% sodium nitroprusside) were mixed. After addition of alkaline sodium hypochlorite reagent (in final concentrations of 0.5% NaOH, 0.042% sodium hypochlorite), the mixture was incubated at 37°C for 20 min and the absorbance at 625 nm was determined. Ammonium contents in the samples were calculated as % in relation to control. The values are means of three replicates \pm SE.

RESULTS AND DISCUSSION

In the present experiment, we have examined the effect of Cd on developing marrow seedlings after 24 h treatment of hydroponics culture.

Nitrate assimilating enzymes: A negative correlation was found between the NR activity and Cd concentrations (Fig. 1). Cd reduced substantially the NR activity in both shoots and roots of the examined plant at all concentrations used. The Cd inhibited the NR activity in shoot by 60% and in root by 80% at 50 μ M Cd^{2+} treatment.

NiR activity, which converts NO_2^- to NH_4^+ , showed a trend similar to that of NR (Fig. 2). With an increase in Cd concentrations, a progressively greater decrease in NiR activity in both plant organs (shoot and root), was observed. The activity of NiR was declined to 58% in shoot and to 38% in root with application of 50 μ M Cd^{2+} .

Ammonium assimilating enzymes: The activities of GS and GOGAT followed a similar pattern in both shoot and root of seedlings (Fig. 3, 4). Both GS and GOGAT activities, showed decrease to a greater extent in shoots and roots of marrow seedlings under Cd stress. The magnitude of decrease was most pronounced with 50 μ M

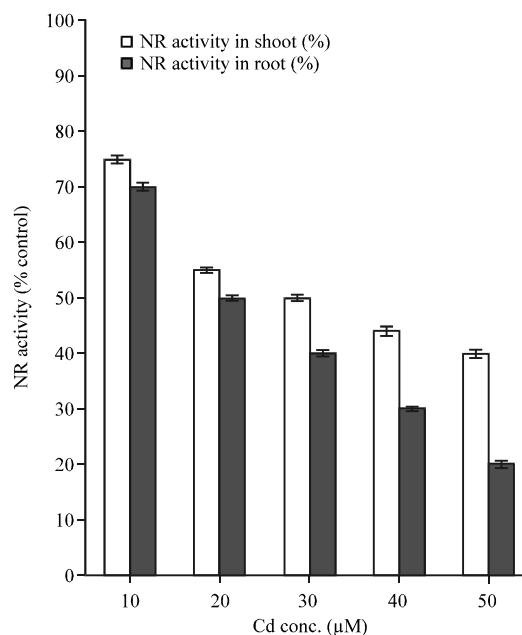


Fig. 1: Changes in NR activity in the shoots and roots of 7 days old marrow seedlings, grown with NO_3^- as N source after 24 h of exposure to different Cd concentrations

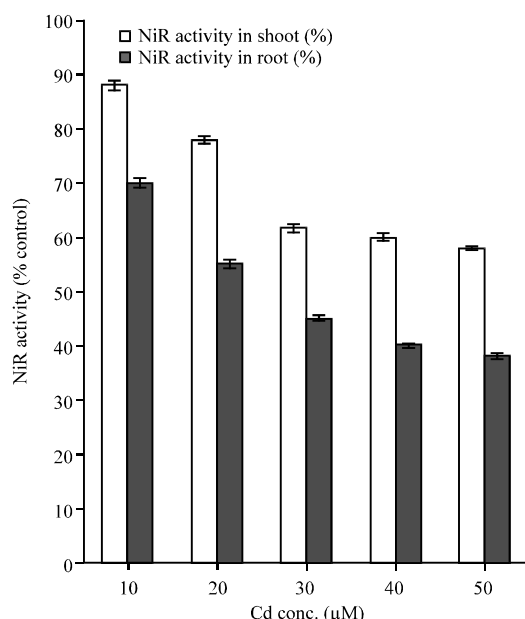


Fig. 2: Changes in NiR activity in the shoots and roots of 7 days old marrow seedlings, grown with NO_3^- as N source after 24 h of exposure to different Cd concentrations

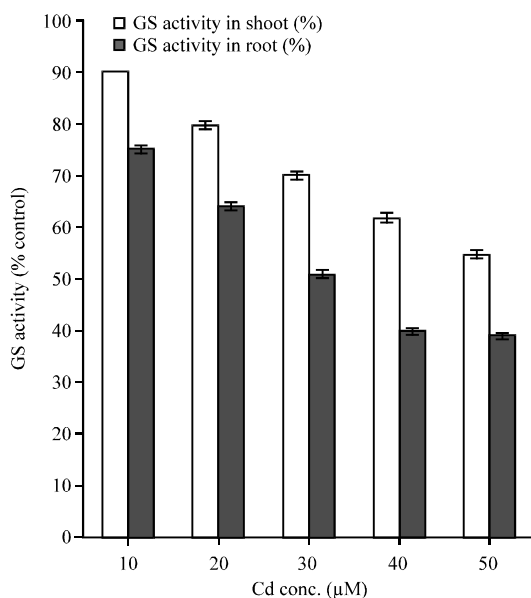


Fig. 3: Changes in GS activity in the shoots and roots of 7 days old marrow seedlings, grown with NO_3^- as N source after 24 h of exposure to different Cd concentrations

Cd^{2+} , whereas the activity of GS was inhibited by 45% in shoot and 61% in root. The inhibition effect of Cd was

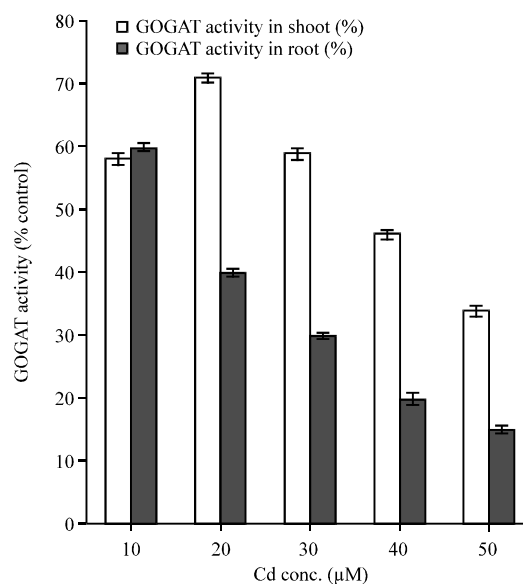


Fig. 4: Changes in GOGAT activity in the shoots and roots of 7 days old marrow seedlings, grown with NO_3^- as N source after 24 h of exposure to different Cd concentrations

more toxic on GOGAT activity, as it declined by 66% in shoot and 85% in root as compared with control.

Glutamate metabolism: The deaminating activity of GDH in both shoot and root of 7 days old marrow seedlings after 24 h Cd stress with different concentrations, was higher substantially than the control (Fig. 5).

It is apparent from Fig. 5 that the values of GDH activities increased to a greater extent under Cd stress. The deaminating activity GDH enhanced by 200% and 140% in shoot and root of plants treated by 50 $\mu\text{M Cd}^{2+}$, as compared with control, respectively.

Ammonium content: Cd application for 24 h, increased ammonium content substantially in both shoot organs (shoot and root) of marrow seedling (Fig. 6). The ammonium content in shoots of seedlings treated with increasing concentrations of Cd, showed, in general, high significant increases. In addition, the ammonium content in roots showed variable increase in response to the different concentrations of Cd. The most effective concentration of Cd which led to the highest ammonium content was 30 $\mu\text{M Cd}^{2+}$.

Most fast-growing plant reduce nitrate in their leaves where the main part of reducing power arises directly from light via ferredoxin (Beevers and Hageman, 1980). Nitrate is reduced to NH_4^+ by the plant enzymes NR and NiR.

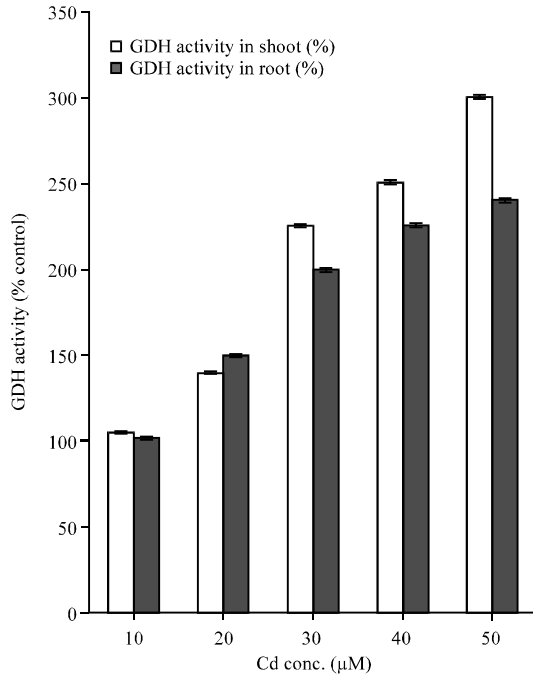


Fig. 5: Changes in GDH activity in the shoots and roots of 7 days old marrow seedlings, grown with NO_3^- as N source after 24 h of exposure to different Cd concentrations

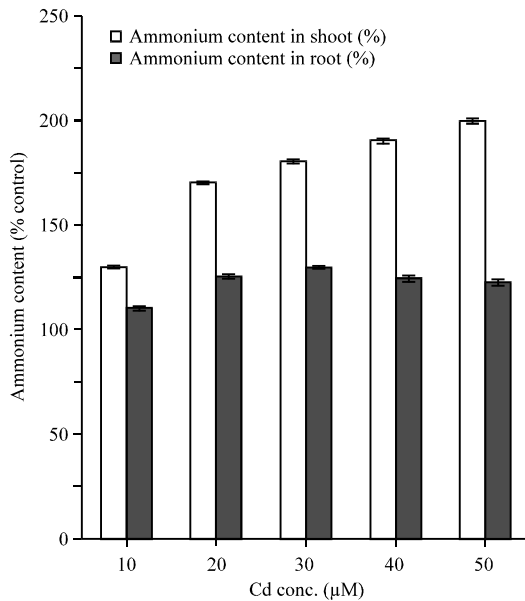


Fig. 6: Changes in levels of ammonium content in the shoots and roots of 7 days old marrow seedlings, grown with NO_3^- as N source after 24 h of exposure to different Cd concentrations

Since NH_4^+ is toxic (Britto Dev and Hubert, 2002) it must be rapidly assimilated into non-toxic metabolites. It is converted to glutamine and glutamate by the enzymes GS and GOGAT (Ireland and Lea, 1999). Even though the GS/GOGAT pathway is the major route in higher plants, the reversible amination of 2-oxoglutarate to yield glutamate. Cd as do other heavy metals, imbalances the water uptake and nutrient metabolism (uptake, transport and use) at the root level interfering with the uptake of Ca, Mg, K and P (Benavides *et al.*, 2005). The inhibition of the root Fe (III) reductase induced by Cd leads to a Fe (II) deficiency in cucumber and sugarbeet (Alcantara *et al.*, 1994).

Many opinions on the change of nitrate content and on the activity of particular enzymes of nitrate metabolism were stated in the presence of heavy metals. Yevdokimova (1994) found that nitrate is accumulated in plants grown in soils with high contents of heavy metals.

The incorporation of Cd into the nutrient solution at different concentrations, led to greater progressively significant decrease in activity of NO_3^- assimilating enzymes (NR and NiR) as clear in Fig. 1 and 2.

The nitrate present in plant cell cytoplasm participates directly in the expression of the NR gene and thus influences the NR induction (Hoff *et al.*, 1992; Tischner *et al.*, 1993). Burzynski (1988) supposed that heavy metals and especially Cd, inhibit nitrate translocation from xylem to cytoplasm. The reduced NR activity in organs of plant photosynthesis is frequently related to the appearance of water deficit in heavy metals contaminated plants (Rauser and Dumbroff, 1981).

Studies of the effects of Cd on N assimilation have frequently constitutes the rate-limiting step of N assimilation catalyzed by nitrate reductase (Gouia *et al.*, 2000). In general, heavy metals toxicity is attributed to binding of heavy metal to enzymes, resulting in alteration and inhibition of metabolism (Assche and Clijsters, 1990). Enzymes of nitrogen metabolism have generally been shown to lose their activity to different extends during Cd stress (Chaffei *et al.*, 2006b).

In different plant species (e.g., tomato, maize, pea and barley) Cd alters the activity of different enzymes involved in nitrogen metabolism (Nussbaum *et al.*, 1988; Boussama *et al.*, 1999). At the root level, the reduction of nitrate absorption may be due to transpiration inhibition. Moreover, both the nitrate reductase and nitrite reductase activity in roots and leaves are affected (Chaffei *et al.*, 2004) as well as nitrate transport from roots to shoots (Di Toppi and Gabbrielli, 1999) leading to a reduced nitrate assimilation by whole plant.

Both NH_4^+ produced in the plant by the reduction of NO_3^- and the supply via fertilizer application are incorporated into organic forms primarily by the enzyme

GS (Srivasankar and Oaks, 1996; Migge and Becker, 1996). This enzyme catalyzes the conversion of glutamate to glutamide. Afterwards, GOGAT catalyzes the reduction of the amide group from glutamine formed by GS to 2-oxoglutarate in order to form two glutamate molecules. One of the glutamate molecules can be incorporated as a substrate for the reaction of GS (Srivasankar and Oaks, 1996). In present experiment, the activities of GS and GOGAT were progressively greater decreased in both organs of marrow seedlings treated by increasing dosages of Cd²⁺.

A significant reduction of GS activity by Cd in various plant species and its complete inhibition in sugar beet plants (Popovic *et al.*, 1996) is sometimes explained by Cd ability to form inactive complexes (the O-, N-, or S-ligands) of natural proteins, inhibiting thus their activities (Assche and Clijsters, 1990). The activity of the enzymes responsible for the incorporation of ammonium molecules into the carbon skeleton (i.e., glutamine synthetase and glutamate synthase) is also compromised (Chaffei *et al.*, 2004).

In the present experiment, Cd enhanced GDH activity in both shoot and root of marrow seedlings (Fig. 5). The stimulation of GDH in response to Cd observed in bean plant grown with two types of N source, has been noted equally by Boussama *et al.* (1999), Assche and Clijsters (1990) and Papazoglou *et al.* (2005). This stimulation of GDH activity under Cd stress resulted to increase of GDH protein content and in induction of the transcription of GDH gene accompanied by an increase of ARNm content (Chaffei *et al.*, 2006a).

On the other hand the activity of the glutamate dehydrogenase (GDH) is enhanced during Cd-stress (Boussama *et al.*, 1999). Because high activity of GDH enzyme has been related with pathogen response and senescence induction (Osuji and Madu, 1996; Msclaux *et al.*, 2000) and changes in nitrogen metabolism due to Cd stress are similar to the ones induced during senescence, it has been hypothesized that Cd induces senescence-like symptoms at least in tomato leading to nitrogen mobilization and a storage strategy (Chaffei *et al.*, 2004).

Some studies support the involvement of GDH in the assimilation of ammonium produced in stress conditions like heavy metallic stress such that induced by Cd (Chaffei *et al.*, 2003; Masclaux-Daubresse *et al.*, 2006). Generally, those conditions bring about disturbances on the activities of enzymes involved in the ammonium assimilation such as an inhibition of GS (Kamachi *et al.*, 1991) and GOGAT (Singh and Srivastava, 1986; Gouia *et al.*, 2000).

The present results demonstrated a concentration dependent effects of Cd on NH₄⁺ content, being stimulated by increasing Cd concentration in both organs of marrow seedlings. With respect to NH₄⁺, the accumulation of this ion was probably due to a direct effect of the application of heavy dosages of N, this perhaps being one of the factors responsible for the sharp fall in fruit yield (Sanchez *et al.*, 2004). Toxicity in NH₄⁺ can result from the following causes: (1) induced nutrient deficiency, blocking ion uptake; (2) inhibited secondary growth, acidifying the root zone; (3) altered intercellular pH and osmotic balance; (4) decoupled electron transport and photophosphorylation, followed by NH₄⁺ accumulation in leaves and (5) altered polyamine and phytohormone metabolism (Gerendas *et al.*, 1997).

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