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RESEARCH ARTICLE

ADJUSTMENTS OF NITROGEN AND CARBON METABOLISMS WERE THE MAINLY STRATEGY FOLLOWED IN AMMONIUM-FED TOMATO TO ALLEVIATE CADMIUM TOXICITY

Afef Hajaji Nasraoui*, Houda Maaroufi Dguimi, Donia Bouthour, Houda Gouia and Chiraz Chaffei Haouari

Research Unit of Nitrogen Nutrition and Metabolism and Stress-related Proteins (99/UR/C 09-20), Faculty of Sciences of Tunis, University of Tunis El Manar, 1060 Tunisia

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ABSTRACT

It was known that cadmium (Cd) toxicity evoked protective reactions that could induce cell death. But the question arises was the effect of nitrogen regime on Cd plant responses. To got more explications, we examined the effects of Cd in NH_4^+-fed tomato plants treated continuously or transitory by 25µM of $CdSO_4$. Reduction of glutamine synthetase (GS) activity by Cd disappeared progressively after transfer of Cd-stressed tomato to control medium. It has been shown that Cd enhanced activity and protein accumulation of cytosolic isoenzyme (GS1) and reduced those of chloroplastid isoenzyme (GS2). When Cd-treated tomato plants were transferred on control medium, GS1 protein level diminished. Whereas, GS2 protein level remained unchanged. Our data showed that Cd stimulated the seven isoenzymes activities of glutamate deshydrogenase (NADH-GDH). Cadmium reduced too, phosphoenolpyruvate carboxylase (PEPC) and isocitrate dehydrogenase (ICDH) activities. Thus, we suggested that CO_2 anaplerotic fixation into organic acids was secondary in leaves. Especially as photosynthetic rate (Amax) and photochemical quenching (qp) were stimulated and non-photochemical quenching was reduced (NPQ) by Cd in leaves of NH_4^+-fed tomato.

INTRODUCTION

Cadmium (Cd) is a metal found in soil which is extracted as part of zinc deposits. It is widely used in the industry of plastics and as a component of batteries. It constitutes an industrial and environmental pollutant released as air contaminant from fertilizers and, more prominently, in the form of wastewater. Cd is not essential for the human body and has no known useful biological functions. Cd is classified in group I of carcinogens by the International Agency of Research on Cancer (IARC, 1993). Soil contamination with heavy metals has become a worldwide problem, leading to losses in agricultural yield and hazardous health effects as they enter the food chain (Guo and Marschner. 1995; Salt *et al*., 1995). Man's energy and chemical consumption is the main cause of trace element pollution in the biosphere. Cadmium negatively affects photosynthesis and growth (Khan *et al*., 2006; Mobin and Khan. 2007). The observed negative effect of Cd on photosynthesis has been attributed to the inhibition of chlorophyll (Chl) biosynthesis (Padmaja *et al*., 1990) or reduction in growth (Ekmekci *et al*. 2008; Iqbal *et al*., 2010). In another way, nitrogen is quantitatively the most essential nutrient for plants and a major limiting factor in plant productivity. Nitrogen regime had a significant effect on plant growth within control and different abiotic stress (Kant *et al*., 2005; Nasraoui *et al*., 2010). The nitrate is the major form of inorganic nitrogen that is available for growth of majority of plants. On the other hand, excess NH_4^+ can apparently be toxic to some plants (Kronzucker *et al*.,2001). Thus, efficient NH_4^+ transport and subsequent assimilation systems should be highly regulated within the roots, suggesting that most of the NH_4^+ can be assimilated by Glutamine Synthetase (GS). Since the discovery of the GS/(GOGAT) cycle by Lea and Miflin (1974), it is now well established that this cycle is the principal way for NH_4^+ assimilation under normal conditions (Ireland and Lea, 1999; Lea and Miflin. 2003).

Glutamate dehydrogenase (GDH) catalyzes the amination of 2-oxoglutarate and the deamination of glutamate; the direction of the activity depends on specific environmental conditions (Melo-Oliveira *et al*., 1996; Pablish, 1996). GDH activity increase under stress conditions has been often observed (Nasraoui *et al.*, 2011; Chaffei *et al.*, 2012). Ammonium assimilation requires a substantial contribution of fixed C in both leaves and roots (Amancio and Santos. 1992). In leaves, triose-P enters the glycolytic pathway directly (Melo-Oliveira *et al.*, 1996). In roots, C is required directly as α-ketoglutarate (by metabolism of sucrose through glycolysis/Krebs) and indirectly by the generation of reducing power (reduced Fd) in the plastids. Phosphoenolpyruvate derived from glucose reacts with carbon dioxide from the atmospher to yield oxalacetic acid through phospho enolpyruvate carboxylase (phosphate:oxaloacetate-carboxy-lyase; EC 4.1.1.31). The carboxylation of phosphoenolpyruvate in roots is enhanced

Corresponding author: hajajiafef@yahoo.fr

when ammonium is the major source of nitrogen (Arnozis *et al.*, 1988). Therefore, PEPC may be an important interface between carbon and nitrogen metabolism. In this paper, we studied the effects of cadmium supply period on the ammonium assimilation in tomato plants. This species was chosen for its agronomic interest and its different comportment in normal conditions and under cadmic stress. Therefore, the aim of this paper was to analyze principal enzymes involved in nitrogen and carbon metabolisms which could help to elucidate the tolerance ability acquired by tomato plants grown under ammonium nutrition.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of tomato (*Solanum lycopersicon*) were germinated in petri dishes in the dark. Seedlings were transferred and grown under continuous aeration in a nutrient solution containing 0.1 mM KNO_3, 0.5 mM $CaSO_4$, 1 mM $MgSO_4$, 2.5 mM K_2SO_4, 1 mM KH_2PO_4, 1 µM $ZnSO_4$, 5 µM $MnSO_4$, 30 µM H_3BO_3, 1 µM $CuSO_4$, 30 µM Fe-K-EDTA, 1 µM $(NH_4)_6MO_7O_{24}$. Plants were grown in a growth chamber under controlled conditions: a 16h-light (150 µmol m^{-2} s^{-1} PAR)/8h dark cycle, 22 °C (light) /18 °C (night) and 65 % relatively humidity. The 7-day-old seedlings were supplied with nutrient solution containing 5mM of NH_4^+. Cadmium was added to the medium as $CdCl_2$ at 25 µM. After one week half of Cd-treated plants were transferred to culture medium (without Cd). Plants were harvested every 72h. Fresh weight of leaves was determined before storage in liquid N_2.

Determination of leaf area

The fresh leaves were collected and there area was scanned. After that, total leaf area of each plant was determined.

Analysis of ammonium content

Ammonium was measured after extraction of plant tissues (0.5 to 1 g FW) at 4 °C in 2 cm^3 of H_2SO_4 (0.3 mM) and 0.5 % (w/v) Polyclar AT. Homogenate was then clarified by centrifugation for 15 min at 30,000 g. Ammonium was quantified by the Berthelot reaction modified according to Weatherburn (1967).

Enzymes activities

Glutamine synthetase (GS) activity was determined using hydroxylamine as substrate, and the formation of γ-glutamylhydroxylamine (γ-GHM) was quantified with acidified ferric chloride (O'Noel and Joy. 1973). The γ-GHM was quantifed using commercial glutamine as a standard after reading the absorbance of the incubation at 540 nm. Glutamate dehydrogenase (NADH-GDH) activity was measured as described by Turano *et al.* (1997). Frozen samples were homogenized in a cold mortar and pestle with 100 mM Tris-HCl (pH 7.5), 14 mM β-mercaptoethanol and 1 % (W/V) PVP. NADH-dependent activity was determined by following the absorbance changes at 340 nm. Isocitrate dehydrogenase activity (ICDH) activity was measured in 1 ml reaction set containing 0,1 potassium phosphate buffer (pH 7.6), 50 mM $MgCl_2$, 50 mM isocitrate, 5 mM $NADP^+$ and appropriate amount of crude extract. The reaction was initiated by adding isocitrate and the NADPH formed was followed at 340 nm (Gálvez and Gadal. 1994). Phosphoenolpyruvate carboxylase (PEPC) activity was measured spectrophotometrically at 340 nm, in a final volume of 1 ml containing 100 mM HEPES-HCl, 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 0.2 mM NADH at the optimal pH (8.0) and at 2 mM PEP (Echerevarria *et al.*, 1994). Assays were initiated by the addition of plant extracts.

Gel electrophoresis, protein gel blot analysis and gel staining procedure

Proteins were extracted as described above for GS and GDH assays. Proteins were separated by SDS-PAGE (Laemmli, 1970). An equal amount of protein was loaded in each track. The percentage of polyacrylamide in the running gels was 10 % for both GS and GDH. Denatured proteins were electrophoretically transferred to nitrocellulose membranes for GS detection or directly stained with Coomassie Blue. Polypeptide detection was performed using polyclonal antiserum raised against grape leaf GS and GDH (Loulakakis and Roubelakis-Angelakis. 1992). Relative GS and GDH protein amounts were determined by densitometric scanning of Western blot membranes.

Native polyacrylamide gel electrophoresis

Native-PAGE was performed in slab gels containing 7 % acrylamide by the method of David and run at 25 volts for 20h in a refrigerator. At the completion of electrophoresis, bands containing GDH activity (deamination) were visualized with a tetrazolium assay (Hartman *et al.* 1973). After incubation at 25 °C for 20 min, the gel was distained with distilled water at 4 °C and photographed.

Measurements of gas exchange and chlorophyll fluorescence

Measurement of net assimilation rate (A_{max}) was made with a CIRAS-1 gas exchange system (PP Systems, Hitchin, UK). Chlorophyll fluorescence emission from the upper surface of the leaves of intact plants was measured by modulated fluorimeter (MINI-PAM) Photosynthesis Yield Analyser (Walz, Effeltrich, Germany). Leaves previously selected for measurement of gas exchange were used for fluorescence measurements. Non-photochemical quenching of fluorescence (NPQ), which is proportional to the rate constant of thermal energy dissipation was calculated following (Van Kooten and Snel. 1990). The photochemical quenching (q_p) was calculated following (Bjorkman and Demmig. 1987).

Statistical analysis

The data are presented in the figures as the average of at least six replicates per treatment and means ± confidence limits at p= 0.05 level.

RESULTS

When compared to values found in control plants, leaf area was more important in tomato plants treated transitory or continuously by Cd (Fig 1A). Parallel, photosynthetic activity increased in Cd-treated tomato. In leaves derived from tomato transitory treated with Cd, the degree of increase was higher (Fig 1B). Results (Fig 2A) showed that photochemical

quenching (qp) was enhanced in leaves derived from transitory Cd-treated tomato compared to those continuously treated then controls. Thus we observed that the non photochemical quenching (NQP) was reduced in Cd-treated tomato regardless the period of treatment (Fig 2B). Cadmium increased ammonium contents in leaves derived from tomato plants fed with ammonium as nitrogen source (Fig 3A). The ammonium contents diminished when plants were pretreated with Cd and transferred on culture medium with out metal.

In order to investigate the influence of cadmium on nitrogen management, we measured activity and proteins contents for glutamine synthetase (GS). The presence of Cd in the culture medium resulted in a decrease of total GS activity (Fig 3B). GS2 protein accumulation was more important in control plants. Presence of Cd induced GS1 accumulation and inhibited the GS2 one (Fig 3C). The glutamate dehydrogenase activity increased progressively in control and treated plants (Fig 4A). Protein accumulation was more pronounced in Cd-treated leaves compared to in control ones (Fig 4B). Native activity demonstrated that the different GDH isoforms were stimulated (Fig 4C). But, the density of the different bands were more intensive in treated leaves than in control ones. Activities of the two principal enzymes involved in carbon metabolism in leaf extracts of tomato plants were measured. Activities of phosphoenolpyruvate carboxylase (PEPC) and isocytrate defydrogenase (ICDH) increased progressively in control leaves tomato. Leaf extracts from plants grown continuously with Cd showed significant decreases in the activities of both PEPC and ICDH (Fig 5A and B). But, when tomato plants were transferred in control medium after exposure to Cd the activities of PEPC and ICDH were more inhibited.

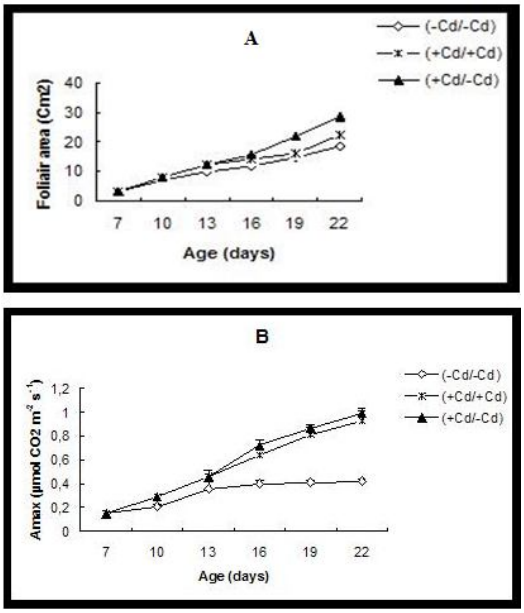


Figure. 1 Leaf area (A) and photosynthetic activity (B) of leaves of tomato plants grown in absence (□) of Cd. When present, Cd (25µM Cd) was supplied continuously (x) or transitory (▲) in culture medium. Data are means of six replicates SE at 0.05 level (Tukey's test).

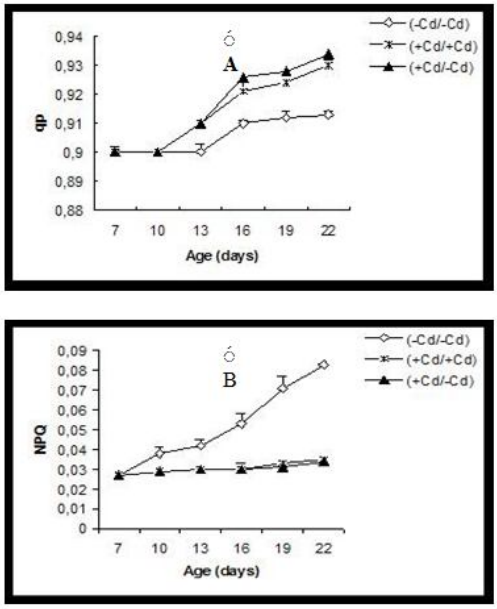
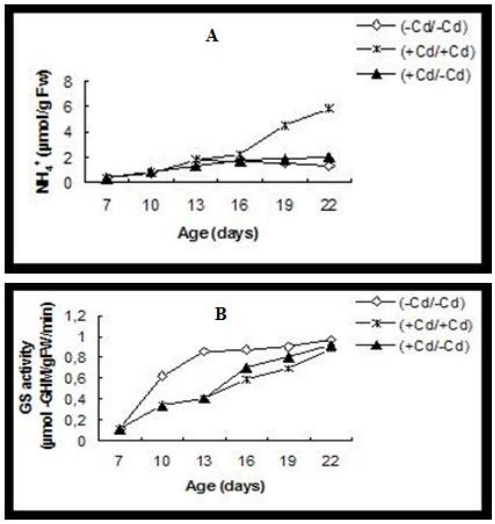


Figure. 2 Photochemistry quenching (A) and non photochemistry quenching (B) in leaves of tomato plants grown in absence (□) of Cd. When present, Cd (25µM Cd) was supplied continuously (x) or transitory (▲) in culture medium. Data are means of six replicates SE at 0.05 level (Tukey's test).



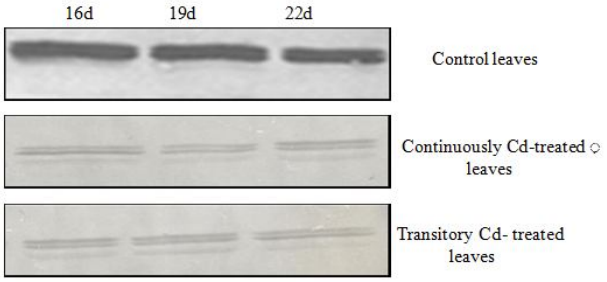


Figure. 3 Ammonium tenor (A), GS activity (B) and GS protein content (C) in leaves of tomato plants grown in absence (□) of Cd. When present, Cd (25µM Cd) was supplied continuously (x) or transitory (▲) in culture medium. Data are means of six replicates SE at 0.05 level (Tukey's test).

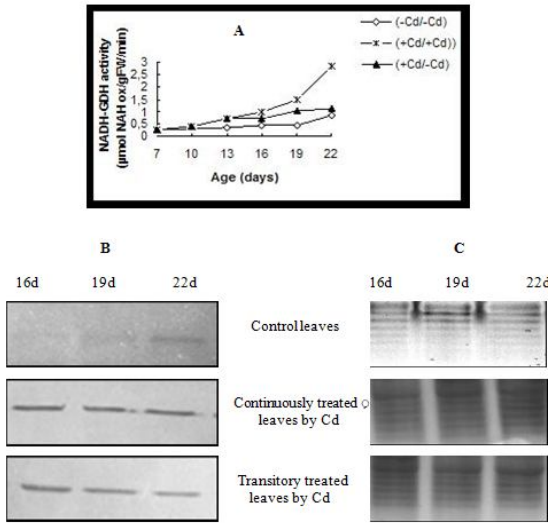


Figure. 4 NADH-GDH activity (A), GDH protein content (B) and GDH native activity (C) in leaves of tomato plants grown in absence (□) of Cd. When present, Cd (25µM Cd) was supplied continuously (x) or transitory (▲) in culture medium. Data are means of six replicates SE at 0.05 level (Tukey's test).

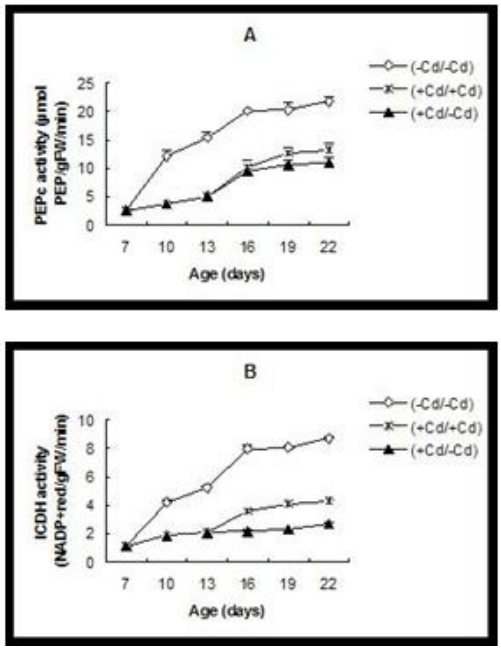


Figure. 5 PEPC (A) and ICDH (B) activities in leaves of tomato plants grown in absence (□) of Cd. When present, Cd (25µM Cd) was supplied continuously (x) or transitory (▲) in culture medium. Data are means of six replicates SE at 0.05 level (Tukey's test).

DISCUSSION

The main question about how to alleviate heavy metal toxicity, above all, is how plants resist heavy metal stress in the long term and decrease metal translocation to the shoots. Tomato reported to be a root Cd-accumulator (Lopez *et al.*, 2009; Nasraoui *et al.*, 2010) and Cd was more absorbed and accumulated when grown with nitrate (Nasraoui *et al.* 2010). Previous study indicated that NH_4^+ supply decreased the biomass of different tomato organs (Nasraoui *et al.*, 2011). More that, it is shown that growth inhibition by Cd was markedly reduced by use of ammonium as nitrogen source compared to nitrate form. Here, we demonstrated that photosynthetic activity and photochemical quenching rose by Cd, accordingly, the non photochemical quenching was reduced in leaves tomato grown in our culture conditions. The positive effects of NH_4^+ on tomato growth under cadmic condition, led us to examine NH_4^+ assimilation pathways. Cd caused a large decrease in total GS activity which played the major role in ammonium assimilation in control conditions (Tobin and Yamaya. 2001; Miflin and Habash. 2002). The Data found showed that GS activity increase progressively parallel to NH_4^+ accumulation. This led to suggest that GS was directly stimulated by ammonium accumulation. Activity of this enzyme has also been shown to decrease in response to Cd condition. In all tested leaves, several GS protein arrangements could be distinguished. Only two cytosolic GS2 isoforms (GS2a, GS2b) seemed to be active in control plants (Chaffei *et al.*, 2009). The Presence of Cd resulted in loss of cytosolic GS accumulation and enhancement of the chloroplastic one (GS2). Cytosolic GS isoforms were suggested to be restricted to functioning to generate glutamine for sink organs such as developing or reproductive organs (Miflin and Habash. 2002). It was clear that under cadmic condition GS1 isoform was largely stimulated by ammonium accumulated in leaves. We also showed that GS1 protein was more abundant in control samples compared to those treated transitory or continuously by Cd. Ammonium nutrition provided to tomato plants could resemble leaf senescence situation described in another tomato cultivar (Pe´rez-Rodrı´guez and Valpuesta. 1996).

These results suggest that GS1 isoform may be affected by Cd supplied during treatment. In another words, GS1 expression in Cd-treated tomato is modulated by the increase of ammonium content under stress conditions. Furthermore, the preferential accumulation of GS1 isoform in leaves tomato treated with Cd indicates that GS1 protein level and total GS activity were correlated. It has been reported that overexpression of chloroplastic GS leads to increased tolerance of plants to different abiotic stress. In short-term experiments using tobacco leaf discs Masclaux- Daubresse *et al.*, (2005) showed that glutamate induced GS1 and, while it inhibited GS2 mRNA and glutamine accumulation caused only a transitory induction of GS1. On the other hand, during periods of growth under stress conditions, nitrogen is remobilized. That is, carbon and nitrogen are recycled in leaves by protein breakdown (Fukutoku and Yamada. 1984; Gouia *et al.*, 2000). The amino acids thus liberated may be recycled by glutamate dehydrogenase (GDH) activity (Mena-Petite *et al.*, 2004).

Although GDH could operate in the direction of ammonium assimilation (Oaks, 1995), particularly under various stress conditions or when an increase of ammonia in leaves is produced as a consequence of GS activity inhibition, it is more extensively assumed that it operates in the direction of glutamate deamination (Miflin and Habash. 2002). NADH-GDH activity increase was accompanied with protein accumulation which remained largely important in Cd-stressed samples. These observations were similar in leaves derived from Cd-treated tomato transferred or not on control medium. Our data showed that the seven different isoforms of GDH

were stimulated either by NH_4^+ supply as nitrogen source or by Cd presence. In fact, despite the controversy over the role of GDH in higher plants, it is evident from our data that GS and GDH activities are inversely related. That is, there is a correlation between the decrease in GS activity and the rise in GDH one. However, as be shown in this study, the NADH-GDH activity was higher than GS one. Thus, the GDH activity increase may be able to assimilate some of not assimilated ammonia by GS activity, it would be sufficient to compensate the GS activity loss. On the other hand, it seemed that ammonium assimilation enzymes tended to lose their activity during senescence (Peeters and Laere. 1992), except GDH, which was more stable than GS (Schlce *et al*. 1994). Thus, under stress conditions such as in the cadmium condition, there is a demand to obtain carbon from amino acids (Miflin and Habahs. 2002). When tissues become carbon-limited as occurs during storage, tricarboxylic acid intermediates (Dubois *et al.,* 2003) were supplied by the oxidative deamination of glutamate catalysed by GDH activity (Aubert *et al.,* 2001). However, GDH has a predominantly deaminating activity (Robinson *et al.*, 1991), providing carbon skeletons for optimal tricarboxylic acid cycle process (Aubert *et al.*, 2001), and for proteins when there is a carbohydrate deficiency (Lea *et al.*, 1992). In our experiment, we observed a general increase in GDH activity that was paralleled to GS activity fall. We have observed in previous study (Nasraoui *et al.*, 2011) that transferring Cd-treated tomato to control medium could induce the recovery of many physiological parameters, including biomass production, foliar area, roots length and, soluble proteins contents. Similarly, the present findings show the likelihood of recovery of protein content and GDH activity. However, the rate of physiological recovery is dependent on the cadmium level severity and treatment period. As the contamination period is prolonged, the GS protein level and activity recovery rates decrease whereas, those of GDH increase.

Several studies showed that PEPC and ICDH were increased by Cd in tomato leaves. This, in order to generate reducing power, which may play a role in redox mechanisms in plant cells (Chaffei *et al*. 2009; Lopez-Milan *et al.* 2009). It has been widely described that Cd exposure causes oxidative stress, and accordingly several enzymes and metabolites involved in defense mechanisms against oxidative stress elicited by Cd (Dong *et al*. 2006; Lin *et al*. 2007). Data presented showed that in ammonium tomato leaves, PEPC and ICDH activities decreased under Cd conditions. Thus, we suggested that in such condition of nitrogen nutrition, anaplerotic fixation of CO2 was restricted to roots (Unpublished result). But, in leaves, photosynthesis is the major way of carbohydrates synthesis. It was shown previously that, different photosynthetic parameters were enhanced in those organs (Nasraoui *et al.* 2010). Thus, the non-photosynthetic carbon incorporation into metabolism which is dealt mainly to PEPC and ICDH activities seems to be the non correct way in this case of growth conditions.

Overall these results suggest that tomato plants adjust GS and GDH activities to the alleviate Cd contribution in to ammonium accumulation. GS activity inhibition reflected degradation of cytosolic isoform (GS1), regardless the slightly accumulation of chloroplastid isoform (GS2). In the other hand, protein accumulation, stimulation of GDH deaminating activity by activation of seven different isoforms highlight the capacity of tomato plants to tolerate ammonium detoxification and cadmic stress. PEPC and ICDH activity also decreased in leaves of tomato grown under Cd treatment, suggesting that leaves did not need to enhance anaplerotic CO2 fixation to product carbon skeletons, while photosynthesis was enhanced. Thus we could suppose that NH_4^+-fed tomato was probably well protected against Cd treatment.

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