RESEARCH ARTICLE

Foliar nitrogen and changes in free amino acid composition of *Solanum lycopersicum* under cadmium toxicity: kinetics of $^{15}NH_4^+$

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ABSTRACT

Glutamate metabolism and amino acid translocation were investigated in the control and cadmium stressed shoots of tomato (*Solanum lycopersicum* – 63/5 F1), using (^{15}N) ammonium and (^{15}N) glutamate tracers. Regardless of organ type, [^{15}N] ammonium assimilation occurred via glutamine synthetase (EC 6.3.1.2), in the control and stressed plants, and it did not depend on glutamate dehydrogenase (EC 1.4.1.2). The [^{15}N] ammonium and ammonium accumulation patterns support the role of glutamate dehydrogenase in the deamination of [^{15}N] glutamate to provide 2-oxoglutarate and [^{15}N] ammonium. In the presence of cadmium, excess [^{15}N] ammonium was incorporated into asparagine, which served as an additional detoxification molecule. In the presence both of methionine sulfoximine and cadmium, glutamate, alanine, and γ-amino butyrate of leaf tissue continued to become labelled with ^{15}N. The labelling kinetics of amino acids in leaves of tomato plants in the presence of cadmium show that continued assimilation of [^{15}N] ammonium can occur when the glutamine synthetase-glutamate synthase cycle is inhibited. The data provided evidence that the glutamine synthetase pathway and glutamate dehydrogenase play distinct roles in the source-sink nitrogen cycle of tomato leaves under cadmium stress conditions.

Keywords: ammonium, glutamate dehydrogenase, methionine sulfoximine, ^{15}N-NH_4^+.

INTRODUCTION

Ammonium is the final form of inorganic nitrogen, which is prior to the synthesis of organic nitrogen compounds. It is also produced via internal metabolic reactions, including photorespiration, hydrolysis of nitrogen carrying and storage molecules, and amino acid conversion (Ireland and Lea, 1999). In nonleguminous C3 plants, such as tomato (*Solanum lycopersicum*), the photorespiratory ammonium production by the oxidative decarboxylation of Gly exceeds by about ten-fold the primary nitrate reduction in the vegetative leaves (Foyer et al., 2009).

In senescing leaves, a large amount of ammonium is produced as a result of protein hydrolysis (Hörteinsteiner and Feller, 2002). Therefore, it is essential that toxic ammonium is immediately reassimilated into organic molecules for nitrogen cycling. Ammonium is assimilated into the Gln amide group, which is then transferred to the position of 2-oxoglutarate, yielding two molecules of glutamate (Glu) by the concerted reaction of glutamine synthetase (GS) and Glu synthase (Fd-GOGAT; EC 1.4.7.1; NADH-GOGAT; EC 1.4.1.14). Nitrogen is then incorporated into Asp, Ala, Asn, and other amides and amino acids. Gln-dependent asparagine synthetase

(AS; EC 6.3.5.4) provides Asn, which serves as a nitrogen carrier together with Gln and Glu.

Several studies have been carried out to define the roles of enzymes in nitrogen assimilation and remobilization, tightly interrelated processes during plant growth and development (Miflin and Habash, 2002). It was proposed that ammonium might be directly incorporated into Glu by amination of 2-oxoglutarate via mitochondrial Glu dehydrogenase (NADH-GDH; EC 1.4.1.2) and subsequently into Gln by cytosolic GS1 under particular physiological conditions.

Studies on source-sink relations have shown that the GDH is induced in plants when nitrogen remobilization is maximal (Masclaux et al., 2000). This led to the proposal that the physiological role of GDH is to synthesize Glu for translocation in senescing leaves (Miflin and Habash, 2002). However, there is no evidence to discern a redundant or indispensable role of GDH and GOGAT for Glu synthesis and nitrogen remobilization (Chaffei et al., 2004; Gouia et al., 2008). In addition, GDH catalyzes the reversible oxidative deamination of Glu in order to supply 2-oxoglutarate and ammonium (Aubert et al., 2001; Chaffei-Haouari et al., 2011).

Cadmium is a nonessential heavy metal, which does not have any metabolic function in higher plants. Under natural conditions, it exists at low concentrations in most soils. It enters the soil with phosphorus fertilizers, sewage sludge, and air pollutants. It has a great mobility in the soil as compared with other heavy metals, and is taken up in varying degrees by plants (Bavi et al., 2011). The increasing amount of cadmium in the environment affects various physiological and biochemical processes in plants (Nagajyoti et al., 2010). Reductions in both biomass production and nutritional quality have been observed in crops grown on soils contaminated with moderate levels of heavy metal (Odjegba and Fasidi, 2007). Even at low concentrations, it inhibits plant growth and disturbs photosynthesis, sugar metabolism, sulphate assimilation, and several enzyme activities (Astolfi et al., 2011; Chen et al., 2008).

To better understand the role of GDH and GOGAT in Glu metabolism in the coordinated reaction with GS, we studied the kinetics of *in vivo* turnover of [^{15}N]Glu fed to leaf samples of cadmium-treated tomato plants. The time course of [^{15}N] ammonium assimilation into the amino acids was then determined *in vivo* in control and stressed young leaves.

MATERIAL AND METHODS

Plant material and growth conditions: Seeds of tomato (*Solanum lycopersicum* Mill cv. 63/5F1) were sterilized in 10% (v/v) hydrogen peroxide for 20 minutes, they were also washed abundantly in distilled water afterwards. After imbibition, seeds were germinated on moistened filter paper at 25ºC in the dark. After seven days, uniform seedlings were transferred to 6 L plastic beakers (eight seedlings per beaker), which were filled with continuously aerated, basal nutrient solutions of an initial pH 5.8 to 6, containing 3 mM KNO_3, 0.5 mM Ca $(NO_3)_2$, 2.4 mM KH_2PO_4, 0.5 mM $MgSO_4$, 100 µM Fe-K_2-EDTA, 30 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, and 1 µM $(NH_4)_6Mo_7O_{24}$. Plants were grown in a growth chamber (26ºC/70% of relative air humidity during the day, 20ºC/90%, at night). The photoperiod was of 16-hour daily with photosynthetic photon flux of 150 µmol m^{-2} s^{-1} at the canopy level. Ten days after transplant, cadmium was added to the medium as $CdCl_2$ at 0 and 50 µM, for seven days.

^{15}N-labelling experiments, amino acid analysis, and gas chromatography-mass spectrometry measurement: ^{15}N-labeling experiments were performed using shoots of 17 days-old tomato plants, which were prepared from plants treated with or without 50 µM of cadmium (control plants). Such samples were floated on 10 mM MES buffer, pH=6.5, containing 10 mM $CaCl_2$, 40 mM KCl, and 2% (w/v) polyethylene glycol, both in the light (photosynthetic photon flux of 250 μmol photons m^{-2} s^{-1}) or in the dark, with or without 1 mM MSX for one hour. Afterwards, [^{15}N] ammonium (99% enrichment) or [^{15}N] Glu (99% enrichment; Euriso-top S.A.) was added to the medium. Shoot part was dipped into the labelling solutions, quickly transferred, and rinsed with a large volume of water before freezing in liquid nitrogen (time 0). Shoot samples were further incubated in labelled medium ([^{15}N] ammonium or [^{15}N] Glu) and collected at four and eight hours. Samples were frozen in liquid nitrogen prior to analysis.

Total amino acids and ammonia were extracted with 2% (w/v) sulfosalicylic acid. Extracts were centrifuged at 17,500 g for 20 minutes in order to eliminate cellular debris. Amino acids were applied to a column (AG 50W-X8 resin, 100 to 200 mesh, H^+-form, 5 x 0.5 cm; Bio-Rad Laboratories), washed with 4 mL water, and eluted with 2.5 mL of 6 M NH_4OH, then with 1 mL water. Total amino acids were determined according to Rosen (1957) and ammonium content by the Berthelot reaction. One-half of the supernatant was adjusted to pH=2.1 with LiOH, and amino acids were separated and quantified by ion exchange chromatography on a Biotronic LC5001 analyzer using a standard amino acid mixture by Perkin-Elmer Nelson 2100 software (Rochat and Boutin, 1989). From the remaining supernatant, amino acids were derivatized with either *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide in acetonitrile (Chaves das Neves and Vasconcelos, 1987). The atom percentage of amide and of amino ^{15}N were determined by gas chromatography (GC)-mass spectrometry (MS) analysis (model MD800; Fisons). [^{15}N] Ammonium released

from [^{15}N]Glu was determined after purification and derivatization steps, as described by Fujihara et al. (1986).

Metabolite extraction and analysis: Amino acid and NH_4^+ were determined after extraction in a 2% solution of 5-sulfosalicylic acid (50 mg FW ml^{-1}). Total amino acid content was assayed by Rosen et al. (1969) colorimetric method, using glutamine as a reference. Individual amino acid composition was determined, using ion exchange chromatography (Rochat and Boutin, 1989). Free NH_4^+ was determined by the phenol hypochlorite colorimetric method (Berthelot reaction) using $(NH_4)_2SO_4$ as a reference.

Protein extraction and enzyme assays

Glutamine synthetase: Enzyme was extracted with 25 mM tris-HCl buffer (pH=7.6), 1 mM EDTA, 1 mM $MgCl_2$, 14 mM β-mercaptoethanol, and (1%) polyclar. GS activity was measured according to O'Neal and Joy (1973). The homogenate was centrifuged at 28,000 g for 20 minutes at 4°C. GS activity was determined using hydroxylamine as the substrate, and the formation of γ-glutamylhydroxamate (γ-GHM) was quantified with acidified ferric chloride.

Glutamate dehydrogenase: GDH extraction was performed according to Masclaux-Daubresse et al. (2000). 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) polyclar. The extract was centrifuged at 12,000 g for 15 minutes at 4°C. NADH and NAD^+-GDH activities were determined by following the absorbance changes at 340 nm. Enzyme activities were expressed per min and g FW. Total soluble protein was determined using a commercially available kit, according to Bradford (1976).

Statistical analysis: Values presented were means ± standard deviation (SD) of three replicates. Statistical analyses were carried out by analysis of variance, using SPSS10.0 software. Differences between treatments were analyzed by the Duncan's multiple range test.

RESULTS

To investigate Glu behaviour in cadmium-treated plants, leaf samples were incubated with [^{15}N]Glu, either in control or in treated plants. Total ammonium levels were higher in cadmium-treated than in control shoots (Figure 1). A remarkable difference was detected in ammonium levels between four or eight hours after [^{15}N] Glu incubation conditions. The addition of Met-sulfoximine (MSX) drastically inhibited GS activity, whereas it did not affect the aminating and deaminating activities of GDH (data not shown).

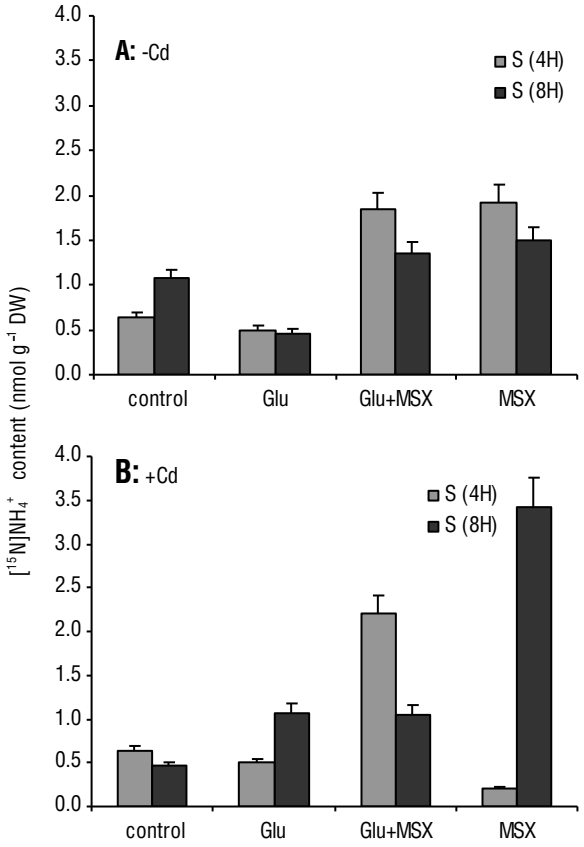

Figure 1. Changes in the level of [^{15}N] ammonium in shoot tissues treated (A) or not treated with cadmium (B). Shoot samples from 17-days-old tomato plants were incubated into the solutions with or without 1 mM MSX, were harvested at four and eight hours. [^{15}N] Ammonium content was expressed as nmol g^{-1} DW. Values represent the means of analysis from five independent plants. DW: dry weight.

Following MSX treatment, ammonium reassimilation through GS was inhibited and the increase in total ammonium content via photorespiration became apparent, as the difference between the control and cadmium treatments shoots (Figure 1B). Release of [^{15}N] ammonium from [^{15}N] Glu was higher in the stressed plants when compared to the Control ones (Figure 1). The addition of MSX led to similar rates of [^{15}N] ammonium release from [^{15}N] Glu in treated and not treated shoots with cadmium (Figures 1A and B). The results suggest that an extra [^{15}N] ammonium was released from [^{15}N] Glu deamination in the cadmium-treated plants by the reaction independent of photorespiration in the shoots.

The labelling kinetics of amino acids in shoots of tomato plants supplied with 1 mM MSX for two hours and then with 5 mM [^{15}N] Glu for subsequent 12 hours shows that continued assimilation of [^{15}N] H_4^+ can occur when the GS/GOGAT cycle is inhibited (Figure 2).

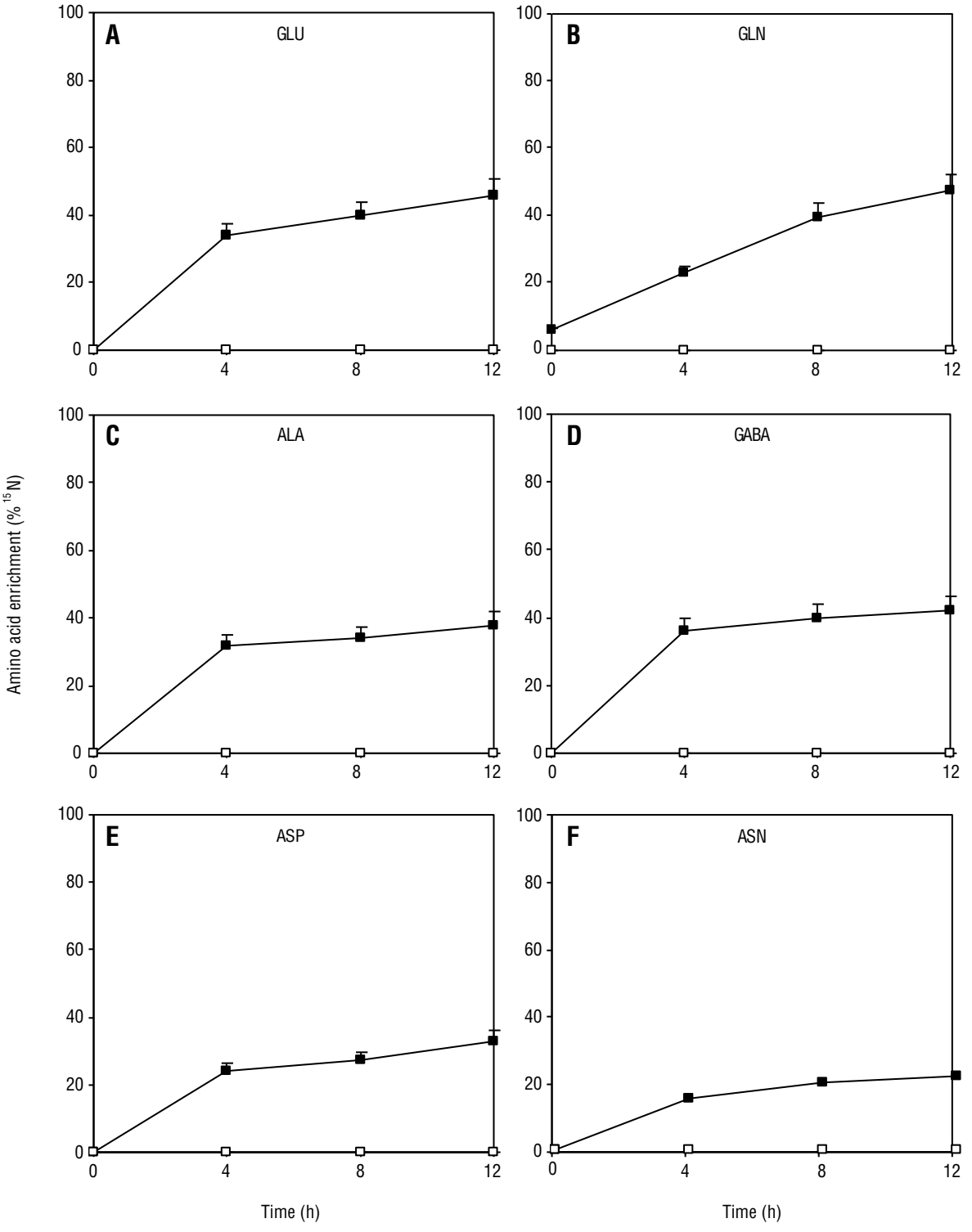

Figure 2. Kinetic analysis of [^{15}N] ammonium incorporation in to amino acids in absence of cadmium in the culture medium. Shoot samples from 17 days-old tomato plants were floated on incubation buffer. After addition of [^{15}N] ammonium (time 0), samples were incubated and harvested at 4, 8, and 12 hours from the solution without or with MSX (GS inhibitor). ^{15}N labelling in amide and amino nitrogen was determined by a GC-MS analyser. Values represent the means of analysis on shoot samples from five independent plants.

It is worth noting that in shoots in the absence of MSX, glutamate and GABA are much more heavily labelled than glutamine amino-N after two hours of exposure to [^{15}N] Glu (Figures 2A and B). This would not be inconsistent with some primary ammonia assimilation via GDH. An alternative explanation for these results is that, in the presence of MSX, direct assimilation of ammonia into alanine occurs via an alanine dehydrogenase in tomato shoots, with alanine transaminated to glutamate and/or GABA. However, in the absence of MSX, glutamate and GABA are much more heavily labelled that alanine at early time points (Figure 2B). Therefore, it is unlikely that alanine is a precursor of glutamate and GABA. It is evident that it is an MSX-insensitive pathway of ammonia assimilation in operation in tomato shoots, with GDH remaining a leading candidate for this pathway. However, this rate could be underestimated as a result of protein turnover and associated isotopic dilution of the free glutamate and alanine pools by ^{15}N-amino acids released from protein, and therefore underestimated if MSX has secondary effects on photosynthesis and carbon economy, which in turn affects the supply of 2-oxoglutarate to GDH *in vivo*. The pools of glutamine, glutamate, alanine, aspartate, and asparagines after 12 hours exposure to 15 NGlu were, on average, five to ten-fold lower in the MSX treated than in the Control (-MSX) plants.

The kinetics of [^{15}N] ammonium assimilation was determined in shoots when cadmium was present (Figure 3). When tomato plants were treated with 20 µM of cadmium (Figure 3), the [^{15}N] enrichment in Glu and Gln was substantially increased in shoot tissues (Figures 3A and B). MSX inhibited completely GS activity, but it did not inhibit the GDH aminating and deaminating activities (Figures 4–6). As the GDH aminating activity was induced in cadmium-treated tomato tissues in order to compensate the lack of GS (Chaffei et al., 2004). The net increase of [^{15}N] Glu in shoots, where tissues were incubated with MSX, suggests that GDH assimilates ammonium in the tomato tissues under stress conditions. In contrast, [^{15}N] enrichment in Ala and GaBa was highly decreased in cadmium-treated shoots (Figure 3A). These results suggest that MSX did not inhibit the total GOGAT activity and that the difference was due to the higher Glu contents. However, the Asn content was increased in shoots (Figures 2F and 3F). The addition of MSX resulted in the complete inhibition of [^{15}N]Gln labelling, and the continued synthesis of [^{15}N] Glu, therefore GS probably was not the sole that catalyzes the efficient entry of ammonium.

In cadmium-treated plants, the high GDH-aminating activities were measured *in vitro* from shoot tissues (Figure 4). The tomato tissues exhibit high *in vivo* rates of ammonium assimilation in the presence of MSX, a potent inhibitor of GS (Figure 4B). To test this hypothesis, plants were supplied with $^{15}NH_4$ in the presence of MSX. The presence of the inhibitor resulted in approximately 90% of inhibition of *in vitro* GS activity (Figure 3A). Figure 7 insert shows the levels of GDH deaminating activities of tomato shoots, with or without MSX. These results support that GDH strongly aminates 2-oxoglutarate in Glu (Figure 4B), but also exerts low deaminating activity (Figure 5B) under stress growth conditions. In addition, it is shown that the increase of aminating/deaminating GDH ratios in cadmium-treated tissue with or without MSX (data not shown) reflects the implication of aminating GDH activity in the balancing of the cellular levels of three major components: ammonium, 2-oxoglutarate and Glu, under cadmium stress conditions. GDH operates in the aminating direction, therefore it may assimilate excessive ammonium in concert with GS/GOGAT cycle (Tobin and Yamaya, 2001). The cadmium-treated tomato tissues overexpressing the *gdh* gene encoding for the α-subunit for GDH showed increased tolerance to heavy metal stress (Damianos et al., 2007).

Although the function of GDH in the cytosol is induced in this cellular compartment, it plays an important role in recycling carbon and nitrogen molecules in source organs and/or at specific stress conditions. This may be the case when the enzyme activity is high, like in mature roots (data not shown), or induced in response to either internal or external supply of ammonium.

DISCUSSION

Nitrogen metabolism in senescing tomato tissues induced by cadmium is characterized by a progressive hydrolysis of stromal proteins and degradation of chloroplasts (Hörteinsteiner and Feller, 2002). Since the main metabolic process in tomato plant senescence consists of nutrient remobilization, toxic free ammonium should be rapidly fixed again into the amino acids to avoid deteriorating effects and to provide nitrogenous forms, which are suitable for source-sink transport. Our ^{15}N-labeling study provides evidence that significant [^{15}N] ammonium was released from [^{15}N] Glu deamination by GDH in the cadmium stress conditions to higher extents in older than in

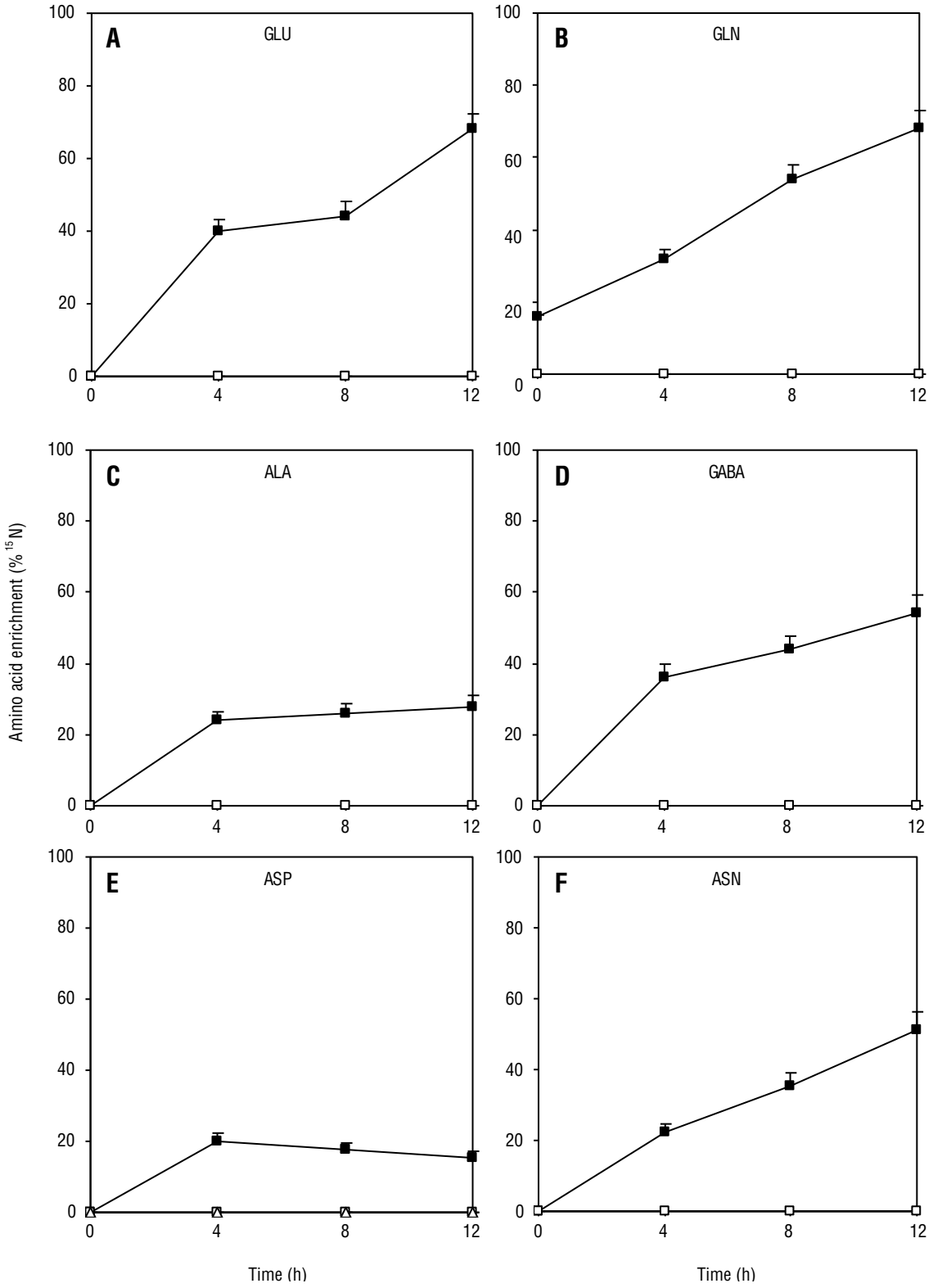

Figure 3. Kinetic analysis of [^{15}N] ammonium incorporation into amide acids in the presence of 20 µM of cadmium in the medium culture. Shoot samples from 17 days-old tomato plants were floated on incubation buffer. After addition of [^{15}N] ammonium (time 0), samples were incubated and harvested at 2, 4, 8 ,and 12 hours from the solution without or with MSX (GS inhibitor). ^{15}N-labelling in amide and amino nitrogen was determined by a GC-MS analyser. Values represent the means of analysis on shoot samples from five independent plants.

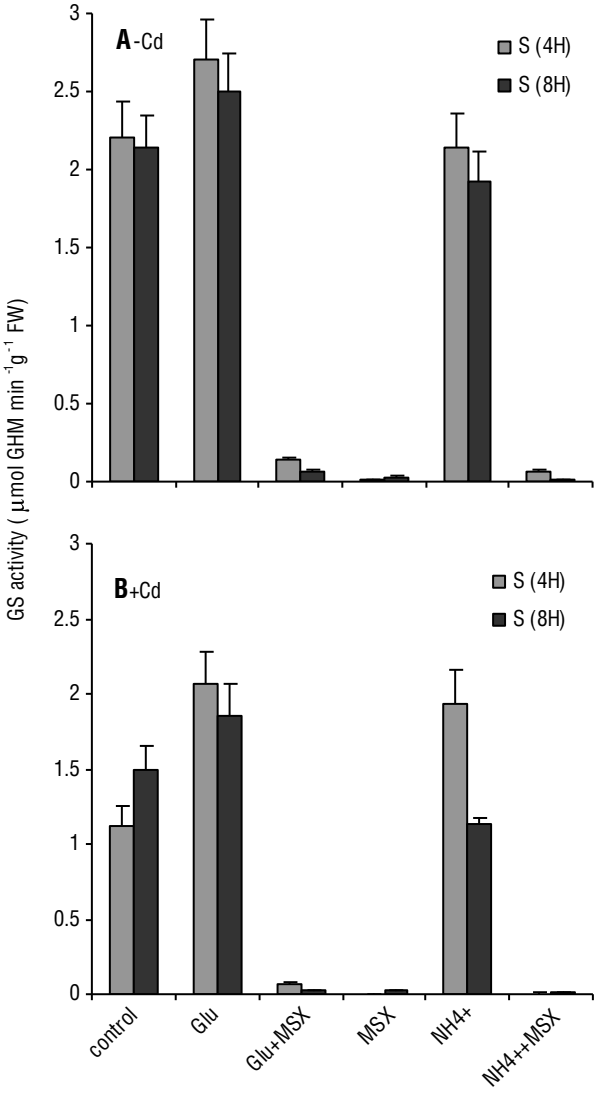

Figure 4. Effect of cadmium on the activity of glutamine synthetase (GS) in shoot tissue of tomato plants, which were treated or not treated with MSX (GS inhibitor). Glutamine synthetase activity was expressed as µmol GHM/min/g FW. Values represent the means of analysis from five independent plants. FW: fresh weight.

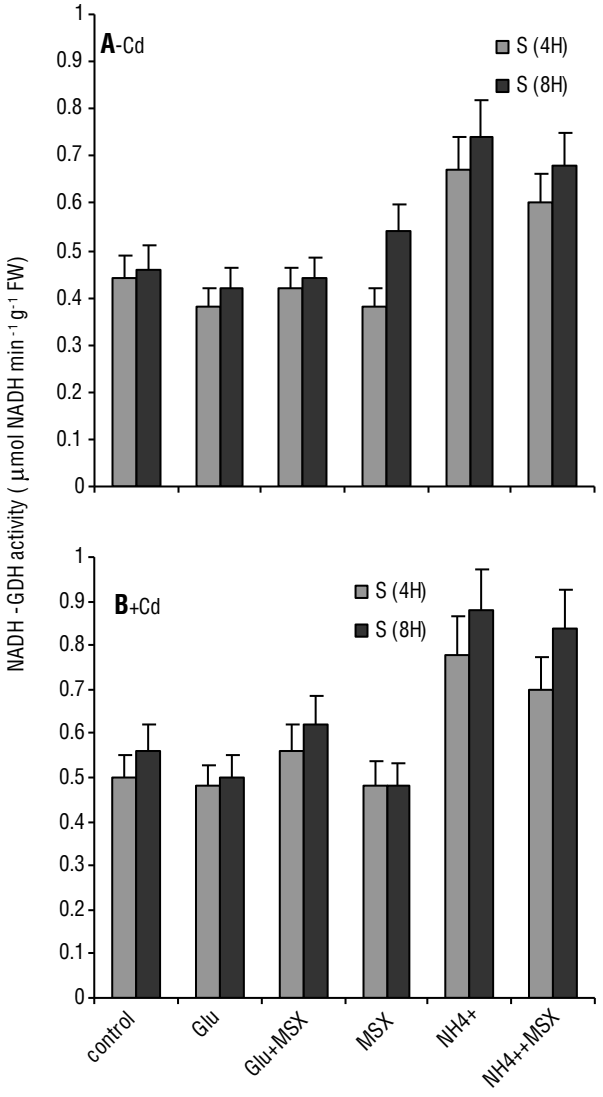

Figure 5. Effect of cadmium on the activity of glutamate dehydrogenase NADH-dependent (NADH-GDH) in shoot tissue of tomato plants, which were treated or not treated with MSX (GS inhibitor). Glutamate dehydrogenase activity was expressed as µmol NADH/min/g FW. Values represent the means of analysis from five independent plants. FW: fresh weight.

young leaves. This is consistent with the higher GDH activity in response to natural senescence (Masclaux-Daubresse et al., 2002; Labboun et al, 2009). It is estimated that up to one-third of the Glu-dependent respiratory rates in isolated mitochondria can be attributed to the GDH deamination reaction (Aubert et al., 2001; Miyashita and Good, 2008).

The carbon flow from Glu oxidation becomes important under conditions of carbon limitation in cadmium stress conditions (Chaffei et al., 2004) and carbohydrate starvation (Robinson et al., 1992; Miyashita and Good, 2008). Thus, GDH isoenzyme profiles may reflect the physiological function of GDH, which seems to be an important link of metabolic adaptation in cells,

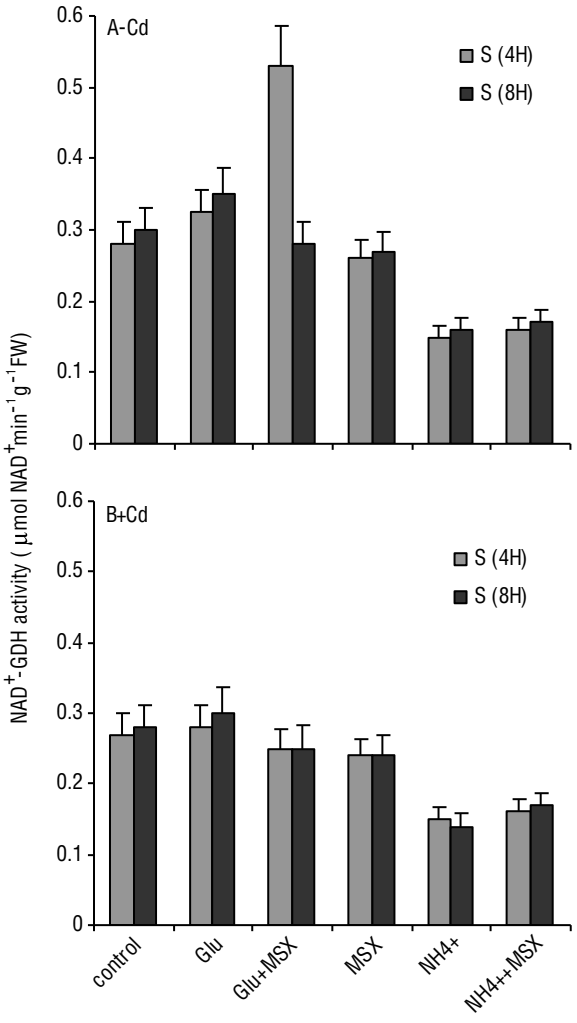

Figure 6. Effect of cadmium on the activity of glutamate dehydrogenase NAD^+-dependent (NADH-GDH) in shoot tissue of tomato plants, which were treated or not treated with MSX (GS inhibitor). Glutamate dehydrogenase activity was expressed as µmol NAD^+ min^{-1} g^{-1} FW. Values represent the means of analysis from five independent plants. FW: fresh weight.

aimed at using carbon sources other than sugar during carbohydrate starvation (catabolic activity of GDH) and at protecting plant tissues against ammonium accumulated due to heavy metal stress (anabolic activity of GDH) (Lehman et al., 2010). Therefore, it is conceivable that GDH supplies 2-oxoglutarate by Glu oxidation for the nitrogen and carbon cycle in stressed shoots.

Kinetics of [^{15}N] ammonium assimilation clearly showed that Fd-GOGAT and/or NADH-GOGAT transferred [^{15}N] from Gln to [^{15}N] Glu taking only two hours for [^{15}N] ammonium feeding in absence of cadmium, while the [^{15}N] Glu labelling by GOGAT was slightly delayed in the presence of heavy metal. Despite the induction of cytosolic GS1 and a partial degradation of chloroplasts in shoot of stressed plants, the chloroplastic GS2 protein remains predominant over the cytosolic GS1 protein in tobacco leaves (Chaffei et al., 2004). This implies that both the chloroplastic GS2 and cytosolic GS1 are involved in ammonium assimilation. Our in vivo ^{15}N-labeling data clearly contrast with the proposal that GDH and cytosolic GS1 play a major role for the synthesis and reallocation of amino acids in senescing leaves (Miflin and Habash, 2002).

Moreover, MSX completely blocked [^{15}N] transfer from [^{15}N] ammonium to the amide and amino groups of Glu and Gln in shoots. These data provide strong evidence that GDH plays a primary route of ammonium assimilation in cadmium stress conditions, and GS-GOGAT cycle plays a minor one. In concert with the GS1 located in the CC-SE complex, Fd-GOGAT presumably plays a complementary role to NADH-GOGAT during development because NADH-GOGAT occurred at higher control plants and decreased with cadmium stress (Chaffei et al., 2004). Consistently, the low level of Fd-GOGAT (15% of wild-type activity) supplies Glu for the normal growth of the transgenic antisense Fd-GOGAT tobacco lines, in which NADH-GOGAT is not detected in shoots (Feraud et al., 2005). Taken together, the data support that the GDH induced takes place in mitochondria and cytosol cells for the biosynthesis of Glu prior to the cycle of amino acids, and that GS-GOGAT cycle does not play a role in Glu supply even in tomato shoot tissues.

Regardless of the effect of ammonium, increases in GDH activity have also been observed following carbohydrate starvation, a process that could be reversed by the presence of cadmium in culture medium. In many studies, both the metabolic environment and the tissue localization of GDH were not really taken into account, since the catalytic function of the enzyme in higher plants was studied *in vivo* by probing ^{15}N-labeled ammonium or Glu leaf organ (Dubois et al., 2003; Tercé-Laforgue et al., 2004; Masclaux-Daubresse et al., 2006).

A study performed on tomato shoots treated with cadmium tended to indicate that shoots were able to incorporate less ^{15}N ammonium into total reduced nitrogen. However, the plant response in addition to inhibition of GS activity (MSX) allowed the conclusion that GDH is solely responsible for the difference found in the rate of ammonium assimilation in stress

conditions, when ammonium becomes toxically accumulated (Aubert et al. 2001; Lehman et al, 2010). Thus, data support the notion that the GS-GOGAT cycle takes place for biosynthesis of Glu and Gln prior to the cycle of amino acids, and that GDH plays a role in Glu supply even in cadmium stressed leaves of tomato, where GDH is induced.

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