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RESEARCH ARTICLE

INHIBITION OF PROTEIN SYNTHESIS BY CYCLOHEXIMIDE OF *Solanum lycopersicon* SEEDLINGS UNDER CADMIUM STRESS CONDITIONS

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ABSTRACT

The effects of cycloheximide on the changes in activity of glutamate dehydrogenase-NADH dependent (NADH-GDH) and NAD^+-dependent (NAD^+-GDH), and glutamine synthetase (GS) were studied during the senescence of tomato seedlings in the presence or absence of cadmium. The GDH and GS1 enzymes seemed to be synthesized at the presence of cadmium. Cycloheximide-treated leaves and roots showed an increase in the expression immunostaining intensity of mRNA band *gdh* and *gs1*, suggesting an increase in neosynthesis of enzymes and genes activation. The rate of synthesis of GDH was clearly higher than that of its degradation. Cycloheximide (CHX) delayed the protein degradation processes in the cadmium stress conditions. Cycloheximide treatments produced lower gs1 and *gs2* enzyme levels than their respective controls especially in leaves. However, the enzyme activity was lower in cycloheximide treated leaves and roots than in the controls. The results are discussed with reference to the requirement for protein synthesis in the cadmium stress conditions.

INTRODUCTION

Heavy metals pollution of soil and water is a very serious environmental problem with potentially harmful consequences for agriculture and human health. Studies carried out in different plant species have revealed that Cd is strongly phytotoxic and causes growth inhibition and even plant death, although the mechanisms involved in its toxicity are still not completely understood (Sandalio *et al.* 2001). Cadmium concentration in shoot and root of maize and pea plants increased consistently with the increasing the concentration of external Cd level in the growth media as reported by Paradiso *et al.* (2008). The inhibition of plant growth by Cd stress is related to its effect on nutrient uptake and distribution within the plant cell as found by Schützendübel and Polle (2001). Furthermore, deleterious effects of heavy metal stress in plants may be coupled to other physiological processes via the stimulation of some enzymatic activities that limit cell growth and consequently, accelerate tissue ageing. Stress-tolerant plants usually contain high constitutive levels of protective metabolites, while the more-sensitive ones show their induction under different kind of stress. Cd treatment can also result in endogenous ammonium increase through deamination of some free amino acids and other N forms. Chaffei *et al.* (2004) demonstrated that Cd treatment produced ammonium accumulation through an increase in protease activity. Glutamine synthetase (GS) is one of the key enzymes in the main pathway of ammonium assimilation in higher plants (Miflin and Habash 2002), and deleterious effects of Cd on its activity have been observed in several species (Miflin and Habash 2001. Wang *et al.* 2008), which reflects a general inhibition of primary N assimilation. In contrast, glutamate dehydrogenase (GDH), an important enzyme in the "shunt" of N metabolism, was induced under Cd stress, but the physiological role of GDH and the effect of its activity increase are still controversial (Wang *et al.* 2008). Conversely, N metabolism is important for the response of plants to Cd toxicity. In the present study, we used cycloheximide, a protein synthesis inhibitor, to assess the importance of protein enzyme synthesis including GS and GDH in nitrogen metabolism under cadmium stress conditions.

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MATERIAL AND METHODS

Plant material and growth conditions: the plant material used in this work was tomato (*Solanum lycopersicon*. Mill. cv. 63/5F1). Seeds were sterilized in 10% (v/v) hydrogen peroxide for 20 min, and washed abundantly in distilled water afterwards. After imbibition, the seeds were germinated on moistened filter paper at 25°C in the dark. After 7 d, the uniform seedlings were transferred to 6 litre plastic beakers filled with continuously aerated, basal nutrient solutions of an initial pH 5.8-6, 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

(26°C/70% relative humidity during the day, 20°C/90% relative humidity during the night). The photoperiod was 16 h with a light irradiance of 150 µmol m^{-2}. s^{-1} at the canopy level. At the age of 17 day after transplant, cycloheximide (30 µM) was added to the medium for two hours. After cycloheximide treatment, plants were separated into control and plants treated with cadmium 50 µM for 5 hours. Tomato seedlings were subdivided into leaves, cotyledonal leaves, steam and roots, then samples were stored in liquid nitrogen for subsequent analysis

Total protein determinations

Soluble protein was determined using a commercially available kit according to Bradford method (1976).

Glutamine synthetase (GS)

Enzyme was extracted with 25mM tris-HCl buffer (pH: 7.6), 1mM EDTA, 1mM $MgCl_2$, 14Mm β-mercaptoethanol and polyclart AT %. GS activity was measured according to the method of O'Neal and Joy (1973). The homogenate was centrifuged at 28,000×g for 20 min 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)

GDH extraction was performed according to the method described by Masclaux *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) PVP. The extract was centrifuged at 12,000×g for 15 min at 4 °C. NADH and NAD^+-GDH activity was determined by following the absorbance changes at 340 nm. Enzyme activities were expressed per min and g FW.

Extraction of total RNA and Northern blot analysis:

Total RNA was extracted as described by Verwoerd *et al.* (1989) from plant material stored at -80°C. Northern blot analysis was performed as described previously (Masclaux et al. 2000). The following 32P-labelled probes were used for mRNA detection: *GS2* from *Nicotiana tabacum* (Becker et al. 1992), *GS1* from *N. tabacum* (Dubois *et al.* 1996), *GDH* cDNA from *N. tabacum* (Masclaux-Daubresse *et al.* 2002) and asparagine synthetase *Ljas1* (accession number X89409) and *Ljas2* (accession number X94184) from *L. japonicum*, kindly provided by Dr. R.N. Waterhouse. The 18S rRNA probe (Deng *et al.* 1991) was used as a constitutive control and the *Glubas* probe, which encodes a basic β-1,3-glucanase, as a stress-related control (Van Kan *et al.* 1992). Since tobacco and tomato genes are highly homologous, hybridization with *GS1*, *GS2* and *GDH* probes was performed under high stringency conditions at 65°C according to Church and Gilbert (1984). Filters were washed with 2×SET (0.06 M Tris-HCl pH 8, 0.3 M NaCl, 4 mM EDTA) at room temperature for 5 min and at 65°C for 10 min. Additional washing was performed successively using 1 x SET and 0.5×SET at 65°C for 15 min before drying and exposure to X-ray film. Heterologous hybridization with a mix of *Ljas1* and *Ljas2* probes was performed in low stringency conditions at 55°C. Filters were washed with 2×SET (0.06 M Tris-HCl pH 8, 0.3 M NaCl, 4 mM EDTA) at room temperature for 5 min and at 55°C for 10 min. Additional washings were performed using 1×SET at 55°C for 15 min, before drying and exposure to X-ray film. The relative amounts of mRNA were determined by densitometric scanning of Northern blot autoradiograms and quantified using the NIH image 1.63 applications (public domain).

RESULTS

Effect of cycloheximide on GDH activity

Under control conditions (0µM of cadmium) (Fig. 1A), the cyclohexemide not delay the NADH-GDH activities and had little effect.

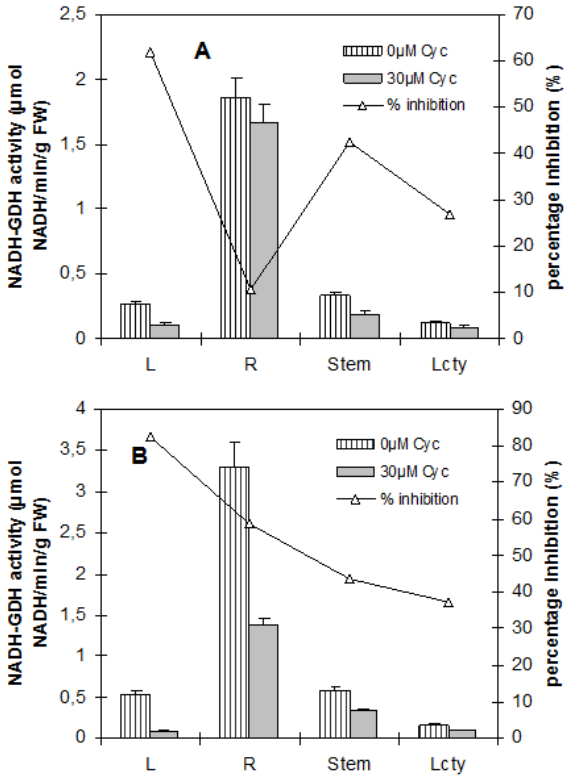

Fig. 1. Cycloheximide Effects on NADH-GDH activity in leaves (L), roots (R), steam (T+P) and cotyledolnal leaves (Lcoty), in absence (A) or in presence (B) of 20 µM cadmium. Results were expressed as percent (%) of control plants. L: 0.2650 ± 0.02, R: 1.863 ± 0.15, T+P: 0.336 ± 0.025, Fcoty: 0.126 ± 0.012 µmol NADH ox/min/g FW.

This effect was provided by the percentage of inhibition with cycloheximide. In the presence of 50 µM of cadmium (Fig. 1B), the inhibitor effect of cycloheximide was more pronounced especially in leaves (80%) and roots (60%). These results correlate to those of Juan Cuello and Sabater (1982). Similar results were obtained for all samples from repeated extractions. The same analysis was made on the diamination activity of the GDH (Fig. 2). The figure 2A and 2B show the net inhibition of the activity NAD^+-GDH by a soaking of 5 hours of tissues in a containing solution 50 µM of cadmium than plants not treated with Cd. So the effect of the cadmium in activities NADH-GDH or NAD^+-GDH is the same that it is made in contribution in the nourishing environment on 7 days of culture of the plantations of tomato, or by soaking of tissues during 5 hours. In plants treated by cycloheximide, NAD^+-GDH activity is sharply decreased whether it is in presence or in abscence of cadmium (Fig. 2A and 2B).

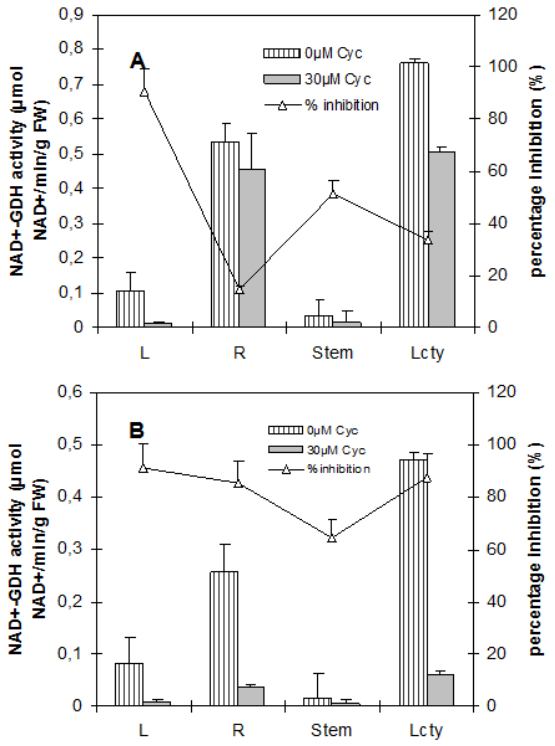

Fig. 2. Cycloheximide Effects on NAD$^+$-GDH activity in leaves (L), roots (R), stem and cotyledolnal leaves (Lcoty), in absence (A) or in presence (B) of 20 µM cadmium. Results were expressed as percent (%) of control plants. L: 0.273 ± 0.02, R: 2.63 ± 0.15, T+P: 1.26 ± 0.025, Lcoty: 0.26 ± 0.012 µmol NADH red/min/g FW.

The percentage of inhibition by the cycloheximide is more raised in all tissues, except in the case of stems treated to the cadmium than in those that have not undergoes this treatment (Fig. 2B). The cadmium and the cycloheximide have an additive effect in all the tissues with the exception of steams.

Effect of cycloheximide on GS activity

The measures of activity glutamine synthetase are presented in figures 3A and 3B. The figure 2B shows that activity GS is globally inhibited by the presence of cadmium in all the tissues, that reproduces effect first noticed in complete plant in culture compared to plants not treated with Cd (Fig. 3A). The presence of a preliminary treatment of tissues by the cycloheximide also inhibits activity GS. Nevertheless this inhibition is lesser in the presence of cadmium than in absence of cadmium, in the case of roots and stems (Fig. 3B). This effect is inverted in the case of shoots and cotyledonal leaves (Fig. 3B). Knowing that the composition of the pools GS1 and GS2 is different in roots and stems cotyledonal leaves, a differential sensibility of specific activities GS1 and GS2 in the cycloheximide can be suggested. To plants treated by Cd, there is a resumption of the synthesis of the protein which is translated by GS1 synthesis, bother of which is inferred by Cd. It against part, the cadmium infers a decline of the expression of the GS2. The effect of cycloheximide will be so more austere that it goes to the sense of that of the cadmium, that is case in organs which produce especially the isoforme GS2 as shoots and cotyledonal leaves. On the other hand, for roots and shoots more than in stem, the effect of the cadmium and the cycloheximine on the GS1 being in opposition, the enrichment of these tissues in GS1 with regard to sheets explains the least sensibility of roots and steam to the cycloheximide in the presence of cadmium. It is so possible to conclude that in the presence of cycloheximine in the environment the positive effect of the cadmium in the GS1 remains sensitive.

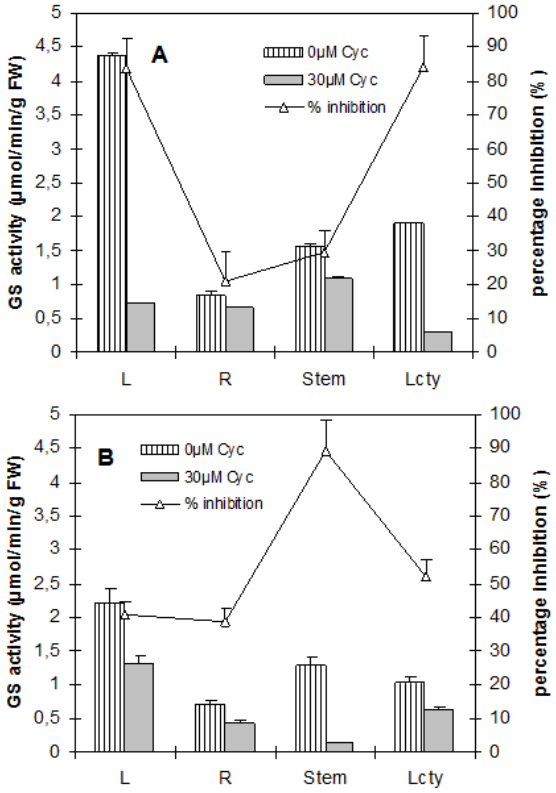

Fig. 3. Cycloheximide Effects on glutamine synthetase (GS) activity in leaves (L), roots (R), stem (T+P) and cotyledolnal leaves (Lcoty), in absence (A) on in presence (B) of 20 µM cadmium. Results were expressed as percent (%) of control plants: L: 2.65 ± 0.08 µmol γ-GHM/g FW/min, R : 1.012 ± 0.202 µmol γ-GHM /g FW/min, stem : 1.55 ± 0.1 µmol γ-GHM /g FW/min, Lcoty: 0.66 ± 0.052 µmol γ-GHM /g FW/min.

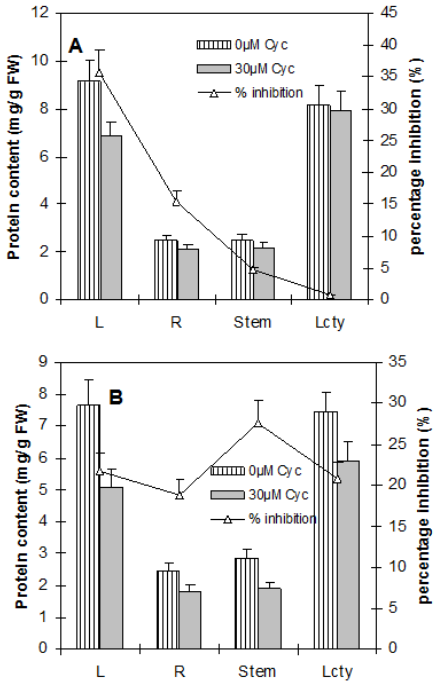

Fig. 4. Cycloheximide Effects on total soluble protein content in leaves (L), roots (R), stem and cotyledolnal leaves (Lcoty), in absence (A) or in presence (B) of 20 µM cadmium. Results were expressed as percent (%) of control plants. L: 9.126 ± 0.92, R: 2.475 ± 0.15, stem: 2.376 ± 0.25, Lcoty: 8.164 ± 0.8 mg/g FW.

Effect of cycloheximide on total soluble protein content

The result of total protein content in different organs was presented in figure 4. We can notice that the cycloheximide affects negatively total protein content (Fig. 4) by blocking the "turn over" proteins synthesis. The inhibition of the protein synthesis seems more marked in leaves than in roots, suggesting a turn over protein contents faster in the leaves than in the roots. Besides, the results of the figure 4B show that the effect of the cycloheximide is more marked in tissues put in the presence of cadmium in the case of stems, roots and cotyledonal leaves.

Effect of cycloheximide in the expression of gs1, gs2 and gdh genes

Inhibition of protein synthesis can affect the neosynthesis of proteins GS and GDH but also that of factors of transcriptions involved in the regulation of the expression of bothers coding these enzymes. That is why we examined the effect of the cycloheximide in the accumulation of these transcripts. This study was led on leaves and steam (Fig. 5). Obtained results show that globally the presence of cycloheximide affects the expression of various genes by decreasing contents in corresponding mRNA (Fig. 5A-D, line 1 and 3). In abscence of cycloheximide (line 2 and 4), the effect of an immerssion of tissues in a containing solution 20 µM of cadmium does not affect significantly contents in mRNA *gdh*, *gs1* or *gs2*.

However in the presence of cycloheximide, a differential effect of the dumping, during 5 hours in the presence of cadmium, is revealed. So it is possible to observe as in the case of activities GDH an opposite effect of the cadmium and the cycloheximide in the accumulation of the mRNA *gdh*. This shows that variations observed in contents in mRNA and for activities NADH-GDH are correlated. This confirms the hypothesis of a regulation transcriptional of the GDH by the cadmium. However this does not exclude either the existence of a regulation post-transcriptional from cadmium on the activity GDH. The notable difficult that the explanation of the diminution of the activity NAD^+-GDH in the presence of cadmium. That this does not seem indeed at all correlated to the accumulation of the mRNA observed. The use of a probe heterologous of Tobacco does not exclude existence to the tomato of another gene *gdh* that *gdh1*, the homology gene of which would not be sufficient to allow one cross-hybridization with the probe of tobacco. In the case of the GS, in leaves and steam, the presence of two cycloheximide and the cadmium seem to have a cooperative effect in the inhibition of RNAm *gs2* synthesis. The quality of hybridizations with the probe gs1 suggests a careful interpretation especially in the case of leaves, for which we would be tried to say that the cycloheximide and the cadmium are ineffective on the mRNA *gs1*. It does not seem that there is a cooperative effect between two substances in the case of the GS1. This suggests that hypothesis illustrated in the plan proposed in the previous paragraph to a regulation transcriptional of the GS2 and post-transcriptional of the GS1 (Fig. 7).

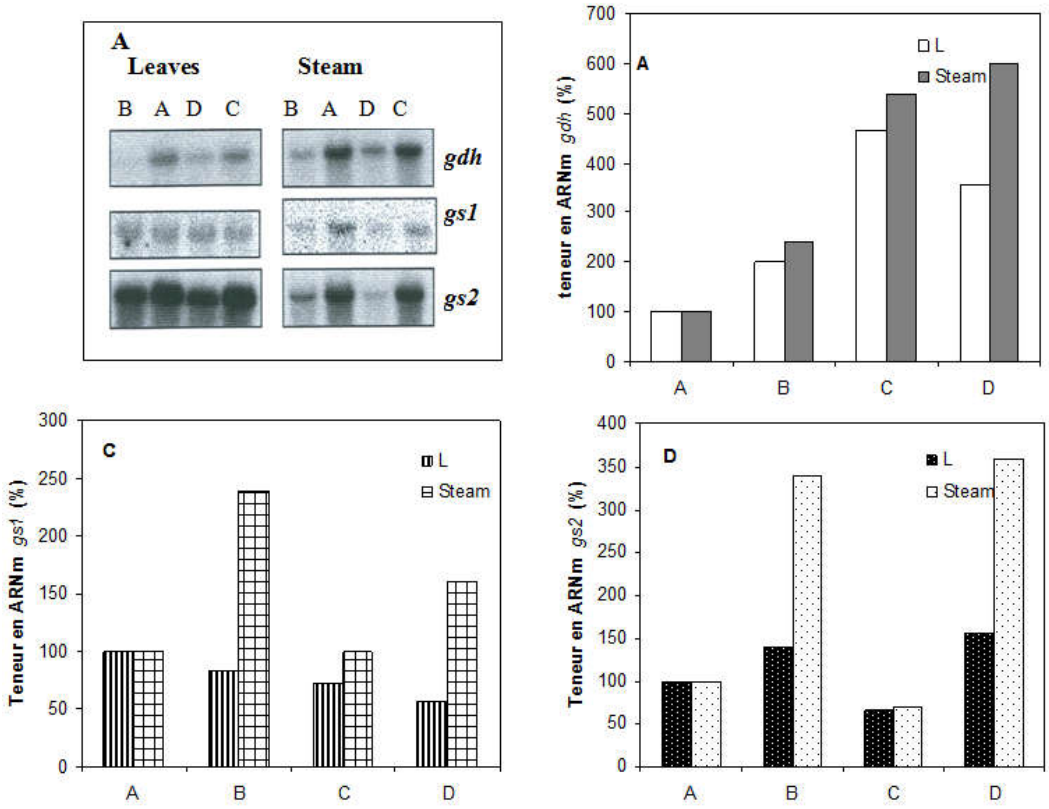

Fig. 5. Cycloheximide Effects on NADH-GDH activity repport, NAD^+-GDH activity repport and GS activity repport: B/A and D/C.
A: -Cd/-Cyloheximide
B: -Cd/+Cycloheximide
C: +Cd/-Cycloheximide
D: +Cd/+Cycloheximide

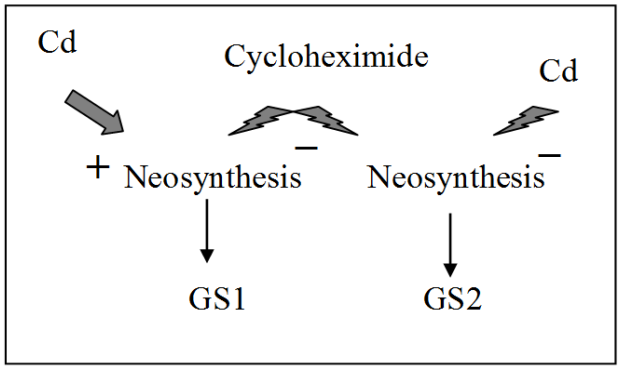

Fig. 6. Cycloheximide Effects on RNAm content of *gdh*, *gs1* and *gs2* content in leaves (L) and steam (T+P), in presence or absence 20 µM cadmium. Results were expressed as percent (%) of control plants.
A: -Cd/-Cyloheximide, B : -Cd/+Cycloheximide
C: +Cd/-Cycloheximide, D: +Cd/+Cycloheximide

DISCUSSION

To better discover the effect of cycloheximide, we established the report of activity NADH-GDH, NAD^+-GDH and GS (Fig. 7A, B and C). These results show that the effect of cycloheximide is more pointed at plants treated with Cd. This negative effect is more important in shoots and roots than stems and cotyledonal leaf. On the other hand stem and cotyledonal foliar seem insensible. These results correlate to those of Hanna *et al.* (2003). The two enzymes of GDH seemed to be synthesized during senescence established with cadmium. The rate of synthesis of GDH was clearly higher than that of its degradation, thus continuously increasing during senescence. Cycloheximide accelerated senescence of tomato plants in all organs. Cycloheximide treatments produced lower enzyme levels than their respective controls in both the light and dark, but the enzyme levels were higher in cycloheximide treated leaves in the light than in the controls in water in the dark. The results are discussed with reference to the requirement for protein synthesis in the different processes of senescence.

Clarification of the physiological role(s) of GDH isoenzymes is of great interest for biologists, because GDH occupies a biochemically critical position at the junction between carbon (2-OG) and nitrogen (Glu) metabolism and participates in the balancing of the cellular levels of three major components: the ammonium ions, 2-OG, and Glu. Until recently, there had been much controversy over the physiological function(s) of GDH isoenzymes in plants (Stitt *et al.* 2002; Dubois *et al.* 2003), although they have been the focus of many projects and were at the center of an ongoing scientific discussion for three decades. The controversial results were largely due to several constrains: (1) in most studies, the in vitro enzymatic activities had been considered as indices of the in vivo function of GDH; (2) the in vitro activity of GDH is readily reversible; (3) the on-gel activity of the seven GDH isoenzymes is assessed using Glu as substrate for activity staining; (4) GDH is expressed in the form of seven GDH isoenzymes consisting of two subunit polypeptides at different ratios (Loulakakis and Roubelakis-Angelakis 1991; Turano *et al.* 1996) encoded by different genes (Loulakakis and Roubelakis-Angelakis 1990b, 1991; Purnell *et al.* 2005); and (5) lack of suitable mutants and transgenic plants with altered genes encoding for the two subunit polypeptides of GDH. In stressed plants (presence of

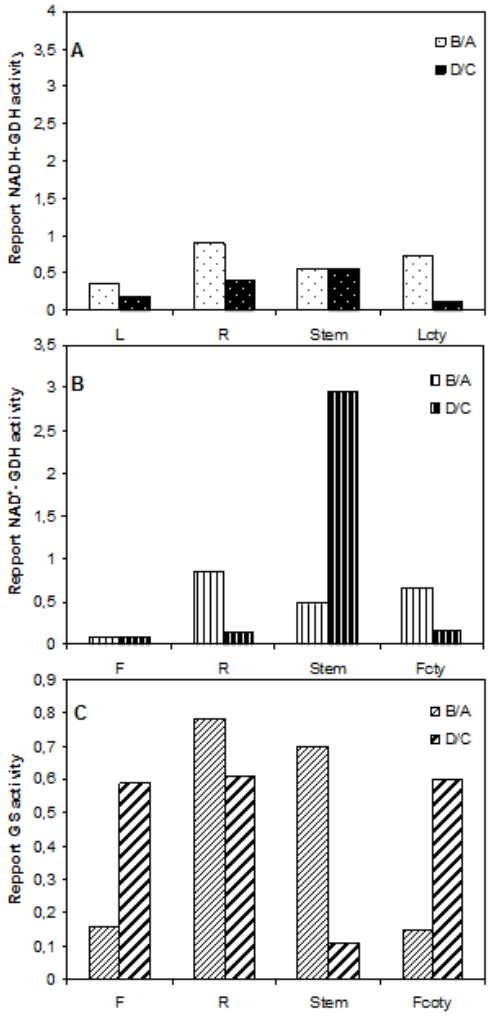

Fig. 7. Schema of the possible effect of cadmium and cycloheximide on activity production of GS1 and GS2.

Cd) high GDH-aminating activities measured in vitro, all overexpressing organs would be expected to exhibit high in vivo rates of ammonium assimilation in the presence of CHX, a potent inhibitor of GS. To test this hypothesis, plants treated with Cd were supplied $^{15}NH_4$ in the presence of CHX. Presence of the inhibitor resulted in approximately 90% inhibition of in vitro GS activity. Results shows the levels of [^{15}N]Glu resulting from the supply of $^{15}NH_4$ to tomato shoots, with or without CHX. From the results presented herein, it is clear that: (1) expression of the plant genes gdh-NAD**;**A is consistent with synthesis of the **α**-subunit polypeptide of GDH (Skopelitis et al., 2007); (2) accumulation of the **α**-subunit polypeptides results in the assembly of the anionic GDH isoenzymes, in agreement with previous results (Loulakakis and Roubelakis-Angelakis 1991; Turano *et al.* 1996); (3) the measured values of in vitro high aminating and low deaminating GDH enzymatic activities do not reflect the in vivo directions of the enzymatic action of GDH and explain the existing paradox and conflict in literature regarding the assessment of the physiological functions of GDH, although high ratios of in vitro aminating to deaminating activities were measured, the reverse was the case in vivo, i.e. very low ratios of aminating to deaminating activities); (4) the only reliable method for the assessment of the in vivo activities of GDH activity is the GC/MS or NMR analysis of the fate of [^{15}N]Glu and $^{15}NH_4$; and (5) results from these studies using the transgenic tobacco plants overexpressing either of the two

genes that encode for the α- and β-subunit polypeptides (Purnell *et al.* 2005; Purnell and Botella 2007) clarified that in vivo, all GDH isoenzymes strongly deaminate Glu to NH_4 under standard growth conditions. From the results presented, it is clear that: (1) expression of the plant genes gdh-NAD, is consistent with the level of GDH activity; (2) accumulation of the **α**-subunit polypeptides results in the assembly of the anionic GDH isoenzymes, in agreement with previous results (Loulakakis and Roubelakis-Angelakis 1991); (3) the measured values of in vitro high aminating and low deaminating GDH enzymatic activities do not reflect the in vivo directions of the enzymatic action of GDH and explain the existing paradox and conflict in literature regarding the assessment of the physiological functions of GDH, although high ratios of in vitro aminating to deaminating activities were measured, the reverse was the case in vivo, very low ratios of aminating to deaminating activities); (4) the only reliable method for the assessment of the in vivo activities of GDH activity is the GC/MS or NMR analysis of the fate of [^{15}N]Glu and $^{15}NH_4$; and (5) results from these studies using the stressed tomato plants overexpressing either of the two genes that encode for the α- and β-subunit polypeptides (Purnell and Botella 2007) clarified that in vivo, all GDH isoenzymes strongly deaminate Glu to NH_4 and only GDH-isoenzyme 7 and the anionic ones exhibit low aminating activity toward synthesis of Glu under standard growth conditions. Our results in this study, that showed that under stress (cadmium) conditions, the generated reactive oxygen species signal the expression of gdh-NAD**,** resulting in synthesis of GDH-isoenzyme, high in vivo aminating activity, leading to synthesis of Glu that in turn is directed toward Pro synthesis, and the available transgenic lines (Purnell *et al.* 2005; Fontaine *et al.* 2006; this work) will further provide a firm platform to answer pending questions about the physiological role(s) of GDH in plant carbon/nitrogen metabolism and to elucidate the developmental, trophic, and environmental cues regulating the expression of GDH genes.

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