Effect of Cadmium Repartition on Nitrogen Metabolism in Tobacco Seedlings

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Abstract

Thirty-day-old tobacco seedlings (*Nicotiana tabaccum*, Bureley v) were subjected during one week to increasing cadmium (Cd) concentrations (0, 10, 20, 50 and 100 µM $CdCl_2$). Increasing Cd stress led to a gradual decrease of dry weight (DW) production, water and nitrate contents. More than the half of Cd accumulated per plant was sequestered in the oldest leaf stage (S_1 leaves). Leaves from S_1 were the least affected by Cd stress. The activities of nitrate reductase (NR, EC 1.6.1.6), nitrite reductase (NiR, EC 1.7.7.1) were the least reduced in S_1 leaves despite of the high presence of Cd ions. At 100 µM Cd, glutamine synthetase activity (GS, EC 6.3.1.2) from S_1 leaves rose to become 2 times more important than control. Western Blot analysis showed that S_1 GS activity induction was correlated to the GS_1 and GS_2 protein accumulation. Young leaves (S_3 leaves) were more affected by Cd stress than old leaves. The GS activity reduction in S_3 leaves was correlated to GS_2 protein decrease detected by western-blot analysis. So, tobacco plant accumulated Cd ions in old leaves (S_1 leaves) to protect younger leaves which are more sensitive to Cd effects. Leaves from S_1 are a target organ to verify an eventual soil contamination per cadmium. This leaves may evolve adaptive process to partially inactivate Cd ions and maintain stable rate of nitrogen metabolism.

Subject Areas

Environmental Sciences

Keywords

Cadmium, Nitrate Reductase, Glutamate Dehydrogenase, Nitrogen Metabolism

1. Introduction

Cadmium (Cd) is the more noxious soil pollutant and its presence in the environment is essentially due to anthropogenic activities [1]. Because of its long biological half life, Cd^{2+} which belongs to the group of non-essential transition metals is highly toxic. Contamination of agricultural land, and so edible plants, is essentially due to the application of Cd-containing fertilizers and sewage sludges, atmospheric deposition or geogenic origin of Cd [2].

Cadmium toxicity is a major factor limiting plant growth in many soils [3]. Cd had many dangerous effects on plants, essentially the reduction of plant growth [3]. Cadmium inhibitor effect on growth could result from photosynthesis rate reduction [4] and the decline in nitrogen metabolism [5] [6] [7] [8]. A great deal of research has established the ability of cadmium to induce reactive oxygen species (ROS) production in plants [1] [9]. Cadmium stress may also impair the plasma membrane integrity by increasing lipid peroxidation [10]. Alternatively, it could alter plasma membrane permeability essentially nitrate and other essential-nutrients uptake. Hyperaccumulators are ideal plant species used for phytoremediation of Cd contaminated soils. Tobacco plants could be considered too, as a cadmium hyperaccumulator plant [8]. A full understanding of metal tolerance mechanisms of hyperaccumulators will facilitate enhancing their phytoremediation efficiency.

Previous work showed that tobacco plants accumulated Cd mostly in leaves. Leaf Cd content was six times more important than root Cd content. However, tobacco leaves were less affected by Cd stress than roots. In this report, we aimed at better understanding the differences in Cd partitioning between roots and different foliar stages to explain how tobacco leaves supported the high Cd level and could protect nitrogen metabolism under stress conditions. To clarify this question, this study investigated the effects of Cd accumulation on the activity of key enzymes involved in nitrogen metabolism in roots and in different foliar stages of tobacco.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Tobacco seeds (*Nicotiana tabaccum*, Bureley V.) were germinated on a moistured filter paper at 25°C in the dark. The uniform seedlings were then transferred to continuously aerated nutrient solutions containing 8 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 32.9 μM Fe-K-EDTA, and micronutrients: 30 μM H_3BO_4, 5 μM $MnSO_4$, 1 μM $CuSO_4$, 1 μM $ZnSO_4$, 1 μM $(NH_4)_6Mo_7O_{24}$. Plants were grown in a growth chamber with a photoperiod of 16 h-light $(m^{-2}s^{-2})$/8h-darck at 26°C and 20°C, respectively. The relative humidity was maintained with 70% and 90% in the light and in dark respectively. After an initial growth period of thirty days, $CdCl_2$ (10, 20, 50 and 100 μM) was added to the medium. After 7 days of heavy metal treatment, plants were assorted into shoots and roots. Roots were rapidly washed three times in 1 L of distilled water

and samples were desiccated at 60°C. The fresh and dry weights of each sample were determined before chemical analysis. Plant materials were kept at −80°C before analysis.

2.2. Determination of Nitrate Content

Nitrate ions were extracted from dry matter with 0.5 N H_2SO_4 at room temperature for 48 h. Nitrate was calorimetrically determined on an automatic analyzer following diazotation of the nitrite obtained by reduction of nitrate on a cadmium column [11].

2.3. Determination of Cadmium Content

Cadmium content in various plant tissues was analyzed by digestion of dried samples with an acid mixture ($HNO_3/HClO_4$, 4/1 v/v). Cadmium concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer, Analyst 300).

2.4. Protein Content

Soluble protein content was quantified using Coomassie Brilliant blue [12]. with bovine serum albumin as a protein standard.

2.5. Sugar Content

Total soluble sugars were determined according to [13].

2.6. Enzyme Assays

2.6.1. Nitrate Reductase

Plant material was homogenized with 100 mM potassium phosphate buffer (pH 7.4) containing 7.5 mM cystein, 1 mM EDTA and 1.5% (w/v) casein. The homogenate was centrifuged at 30,000 g for 15 min at 4°C. Nitrate reductase activity (NRA) was determined according to the method described by [14]. The extract of 0.1 ml was incubated in a reaction mixture containing 0.5 ml of 100 mM potassium phosphate buffer (pH 7.4), 0.1 ml of 0.15 mM NADH, and 0.1 ml of 100 mM KNO_3 at 30°C for 30 min. The extract was incubated with 10 mM $MgCl_2$ (for actual NRA determination) or with 15 mM EDTA (for maximum NRA determination). The reaction was stopped by 0.2 ml of 1000 mM zinc acetate. Nitrite ions were assayed after diazotation with 1 ml of 5.8 mM sulfanilamide, 1.5 N HCl, and 1 ml of 0.8 mM N-naphthyl-ethylene-diamine-dichloride.

2.6.2. Nitrite Reductase

Enzyme extracts were prepared as described above for nitrate reductase. Nitrite reductase was assayed by the method of [15]. The extract of 0.1 mL was incubated in a solution containing 0.4 mL of 100 mM potassium phosphate buffer (pH 7.4), 0.1 mL of 15 mM sodium nitrite, 0.2 mL of 5 mM methyl viologen, 0.2 mL of 86.2 mM sodium dithionite in a 190 mM $NaHCO_3$. The reaction was stopped by a violent agitation on vortex. Nitrite ions were assayed as described

for NRA assay.

2.6.3. Glutamine Synthetase

Samples were homogenized with grinding medium containing 25 mM Tris-HCl buffer (pH 7.6), 1 mM MgCl$_2$, 1 mM EDTA, 14 mM β-mercaptoethanol and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 25,000 g for 30 min at 4°C. GS activity was determined using hydroxylamine as substrate, and the formation of γ-glutamylhydroxamate (γ-GHM) was quantified with acidified ferric chloride [16].

2.6.4. Glutamate Dehydrogenase

GDH extraction was performed according to the method described by [17]. Frozen samples were homogenized in a cold mortar and pestle with 100 mM Tris-HCl (pH 7.5), 14 mM 2-mercaptoethanol, and 1% (w/v) PVP. The extract was centrifuged at 12,000 g for 15 min at 4°C. GDH activity was determined by following the absorbance changes at 340 nm.

2.7. Western-Blot Analyses

Proteins were extracted from frozen leaf material in cold extraction buffer containing 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM MgCl2, PVP 0.5% (w/v), 2mercaptoethanol 0.1% (v/v) and 4 mM leupeptine and separated by SDS-PAGE [18] Equal amounts of protein (25 μg) were loaded in each track. The percentage of polyacrylamide in the running gels was 12%. Proteins were electrophoretically transferred to nitrocellulose membranes for Western blot analysis. Polypeptide detection was done using polyclonal antiserum raised against Arabidopsis GS (Masclaux-Daubresse). Antibodies were raised in rabbits against the synthetic peptide AYGEGNERRLTG by Eurogentec (Seraing, Belgium) and they detected both GS$_1$ and GS$_2$ isoenzymes.

2.8. Statistical Analysis

The data are presented in the figures and in the tables as the average of at least six replicates per treatment and means ± confidence limits at P = 0.05 level. Each experiment was conducted in duplicate.

3. Results

3.1. Growth Response to Cadmium

Cadmium treatment induced a progressive decrease of leaf and root dry weight (DW) production (Figure 1(a)). Since low Cd treatment (10 μM), the root and leaf growth was affected. At high Cd treatment (100 μM), the reduction of root DW production was more than 70%. For the different foliar stages, the growth reduction was 36%, 64% and 76% in S$_1$, S$_2$ and S$_3$, respectively. S$_1$was the oldest foliar stage, S$_2$ and S$_3$ were younger than S$_1$ (Figure 1(a)). Cd belated the emergence of the fourth foliar stage in plants treated by high Cd doses (50 and 100 μM).
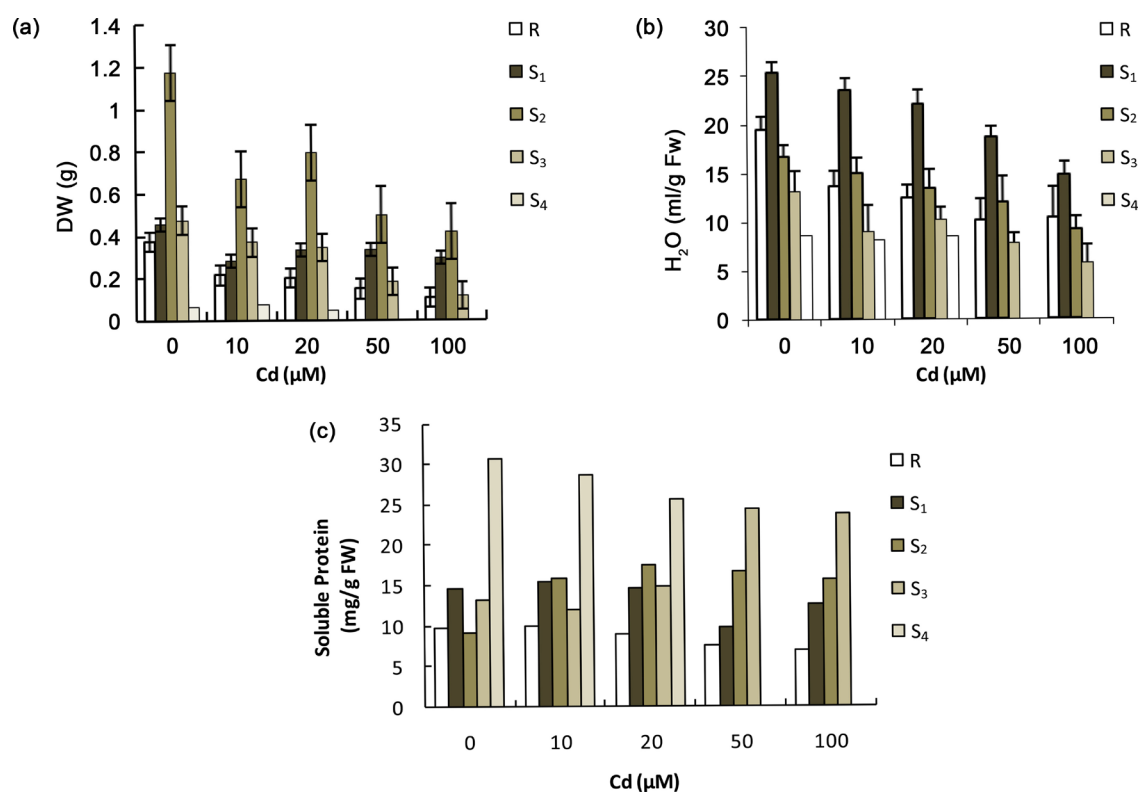
Figure 1. Effects of Cd treatments (0, 10, 20, 50 100 μM) for 7 days on (a) dry weight production (DW), (b) water contents and (c) Soluble protein contents. Data are means of six replicates ± CL at 0.05 levels.

The growth inhibition of tobacco seedlings was accompanied by a decrease in water contents (Figure 1(b)). At 100 μM Cd, root water content was decreased by about 45%. The leaf hydration was significantly reduced: Old leaves (S_1) were less dehydrated than S_3 leaves. The decrease of S_1, S_2 and S_3 water content was respectively about 40%, 45% and 57% (Figure 1(b)).

Soluble protein (SP) contents were gradually decreased with Cd treatment (Figure 1(c)). The highest Cd stress (100 μM) resulted in a decrease in soluble protein contents in roots (20%) and in leaves. The decrease of SP content was more important in S_1 leaves (50%) and lesser in S_3 and S_2 leaves (25%).

3.2. NO_3^-, Cd and Soluble Sugar Contents

In control plants, more than 85% of total NO_3^- ions were accumulated in leaves (Figure 2(a)). Under increasing Cd concentration, NO_3^- contents were greatly decreased in both leaves and roots. At 100 μM Cd, the decrease of root NO_3^- content was 80% with respect to control. In leaves, the reduction of NO_3^- content was, respectively, 60%, 90% and 95% in S_1, S_2 and S_3 leaves. The NO_3^- content of S_1 leaves was the less reduced under high Cd stress (Figure 2(a)).

Cadmium ions were more accumulated in leaves than in roots. At 10 μM Cd, the leaves accumulated more than 90% of the total of cadmium absorbed by plant (Figure 2(b)). In leaves, S_1 accumulated 50% of the total Cd quantity accumulated per plant. S_2 and S_3 accumulated respectively 30% and 3% of the total
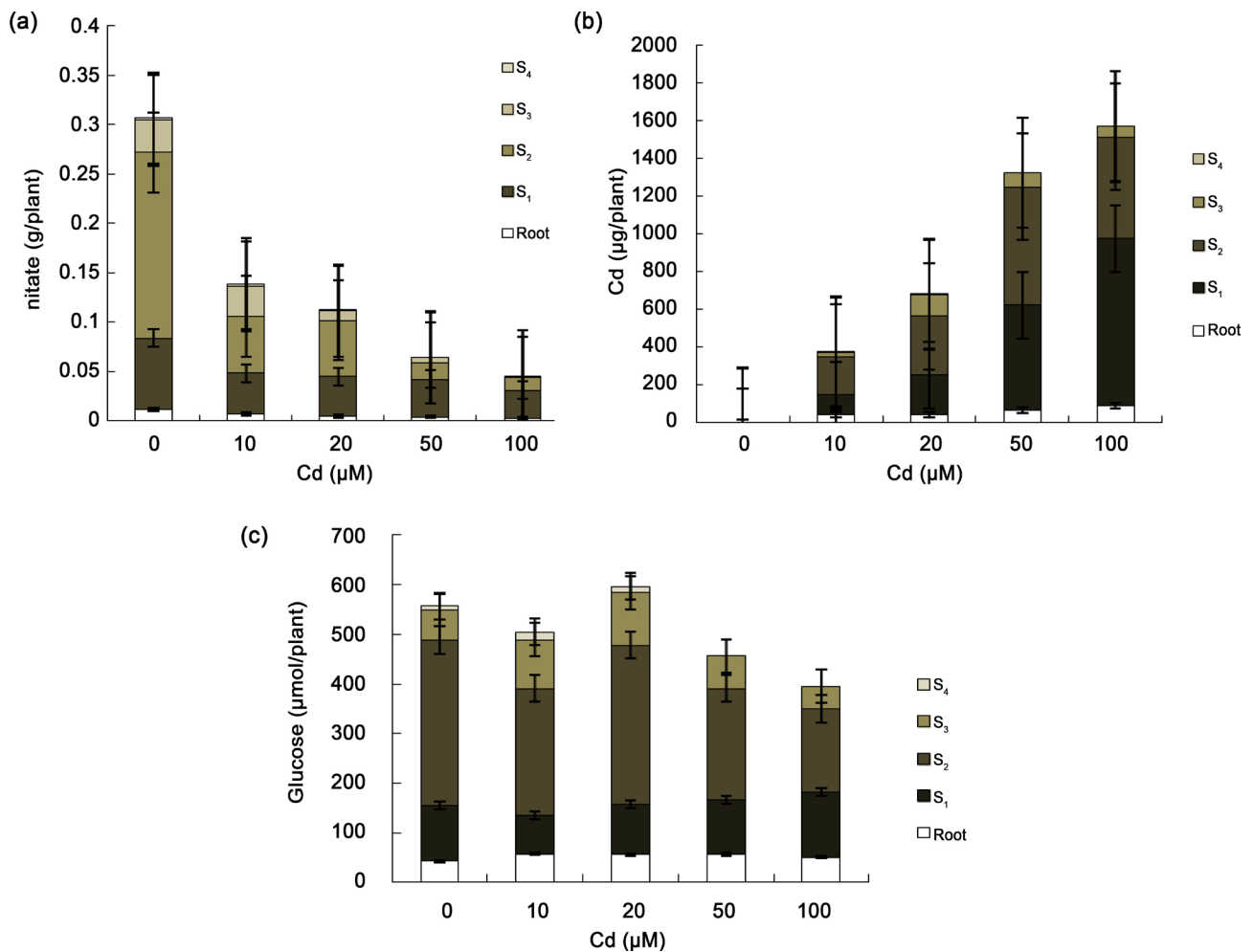
Figure 2. Changes in contents of (a) NO_3^- (g/plant), (b) Cd (g/plant) and (c) soluble sugars (μmol/plant) in different part of plants under Cd treatments (0, 10, 20, 50, 100 μM) for 7 days. Data are means of six replicates ± CL at 0.05 levels

quantity of Cd accumulated in each plant (**Figure 2(b)**).

In control tobacco seedlings, the major quantity of soluble sugars was accumulated in leaves: 80% of the total sugar quantity accumulated per plant. Roots accumulated 6% of the whole sugar quantity accumulated per plant. Under Cd stress, the soluble sugar quantity increased in roots (+20%) and in S_1 leaves (+15%). The soluble sugar quantity decreased apparently in S_2 (50%) and in S_3 (25%) leaves (**Figure 2(c)**).

3.3. Effects of CdCl₂ on the Nitrogen-Assimilating Enzymes

3.3.1. Nitrate Reductase Activity

In control tobacco seedlings, Nitrate reductase (NR) activity was higher in the leaves (80%) than in the roots (**Figure 3(a)**). Under high Cd treatment, root NR activity decreased with 80% with respect to control. In leaves, the reduction of NR activity was respectively 56%, 60% and 40% in S_1, S_2 and S_3. The NR activity reduction was more severe in roots. At 100 μM Cd treatment, root NR activity did not exceed 1, 8% of the whole NR activity for each plant (**Figure 3(a)**).
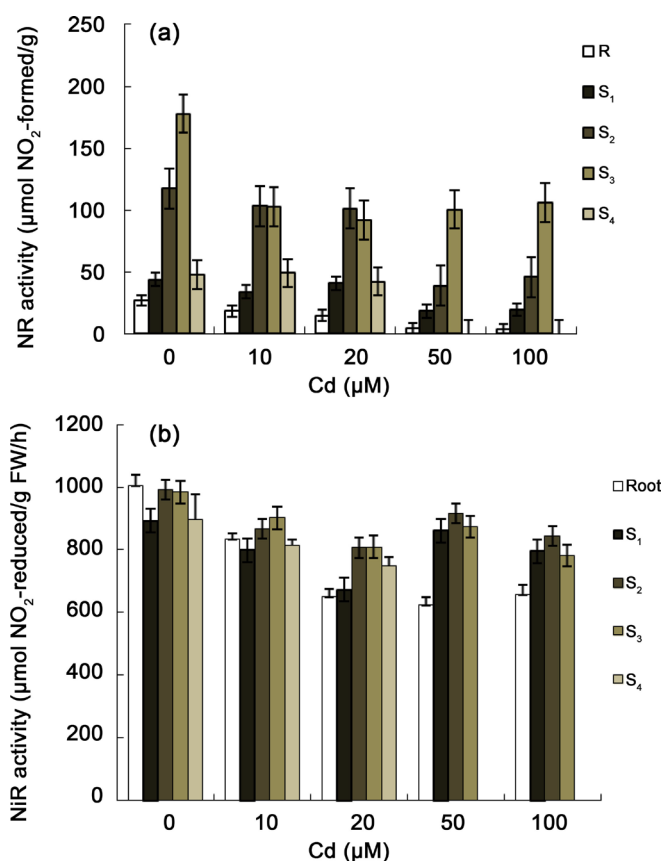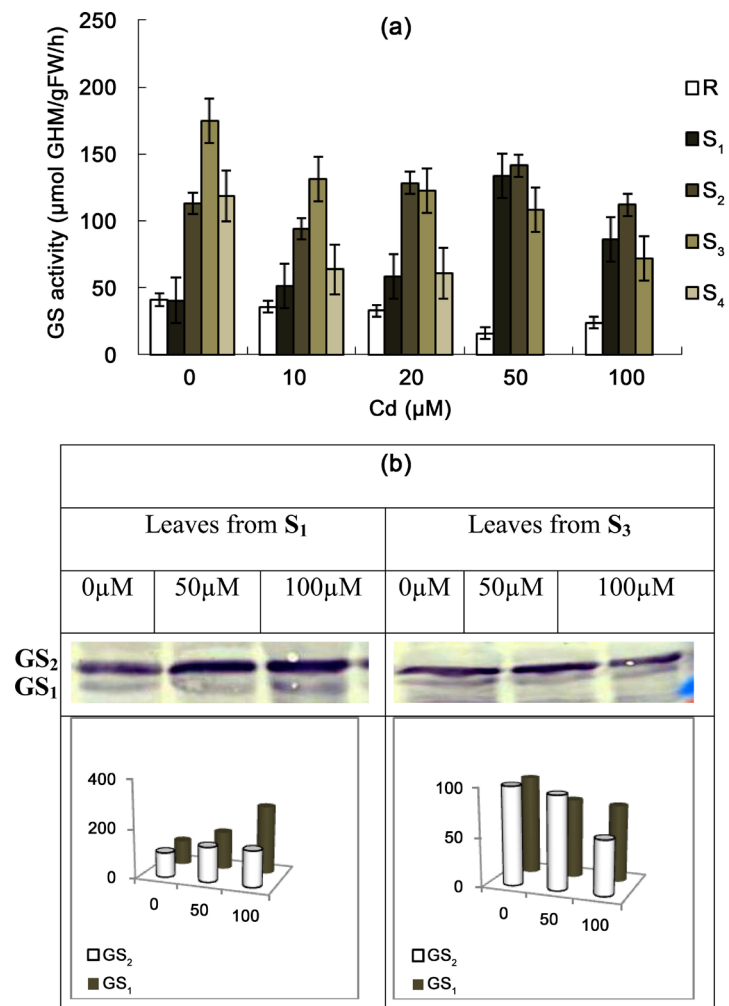
Figure 3. Effects of Cd treatments (0, 10, 20, 50, 100 μM) for 7 days on (a) Nitrate reductase activity (μmol NO_2^- formed. $g^{-1}FWh^{-1}$), and (b) Nitrite reductase activity (μmol NO_2^- reduced. $g^{-1}FWh^{-1}$) in different organs. Data are means of six replicates ± CL at 0.05 levels

3.3.2. Nitrite Reductase Activity

In leaves of control seedlings, NiR activity was more important than in roots; it represented 70% of the whole NiR activity in each plant. The NiR activity in roots was 18% of the total NiR activity per plant. The Cd addition in the culture medium caused a decrease of NiR activity in each plant organ. At 100 μM treatment, root NiR activity was more affected; it was decreased by about 35%. In leaves, the reduction of S_1, S_2 and S_3 NiR activity were correspondingly, 11%, 15% and 20% with respect to control (**Figure 3(b)**). S_1 NiR activity was less affected by high Cd treatment.

3.3.3. Glutamine Synthetase Activity

In the control plants, more than 80% of total GS activity was restricted in leaves. The inhibitory effect of Cd on GS activity appeared in each plant organ except for S_1 GS activity. At 100 μM, GS activity decreased by 40% in roots with respect to control. Leaves from S_1 GS activity increased to become 2 times more important than control. Leaves from S_3 GS activity decreased by 60% with respect to the control (**Figure 4(a)**). Whereas, leaves from S_2 GS activity was even under high Cd treatment. Western blot analysis showed that GS_2 is the major isoen-
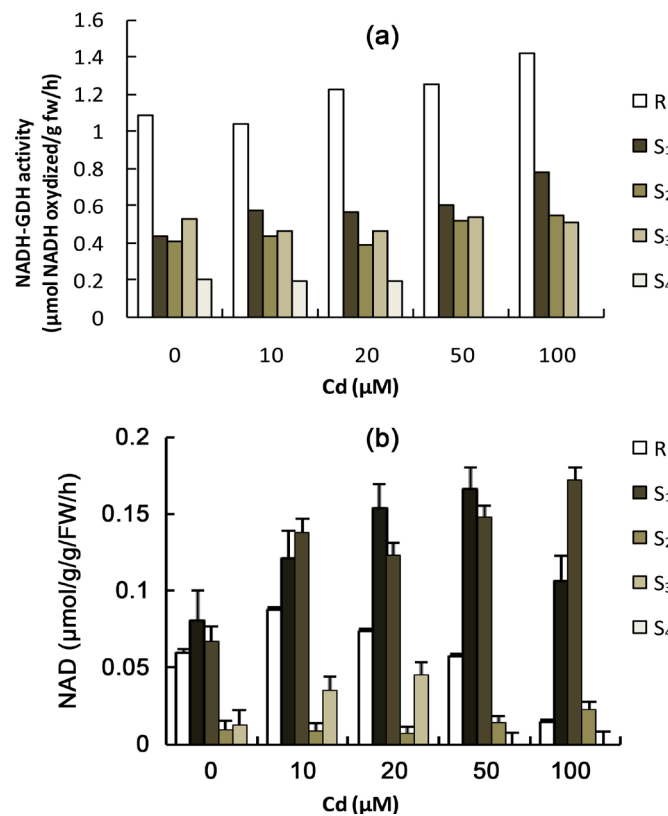
Figure 4. Effects of Cd treatments (0, 10, 20, 50, 100 μM) for 7 days on (a) GS activity (μmol γ-glutamyl hydroxamate. g^{-1} FW h^{-1}) in different foliar stages and roots. Data are means of six replicates ±CL at 0.05 levels. (b)Western-Blot analysis is assayed from S_1 and S_3 leaves in response to 7 days of 0, 50 and 100 μM Cd grown on hydroponic medium. Total proteins are extracted from the seedlings as described in "Materials and Methods". An aliquot of 25 μg of total protein from each sample was loaded onto each line: in Control leaves (CL), stressed leaves (SL).

zyme in tobacco leaves. Cadmium treatment reduced GS_1 and GS_2 protein quantity in S_3 leaves. The reduction of GS protein quantity is correlated to the decrease of GS activity. This reduction may be the result of protein degradation. Cadmium could have inhibitory effect on GS expression. Cadmium stress induced GS_1 and GS_2 protein quantity in leaves from S_1 (**Figure 4(b)**). So, the stimulation of GS activity corresponded to GS protein accumulation.

3.3.4. Aminating and Deaminating GDH Activities

In control plants, GDH aminating activity (NADH-GDH) was more important in roots than in leaves (**Figure 5(a)**). In both organs, aminating GDH activity was more important than deaminating GDH activity (NAD-GDH) (**Figure 5(b)**). Under Cd treatments, the aminating GDH activity was enhanced in the

Figure 5. Effects of Cd treatments (0, 10, 20, 50, 100 μM) for 7 days on (a) NADH-GDH activity (μmol NADH oxidized. $g^{-1}FWh^{-1}$) and (b) NAD-GDH activity (μmol NAD reduced. $g^{-1}FWh^{-1}$) in different foliar stages and roots. Data are means of six replicates ±CL at 0.05 levels.

roots and especially in leaves (**Figure 5(a)**). At 100 μM Cd, the aminating GDH activity was stimulated by 30% in the roots and more than 70% in S_1 leaves, with respect to controls (**Figure 5(a)**). Cd stress induced a slight decrease in aminating GDH activity of young leaves (S_3 and S_4). In stressed seedlings, GDH deaminating activity (NAD-GDH) was stimulated in leaves. At high Cd treatment, NAD-GDH activity in young leaves (S_2, S_3 and S_4) was at least two times higher than controls. In the roots, Cd stress had an inhibitory effect on the deaminating GDH activity which was decreased by 70% at 100 μM Cd treatment (**Figure 5(b)**). Cd stress caused a slight increase in deaminating GDH activity in S_1 leaves (3%) with respect to control.

4. Discussion

Under increasing Cd treatments, we observed a mean biomass decrease in leaves and in roots [19]. This effect was mainly observed in the roots (70%), while leaves were apparently damaged only by the highest Cd concentrations. At 100 μM Cd treatment, the reduction of DW production in leaves from S_1 (oldest foliar stage) was less important than the decrease in leaves from S_2 and S_3. As well, old leaves (S_1) were, less dehydrated than younger leaves (S_3) (**Figure 1(b)**). So, old leaves from S_1 were less affected by Cd than younger leaves (S_2 and S_3).

Ion analysis showed that Cd stress led to an elevated decrease in NO_3^- contents in roots and leaves. The inhibitory effect of Cd on nitrate contents was reported in tomato seedlings [6] in *Solanum nigrum* L. [7] and in rice (Huang and Xiong, 2009). The NO_3^- content decrease was more important in young leaves and roots. Our data showed that Cd content in leaves and roots did not follow the same trends according to Cd exposure in the same variety of tobacco (Bureley) which had been reported in Bovet *et al.* (2006) [19]. Cadmium was accumulated essentially in leaves: at 100 μM treatment, leaves had 90% of Cd accumulated per plant [8] [20]. This data confirm what had been described by [21] [22]. At high Cd treatment, leaves from S_1 accumulated more than the half of current Cd per plant, while S_3 accumulated only 3%. Tobacco plant could adopt exclusive strategy of Cd ions to protect young leaves from Cd accumulation. These old leaves that accumulated the most important quantity of Cd were less affected by Cd stress compared to different foliar stages of tobacco plant (**Figure 1(a)**; **Figure 2(a)** and **Figure 2(b)**).

The obtained decrease in nitrate contents in leaves and roots, could affect the subsequent processes involved in nitrate reduction and assimilation. In fact, NO_3^- regulated the NR and NiR expressions [7] and activities [23]. In tobacco seedlings, leaf NR activity was more important than root NR activity (**Figure 3(a)**). This elevated nitrate reduction was related to the higher leaf NR protein contents [24] and a sufficient availability of light and reducing power [25].

After Cd exposure of tobacco seedlings, NR activity induced a significant decrease [8] [23] which was more pronounced in the roots than in leaves (**Figure 3(a)**). In leaves, NR activity reduction was more pronounced in S_2 leaves. The NR activity decrease in leaves from S_2 was associated to a severe decrease in nitrate content (**Figure 2(b)**). The nitrate content plays a direct role for NR protein production and activation. Soluble sugar content decreased visibly in leaves from S_2 under Cd stress. This reduction of sugar content could cause NR activity decrease in S_2 leaves. Klein *et al.* 2000 [26] reported that low sugar repressed NR gene expression that affected NR protein quantity and NR activity [20]. Thereafter, the Cd-induced inhibition of NR activity in the leaves may result from the low nitrate availability at the enzyme reduction site. The decrease of NR activity in Cd-treated plants could also reflect an increase in the enzyme breakdown induced by toxic oxygen species generated during stress treatment. Indeed free radicals could cause the breakdown of proteins directly by oxidative reaction or indirectly by increasing proteolytic activity. The NiR activity was less affected by Cd stress than the NR activity. This higher NiR activity in the leaves and the roots, disable the toxic accumulation of nitrite ions [27].

With increasing Cd concentration, NiR activity was slightly decreased in the leaves and mainly in the roots (**Figure 3(b)**). The ammonium produced by NiR was then incorporated into an organic form primarily by the GS enzyme. The presence of Cd in the nutrient solution caused a significant decrease in GS activity in roots (**Figure 4(a)**). At 100 μM Cd treatment, GS activity increased in S_1 leaves, to become 2 times more important than control.

GS activity induction is correlated to the stimulation of GS_1 and GS_2 protein accumulation (**Figure 4(b)**). This result was reported previously in tomato seedlings [7]. The GS activity induction and the cytosolic GS isoforme (GS_1) protein increase were probably related to the induction of GLN transcripts. While in S_3 leaves, cadmium treatment reduced GS_1 and GS_2 protein quantity. The reduction of GS protein quantity is correlated to GS activity decrease. Cadmium stress affected the nitrogen enzyme activity by enzyme protein alterations.

At the same time as, Cd stress was found to increase the aminating GDH activity in S_1 leaves and roots, even at high Cd concentrations (100 μM) (**Figure 5(a)**). Conformingly to the increase in S_1 GS activity, aminating GDH activity was stimulated to provide GS activity with Glutamate. This increase of aminating GDH was reported by many authors [7]. Aminating GDH activity seems to be also involved in the ammonium detoxification under stress conditions [9].

Cd stress caused a clear increase in deaminating GDH (NAD-GDH) activity in young leaves. Deaminating GDH activity became 2 times more important than control. This increase in deaminating GDH activity could provide young leaves with carbohydrates. While an important deaminating GDH activity decrease was noted in roots (70%). In S_1 leaves, the NAD-GDH activity reduction was insignificant (3%) with respect to control (**Figure 5(b)**).

We noted that Cd was accumulated in a lessening gradient from basal to apical leaves. This lessening gradient of Cd accumulation was accompanied with a Cd tolerance gradient in the same direction. Thus the response difference of tobacco leaves to the cadmium could be bound either to the leaf mature or/and the contact with important Cd quantities. Although, more work is needed at the molecular level for further information towards the subcellular accumulation of Cd in young and old leaves, phytochelatins accumulation and the Cd effects on protein and gene expression of nitrogen metabolism enzymes. Although foliar Cd accumulation, roots were more affected by Cd stress. The lower sensitivity of S_1 leaves to Cd could be related, at least in part, to a lesser reduction of nitrate reduction and ammonium assimilation, concomitantly with a high increase in aminating GDH activity under Cd stress and an ability of S_1 leaves to accumulate this metal in non-active form. Tobacco plant could be considered as a hyperaccumulator plant used to clean up soil contaminated with cadmium. Ultimately, the large accumulation of Cd in leaves invited tobacco manufactories using leaves for cigarette production, to strictly make sure that exploited soils are not contaminated by Cd or other heavy metals. The reduction of cadmium content can reduce health hazards to smokers by selection of young leaves rather than old leaves and control of pH soil that have an effect on Cd uptake. S_1 leaves are a target organ to verify an eventual soil contamination per cadmium.

References

[1] Di Toppi, L. and Gabbrielli, R. (1999) Response to Cadmium in Higher Plants. *Environmental and Experimental Botany*, **41**, 105-130.
https://doi.org/10.1016/S0098-8472(98)00058-6

[2]   McLaughlin, M.J. and Singh, B.R. (1999) Cadmium in Soils and Plants. Kluwer Academic Publishers, Dordrecht, 1-9. https://doi.org/10.1007/978-94-011-4473-5

[3]   Fediuc, E. and Erdei, L. (2002) Physiological and Biochemical Aspects of Cadmium Toxicity and Protective Mechanisms Induced in *Phragmites australis* and *Typha latifolia. Journal of Plant Physiology*, **159**, 265-271. https://doi.org/10.1078/0176-1617-00639

[4]   Wagner, G.J. and Trotter, M.M. (1982) Inducible Cadmium Binding Complexes of Cabbage and Tobacco. *Plant Physiology*, **69**, 804-809. https://doi.org/10.1104/pp.69.4.804

[5]   Wahid, A., Ghani, A., Ali, I. and Ashraf, M.Y. (2007) Effects of Cadmium on Carbon and Nitrogen Assimilation in Shoots of Mungbean [*Vigna radiata* (L.) Wilczek Seedlings. *Journal of Agronomy & Crop Science*, **193**, 357-365. https://doi.org/10.1111/j.1439-037X.2007.00270.x

[6]   Chaffei, C., Pageau, K., Suzuki, A., Gouia, H., Ghorbel, M.H. and Masclaux-Daubresse, C. (2004) Cadmium Toxicity Induced Changes in Nitrogen Management in *Lycopersicon esculentum* Leading to a Metabolic Safeguard through an Amino Acid Storage Strategy. *Plant Cell Physiology*, **45**, 1681-1693. https://doi.org/10.1093/pcp/pch192

[7]   Wang, L., Zhou, Q., Ding, L. and Sun, Y. (2008) Effect of Cadmium Toxicity on Nitrogen Metabolism in Leaves of *Solanum nigrum* L. as a Newly Found Cadmium Hyperaccumulator. *Journal of Hazardous Materials*, **154**, 818-825. https://doi.org/10.1016/j.jhazmat.2007.10.097

[8]   Mâaroufi, H., Debouba, M., Ghorbel, M.H. and Gouia, H. (2009) Tissue-Specific Cadmium Accumulation and Its Effects on Nitrogen Metabolism in Tobacco (*Nicotiana tabaccum*, Bureley *v.* Fb9). *Comptes Rendue de Biologie*, **332**, 58-68. https://doi.org/10.1016/j.crvi.2008.08.021

[9]   Romero-Puertas, M.C., McCarthy, I., Gómez, M., Sandalio, L.M., Corpas, F.J., del Río, L.A. and Palma, J.M. (2004) Reactive Oxygen Species-Mediated Enzymatic Systems Involved in the Oxidative Action of 2,4-Dichlorophenoxyacetic Acid. *Plant, Cell & Environment*, **27**, 1135-1148. https://doi.org/10.1111/j.1365-3040.2004.01219.x

[10] Mediouni, C., Benzarti, O., Tray, B., Ghorbel, M.H. and Jemal, F. (2006) Cadmium and Copper Toxicity for Tomato Seedlings. *Agronomy for Sustainable Development*, **26**, 227-232. https://doi.org/10.1051/agro:2006008

[11] Henriksen, A. and Selmer-Olsen, A.R. (1970) Automatic Methods for Determining Nitrate and Nitrite in Water and Soil Extracts. *Analyst*, **95**, 514-518. https://doi.org/10.1039/an9709500514

[12] Bradford, M.M. (1976) A Rapid and Sensitive Method for the Quantitative Determination of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, **72**, 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

[13] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric Method for Determination of Sugars and Related Substances. *Analytic Chemistry*, **28**, 350-356. https://doi.org/10.1021/ac60111a017

[14] Robin, P. (1979) Etude de quelques conditions d'extraction du nitrate réductase des racines et des feuilles de plantules de maïs. *Physiologie Végétale*, **17**, 45-54.

[15] Losada, M. and Paneque, A. (1971) Nitrite Reductase. *Methods in Enzymology*, **23**, 487-491. https://doi.org/10.1016/S0076-6879(71)23120-7

[16] Wallsgrove, R.M., Lea, P.J. and Miflin, B.J. (1979) Distribution of the Enzymes of

Nitrogen Assimilation within the Pea Leaf Cell. *Plant Physiology*, **63**, 232-236.
https://doi.org/10.1104/pp.63.2.232

[17] Magalhaes, J.R. and Huber, D.M. (1991) Free Ammonia, Free Amino Acid and En-zyme Activity in Maize Tissue Treated with *Methionine sulfoximine*. *Journal of Plant Nutrition*, **14**, 883-895. https://doi.org/10.1080/01904169109364249

[18] Laemmli, U.K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, **227**, 680-685. https://doi.org/10.1038/227680a0

[19] Bovet, L., Rossi, L. and Lugon-Moulin, N.C. (2006) Cadmium Partitioning and Gene Expression Studies in *Nicotiana tabacum* and *Nicotiana rustica*. *Physiologia Plantarum*, **128**, 466-475. https://doi.org/10.1111/j.1399-3054.2006.00756.x

[20] Wang, R., Guegler, K., LaBrie, S.T. and Crawford, N.M. (2000) Genomic Analysis of Nutrient Response in Arabidopsis Reveals Diverse Expression Patterns and Novel Metabolic and Potential Regulatory Genes Induced by Nitrate. *The Plant Cell*, **12**, 1491-1510. https://doi.org/10.1105/tpc.12.8.1491

[21] Huang, H. and Xiong, Z.T. (2009) Toxic Effects of Cadmium, Acetochlor and Ben-sulfuron-Methyl on Nitrogen Metabolism and Plant Growth in Rice Seedlings. *Pesticide Biochemistry and Physiology*, **94**, 64-67.
https://doi.org/10.1016/j.pestbp.2009.04.003

[22] Lugon-Moulin, N., Zhang, M., Gadani, F., Rossi, L., Koller, D., Krauss, M. and Wagner, G.J. (2004) Critical Review of the Science and Options for Reducing Cadmium in Tobacco (*Nicotiana tabacum* L.) and Other Plants. *Advances in Agronomy*, **83**, 111-180. https://doi.org/10.1016/S0065-2113(04)83003-7

[23] Takabayashi, M., Wilkerson, F.P. and Robertson, D. (2005) Response of Glutamine Synthetase Gene Transcription and Enzyme Activity to External Nitrogen Sources in the Diatom *Skeletonema costatum* (Baillariophyceae). *Journal of Phycology*, **41**, 84-94. https://doi.org/10.1111/j.1529-8817.2005.04115.x

[24] Abd-ElBaki, G.K., Siefritz, F., Man, H.M., Weiner, H., Haldenhoff, R. and Kaiser, W. (2000) Nitrate Reductase in *Zea mays* L. under Salinity. *Plant, Cell and Environment*, **23**, 515-521. https://doi.org/10.1046/j.1365-3040.2000.00568.x

[25] Lillo, C., Meyer, C., Lea, U.S., Provan, F. and Olteda, S. (2004) Mechanism and Importance of Post-Translational Regulation of Nitrate Reductase. *Journal of Experimental Botany*, **55**, 1275-1282. https://doi.org/10.1093/jxb/erh132

[26] Klein, D., Morcuende, R., Stitt, M. and Krapp, A. (2000) Regulation of Nitrate Reductase Expression in Leaves by Nitrate and Nitrogen Metabolism Is Completely Overridden When Sugars Fell below a Critical Level. *Plant, Cell and Environment*, **23**, 863-871. https://doi.org/10.1046/j.1365-3040.2000.00593.x

[27] Ezzine, M. and Ghorbel, M.H. (2006) Physiological and Biochemical Responses Resulting from Nitrite Accumulation in Tomato (*Lycopersicon esculentum* Mill. cv. Ibiza F1). *Journal of Plant Physiology*, **163**, 1032-1039.
https://doi.org/10.1016/j.jplph.2005.07.013

Abbreviations

CL	confidence limit
Chl a	chlorophyll a
Chl b	chlorophyll b
DW	dry weight
Fd-GOGAT	ferredoxine glutamate synthase
GDH	glutamate dehydrogenase
GS	glutamine synthetase
NADH-GOGAT	NADH glutamate synthase
NR	nitrate reductase
NiR	nitrite reductase