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Review Article

Cadmium Toxicity and Genetic Diversity: A Review on Embryo Growth, Reserve Mobilization, Oxidative Stress and Root Damage

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Abstract

Medicago truncatula genotypes differing in cadmium susceptibility were used to test mineral, reserve mobilization, oxidative disorders, defense pathways and stress responses in embryos. Cadmium caused alteration of mineral elements, carbohydrate and free amino acid accumulations. Carbohydrates were determining to susceptible lines growth in control condition; nevertheless, free amino acids enable tolerant lines to counteract cadmium intrusion. Transcriptional changes in response to cadmium treatment were analyzed a gene encoding a monosaccharide transport protein (*Mt*MST). A significant down-regulation was observed in the most susceptible line TN1.11. Glucose was over-consumed in tolerant lines. Thus, glucose metabolism integrity is shown essential to enhance growth under cadmium exposure. Nutrient evaluation in germination medium quantified solute losses extent at the expense of suitable mobilization to the growing embryonic axis and membrane alterations. FAAS and TSS leakages were reduced in case of tolerant lines while monosaccharide losses were accented in susceptible lines. Interactions between root growth inhibition, and the occurrence of oxidative injury suggest differential responses of the genotypes, with susceptible or tolerant accessions. ROS and H_2O_2 did not seem related to tolerance or susceptibility. Oxidative burst impact on cell membrane integrity suggests an active role of this burst in susceptible lines. Ascorbate-glutathione and antioxidative system, secondary metabolism events including phenolic compounds and lignification launching; and developmental modifications were described. Transcriptional changes in response to cadmium treatment were analyzed on target genes involved in (1) ROS-scavenging enzymes, (2) reduced glutathione metabolism and (3) metal chelating metabolism. *In situ* observation illustrated soluble phenolic compounds accumulation under Cd-treatment. However, lignification was restricted to recently created protoxylem elements established in the root tip area usually constituting the elongation zone. CD was increased. In absence of necrotic reactions, developmental changes including lignin deposition increase in cellulose and pectin contents, inter-cellular meatus, condensed and deformed hairs were noticed in Cd-treated roots.

Keywords: *Cadmium; Genetic Diversity; Embryo Growth; Reserve Mobilization; Oxidative Stress; Root Damage*

Introduction

Cadmium, a non-essential trace element, is ubiquitous [1], with a very high affinity to oxygen-, nitrogen- or sulfur-containing ligands [2,3]. Environmental protection agencies have unanimously ranked cadmium as one of the most polluting heavy metals [4]. Its tolerable threshold in crops and edible legumes is 0.1 mg/kg [5].

Growth decline in plants is a major effect of cadmium toxicity and reductions of biomass production and nutritional quality have been often observed in crops [3,5,6].

Germination and early seedling growth are crucial in plant life and extremely responsive to environment alterations [6]. Decreased germination rates have often been observed after exposure to metallic ions [7,8]. The literature reported a correlation between growth decline and numerous disorders in germinative metabolism [9].

Medicago truncatula was used to investigate the effect of Cadmium on germination and early seedling growth. *Medicago truncatula*, as a legume model, is easily grown and maintained under laboratory conditions. The biodiversity of this species should reveal potentially important characters during germination and heterotrophic growth.

Biomass mobilization represents a crucial event during germination and depends on previous water and oxygen absorption. After degradation of polymers, released metabolites are transported to growing embryonic cells. Oligosaccharides and hexoses are energy source for the embryonic axis [10], whereas others can be used for macromolecules synthesis, prosthetic groups, cofactors or activators of enzymatic systems. Metabolite transport depends on the availability of membrane transporters [11] and hydrolases [10] which are significantly stimulated during germination. Their activity leads to perforations in the cell wall, facilitating root emergence.

This research work inspects the impact of imbibition with $CdCl_2$ solution on (1) growth and (2) reserve mobilization. The response to Cd stress was analyzed within six *Medicago truncatula* embryos. Cd-treated seedling showed root growth inhibition with a genotype-dependent pattern. Free amino acid and sugar content as well as leakage into the germination medium were studied to highlight nutrient transport and membrane integrity alterations in growing roots. We also analyzed the mechanisms of metal action on Monosaccharide Transporter (MtMSt) gene expression.

Under Cd-exposure, ROS overproduction is as important as the antioxidative defense mechanisms may be lacking or altered [12]. Therefore, ROS occurrence tends to pull electrons from other molecules such as phospholipids and result in lipoperoxidation enhancement evidenced by high malondialdehyde content in pea [10]. When diffusing through cells, cadmium changes redox status and polarity of neighboring cell and would stimulate antioxidant defense mechanisms. ROS enhancement may have several origins; for instance, glutathione and protein-bound sulfhydryl-groups depletion and prooxidant status enhancement, calcium-dependent systems activating and iron-mediated processes affecting or photosynthetic electron chain disruption [13-15].

As antioxidants; antioxidative enzymes in concert with glutathione, ascorbate and superoxide dismutase (SOD), ascorbate peroxidases (APX), and catalases (CAT) regulates reactive oxygen species levels. Ascorbate reprocess is realized by monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DAR), and glutathione reductase (GR).

To allow its transport, Cd is chelated by thiol ligands: such as glutathione (GSH) and its derivatives namely phytochelatins (PCs) [16]. GSH can regulate the cell redox status through signaling pathways inducing the expression of genes encoding antioxidant enzymes. It is also involved in xenobiotic detoxification mechanisms [17]; as reductant by glutathione reductase (GR), glutathione transferase (GST), glutathione peroxidase (GPX) and some peroxiredoxins (PRX). For instance, GST maintains a protective role against damage from oxidative stress initiated by hydroxyl radicals which induces lipoperoxides and membrane damage [18]. These compounds are conjugated with

glutathione through GST intermediate allowing their elimination [19]. GR enzymes, on the other hand, coordinate with GPX maintaining the balance of the glutathione system. Moreover, GSH is required for the synthesis of PCs. In most plant families, PCs are synthesized enzymatically from glutathione by phytochelatin synthase (PCS) [20]. PCS bind heavy metals and form metal-PC complexes reducing metal toxicity [21].

We secondly describe: (1) the oxidative stress enhanced responses and (2) detoxification mechanisms alterations. H_2O_2 enhancement and ROS *in situ* observation were measured. Membrane integrity was analyzed using MDA and solute leakage estimations.

Gene expression of anti-oxidative and detoxification capacities alterations was analyzed in (1) the sequence of physiological reactions including ROS-scavenging enzymes and ascorbate-glutathione-related antioxidant systems, (2) genes involved in reduced glutathione metabolism (Glutathione-S-transferase (EC: 2.5.1.18) and Glutathione reductase (EC 1.8.1.7) and (3) a gene implicated in metal chelating metabolism; PCS.

However, under Cd-exposure common defense pathways are launched in plant cells likewise to other biotic or abiotic stresses. Acting as a signaling molecule, H_2O_2 accumulation is the common first event of these pathways. In plant-pathogen interactions, a coordinated succession of reactions relating peroxidases activation, secondary metabolism enhancement, structural changes such as lignin deposition, and eventually cell death (CD) are induced by H_2O_2 [22]. Actually, Fojtova and Kovarik (2000) illustrated apoptotic alterations in suspension cultures of tobacco cells after Cd-exposure, which were characterized by DNA fragmentation. It must be recalled that CD is a process that cannot be inverted.

After cadmium exposure, unusual development leading to xylogenesis and ultimate lignifications establishment were observed [23]. All the same, lignified xylem elements were established too near to the root tip at the expense of the elongation zone [24].

In case of heavy metal stress, development alterations may increase heavy metal potential to bind cell walls by increasing their cation exchange capacity. Thus, cell wall composition changes due to Cadmium stress may be involved in cell response optimization [25]. It is important to consider that the first path of metal uptake is simple diffusion through the apoplast of the root cortex and the endoderm, and through pores in networks of cellulose, hemicellulose and glycoproteins. However, some ions may be absorbed by the negative surface charges (polygalacturonic acids, pectins) which act as ion exchangers [26].

Similarly, ROS accumulation and peroxidases stimulation are involved in defense polymers polymerization in cell wall; forming a mechanical barrier to stress and ensuring cell wall rigidity [27,28].

Therefore, we finally describe (1) secondary metabolism (phenolics and lignification) and (2) developmental changes and CD, occurring in roots after Cd exposure.

Results

Root growth and reserve mobilization [32]

Root growth Cd treatment affected root length of germinating *Medicago truncatula* seedlings Lines A strong decrease in embryo root length by cadmium treatment was due to faster growth in control condition.

Mineral reserve distribution: Macroelements (k and Ca) and microelements (Fe, Zn and Cu) were studied. Taken together, mineral mobilization was affected depending on the genotype and the ion. PCA analyses confirmed that for none of the macro- and microelements did their levels seem to be related to cadmium tolerance of the accessions (Figure 1).

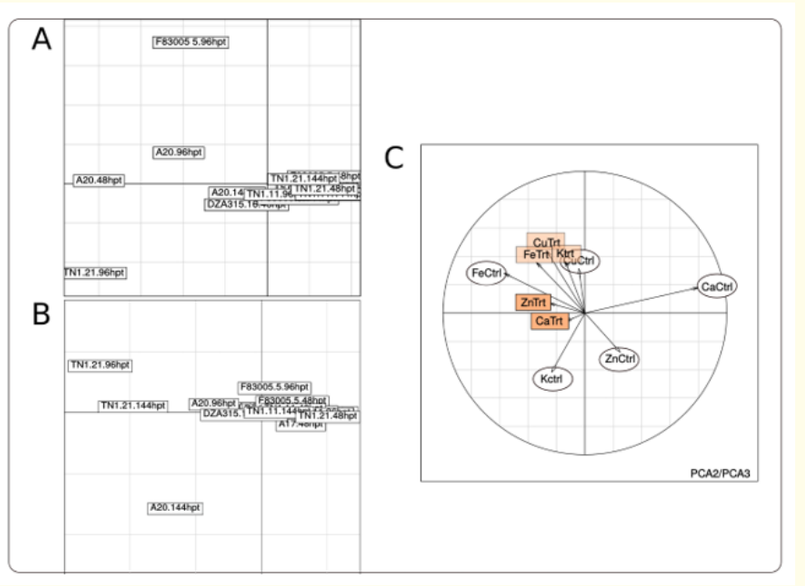

Figure 1: *PCA analysis of mineral contents in roots of Medicago truncatula seedlings imbibed with H2O (0 µM) or Cd (100 µM) (Lines A17, A20, DZA315.16, F83005.5, TN1.11 and TN1.21) at 48h, 96h and 144h. and correlation circle of mineral contents (C). A. PCA1: 64%, PCA2: 22% of inertia. B. PCA1: 75%, PCA2: 14.9% of inertia. C. PCA plot of mineral reserves, under control (ellipse) or Cd-treated (squares) conditions. PCA2: 22%, PCA3: 10% (PCA1, 64% of inertia, represented a size effect among the data, where all the mineral contents were positively correlated) [50].*

Organic reserves distribution

Free amino acids (FAAS) and total soluble sugars (TSS)

The results strongly suggest that each accession is using its global organic reserves in a proper way. In cadmium conditions, the independence between leakage and plantlet content of organic reserves is also observed, suggesting that cadmium does not ubiquitously promote leakages, through cell membrane damages, of FAAS and TSS from the different accessions (Figure 2).

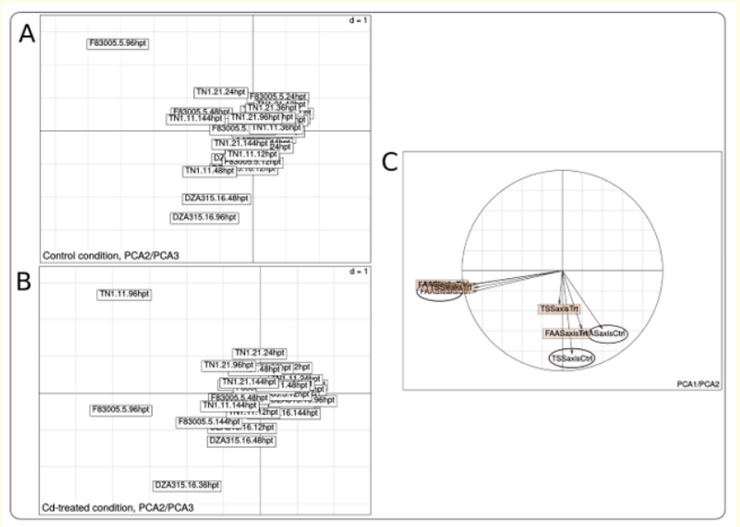

Figure 2: *Free amino acids and total soluble sugars contents in roots (A) and germination medium (B), and PCA analysis of them of Medicago truncatula seedlings imbibed with H2O (0 µM) or Cd (100 µM) (Lines A17, A20, DZA315.16, F83005.5, TN1.11 and TN1.21). (A) and (B) Data are mean values of 3 independent experiments. Values are means of 4 measures. Each measure was carried out with 12 seeds. Error bars (SE) indicated when large enough to be shown. (C) PCA analysis at 48h, 96h and 144h, and correlation circle (C). A. PCA: 27%, PCA3: 21% of inertia (PCA1, 44% of inertia, represented the contribution of TN1.21 to the dataset). B. PCA2: 34%, PCA3: 15% of inertia (PCA1, 46% of inertia, represented the contribution of TN1.21 to the dataset). C. PCA plot of free amino-acids and total soluble sugars in plantlets, and corresponding leakages, under control (ellipse) or Cd-treated (squares) conditions. PCA1: 44%, PCA2: 27%. FAAS: Free Amino Acids Contents, TSS: Total Soluble Sugars, Ctrl: Control Plants, Ttr: Cd-Treated Plantlets, axis: Contents in plantlets, leaks: Leakage in medium [50].*

However, TSS accumulation which is roughly correlated with cadmium susceptibility suggests another plausible explanation, namely a selective mobilization of substrates. Considering that TN1.11 shows a higher growth rate than the tolerant line DZA315.16 under control condition, a difference between sensitive and tolerant genotypes might be that the first line invests in growth and mobilize carbohydrates as energy substrates, while the latter mobilizes less sugars and would establish defense systems at the expense of growth in the first hours or days of germination and early-seedling growth (Figure 2).

Glucose, fructose and sucrose: PCA of simple sugars contents and leakages in control conditions (Figure 3) allows readily identifying the three previously described groups for response to cadmium stress. This suggests that carbohydrate metabolism is a key component of this response. Tolerant accessions are characterized, in control conditions, by a high content in glucose and/or sucrose in embryonic axes, while susceptible lines show high fructose content in embryonic axes. When submitted to Cadmium treatment, the grouping patterns is conserved, even if slightly modified: tolerant accessions still present high levels of glucose and sucrose in the axes, while susceptible ones have high fructose content.

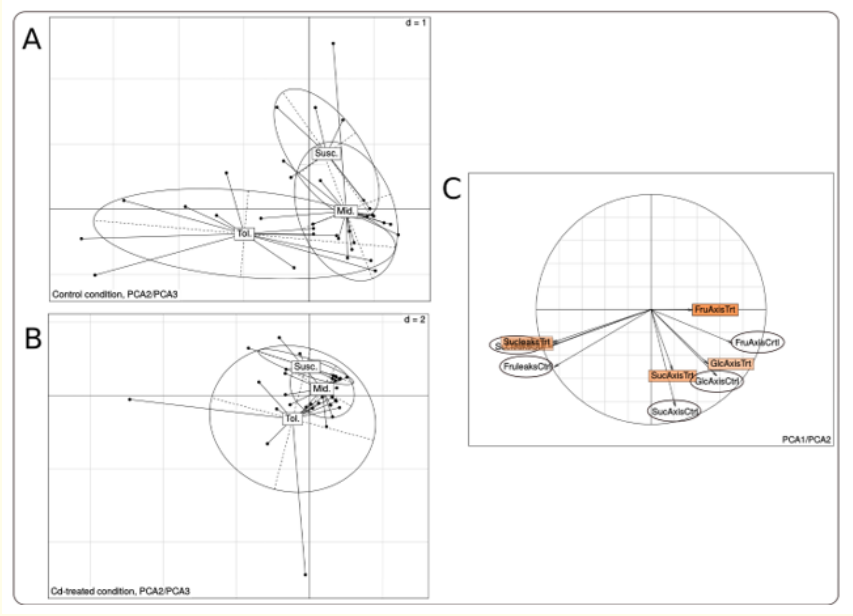

Figure 3: *Glucose, fructose and sucrose contents in roots (A), in germination medium (B) of Medicago truncatula germination medium and PCA analysis of them (C) imbibed with H2O (0 µM) or Cd (100 µM) (Lines A17, A20, DZA315.16, F83005.5, TN1.11 and TN1.21). (A) and (B) Data are mean values of 3 independent experiments. Values are means of 4 measures. Each measure was carried out with 12 seeds. Error bars (SE) indicated when large enough to be shown. (C) PCA analysis at 48h, 96h and 144h, and correlation circle (C). A. PCA2: 30%, PCA3: 11% of inertia (PCA1, 48% of inertia, represented the contribution of DZA315.16 to the dataset). For simplicity, the barycentric coordinate of each group of accessions is plotted, and related measurements are identified by edges. The groups for tolerant (Tol.), intermediate (Mid.) and susceptible (Susc.) lines to cadmium stress were defined by Rahoui., et al. [29]. Tol.: DZA315.16, F83005.5; Mid.: A17, A20, TN1.21; Susc.: TN1.11. B. PCA2: 24%, PCA3: 20% of inertia (PCA1, 45% of inertia, represented the contribution of DZA315.16 to the dataset). Accessions groups are defined as in A. C. PCA plot of simple sugars contents in plantlets, and corresponding leakages, under control (ellipse) or Cd-treated (rectangles) conditions. PCA1: 48%, PCA2: 30%. Glc: Glucose, Suc: Sucrose, Fru: Fructose, Ctrl: Control Conditions, Ttr: Cd-Treated Plantlets, Axis: Contents in plantlets, leaks: Leakage in medium [50].*

Analysis of simple sugar during cadmium treatment reveals that leakages are almost independent of sugar contents within the embryonic axes. As for FAAS and TSS, this suggests that cadmium does not ubiquitously promote leakages of sugars from the different accessions and point to active response mechanisms. In all case but fructose, the amount of sugars in cadmium treated conditions is correlated to the sugar content in control conditions. These suggest that modifications of fructose metabolism or leakage may be related to the response to cadmium treatment. Whether or not the particular pattern of fructose leakage in the resistant line DZA315.16 is related to its tolerance to cadmium deserves to be studied.

Nutrient leakage amounts changed in Medicago truncatula depending on the concentration of metal present in growth media. These leakages, differing in quantity and quality between lines, highlight the importance of these solutes as exclusive substrates for growth (sugars) and the establishment of protein and defense systems (amino acids). These results lead us to consider different responses induced after exposure to cadmium. In one hand, sensitive lines favor growth and this is evidenced by the preferential mobilization of sugars as essential energy substrate for growth. These lines would be unable to adequately regulate amino acids losses which are higher in comparison with treated tolerant lines. In the other hand, tolerant lines, mobilize amino acids to develop defense mechanisms, extremely restrict solute losses, and reduce growth as a cost of tolerance.

Sugar transporter gene expression: Cadmium altered gene regulation in a genotype-dependent way. MtMST was over-expressed at 24h in 5 lines except TN1.11, with highest induction ratios in A20 and A17. This up-regulation decreased at 96h and was under the threshold of induction factor 2 in three lines. In line TN1.11, gene expression was down-regulated by cadmium treatment after 96 hours.

Considering TSS, an increasing "metal inhibition" was noticed. The most tolerant lines were mobilizing less total soluble sugars. TN1.11, a susceptible line, contained large levels of these substrates. These results might be related to the reduction of sugar transport to the growing cells. Indeed, the expression profile of monosaccharide transport gene MtMST showed an up-regulation under Cd treatment in 5 Medicago truncatula lines except for TN1.11 where this gene was completely repressed at 96h. The down-regulation of MtMST in TN1.11 was correlated to relatively high Glc-levels in TN1.11 treated roots at 144h. Indeed, Glc-accumulation in treated embryonic tissues and the failure to use this nutrient should be associated to the transporter inhibition after Cd-exposure. This result led to the hypothesis that sugar is previously transported to the roots, as a result of the degradation of starch in cotyledons. Sugar accumulation at this time is an indication of an abnormal function of MtMST transporter. Glc was made available but not accessible to degradation as transport in roots is altered after metal treatment. It should be noted that in TN1.11, the most susceptible line, Glc and Fru contents are significantly increased after Cd treatment, compared to controls. On the other hand, after a transient early increase, these sugars and sucrose were depleted in DZA315.16, a Cd-tolerant line.

These results highlight the importance of glucose metabolism as a key step determining the genotype susceptibility or tolerance. Glc is an essential energy substrate for respiration resumption during germination, and a line that would be able to metabolize Glc under stress conditions, could tolerate cadmium poisoning.

Antioxidative systems and antioxidant genes regulation changes [29]

It was proposed that seedling may survive in Cd-contaminated medium through the efficiency of some components of the antioxidative system to avoid an oxidative burst, through reduced metal uptake by Cadmium chelating within roots and the induction of antioxidant defenses during germination. Differential responses among accessions suggested that either there are different tolerance mechanisms that are not shared by all accessions, or that tolerance mechanisms are common to all accessions but differ in their levels or intensities. In the present study, biochemical and transcriptional measurements of antioxidative system and developmental changes were performed, thus enabling in-depth investigation on the control of Cd tolerance in the model legume *Medicago truncatula*.

Oxidative stress status and membrane integrity: H_2O_2 and ROS accumulations in root tissues and a slight increase in MDA and solute leakage as a result of putative membrane integrity alteration were showed. Slight increases of MDA production were observed in Medicago genotypes, with a complex pattern in function of time. An additional Cd-induced damage was a pronounced and prejudicial solute wasting in imbibition medium.

Our results showed H_2O_2 production in both control and treatment conditions in all genotypes, with complex patterns. Yet, the two most tolerant accessions, DZA31516 and TN1.21 showed relatively higher levels of H_2O_2 at 144h, with no significant differences between Cd-treated and control conditions at this time point. This suggests that these lines constitutively present a high level of oxidative mechanism.

Antioxidant system regulation: The deleterious effects resulting from ROS accumulation and altered cellular oxidative state may be alleviated by detoxifying enzymes, such as superoxide dismutase (SOD), catalase (CAT) and enzymes of glutathione-ascorbate (ASC-GSH). The analysis of enzyme activity in the present study showed that Cd induced an enhancement of total SOD in 4 genotypes and a decrease in line TN1.11. Nevertheless, Cd caused a differential sensitivity to Cd-stress.

Gene expression analysis showed that Cd induces an up-regulation of SOD. PRX activity and CAT activities were enhanced and A17 did not respond with a significant increase. In contrast to its SOD response, line TN1.11 showed induction of these two enzymes. These results are in agreement with the induction of PRX gene expression by Cd treatment in all genotypes. Our results show that Cd stress induces an up-regulation of PRX, CAT and total SOD activities to counteract ROS and oxidative burst.

Cd treatment affected APX and MDAR and contrasting results were obtained in different genotypes. APX was slightly inhibited in A17, not significantly affected in TN.11 under Cadmium-exposure, but enhanced in the other genotypes. In contrast, Cd enhanced MDAR activity in all tested genotypes.

Glutathione metabolism and metal sequestration

Glutathione reductase and glutathione-s-transferase gene expression: We discussed cadmium impact on glutathione pathway by testing GR and GST genetic regulation. The results show that GR is overexpressed in all lines with minimal expression in TN1.11 calling for the sensitivity of the latter. While, DZA315.16 notices similar up-regulation levels to those noted in TN1.11. GST gene expression shows an up-regulation under cadmium stress in all lines even in TN1.11. The lowest up-regulation in TN1.11 calls for its vulnerability compared to other genotypes.

Phytochelatin-synthase gene expression: Gene expression patterns were clearly different in the susceptible TN1.11 accessions, with consistent default in activating gene expression or non-timely expression, as in the case of PPX or PCS. It is more difficult to identify consistent pattern that may be related to the tolerant status of DZA315.16 or TN1.21 lines.

Cadmium-induced differentiation in roots [33]

Cell death: Although line TN1.21 responded to Cd stress by induction of cell death as assessed by Evans blue, the absolute values were lowest in controls and Cd-treated roots compared to the other genotypes. It should be noted that line TN1.21 was considered as tolerant based in root growth inhibition [29].

Secondary metabolism and differentiation

Phenolic compounds and lignification: Cd-induced phenolic compounds varied and was particularly enhanced in the more sensitive lines (A17 and TN1.11). Cd triggered cytosolic soluble phenolics accumulation. A response which was much earlier than lignification and extended over the entire root cross section.

Pectin and cellulose: Cell wall composition varied under Cadmium-exposure as shown by observations of cellulose and pectin staining. Slight increases in pectin content were observed.

Root differentiation: Cd has described to reduce root growth and increase root hair production near the apex.

Discussion

Germination inhibition is among the well-known effects of toxic impact of heavy metals and key processes are depressed [6,7]. Reduction of early seedling growth was reported to be due to toxic effects on capacity of moisture, resumption of respiration, reserve mobilization, nutrients availability and oxidative status, in germinating seeds of faba bean and pea [7,8].

Negative interference between Cd and mineral absorption and transport was shown during legume germination. Therefore, the effect on mineral mobilization was not correlated to tolerance or susceptibility as described for the early seedling growth test.

Cd-induced ionic composition disorders can be related with trace and macro-element transport which is essential for growth and plant development [34,35]. Thus, nutritional disorders induce physiological and biochemical disturbances after Cd poisoning symptoms. For instance, root browning and short lateral roots formation were assumed to be due to a calcium deficiency [38]. Potassium and magnesium absorption may also be negatively affected by Cd [37,38]. Indeed, Cd is supposed to use cation transport systems usually involved in essential elements assimilation such as zinc transporters (ZIP and IRT), iron (Nramp) [38-40], or Ca-channels and Ca-transporters [41].

Germination *sensus stricto*, starting with imbibition and ending with radicle emergence, occurs in one to two days, depending on genotype and temperature [42]. During early seedling growth, sprouts are heterotrophic and depend on storage tissues reserves. Nitrogen and carbon metabolisms are activated at early stages of germination as revealed by amino acid and soluble sugar content changes [43]. Comparing FAAS pool and protein compositions, amino acids interconversion occurs in cotyledons after protein degradation and in embryonic axis after amino acids import. In fact, ammonium amount is low at germination early stages and increases sharply after the onset of radicle mainly in the axis of the embryo [43].

FAAS content changed in control and treated roots. The "normal" tendency observed in control roots was significantly altered in treated F83005.5, TN1.11 and DZA315.16 at different time points and did not seem to be correlated to the line's Cd susceptibility. Protease activation and/or induction initiates amino acid release. The observed effect of Cd on FAAS might result from inhibitory action on proteolysis or/and on metabolite transport. It has been observed that Cd-treated storage tissues have less FAAS compared to controls [8,7,103].

During seed germination, storage tissues rapidly mobilize large sugar amounts to the growing embryonic axis [44]. In *Medicago truncatula*, Glc and Fru amounts were reported to be very low before root emergence. They were barely detectable in the cotyledons, while they increased sharply in the embryonic axis. Meanwhile, Suc amount significantly increased in the embryonic axis during germination, before increases of Glc and Fru [43].

Heavy metal-induced nutrient leakages have been demonstrated in legume species. It was noticed that the amount of leakage increased with increasing Cd-concentrations in the germination medium [7,8].

Solute leakages were reported to be related to lipoperoxidation and electrical conductivity in other species [45]. These membrane changes are a consequence of the direct action of the metal on the composition of the membrane [8], induced antioxidant status changes or the stimulation of lipoxygenase activity [29].

Research literature reported the "principle of allocation" referring that there is a coast in each allocation of energy between morphological plant compartments [46]. Researchers suggested that plant structures represent carbon allocation, but so do physiological pro-

cesses and biochemical characters. Therefore, adaptive responses in one direction must be constrained by a loss in some other variable [47]. This was noticed on herbicide resistance [48] or on heavy-metal tolerance genotypes showing restricted growth in contaminated medium [49].

Therefore a "metallo-inhibition" of carbohydrate transport from "source" to "sink" tissues has been demonstrated across the entire plant in bean [50]. It has also been reported that Cd reduced sugar transfer from cotyledons during the germination of faba bean [7,8].

Cadmium impact on hydrolytic activities could also explain monosaccharide decrease in growing embryonic cells. Indeed, it has been observed that Cd alters α-amylase activity in cotyledons of faba bean seeds during germination [7,8]. Similarly, Fru levels are closely associated to invertase-activity, and depend on numerous and sophisticated metabolic pathways [51].

ROS accumulation could be explained by enzymes activation such as SOD [52] and stimulating the activity of parietal NADH-oxidase may explain, in part, apoplasmic H_2O_2 accumulation [52]. Similarly, H_2O_2 overproduction might be involved in oxidative polymerization of some defence polymers at the cell wall forming a mechanical barrier against the stress [54].

However, oxidative stress is one of the underlying tissues injury origins following exposure to Cd [55]. Consequently, changes in lipoperoxides concentration can be a good indicator of membrane integrity. Slight increases of MDA production were observed in Medicago genotypes. Many studies have shown also that cadmium causes the loss of membrane integrity following lipoperoxidation process [56].

Cd induced a pronounced solute wasting in imbibition medium showed by a supplemental exudation after Cd treatment, probably at the expense of the suitable transport to growing embryonic axis. Solute leakage, increasing with metal concentration in germination medium, was described in other legumes [7,8,10]. These leaks are due to cell membrane damages and are evidenced by lipoperoxides overproduction [103]. During stress, membrane integrity maintenance is crucial to determine plant tolerance towards heavy metal [57].

In seed physiology, H_2O_2 is usually considered as a toxic molecule [58]. Similarly, it should be noted that, under both natural conditions and stress, ROS overproduction plays a key role in seeds; especially, during germination. Indeed, Foyer and Noctor [59] have proposed to reassess the concept of oxidative stress by distinguishing oxidative signalling and oxidative stress. Actually, the oxidative burst alters the cellular redox status and acts as a "switch" of many enzymes involved in particular signalling events (protein kinases, calcium signaling) and in cell cycle control. So, redox status is considered as a real sensor of the cell physiological state, considering that every oxidative burst is a stress by-product. ROS occurrence and detoxification systems redundancy allow a fine control of transmitted signal and an appropriate cell response. In fact, the antioxidant system maintains intracellular redox homeostasis, preventing accumulation of toxic ROS, while allowing ROS-mediated signalling to occur [59].

ROS accumulation might be due to the activation of enzymes such as SOD [60] and cell wall NADH-oxidase which could lead, in part, to H_2O_2 accumulation at the apoplasmic level [53,61].

Solely higher total SOD activity was detected in Cd-tolerant transgenic *Nicotiana tabacum*; while MnSOD activity and gene expression levels didn't differed in both control and Cd-treated plants [62]. Inhibition of Cu-Zn SOD activity was observed in leaves and roots after a heavy metal stress and was attributed to a metabolic disorder caused by the accumulation of hydrogen peroxide which acts as an inhibitor of this isoform [63]. The inhibition of Cu-Zn SOD activity can also be attributed to its sensitivity to zinc deficiency and its possible substitution by Cd [64].

Under Cadmium exposure PRX activity and CAT activities were enhanced in all in 4 out of 5 lines, A17 did not respond with a significant increase. In contrast to its SOD response, line TN1.11 showed induction of these two enzymes. These results are in agreement with the induction of PRX gene expression by Cd treatment in all genotypes. Our results show that Cd stress induces an up-regulation of PRX, CAT and total SOD activities to counteract ROS and oxidative burst.

This join up this fact showing that Cd stress induces an up-regulation of PRX in agreement with SOD to counteract ROS and oxidative burst. Indeed, SOD and PRX activities increased in response to plomb stress in rice seedlings [65]. In contrast, after Cd treatment, rice seedlings showed PRX activity induction while SOD activity was not affected [66]. Moreover, PRX up-regulation seems to be correlated to root growth alterations and cell wall rigidification [67].

Antioxidative enzymes (SOD and CAT) together with ascorbate-peroxidases (APX) and glutathione control cellular concentrations of H_2O_2 and O_2 [54]. Recycling of ascorbate and GSH is achieved by monodehydroascorbate radical reductase (MDAR), dehydroascorbate reductase (DAR), and glutathione reductase (GR).

Cd treatment affected APX and MDAR and contrasting results were obtained in different genotypes. APX was slightly inhibited in A17, not significantly affected in TN.11 under Cadmium-exposure, but enhanced in the other genotypes. In contrast, Cd enhanced MDAR activity in all tested genotypes. Ascorbate-glutathione associated defense enzymes were reduced in leaves of Cd-exposed Helianthus annuus plants [68]. Elevated APX activities were reported in roots and leaves of Phaseolus vulgaris as well as in suspension cultures of Nicotiana tabacum cells after Cd treatment [69,70]. MDAR was upregulated in plants with reduced CAT and APX levels [71]. Nevertheless, Cd repressed H_2O_2 detoxication systems such as GR, CAT and APX activities and improved SOD activities in Scots pine roots [72].

The results demonstrate the importance of glutathione metabolism as a non-specific response to oxidative injury and an up-regulation of GR and GST after metal exposure and its key role in ROS homeostasis. During seed germination and seedling growth in presence of metals, GSH changes levels have been reported. Here we discuss cadmium impact on glutathione pathway by testing GR and GST genetic regulation. In other works, it has been demonstrated a similar result after exposure to metal stress. For instance, rice seedlings showed increased activities of GR in response to Pb stress [65].

The presence of phytochelatins in seeds is scarce, which perhaps reflects the low levels of metals within seeds compared to other tissues. Expression of phytochelatin-synthase has been reported in the leaves, roots, stems and cotyledons of *Arabidopsis*, and in tomato roots and stems [73]. In *Arabidopsis*, phytochelatin-synthase is transcriptionally regulated during the early stage of seedling development. After 15 germination days, PCS is constitutively expressed and regulated instead by substrate abundance [74]. Therefore, our results are consistent with the involvement of Cd in the early induction of chelating and transport mechanism in case of *Medicago truncatula* germinating seeds. In fact, within plant cells, Cd is bound to S-containing ligands, such as those present in glutathione, metallothioneins, and phytochelatins [75]. In many plant species, the synthesis of phytochelatins is induced by Cd exposure [76-78] and Cd is often sequestered in the vacuole as Cd-phytochelatin complexes [39,78]. The gene repression at 96h can be explained by metal supply depletion. In fact, phytochelatin-synthase substrates are metal GSH thiolates molecules in with a blocked thiol group when binding to a metal ion such as Cd. Therefore, phytochelatin synthesis stops when the supply of metal ions is exhausted [79].

CD might be due to one or more of several factors, namely, Cd-mediated oxidative injury altering membrane integrity, lipid peroxidation and loss of essential nutrients [103]. Taken together, the higher penetration of Evans blue and the increase of ion leakage in Cadmium-exposed plants could be an indicator of oxidative deterioration and accelerated senescence induced by Cadmium [72,80].

While our data only show toxic effects of Cd on cell viability but are no proof of CD, other studies have demonstrated that heavy metal induced CD. Schutzendubel., *et al*. [72] reported that high Cd concentrations led to transient increase in CD. Fojtova and Kovarik [32] reported that Cd activated apoptotic alterations in tobacco cells suspension cultures that were illustrated by DNA fragments observed 48h after Cd-treatment.

CD occurs as part of plants normal developmental program and is tightly controlled in committed cells all through xylogenesis [81]. H_2O_2 is an intermediate signal molecule in CD [22] and a rapid H_2O_2-mediated strengthening of cell walls would explain the fast abolishment of growth due to Cd-treatment which was noticeable after less than 48 hours.

Unlike necrosis, CD is severely controlled and once cells are dedicated to CD, the process cannot be upturned [72]. In our study, we did not observed arbitrary injury in cross sections of roots as an expectation for necrotic reactions suggesting that Cd induced loss of viability which might correspond to CD rather than to necroses.

Phenolics may protect from oxidative injury. In fact, in concert with ascorbate and APX, phenolics participate to H_2O_2 annihilation [82]. Phenolic compounds are electron donors for NADH-oxidase and coniferyl-alcohol-peroxidase enzymes. They are known for their protective role in plant and participate in several compounds biosynthesis such as lignin, suberin or flavonoids [83,84].

After heavy metal exposure, cell wall lignification was revealed strictly related to ROS levels and their oxidative injury [79] exemplifying that ROS could be a switch signal that induces lignin metabolism activation as a defense mechanism under stress exposure to restore unbalanced cellular oxidative system [90].

In addition to lignification, deposition of proteins, glycoproteins and polysaccharides can lead to cell wall reinforcement [85,86].

The main mechanism of metal uptake relies on a simple diffusion through the root cortex apoplast and the endoderm, and namely through pores in the network of cellulose, hemicelluloses and glycoproteins. Yet, some ions can be absorbed by negative charges at the cell wall surface which act as ion-exchangers [26]. In response to a heavy metal stress, cell wall modifications could increase its potential for entrapping metals by increasing for example the capacity of cationic exchange. Heavy metal binding to cell wall depends hugely on the negative charges density, which itself depends on pectin methylation. These charges density and their distribution within the parietal compartment determine then the cell wall CEC [87]. Muschitz., *et al*. [88] have shown induced changes in cell wall cationic exchange capacity (CEC) in response to Zn-stress.

Increases in pectin levels were correlated with important aluminum levels in Zea mays cells [89]. In the same way, it has been noted a pectin increase in the walls of a tobacco cell suspension [90] or for As-treated wheat seedlings [91]. Studies relating the effect of abiotic stress on the organization and distribution of polymers in cell wall are rather scarce. However, it seems plausible that the changes in the walls composition following a stress brought on by heavy metals could be involved in optimizing the cellular response. The role of cell walls in the subcellular compartmentalization of Zn, Cd and Cu was highlighted in many works [92].

Cell wall lignification leads to growth inhibition. The increase of peroxidase activity and the decrease of root elongation were reported to be correlated [93]. Heavy metals stimulate the lignin polymerization, leading to an increase of cell wall mechanical resistance [94,95]. The thickening of the casparian band under metallic stress is a form of defense adopted by plants [94].

In addition to early development of endoderm and ectoderm, many changes in root anatomy are recorded when it is exposed to high Cd concentrations [96].

In agreement with others observations in maize, radish, barley, sorghum and Rhodes grass suggesting that Cd accelerates cell senescence [24,97,98]. Cd treatment also disintegrated rhizodermis and external cortical cell layers which was accompanied by loss of cell turgor, intercellular air-carrying meatus and cortical cells whose epidermis was irregularly shaped in line with other research works [97-101].

In contrast to its effect on cell viability, Cd induced xylogenesis since “normal” protoxylem elements were observed. Nevertheless, in contrast to normal growth suggesting that lignification is the final step in this process [82], lignified xylem elements were established here already at a very short distance from the root tip, usually corresponding to the elongation area. In line with our results, several studies have shown that the root becomes shorter and thicker without setting off necrotic reactions under Cadmium stress [100,102]. Maksimovic., *et al*. [102] explain this by an increase of the size of the parenchyma cells and suggest that the expansion of the cortical tissue has a functional role in increasing the resistance to radial flow of water and solutes.

Vitoria., *et al*. [101] observed the proliferation of cambium cells followed by loss of the organization of the cambial region in radish roots exposed to Cd. These authors suggested that Cd accelerates the maturation of the root with the development of the xylem element in the central cylinder. Consistent with this interpretation, Schutzendubel., *et al*. [72] found that exposure to 50 µM Cd causes accelerated protoxylemic lignification elements near radical apex in Scots pine (*Pinus sylvestris* L.), and Durcekova., *et al*. [24] observed a premature xylogenesis in barley roots exposed to Cd.

Conclusion

Cadmium reduces root growth among a tolerance order, suggesting that different adaptive strategies may be present in different accessions.

Growth inhibition in different genotypes could be correlated to nutritional deficiency and disorders in process of reserves mobilization from cotyledons to embryonic axis. Mineral distribution was affected by cadmium but not correlated to genotype susceptibility.

Organic biomass mobilization was dependent on the cadmium-response genotype. Namely, carbohydrates were mobilized in susceptible lines more than in tolerant ones. In contrast, tolerant lines showed preferential FAAS mobilization, and Glc over-consumption. In agreement with Glc accumulation at the end of the experiment, MtMST expression was completely repressed in TN1.11. These results implied that genotypes react among a specific outline defining metabolite consumption and install growth processes or defense mechanisms. This was evidenced by, in one hand, huge root growth in control condition in susceptible lines and comparative root growth in control and treated tolerant genotypes and in the other hand, by TSS high leakage in tolerant lines and FAAS in susceptible ones.

Hence, our results explain the avoidance of cadmium stress in relatively tolerant germinating Medicago truncatula lines through keeping safe respiration process and defense mechanisms induction through amino acids conversion. These mechanisms enable seeds to survive and seedlings to grow in cadmium metal-contaminated media as it was evidenced by the efficiency of defense and antioxidative systems in the same studied genotypes after Cd-exposure. Furthermore, our research work suggests that the genotype-dependent negative effect of Cd on reserve mobilisation, respiration recovery and nutrient transport during the heterotrophic stage of seed germination might explain the observed differences in susceptibility to this heavy metal.

Despite the biochemical and histological changes, growth inhibition scale in different genotypes cannot be dependent on the overproduction of ROS, H_2O_2 or MDA. The results indicate that higher H_2O_2 levels at 144h and lower MDA accumulation may be related to Cd-tolerance in seedlings, as exemplified with the DZA315.16 and TN1.21 performances. Total ROS accumulation highlights triggered oxidative injury. Solute leakages, as an indicator of membrane integrity, should reveal induced membrane damage after oxidative injury but is not strongly related to genotype sensitivity. Our results explain, in part, the avoidance of cadmium stress in relatively tolerant germinating *Medicago trunctula* through avoiding a possible oxidative burst by the induction of antioxidant defenses during germination that enable seeds to survive and seedlings to establish in cadmium metal-contaminated media. Nevertheless, we demonstrated a relative efficiency of the studied components of the antioxidative system that cannot explain the scale of root growth inhibition.

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