University of São Paulo
“Luiz de Queiroz” College of Agriculture

Root responses of contrasting tomato genotypes to cadmium-induced stress

Karina Lima Reis Borges

Thesis presented to obtain the degree of Doctor in
Science. Area: Genetics and Plant Breeding

Piracicaba
2017

Karina Lima Reis Borges
Bachelor of Biological Sciences

Root responses of contrasting tomato genotypes to cadmium-induced stress
versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
Prof. Dr. **RICARDO ANTUNES DE AZEVEDO**

Thesis presented to obtain the degree of Doctor in
Science. Area: Genetics and Plant Breeding

Piracicaba
2017

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Borges, Karina Lima Reis

Root responses of contrasting tomato genotypes to cadmium-induced stress / Karina Lima Reis Borges. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2017.

100 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura "Luiz de Queiroz".

1. Metais pesados 2. Antioxidantes 3. Estresse oxidativo 4. Eficiência de uso de nutrientes 5. Proteômica I. Título

With love,
to my dear husband Bruno.

ACKNOWLEDGMENTS

To the University of São Paulo, specially the “Luiz de Queiroz” College of Agriculture – ESALQ for the provision of physical and intellectual infrastructure to develop this project.

To National Council for Scientific and Technological Development (CNPq) for the scholarship and financial support.

To Genetics and Plant Breeding graduate program and all professors who contributed to my professional training. To all the department employees, specially Fernandinho, Leia, Claudio Segatelli, Valdir Próspero and Berdan. Thanks for the help!

To Dr. Ricardo Antunes de Azevedo for the guidance, encouragement, for sharing his knowledgments and experiences. I'll always be grateful for the opportunity to be in his lab.

To all lab colleagues, specially Salete Gaziola, Patrick Nunes, Mônica Franco, Berenice Alcântara, Fabiana Belini, Lucélia Borgo, Flávio Rabelo, Lucas Souza and Fernando Piotto, for sharing many coffees and conversations.

To my dear friends Marina Otto, Selene Castilho, Lucas Riboldi, Gabriel Daneluzzi, Diogo Capelin, Natália Teixeira, Maria Tereza Alvarenga, Thalita Rodrigues, Fernanda Lima Vilas Boas, Evellyn Couto, Letícia Lara and Victor Emmanuel.

To Dr. Fernanda Salvato for the partnership in our proteomic adventure, for the advices and for sharing her knowledgments and experiences with me. Our talks always made me think beyond. Thanks a lot.

To Dr. Claudia Barros Monteiro Vitorello for the good advices and for encouraging me to move on, even in the most difficult times.

To Dr. David Muddiman and Dr. Philip Loziuk for the collaboration in obtaining and analyzing proteomic data.

To Rafael Nalin and Franz Hippler for the partnership in papers. Ours talks made me grow professionally. Thank you guys.

Specially, I thank my parents, Ofini and Jane, for being my safe haven, for encouraging my choices and believe in my potential. You are examples of hardwork and honesty.

To all my family, specially to my sisters Larissa and Valéria and to my niece Luiza I'm thankful for sharing such great times.

To my grandmothers Carmen and Josefina, for always think of me in their prayers.

To my parents in law, Boanésio and Edna, for the care and the many special moments we shared.

To the most special person I have ever met: my husband Bruno Borges, for the companionship and love that made these years pass too fast. For the countless times he made me believe that I could do it. A new journey starts now, and I can't wait to share more special moments with you. Love you, honey!

Finally, thanks God for all wonderful people I met in my way, all the opportunities I had and for being such a blessed woman.

“Genius is 1% talent and 99% hard work.”

Albert Einstein

SUMMARY

RESUMO.....	8
ABSTRACT.....	9
1. INTRODUCTION.....	11
REFERENCES.....	12
2. TEMPORAL DYNAMIC RESPONSES OF ROOTS IN CONTRASTING TOMATO GENOTYPES TO CADMIUM TOLERANCE.....	15
ABSTRACT.....	15
2.1. INTRODUCTION.....	15
2.2. METHODOLOGY.....	17
2.2.1. Plant material, growth conditions and treatments	17
2.2.2. Dry matter weight and Cd content	18
2.2.3. Quantification of stress indicators	19
2.2.4. Enzyme extraction and activity determination	19
2.2.4.1. SOD assay.....	19
2.2.4.2. CAT assay.....	20
2.2.4.3. APX assay.....	20
2.2.4.4. GR assay.....	20
2.2.4.5. GST assay.....	20
2.2.5. Polyacrylamide gel electrophoresis (PAGE).....	21
2.2.5.1. SOD activity staining.....	21
2.2.5.2. CAT activity staining	21
2.2.5.3. GR activity staining.....	22
2.2.6. Glutathione content	22
2.2.7. Statistical analysis.....	22
2.3. RESULTS.....	23
2.3.1. Cd accumulation in tomato tissues and impact on plant growth	23
2.3.2. Lipid peroxidation level.....	25
2.3.3. Antioxidant responses to Cd-induced stress	26
2.4. DISCUSSION.....	30
2.4.1. Tomato tissues can accumulate Cd and reduce growth.....	30
2.4.2. Cd induce lipid peroxidation in tomato.....	31
2.4.3. Antioxidant enzymes responding to Cd-induced stress.....	32
REFERENCES.....	35
3. ROOT MORPHOLOGY AND NUTRIENT USE EFFICIENCY IN TOMATO UNDER CD-INDUCED STRESS: COMPARING CONTRASTING GENOTYPES FOR METAL-TOLERANCE.....	41
ABSTRACT.....	41
3.1. INTRODUCTION.....	41
3.2. METHODOLOGY.....	44
3.2.1. Plant material, growth conditions and treatments	44
3.2.2. Plant growth and root morphology	44
3.2.3. Nutritional status and nutrient absorption and use efficiency.....	45
3.2.4. Statistical analysis	45
3.3. RESULTS.....	45

3.3.1 Cd accumulation and impact on plant growth.....	45
3.3.2 Cd effect on root morphology parameters.....	48
3.3.3 Nutrients absorption and use efficiency changes under Cd-induced stress.....	50
3.4. DISCUSSION.....	55
3.4.1. Cd accumulation and impact of stress on plant growth rate and biomass partitioning.....	55
3.4.2. Cd affected root morphology.....	56
3.4.3. Nutritional status changes by Cd-induced stress.....	57
REFERENCES.....	60
4. ROOT PROTEOMICS OF TWO TOMATO GENOTYPES WITH DIFFERENCES IN TOLERANCE TO CADMIUM-INDUCED STRESS.....	67
ABSTRACT.....	67
4.1. INTRODUCTION.....	67
4.2. METHODOLOGY.....	69
4.2.1. Plant growth, treatments and sample collection	69
4.2.2. Protein extraction, quantification and digestion	70
4.2.3. LC-MS/MS analysis.....	71
4.2.4. Protein identification	71
4.2.5. Protein quantification and statistical analysis.....	72
4.2.6. Protein annotation and functional classification.....	72
4.3. RESULTS.....	73
4.3.1. Cd impact on tomato growth.....	73
4.3.2. Cd increased MDA and H ₂ O ₂ contents in tolerant roots.....	74
4.3.3. Large scale tomato roots proteome identification and quantification	75
4.3.4. Functional classification and clustering analysis of DAP	77
4.4. DISCUSSION.....	88
4.4.1. Cd impaired tomato growth in both genotypes and increased MDA and H ₂ O ₂ contents in tolerant roots.....	88
4.4.2. Root proteome profile of tomato genotypes under Cd-induced stress.....	89
4.4.3. Cd induced alterations in proteins related to cell wall.....	89
4.4.4. Cd induced alterations in proteins related to redox balance.....	90
REFERENCES.....	93
5. FINAL CONSIDERATIONS.....	99

RESUMO

Respostas radiculares de genótipos contrastantes de tomateiro sob estresse induzido por cádmio

Esta tese apresenta um aprofundamento do conhecimento global sobre as respostas radiculares de genótipos contrastantes de tomateiro ao estresse induzido por cádmio (Cd). Os genótipos cultivados em meio hidropônico contendo 35 μM de CdCl_2 durante sete dias exibiram acúmulo de metal tanto em raízes como em parte aérea em ambos os genótipos, mas com um aumento do acúmulo de Cd principalmente nas raízes. Pusa Ruby (PR, tolerante) acumulou menores níveis de Cd na parte aérea, exibindo maiores taxas de crescimento e acúmulo maior de MDA comparado ao Calabash Rouge (CR, sensível). No entanto, o genótipo PR parece ter um sistema mais eficiente para lidar com o estresse induzido pelo Cd. Os ensaios enzimáticos revelaram que a presença de Cd alterou o conteúdo de GSH nas raízes de ambos os genótipos, com aumentos nas atividades de APX, GR e GST, que em conjunto podem ser os principais elementos responsivos na defesa contra o estresse oxidativo no genótipo tolerante. Seguindo a caracterização dos sistemas radiculares dos genótipos de tomateiro, o segundo capítulo contém as análises morfológicas. A exposição ao Cd provocou decréscimos nos parâmetros avaliados como comprimento radicular, área superficial e volume, sendo mais severos no genótipo CR. As raízes de tomate expostas ao Cd mostraram alterações na eficiência do uso de nutrientes para vários macros e micronutrientes, sendo menor no CR em comparação com PR. Esses resultados reforçam a explicação de que o fenótipo tolerante observado no genótipo PR pode estar relacionado a um melhor gerenciamento nutricional aliado aos menores danos na morfologia da raiz sob estresse. Finalmente o último capítulo contém dados de proteômica quantitativa, uma abordagem que foi empregada para determinar as alterações provocadas pelo Cd no perfil proteico das raízes de tomateiro. As plantas cresceram em hidroponia e foram expostas ao metal por quatro dias. A contagem espectral revelou um total de 380 proteínas diferencialmente acumuladas (DAP), das quais 62 foram compartilhadas entre os genótipos e apresentaram alterações similares após exposição ao metal. Em geral, CR apresentou maior número de DAP comparado ao PR. DAP foram alteradas em diversas vias, incluindo proteínas envolvidas em parede celular, resposta a estresse e atividades redox. Os resultados obtidos neste estudo contribuíram para aumentar o entendimento da tolerância ao Cd em tomateiro.

Palavras-chave: Metais pesados; Antioxidantes; Estresse oxidativo; Eficiência do uso de nutrientes; Proteômica

ABSTRACT

Root responses of contrasting tomato genotypes to cadmium-induced stress

This thesis presents a more in-depth understanding of global root responses of contrasting tomato genotypes to cadmium-induced stress. Tomato genotypes growing in hydroponics containing 35 μM CdCl_2 over seven days showed metal accumulation in roots and shoots of both genotypes, but with increased Cd allocation over time mainly in roots. Pusa Ruby (PR, tolerant) accumulated lower levels of Cd in the shoots, exhibiting higher growth rate and higher levels of MDA in roots compared to Calabash Rouge (CR, sensitive). Therefore, the PR genotype appears to have a more efficient mechanism to cope with Cd-induced stress. Enzymatic analysis revealed that the presence of Cd altered GSH content in roots of both genotypes, whilst increased the activities of APX, GR and GST, which in turn, together may be the main players against oxidative stress in the tolerant genotype. Following the characterization of tomato roots systems against Cd exposure the second chapter brings the root morphology parameters analysis. Cd exposure decreased the root length, the surface area and the volume in both genotypes, being more severe in the CR genotype. Tomato roots exposed to Cd showed nutrient use efficiency for many macro and micronutrients in the CR genotype lower than verified for the PR. These findings reinforce the explanation that the tolerant phenotype observed in PR plants could be related to a better nutrient management and minor damages in root morphology under Cd stress. Finally, the last chapter brings a large scale quantitative proteomic approach employed to determine alterations in the protein profile of tomato roots exposed to Cd. Tomato genotypes were grown in hydroponics and exposed to Cd over four days. The spectral counting revealed a total of 380 differentially accumulated proteins (DAP), which 62 were shared between both genotypes and showed similar alterations after metal exposure. In general, CR genotype presented higher number of DAP compared to PR. DAP showed alterations in diverse pathways, including proteins involved in cell wall, stress response, and redox activities. The results obtained in this study contributed to increase the understanding of Cd-tolerance in tomato plants.

Keywords: Heavy metals; Antioxidants; Oxidative stress; Nutrient use efficiency; Proteomics

1. INTRODUCTION

Heavy metals naturally occur in the soils as rare elements, but their excessive amounts in the environment are mainly consequence of anthropogenic activities such as mining, smelting, burning of fossil fuels and intensive use of fertilizers (Candeias *et al.*, 2015). Nowadays, numerous metallic elements are treated as widespread environmental pollutants and a very good example is cadmium (Cd), one of the most highly toxic substances that has been ranked no. 7 among top 20 toxicants (Birke *et al.*, 2017; Gill *et al.*, 2012). Cd is widely considered a non-essential transition metal since to date there is only one documented example of a Cd-specific carboanhydrase, a protein requiring Cd as a cofactor, found in the marine diatom *Thalassiosira weissflogii* (Lane *et al.*, 2005).

Due to increase of agricultural contaminated area by heavy metals, the problem of Cd toxicity worsens every year, however the amount of plant species with some tolerance degree to this metal is extremely restricted (Choppala *et al.*, 2014). Plants have evolved tolerance mechanisms to deal with heavy metals which means that tolerant plants survive in the presence of high internal metal concentration having, therefore, other cellular mechanisms such as vacuole compartmentalization (Song *et al.*, 2014) to cope with metal induced stress, such mechanisms still unknown to date. Another tolerance strategy is related to ion-excluders and involves decreasing the amount of Cd entering the cell by extracellular precipitation, absorption by cell walls, reduced uptake or increased efflux (Sarwar *et al.*, 2010). According to Choppala *et al.* (2014) tolerance mechanisms include: (i) synthesis of phytochelatins and metallothioneins; (ii) competition between micronutrients and Cd for the same membrane transporters; (iii) alleviation of oxidative stress by antioxidant production; and (iv) restoration of chlorophyll structure damaged by Cd toxicity.

Among cultivated crops the vegetables are more likely to be grown in Cd-contaminated areas, since the disposal of agro-industrial wastes usually occurs around large urban centers (Gallego *et al.*, 2012) where the green belts are located. Among the vegetables, tomato was chosen as a model study because of many reasons such as the fast life cycle and small size. In addition, the recent publication of the tomato genome (The Tomato Genome Consortium, 2012) has offered the possibility of applying modern molecular approaches to get information on the molecular mechanisms that lead to the accumulation, detoxification and allocation of nutrients and toxic elements such as Cd. The lab team of Dr. Ricardo Antunes de Azevedo has developed researches evaluating the tomato responses to heavy metals and since 1998 has been studying the heavy metals impacts in crops, especially Cd,

aiming to evaluate effects on antioxidant metabolism of cells. Since 2004, Azevedo's team adopted Micro-Tom (MT) cultivar as model studies, and more recently other commercial tomato varieties that have different levels of tolerance to Cd. Several studies have been developed by Azevedo's group with the theme Cd and tomato: Gratão *et al.* (2008), Gratão *et al.* (2009), Monteiro *et al.* (2011), Gratão *et al.* (2012), Dourado *et al.* (2013), Piotto *et al.* (2014), Nogueirol *et al.* (2016), Pompeu *et al.* (2017), however no study comparing contrasting genotypes to Cd-induced stress were performed to date. From the evaluation of a core collection of tomato accessions accomplished by Piotto (2012) it was possible identify plants with a high tolerance degree as well as genotypes extremely sensitive to Cd. Based on these results, the research group has concentrated efforts trying to characterize the screened genotypes. Therefore, the identification of tomato genotypes with contrasting phenotypes and different Cd allocation patterns will allow to explore the possible mechanisms involved in Cd-tolerance.

The roots play a significant role in the water and nutrients acquisition from soil solution. However, heavy metal excess in the root medium cause damages in the root functions by reducing its growth and integrity (Lux *et al.*, 2011). Nonetheless this thesis presents a joint approach and tools to characterize the root system of tomato genotypes with differential tolerance to Cd, using enzymatic and non-enzymatic assays, root morphological parameters, nutritional status and proteomics aiming to contribute for understanding the Cd dynamics in roots and to suggest the potential mechanisms involved in Cd-tolerance in tomato plants.

References

- Birke M., Reimann C., Rauch U., Ladenberger A., Demetriades A., Jähne-Klingberg F., Oorts K., Gosar M., Dinelli E., Halamić J. (2017) GEMAS: Cadmium distribution and its sources in agricultural and grazing land soil of Europe — Original data versus clr-transformed data. *Journal of Geochemical Exploration*, **173**, 13-30.
- Candeias C., Ávila P., Silva E., Ferreira A., Durães N., Teixeira J. (2015) Water-Rock Interaction and Geochemical Processes in Surface Waters Influenced by Tailings Impoundments: Impact and Threats to the Ecosystems and Human Health in Rural Communities (Panasqueira Mine, Central Portugal). *Water, Air & Soil Pollution*, **226**, 1-30.
- Choppala G., Saifullah, Bolan N., Bibi S., Iqbal M., Rengel Z., Kunhikrishnan A., Ashwath N., Ok Y. S. (2014) Cellular Mechanisms in Higher Plants Governing Tolerance to Cadmium Toxicity. *Critical Reviews in Plant Sciences*, **33**, 374-391.
- Dourado M. N., Martins P. F., Quecine M. C., Piotto F. A., Souza L. A., Franco M. R., Tezotto T., Azevedo R. A. (2013) Burkholderia sp. SCMS54 reduces cadmium toxicity and promotes growth in tomato. *Annals of Applied Biology*, **163**, 494-507.

- Gallego S. M., Pena L. B., Barcia R. A., Azpilicueta C. E., Lannone M. F., Rosales E. P., Zawoznik M. S., Groppa M. D., Benavides M. P. (2012) Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environmental and Experimental Botany*, **83**, 33-46.
- Gill S. S., Khan N. A., Tuteja N. (2012) Cadmium at high dose perturbs growth, photosynthesis and nitrogen metabolism while at low dose it up regulates sulfur assimilation and antioxidant machinery in garden cress (*Lepidium sativum* L.). *Plant Science*, **182**, 112-120.
- Gratão P. L., Monteiro C. C., Antunes A. M., Peres L. E. P., Azevedo R. A. (2008) Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. *Annals of Applied Biology*, **153**, 321-333.
- Gratão P. L., Monteiro C. C., Carvalho R. F., Tezotto T., Piotto F. A., Peres L. E. P., Azevedo R. A. (2012) Biochemical dissection of diageotropica and Never ripe tomato mutants to Cd-stressful conditions. *Plant Physiology and Biochemistry*, **56**, 79-96.
- Gratão P. L., Monteiro C. C., Rossi M. L., Martinelli A. P., Peres L. E. P., Medici L. O., Lea P. J., Azevedo R. A. (2009) Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium. *Environmental and Experimental Botany*, **67**, 387-394.
- Lane T. W., Saito M. A., George G. N., Pickering I. J., Prince R. C., Morel F. M. M. (2005) Biochemistry A cadmium enzyme from a marine diatom. *Nature*, **435**, 42-42.
- Lux A., Martinka M., Vaculik M., White P. J. (2011) Root responses to cadmium in the rhizosphere: a review. *Journal of Experimental Botany*, **62**, 21-37.
- Monteiro C. C., Carvalho R. F., Gratao P. L., Carvalho G., Tezotto T., Medici L. O., Peres L. E. P., Azevedo R. A. (2011) Biochemical responses of the ethylene-insensitive Never ripe tomato mutant subjected to cadmium and sodium stresses. *Environmental and Experimental Botany*, **71**, 306-320.
- Nogueirol R. C., Monteiro F. A., Gratao P. L., da Silva B. K. D., Azevedo R. A. (2016) Cadmium Application in Tomato: Nutritional Imbalance and Oxidative Stress. *Water Air and Soil Pollution*, **227**, 20.
- Piotto, F. A. Avaliação de tolerância ao Cádmiio em tomateiro (*Solanum lycopersicum* L.). 2012. Tese (Doutorado em Genética e Melhoramento de Plantas) - Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, 2012. doi:10.11606/T.11.2012.tde-18092012-134028.
- Piotto F. A., Tulmann-Neto A., Franco M. R., Boaretto L. F., Azevedo R. A. (2014) Rapid screening for selection of heavy metal-tolerant plants. *Crop Breeding and Applied Biotechnology*, **14**, 1-7.
- Pompeu G. B., Vilhena M. B., Gratão P. L., Carvalho R. F., Rossi M. L., Martinelli A. P., Azevedo R. A. (2017) Absciscic acid-deficient sit tomato mutant responses to cadmium-induced stress. *Protoplasma*, **254**, 771-783.
- Sarwar N., Saifullah, Malhi S. S., Zia M. H., Naeem A., Bibi S., Farid G. (2010) Role of mineral nutrition in minimizing cadmium accumulation by plants. *Journal of the Science of Food and Agriculture*, **90**, 925-937.
- Song W. Y., Mendoza-Cozatl D. G., Lee Y., Schroeder J. I., Ahn S. N., Lee H. S., Wicker T., Martinoia E. (2014) Phytochelatin-metal(loid) transport into vacuoles shows different substrate preferences in barley and Arabidopsis. *Plant Cell and Environment*, **37**, 1192-1201.
- The Tomato Genome Consortium. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature*, **485**, 635-641, 2012.

2. TEMPORAL DYNAMIC RESPONSES OF ROOTS IN CONTRASTING TOMATO GENOTYPES TO CADMIUM TOLERANCE

Abstract

Despite numerous studies of cadmium (Cd) uptake and accumulation in crops, relatively few information is available considering the temporal dynamic of Cd uptake and responses to stress focused on root system. Here we highlighted responses to Cd-induced stress in roots of two tomato genotypes contrasting in Cd-tolerance: the tolerant Pusa Ruby and the sensitive Calabash Rouge. Tomato genotypes growing in the presence of 35 μM CdCl_2 showed a similar trend of Cd accumulation in tissues, mainly in the root system and overall plants exhibited reduction in the dry matter weight. Both genotypes showed similar trends for MDA and H_2O_2 accumulation with increases when exposed to Cd, being this response more pronounced in the sensitive genotype. Regarding to antioxidant machinery, in the presence of Cd GSH content was decreased in roots while APX, GR and GST activities were increased in the presence of Cd in the tolerant genotype. Altogether these results suggest APX, GR and GST as the main players of the antioxidant machinery against Cd-induced oxidative stress.

Keywords: Antioxidants; Cadmium; Heavy metals; Oxidative Stress; *Solanum lycopersicum*

2.1. Introduction

Heavy metals occur naturally in the soil and can also be released into the environment from anthropogenic activities such as mining, phosphate fertilizers applications and disposal of agro-industrial wastes (Gallego *et al.*, 2012). Due to the increase of agricultural areas contaminated by heavy metals, cadmium (Cd) toxicity is getting worse every year. However, the number of plant species that have some tolerance degree is restricted (Bianconi *et al.*, 2013).

The knowledge about interaction between plants and heavy metals is important to reduce the probability of the introduction of these toxic elements into the food chain (Benavides *et al.*, 2005; Azevedo *et al.*, 2012; Teklić *et al.*, 2013). Such interaction can be understood as the ability that some plant species have to uptake and accumulate heavy metals, associated with the effects caused by these elements in the plant development and metabolism (Kumar *et al.*, 2013). Plant tolerance to Cd is also a relevant issue due to the negative effects on food production and quality, allied to the risks to animal and human health caused by the consumption of contaminated products (Bianconi *et al.*, 2013; Teklić *et al.*, 2013). It is estimated that more than 80% of Cd contamination occurs by ingestion of vegetables and cereals (Egan *et al.*, 2007).

In addition to the well-known negative effects on plant growth, Cd is considered a toxic metal due to its high mobility, disturbing basic physiological and metabolic processes such as uptake and transport of water and nutrients, photosynthesis and respiration (Benavides *et al.*, 2005; Arasimowicz-Jelonek *et al.*, 2011; Nogueirol *et al.*, 2016). Root system is the main way to Cd²⁺ ions uptake by plants from the soil solution, where they are quickly translocated by the xylem to the shoot (Lux *et al.*, 2011). In this context, roots play a significant role in the active uptake of metallic ions and the Cd effects on plant growth may be variable among species (Wei *et al.*, 2012), ecotypes (Li *et al.*, 2009) or cultivars (Kubo *et al.*, 2011). Some plants have the ability to tolerate and accumulate high concentrations of Cd in the shoots, being designated as hyperaccumulators (Milner and Kochian, 2008). Nonetheless, in the large majority of plant species Cd accumulates mainly in the root system with only limited amounts being translocated to the upper parts of the plants (Vitória *et al.*, 2001; Pereira *et al.*, 2002).

According to Dalcorso *et al.* (2010) there are different signaling pathways for heavy metal stress responses. Cd can induce oxidative damage in plants by increasing the production of reactive oxygen species (ROS), which are extremely cytotoxic to all organisms (Gratão *et al.*, 2005; Soares *et al.*, 2016) and whose yield should be minimized (Xu *et al.*, 2010; Perez-Chaca *et al.*, 2014). ROS can cause protein oxidation, DNA damage and lipid peroxidation, being the latter considered the most drastic effect of heavy metals to the plant cells (Gratão *et al.*, 2005). Plant cells have a number of protective mechanisms to eliminate or reduce ROS (Cuypers *et al.*, 2016; Wang *et al.*, 2015), one of them is through the activation of the enzymatic antioxidant system, which operates by the sequential and simultaneous actions of enzymes including superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.11.1.9), among many others (Gratão *et al.*, 2015; Alves *et al.*, 2017). Another potential mechanism includes non-enzymatic compounds, such as low molecular weight thiols that show high affinity for toxic metals (Yadav, 2010). One of the most critical low molecular weight biological thiol is glutathione (GSH), which is a vital part of the antioxidant armory of plant cells against oxidative stress and plays multifaceted roles by contributing to the cellular defense (Mendoza-Cozatl *et al.*, 2011; Anjum *et al.*, 2012). For instance, Tausz *et al.* (2004) have suggested the use of GSH as a marker for oxidative stress in plants. An optimum level of GSH pool, required for normal metabolic activities, is maintained in plants cells growing under non-stressful conditions (Cuypers *et al.*, 2001; Jozefczak *et al.*, 2012; Kandziora-Ciupa *et al.*, 2016). Additionally, the concentration and

redox state of GSH are suggested to be important in transducing oxidative signals originating from ROS in order to activate the antioxidative response (Ball *et al.*, 2004). Cd shows a high affinity for thiols, especially GSH, which is a primary target for free Cd-ions (Cuypers *et al.*, 2010), therefore under Cd-stress with depletion of GSH pool, some enzymes from the glutathione metabolic pathway such as glutathione-S-transferase (GST, EC 2.5.1.18) operate in plant tolerance response to oxidative stress induced by heavy metals (Gill and Tuteja, 2010; Ghelfi *et al.*, 2011).

Most of the information available about Cd responses in plants comes from studies with model plants like the Cd-hyperaccumulator *Thlaspi caerulescens* (Lombi *et al.*, 2002) and Cd-tolerant plants such as *Arabidopsis halleri* (Weber *et al.*, 2006; Zhao *et al.*, 2006), whereas there is less information about Cd accumulation and its effects on cell physiology, especially for important commercial crops such as tomato. A large part of this crop is produced in greenhouses, frequently using reutilization of water, therefore implying an increased risk of heavy metal contamination by the use of Cd-contaminated water (López-Millán *et al.*, 2009). Studies about the effects caused by Cd-exposure on plant development and antioxidant system of cultivated plants have been carried out based on the evaluation after a certain period of exposure (Gratão *et al.*, 2008a; Gratão *et al.*, 2009; Monteiro *et al.*, 2011; Gratão *et al.*, 2015; Nogueirol *et al.*, 2016; Alves *et al.*, 2017; Pompeu *et al.*, 2017). Despite numerous studies of Cd uptake and accumulation in crops, relatively little information is available considering the temporal dynamic of Cd uptake and responses to Cd-induced stress focused on the root system. Therefore, the study of temporal dynamic of antioxidant response plus root development may help to highlight tolerance mechanisms, which in turn involve necessary time for plants to recognize the stress, unleashing machinery to cope with Cd-induced stress in tissues as well as signaling to shoots. In this work, we investigated the temporal dynamic of Cd uptake and response in roots to Cd-induced stress of two tomato genotypes contrasting in Cd-tolerance. For this, plant growth evaluations were performed paralleled with the characterization of the responses of the enzymatic antioxidant and non-enzymatic defense systems in tomato roots treated with Cd.

2.2. Methodology

2.2.1. Plant material, growth conditions and treatments

Two tomato genotypes with differential tolerance to Cd-induced stress were selected based on previous studies (Piotto *et al.*, 2014): *Solanum lycopersicum* cv. Calabash

Rouge (CR) and *Solanum lycopersicum* cv. Pusa Ruby (PR), characterized as sensitive and tolerant to Cd, respectively. Seeds were sterilized with 5% hydrogen peroxide (H₂O₂) for 15 min and washed with double distilled-deionized water, and then, seeds were sown in trays with vermiculite substrate and maintained with Peters commercial solution (JR Peters Inc. 20-20-20 at 1 g L⁻¹) applied during 20 days in order to maintain seedling development. The seedlings were cultivated in a greenhouse under the following conditions: an average mean temperature of 27°C, 11.5 h/13 h (winter/summer) photoperiod, and 250-350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR irradiance (natural radiation reduced with a reflecting mesh (Aluminete-Polysack Industrias Ltda, Leme, Brazil)). Twenty-day-old seedlings were removed from the trays and transferred to a hydroponic system (10-L trays) containing a 10% ionic strength solution (Hoagland and Arnon, 1950), pH 6.5 checked daily, and the total volume maintained at a constant level by using distilled-deionized water. Plants were maintained in hydroponics for 24 hours as an adaptation period in order to mitigate stress generated by seedling transplanting, and also to increase nutrient concentration from 10 to 50% ionic strength. This procedure (gradual increase of salt concentration) was carried out in order to diminish plant stress due to an increased content of salts in solution. Afterwards for stress induction, a 35 μM cadmium chloride (CdCl₂) treatment was used and plants were sampled in three times predetermined in preliminary experiments: 1, 4 and 7 days after Cd addition, designated as T1, T2 and T3, respectively. Moreover, T0 samples (day zero treatment) were collected representing the initial condition before applying the stress. The concentration chosen resembles the limiting amount of Cd (3.6 mg of Cd per kg of dried weight) allowed in agricultural soils in São Paulo State, Brazil (CETESB, 2016). Four biological replicates composed of three plants each were collected. The control was cultivated in trays without Cd application (-Cd) whilst Cd treatments were represented as +Cd. Plant roots and shoots for enzymatic assays and stress indicators were collected and immediately frozen in liquid nitrogen and subsequently stored at -80°C for further analysis. Additionally, four biological replicates composed of four plants each were collected for dry matter weight measurements.

2.2.2. Dry matter weight and Cd content

Roots and shoots were dried at 55°C for five days to reach a constant weight followed by precision weighing. Plant tissues were ground to pass a 200-mesh sieve and Cd concentration was determined by plasma emission spectrometry (ICP-OES, Perkin-Elmer 5100 PC, Norwalk, CT, USA) after nitro-perchloric digestion (Bataglia *et al.*, 1983).

2.2.3. Quantification of stress indicators

The measurements of the malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents were performed in the same extraction, where powdered tissues (200 mg of fresh weight) were homogenized in a pestle and mortar with 20% (w/v) of insoluble polyvinylpyrrolidone (PVPP) and 2 mL of 0.1% trichloroacetic acid (TCA). For MDA the homogenate was centrifuged at 10,000 g for 10 min and 250 μL of the supernatant was added to 1 mL of 0.5% 2-thiobarbituric acid (TBA) and 20% TCA solution and incubated in a water bath at 95°C for 30 min. The concentration of MDA was calculated from the absorbance at 535 nm by using the absorbance coefficient $155 \text{ mM}^{-1} \text{ cm}^{-1}$, following a correction for unspecific turbidity determined by the absorbance at 600 nm (Heath and Packer, 1968).

H_2O_2 was measured spectrophotometrically after reaction with potassium iodide (KI) as described by Alexieva *et al.* (2001). The reaction mixture consisted of 200 μL of 0.1% TCA leaf and root extracts supernatants, 200 μL of 100 mM potassium phosphate buffer and 800 μL of 1 M KI. The reaction was developed for 1 h in darkness at room temperature and absorbance measured at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared with known concentrations of H_2O_2 .

2.2.4. Enzyme extraction and activity determination

Tomato roots (1 g of fresh weight) were homogenized in a pestle and mortar with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v) insoluble PVPP (Azevedo *et al.*, 1998). The homogenate was centrifuged at 10,000 g for 30 min and the supernatant was stored in separate aliquots at -80°C for further enzymatic assays. The total protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard (Bio-Rad Protein Dye Reagent).

2.2.4.1. SOD assay

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was carried out as reported by Constantine and Ries (1977) by the inhibition of 4-nitroblue tetrazolium (NBT) chloride photoreduction. The assay was carried out using the following reaction mixture: 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM riboflavin, 50 mM methionine, 1 mM NBT, 10 mM EDTA and 15 μL of plant extract. The reaction mixture was illuminated (50 μmol

$\text{m}^{-2} \text{s}^{-1}$) at 25°C for 10 min and the absorbance measured at 560 nm. One unit of SOD activity was defined as the amount of the enzyme required to inhibit the reduction of NBT by 50% under the specified conditions. SOD activity of the extracts was expressed as U mg^{-1} protein.

2.2.4.2. CAT assay

Catalase (CAT, E.C. 1.11.1.6) activity was determined as described by Kraus *et al.* (1995) with modifications as described by Azevedo *et al.* (1998). CAT was assayed spectrophotometrically at 25°C in a 10 ml reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5) and 25 μl H_2O_2 (30% solution). The reaction was initiated by the addition of 25 μl plant extract and determined by monitoring H_2O_2 degradation at 240 nm over 1 min. CAT activity was calculated using an extinction coefficient for H_2O_2 of $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ and results was expressed as $\mu\text{mol}^{-1} \text{ min}^{-1} \text{ mg}$ protein.

2.2.4.3. APX assay

Ascorbate peroxidase (APX, E.C. 1.11.1.11) activity was determined by the addition of 50 μl plant extract to 1 ml of a medium containing 80 mM potassium phosphate buffer (pH 7.0), 5 mM ascorbate, 1 mM EDTA and 1 mM H_2O_2 (Nakano and Asada, 1981). APX activity was determined by monitoring the rate of ascorbate oxidation at 290 nm at 30°C. APX activity was expressed as $\mu\text{mol}^{-1} \text{ min}^{-1} \text{ mg}$ protein.

2.2.4.4. GR assay

Glutathione reductase (GR, E.C. 1.11.1.9) activity was assayed at 30°C in a mixture consisting of 1 ml 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1 mM oxidized glutathione (GSSG) and 1 mM NADPH (Smith et al. 1988). The reaction was started by the addition of 30 μl of plant extract. The rate of reduction of GSSG was followed in a spectrophotometer by monitoring the increase in absorbance at 412 nm over a 1 min period. GR activity was expressed as $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ protein.

2.2.4.5. GST assay

Glutathione-S-transferase (GST, E.C. 2.5.1.18) was assayed by spectrophotometer using the method of Habig and Jakoby (1981) with some modifications (Ghelfi *et al.*, 2011).

The assay mixture contained 100 mM potassium phosphate buffer (pH 6.5), 100 mM reduced glutathione (GSH), 40 mM 1-chloro-2,4-dinitrobenzene (CDNB) prepared in 100% ethanol and 25 μ l of plant extract, in 1 mL final volume. Reactions were initiated with addition of the CDNB substrate. The enzymatic formation of 2,4-dinitrophenyl-S-glutathione at 340 nm was monitored for 1 min. GST activity was calculated using an extinction coefficient of 9.6 mM⁻¹ cm⁻¹ and the results were expressed as μ mol⁻¹ min⁻¹ mg protein.

2.2.5. Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was carried out using 12% polyacrylamide gels, with a 4% stacking gel, as previously described (Laemmli, 1970). Sodium dodecyl sulfate (SDS) was omitted for non-denaturing gels. Electrophoresis was carried out at 4°C at a constant current of 15 mA per gel during 3 h for gels stained for SOD activity, 22 h for CAT activity and 4 h for GR activity. Equal amounts of protein (30 μ g) were loaded onto each gel lane. Densitometry was performed using ImageJ software, version 1.47 (National Institutes of Health, U.S.A; <http://imagej.nih.gov/ij>) with relative percentage calculated based on the activity of the respective standard in each gel.

2.2.5.1. SOD activity staining

SOD activity staining was carried out as described by Beauchamp and Fridovich (1971) and optimized by Azevedo *et al.* (1998). After 3 h non-denaturing-PAGE separation, the gel was rinsed in distilled-deionized water and incubated in the dark, at room temperature, in 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM riboflavin, 0.1 mM nitroblue tetrazolium, 1 mM EDTA, and 3% N,N,N,N-tetramethylethylenediamine (TEMED). After 30 min, the gels were rinsed with distilled water and then illuminated in water until the achromatic bands of SOD activity were visible on a purple-stained gel. SOD isoenzymes were distinguished and classified by their sensitivity to inhibition by 2 mM potassium cyanide and 5 mM H₂O₂, according to Azevedo *et al.* (1998).

2.2.5.2. CAT activity staining

CAT activity staining in native PAGE gels was determined as described by Medici *et al.* (2004). The gels were incubated at room temperature in 0.003% H₂O₂ for 10 min and developed in a 1% (w/v) FeCl₃ and 1% K₃Fe(CN)₆ (w/v) solution for 10 min. The relative

percentage was calculated based on the activity of the standard in the gel (CAT from bovine liver, Sigma C-100 lot 20K7010) as described by Alcântara *et al.* (2015).

2.2.5.3. GR activity staining

GR activity staining in native PAGE gels was determined as described by Lee and Lee (2000). The gels were incubated in the dark for 30 min at room temperature in 50 mL of a reaction mixture containing 250 mM Tris buffer (pH 7.5), 3.4 mM GSSG, 0.5 mM NADPH, 50 mg of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 10 mg of 2,6-dichloro-N-(4-hydroxyphenyl)-1,4-benzo-quinoneimine sodium salt (DPIP). The relative percentage was calculated based on the activity of the standard in the gel (GR from yeast, Sigma G3664) as described by Alcântara *et al.* (2015).

2.2.6. Glutathione content

The reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were determined spectrophotometric according to Anderson (1985) with some modifications. Fresh tissues (100 mg) were homogenized in a pestle and mortar with sulfosalicylic acid 5% (1 mL). The homogenate was centrifuged at 10,000 *g* at 4°C during 20 min and 200 µL of the supernatant was added to 1.8 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA and 100 µL of 3 mM 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB). The mixture was kept in the dark for 5 min at room temperature and the absorbance was monitored at 412 nm. Next, the same cuvettes received 100 µL of 0.4 mM NADPH and 2 µL GR (205 U mg⁻¹) and the mixtures were kept in the dark during 20 min. At the end of this period the absorbance was measured at 412 nm. The GSH content was calculated from a standard curve constructed using concentrations of GSH over the 0-1 mM range.

2.2.7. Statistical analysis

All experiments were performed in completely random design. Data from figures represent established linear, quadratic or cubic regressions, showing the trends of the measured parameters during the experimental period in Cd-treated and untreated (control) plants, whereas the Time was considered as independent variable. The choice of the best model was based on Akaike's Information Criterion (AIC) (Akaike, 1974). Data from tables represent means from four biological replicates ($n = 4$) $\pm$ standard deviation (SD). Means

corresponding to the tomato genotypes were compared to the respective control plants within each time period (T1, T2 or T3) by Student's t-test. Differences were considered significant when $p \leq 0.05$. All analyses were performed using the R software version 2.15.1 (R, 2015).

2.3. Results

2.3.1. Cd accumulation in tomato tissues and impact on plant growth

Tomato genotypes growing in the presence of 35 μM CdCl_2 showed a similar trend of Cd accumulation in tissues, mainly in the root system (Figure 1a), with greater increases of this metal until T2 and following a more constant tendency of Cd increments until T3 in both genotypes. Regarding the shoot, the highest accumulation was observed at T2 for the tolerant genotype PR and at T3 for the sensitive genotype CR (Figure 1b). Although the amounts of Cd in the roots have been similar in both genotypes over time, it is evident that after 7 days of Cd-exposure (T3), the CR genotype accumulated more Cd in the shoots than PR.

Figure 1. Cd accumulation ($\mu\text{g g}^{-1}$ dry weight) in tissues of tomato genotypes. Mean values in roots (a) and shoots (b) are shown. Solid black lines represent control condition of tolerant genotype Pusa Ruby in root ($R^2 = 0.24$, $p = 0.11$) and shoot ($R^2 = 0.30$, $p = 0.01$). Dotted black lines represent stress condition of tolerant genotype in root ($R^2 = 0.96$, $p < 0.01$) and shoot ($R^2 = 0.95$, $p < 0.01$). Solid grey lines represent control condition of sensitive genotype Calabash Rouge in root ($R^2 = 0.30$, $p < 0.01$) and shoot ($R^2 = 0.60$, $p < 0.01$). Dotted grey lines represent stress condition of sensitive genotype in root ($R^2 = 0.95$, $p < 0.01$) and shoot ($R^2 = 0.93$, $p < 0.01$). Time indicate 1, 4 and 7 days growing in the presence of CdCl_2 , named as T1, T2 and T3 respectively. T0 represent initial condition before applying stress. DW: dry weight

Cd exposure inhibited the growth of tomato plants with an overall reduction in the dry matter weight of plants during the time length of experiment (Figure 2).

Figure 2. Impact of Cd on plant growth expressed as dry matter weight (mg plant⁻¹) in tomato tissues. Mean values in roots (a), shoots (b) and root + shoot (c) are represented. Solid black lines represent control condition of tolerant genotype Pusa Ruby in root ($R^2 = 0.96$, $p = 0.11$), shoot ($R^2 = 0.96$, $p = 0.01$) and root + shoot ($R^2 = 0.97$, $p < 0.01$). Dotted black lines represent stress condition of tolerant genotype in root ($R^2 = 0.94$, $p < 0.01$), shoot ($R^2 = 0.97$, $p < 0.01$) and root + shoot ($R^2 = 0.97$, $p < 0.01$). Solid grey lines represent control condition of sensitive genotype Calabash Rouge in root ($R^2 = 0.91$, $p < 0.01$), shoot ($R^2 = 0.94$, $p < 0.01$) and root + shoot ($R^2 = 0.94$, $p < 0.01$). Dotted grey lines represent stress condition of sensitive genotype in root ($R^2 = 0.51$, $p < 0.01$), shoot ($R^2 = 0.74$, $p < 0.01$) and root + shoot ($R^2 = 0.72$, $p < 0.01$). Time indicate 1, 4 and 7 days growing in the presence of CdCl₂, named as T1, T2 and T3 respectively. T0 represent initial condition before applying stress.

Both genotypes presented a similar pattern of growth decrease caused by Cd exposure, with larger declines in the sensitive genotype. The root growth rate over time was similar for both genotypes in the absence of Cd (Figure 2a). On the other hand, the roots exposed to Cd exhibited lower accumulation of dry matter due to the presence of the metal, with CR plants exhibiting lower growth rate than PR. In other words, the sensitive genotype had its development more affected by Cd than the tolerant genotype. Similarly to what was observed in the roots, shoot growth was also reduced in the presence of Cd (Figure 2b). There was a small trend of increased dry mass accumulation in the PR genotype when

compared to CR in stressed plants (Figure 2c), although the overall trend of shoot development under Cd-induced stress has been practically the same (Figure 2b).

2.3.2. Lipid peroxidation level

The increase in MDA content was gradual in plants exposed to Cd and first detected in roots of the tolerant genotype PR (Figure 3a). MDA accumulation in PR roots treated with Cd was always greater compared to CR and followed by a linear accumulation until the end of the experiment period. The highest MDA contents were detected in shoots after Cd-exposure, notably at T2 in CR genotype (Figure 3b). Both genotypes showed a similar trend for MDA accumulation when exposed to Cd, increased after T1 and decreased after T2 in shoots, being more pronounced in the sensitive genotype (CR).

Figure 3. Oxidative damage induced by Cd-exposure in tomato tissues expressed as MDA content (nmol g⁻¹ fresh weight) and H₂O₂ (μmol g⁻¹ fresh weight). Mean values of MDA in roots (a) and shoots (b), H₂O₂ in roots (c) and shoots (d) are shown. Solid black lines represent control condition of tolerant genotype Pusa Ruby in root (a: R² = 0.75, p < 0.01; c R² = 0.55, p < 0.01) and shoot (b: R² = 0.52, p < 0.01; d: R² = 0.54, p < 0.01). Dotted black lines represent stress condition of tolerant genotype in root (a: R² = 0.52, p < 0.01; c R² = 0.53, p < 0.01) and shoot (b: R² = 0.80, p < 0.01; d: R² = 0.73, p < 0.01). Solid grey lines represent control condition of sensitive genotype Calabash Rouge in root (a: R² = 0.64, p < 0.01; c R² = 0.65, p < 0.01) and shoot (b: R² = 0.68, p < 0.01; d: R² = 0.54, p < 0.01). Dotted grey lines represent stress condition of sensitive genotype in root (a: R² = 0.64, p < 0.01; c R² = 0.84, p < 0.01) and shoot (b: R² = 0.73, p < 0.01; d: R² = 0.57, p < 0.01). Time indicate 1, 4 and 7 days growing in the presence of CdCl₂, named as T1, T2 and T3 respectively. T0 represent initial condition before applying stress. FW: fresh weight.

H₂O₂ content presented a trend of gradual increases for both genotypes and tissues (Figure 3c and 3d), with major contents detected in roots of both genotypes between T2 and T3 of Cd exposure (Figure 3c). The sensitive genotype presented higher contents of H₂O₂ mainly after T2 in both tissues. When shoots are concerned, higher H₂O₂ contents were detected between T2 and T3, following a decrease tendency at the last time (Figure 3d). Roots and shoots growing without Cd presented similar patterns of H₂O₂ accumulation.

2.3.3. Antioxidant responses to Cd-induced stress

A trend of reduction in SOD activity in root tissue was observed in both genotypes when exposed to Cd, mainly in the tolerant one (Figure 4a).

Figure 4. Specific activity of the antioxidant enzymes in tomato roots under Cd-induced stress. (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) ascorbate peroxidase (APX), (d) glutathione reductase (GR), (e) glutathione-S-transferase (GST). Time indicate 1, 4 and 7 days growing in the presence of CdCl_2 , named as T1, T2 and T3 respectively. T0 represent initial condition before applying stress. Values represent the means of four biological replicates ($n=4$) $\pm$ SD of each treatment (-Cd, without Cd, +Cd, treated with Cd). *Statistically significant differences by Student's t -test ($P \leq 0.05$) compared to respective control. Dotted and solid lines represent +Cd and control conditions respectively. Solid black lines represent control condition of tolerant genotype Pusa Ruby (a: $R^2 = 0.04$, $p = 0.46$; b: $R^2 = 0.11$, $p = 0.16$; c: $R^2 = 0.27$, $p < 0.01$; d: $R^2 = 0.22$, $p < 0.01$; e: $R^2 = 0.36$, $p < 0.01$). Dotted black lines represent stress condition of tolerant genotype (a: $R^2 = 0.68$, $p < 0.01$; b: $R^2 = 0.45$, $p < 0.01$; c: $R^2 = 0.46$, $p = 0.02$; d: $R^2 = 0.78$, $p < 0.01$; e: $R^2 = 0.71$, $p < 0.01$). Solid grey lines represent control condition of sensitive genotype Calabash Rouge (a: $R^2 = 0.04$, $p = 0.04$; b: $R^2 = 0.23$, $p = 0.45$; c: $R^2 = 0.47$, $p < 0.01$; d: $R^2 = 0.75$, $p < 0.01$; e: $R^2 = 0.65$, $p = 0.55$). Dotted grey lines represent stress condition of sensitive genotype (a: $R^2 = 0.02$, $p = 0.42$; b: $R^2 = 0.04$, $p = 0.47$; c: $R^2 = 0.56$, $p < 0.01$; d: $R^2 = 0.71$, $p < 0.01$; e: $R^2 = 0.78$, $p < 0.01$). FW: fresh weight.

Similar results for the tolerant genotype were observed for CAT activity (Figure 4b), whilst the sensitive genotype exhibited no alteration in the enzyme activity pattern. APX activity was increased over time in Cd exposed roots of both genotypes with similar trends of increase found in both genotypes, with higher activity in tolerant roots (Figure 4c). An early response of APX induced by Cd was detected at T1 in both genotypes, reaching a peak at T2 followed by a downward trend after seven days of exposure to the metal (T3). GR activity was shown to be gradually increased during the time of exposure to Cd, with a similar trend exhibited by APX activity (Figure 4d). GR was induced by Cd reaching the maximum activity at T2 for both genotypes showing at least 58% increase when compared to the controls. At T3, GR activity in roots of both genotypes remained higher than in the controls, suggesting enzyme activation induced by Cd. As well as GR, GST activity was stimulated by Cd earlier in CR roots (T1), whose high activity was maintained in both genotypes (Figure 4e), followed by a downward trend, exhibiting lower values between T2 and T3 for both genotypes. The activity of antioxidant enzymes of tomato roots grown over seven days in the presence of Cd were also estimated based on the separation of isoenzymes by non-denaturing PAGE (Figure 5).

Figure 5. Activity staining of CAT, SOD and GR following non-denaturing polyacrylamide gel electrophoresis of tomato roots. The sensitive genotype Calabash Rouge (CR) and the tolerant Pusa Ruby (PR) after 1, 4 and 7 days growing in the presence of CdCl₂, named as T1, T2 and T3 respectively. The treatments are with Cd (+Cd) and without Cd (-Cd). I, II, III, IV and V represents SOD (I-V) and GR isoforms (I-III) in the respective gels.

The different bands of activity were identified using isoform selective staining (data not shown) (Vitória et al., 2001). Five SOD isoenzymes were detected: Mn/SOD (SOD I and II), Fe/SOD (SOD III) and Cu-Zn/SOD (SOD IV and V). SOD I and SOD IV bands were apparently not altered during Cd stress in both genotypes, while SOD II appeared to be induced by Cd in CR roots (Figure 5). SOD III became more evident in the presence of Cd at T2, but it is hardly noticeable in control roots of both genotypes. SOD V was barely noticed in PR roots, whilst in CR it was unaltered after Cd exposure. When CAT activity is concerned, only one CAT isoenzyme was found and its activity was apparently not altered by Cd treatment over time in both genotypes (Figure 5). Three GR isoenzymes were detected in both genotypes with a gradual increase on band intensity of GR III after Cd exposure (Figure 5). In general, the pattern found in the gels is in accordance with the results measured spectrophotometrically (Figure 4).

Under Cd exposure a reduction trend of GSH and increased of GSSG were detected in the sensitive roots at T1 and T2, whereas the opposite were observed in the tolerant genotype (Table 1).

Table 1. Glutathione forms in plant roots (%). GSH (reduced glutathione), GSSG (oxidized glutathione) and GSSG/GSH ratios. The tolerant genotype Pusa Ruby (PR) and sensitive genotype Calabash Rouge (CR) after 1, 4 and 7 days growing in the presence of CdCl₂, named as T1, T2 and T3 respectively. The treatments are with Cd (+Cd) and without Cd (-Cd).

Genotype	Time	Treatment	GSH	GSSG	GSSG/GSH
Pusa Ruby (tolerant)	T1	-Cd	69.13	30.87	0.45
		+Cd	76.65*	23.35*	0.30
	T2	-Cd	42.36	57.64	1.36
		+Cd	43.59	56.41	1.29
	T3	-Cd	53.87	46.13	0.86
		+Cd	87.02*	12.98*	0.15
Calabash Rouge (sensitive)	T1	-Cd	71.22	28.78	0.40
		+Cd	69.03*	30.97*	0.45
	T2	-Cd	52.52	47.48	0.90
		+Cd	45.39*	54.61*	1.20
	T3	-Cd	46.72	53.28	1.14
		+Cd	83.77*	16.23*	0.19

Values represent the means of 4 replicates (n = 4).

*P ≤ 0.05 by Student's t-test.

However, the GSSG/GSH ratio for tolerant genotype was lower under stress (T1), indicating higher initial GSH content in the roots. At T3 increases of GSH content followed by decreases of GSSG were observed for both genotypes (Table 1).

2.4. Discussion

2.4.1. Tomato tissues can accumulate Cd and reduce growth

PR and CR tomato genotypes were previously characterized as Cd-tolerant and Cd-sensitive genotypes, respectively (Piotto *et al.*, 2014). In the present work, using the same genotypes, we aimed at focusing on temporal dynamic response of the antioxidant system to Cd-induced stress in order to shed some light on possible mechanisms involved in Cd tolerance in these tomato genotypes. It is well known that Cd accumulation varies considerably among plant species and also among cultivars within species (Gratão *et al.*, 2008a). Accumulation of Cd in tomato tissues notably occurred within the first 24 hours of Cd exposure (T1) and continued over time, mainly in the roots (Figure 1a). Similar results have been reported for the large majority of plant species studied (Gallego *et al.*, 2012) including maize (Anjum *et al.*, 2015), soybean (Perez-Chaca *et al.*, 2014), radish (Vitória *et al.*, 2001), *Crotalaria juncea* (Pereira *et al.*, 2002) and tomato (Gratão *et al.*, 2008a; López-Millán *et al.*, 2009). The root system is the first tissue of contact with the metal and through which it enters the plant system, suggesting that roots may be the first place where defense mechanisms against stress occurs, for instance by heavy metal immobilization in the vacuoles (Mendoza-Cozatl *et al.*, 2011). Vitória *et al.* (2001) working with radish suggested that Cd-binding proteins would be produced in the roots to control Cd eventual stress. The increase of Cd allocation in roots after seven days of Cd-exposure (T3) explains a possible mechanism used by the plants to protect the shoots against excess of Cd, since it can affect photosynthesis (Bashri and Prasad, 2015; Khan *et al.*, 2007). When Cd accumulation in tomato is concerned, the majority of the studies have confirmed that Cd accumulates in larger amounts in the roots (Hediji *et al.*, 2010), which can be related to the avoidance of translocation, but one report has also shown that Cd could also accumulate in the fruits, which is dependent upon the Cd exposure system used (Gratão *et al.*, 2008a). According to Lux *et al.* (2011) tolerant plants develop extracellular barriers and can store Cd-chelates in the vacuoles, which might be occurring in tomato roots. In our study, we observed at T1 a rapid metal increase in roots tissue, whilst shoots exhibited less Cd accumulation in both tomato genotypes. Accordingly, high proportion of Cd in the soluble fraction as well as in its chemical form could limit root-to-shoot translocation of Cd, resulting in lower Cd levels in the shoots (Su *et al.*, 2014). Gratão *et al.* (2015) reported low Cd accumulation in shoots of the Micro-Tom (MT) tomato cultivar and suggested it may be driven by transpiration. In other study with *Vaccinium myrtillus* L. (bilberry) and *Vaccinium vitis-idaea* L. (lingonberry)

growing in contaminated soils Cd accumulated especially in the shoots, due to the fact that evergreen species maintain heavy metals storage mainly in these organs (Kandziora-Ciupa *et al.* 2017). Apparently effective root-to-shoot translocation of metals indicates a tolerance strategy that may be employed by the PR genotype, once this genotype exhibited lower Cd accumulation in shoots than the CR genotype (Figure 1b), confirming that tolerant genotypes have lower Cd content in the shoots than sensitive genotypes (Hossain *et al.*, 2012).

Growth inhibition was observed in tomato genotypes (Figure 2). The sensitive genotype exhibited a remarkable growth decrease during the stress period, suggesting that the existence of intracellular detoxification or defense strategies may be different from those acting in the tolerant genotype. These findings are in accordance with Lu *et al.* (2013) who reported that root and shoot biomasses of peanut cultivars were mainly affected after Cd exposure. In this work, similar growth rates for both genotypes were observed in the absence of Cd, whilst plants exposed to Cd showed impaired growth induced by metal exposure. At T3, reduction of total biomass (Figure 2c) by 65% and 54% for CR and PR respectively, were observed suggesting that PR plants may have better mechanisms to cope with Cd-induced stress, controlling its growth and development even under stress.

2.4.2. Cd induce lipid peroxidation in tomato

The effect of Cd toxicity can be indirectly determined by evaluating MDA accumulation, a product of lipid peroxidation in plant tissues. Our results confirmed that Cd may increase the rate of lipid peroxidation and membrane damage since MDA levels were altered in the Cd-stressed plants of both genotypes and tissues analyzed (Figure 3a and 3b). Similar increases of MDA have been observed after Cd-exposure in maize (Anjum *et al.*, 2015), wheat (Khan *et al.*, 2007), rice (Sebastian and Prasad, 2014) and tomato (Gratão *et al.*, 2008a). ROS generation may affect the auxin physiology that consequently leads to a reduction of plant biomass (Bashri and Prasad 2015). Increased lipid peroxidation induced by Cd was observed in the tomato MT sitiens mutant (ABA-deficient mutant), which was more pronounced in the roots of the mutant than in the wild-type (MT), suggesting that ABA deficiency amplifies the stress response to Cd (Pompeu *et al.*, 2017). Analyzing shoots at T2, we observed a peak of stress considering the changes in MDA content for both genotypes (Figure 3b), with increases by 56% and 42% for CR and PR, respectively. This may be an indication of different levels of shoot capability to cope with Cd-induced stress leading to the increased MDA contents observed, particularly in CR, which faced a higher

level of oxidative stress and in accordance with the activities of antioxidant enzymes, that showed different mechanisms triggered in Cd-sensitive and Cd-tolerant genotypes (Figure 4).

An analysis over time suggests that H_2O_2 production was increased in plants treated with Cd, especially in roots, for both tomato genotypes (Figure 3a). A major increase of H_2O_2 content was detected in roots of the CR genotype at T3, which appears to be related to enzyme activities observed in the root tissue (Figure 4) since this genotype at T3 showed higher SOD activity compared with PR genotype resulting in higher H_2O_2 content (Figure 3c). Comparing shoots in the presence of Cd we observed a decrease of H_2O_2 content at T3 in both genotypes, which may be a strategy to protect photosynthetic function from Cd-induced oxidative stress (Dixit *et al.*, 2001), by activation of antioxidant enzymes and other protective mechanisms. Stress indicators (MDA and H_2O_2) exhibited higher values for the CR genotype under Al-stress cultivated in sandy and clay soils (Nogueirol *et al.*, 2015). In accordance with these authors, once H_2O_2 plays a role in cellular expansion facilitating the expanding abilities of the cell wall, maybe growth could restrict the greater impact of H_2O_2 , as shown by the more pronounced growth of CR plants. Higher H_2O_2 contents in CR roots and shoots at T2 and T3 under Cd-induced stress were observed, but in contrast, lower growth rate of CR genotype at T3 was observed with root growth decreased by 2-fold when compared with the PR genotype (Figure 2). This evidence reinforce the tolerance degree exhibited by the PR genotype as originally observed and reported by Piotto *et al.* (2014) when testing tomato cultivars in the presence of Cd.

2.4.3. Antioxidant enzymes responding to Cd-induced stress

To gain a better insight into the possible role of oxidative stress induced by Cd we focused on some antioxidative enzymes such as SOD, CAT, APX, GR and GST. SOD specific activity decreased in the presence of Cd over time only in tolerant roots (Figure 4a) and accordingly, lower H_2O_2 concentrations were found in Cd-treated plants in the PR genotype (Figure 3b). Previous reports showed variable responses of SOD activity in plants exposed to Cd (Gratão *et al.*, 2008a; Gratão *et al.*, 2015; Semane *et al.*, 2007; Tamas *et al.*, 2016). Although no change in SOD total enzymatic activity was observed, PAGE analysis showed changes in some SOD isoenzymes which were time-, treatment- and genotype-dependents (Figure 5). Five SOD isoenzymes (SOD I, II, III, IV and V) were observed in PAGE of tomato roots. SOD III (classified as Fe/SOD) was activated in the presence of Cd when compared to the control at T3 but was barely noticeable in control roots for both

genotypes. This result is very similar to that reported by Nogueirol *et al.* (2016) who observed enhancement in SOD III activity in CR roots following increase in Cd concentration. Jozefczak *et al.* (2014) observed Fe/SOD activity in roots of *Arabidopsis thaliana* only after three days of Cd exposure, whereas Cu-Zn/SOD (SOD IV and V) activity was reduced in a time- and dose-dependent manner in roots starting after 24 h exposure. No visible changes in the activity of SOD I and SOD II isoenzymes were detected in tomato roots (Figure 5).

The accumulation of H_2O_2 , a strong oxidant, is prevented in plant cells either by CAT and a number of peroxidases or by the ascorbate-glutathione cycle where APX reduces it to H_2O (Gratão *et al.*, 2005). Gratão *et al.*, (2008b) studying antioxidant responses of tobacco to Cd-stress reported increased CAT activity in the second half of the experimental period, which was clearly accompanied by a shift in the activity of peroxidases that operated during the Cd stress applied to the cells. In this work, CAT specific activity decreased at T3 in PR roots (Figure 4b), suggesting that the detoxification of H_2O_2 was not prevented by CAT activity in both genotypes. Increased activity of APX in stressed roots was noticed mainly in the tolerant genotype at all time points of the experiment (Figure 4c), suggesting that is actively working to break the H_2O_2 produced in excess. The later response of CAT to Cd in PR roots appeared to have been compensated by the increased activity of APX, which in our study, appears to be a main player in the defense response to the oxidative stress in tomato roots cells.

GR was activated in Cd-treated tomato roots of both genotypes studied, with higher proportions at T2 (Figure 4d). Cd stress induced the enzymatic activity in both genotypes earlier (T1), while at T2 the maximum activity of GR was observed in both genotypes, remaining higher in stressed plants until the end of the experiment (Figure 4d). In gel analysis (Figure 5), the GR III showed similar behavior of the spectrophotometer analysis, suggesting that this specific isoenzyme might have been the main contributor to the general changes in GR activity. We hypothesize that perhaps sensitive roots were responding to Cd-induced stress earlier than the tolerant roots and so the GR III was activated first in this sensitive genotype (CR). Increasing GR activity has strengthened the defense mechanism in wheat roots, protecting them against Cd-induced oxidative stress (Yannarelli *et al.*, 2007), which agrees with the data obtained for the tomato genotypes. According to Ding *et al.* (2009) GR plays an important role in the regeneration of GSH prior to incorporation into phytochelatins (PCs), protecting against oxidative stress and

maintaining the ascorbate pool as well. Our work suggests that GR and APX together may be the main players against oxidative stress in tomato genotypes exposed to Cd.

The detoxification activity of GST has been reported mainly related to oxidative stress, xenobiotics and heavy metals (Kumar *et al.*, 2013). GST activity was stimulated by Cd-stress particularly after T2 for both genotypes (Figure 4e), exhibiting the same activity pattern of GR, possibly indicating that there was a balance in GSH utilization by these antioxidant enzymes. It also indicates that Cd caused a rapid induction of both GR and GST (as compared to SOD and CAT) with GST probably operating in the detoxification process using GSH as a substrate. In the present study, the activities of APX, GR and GST were increased in the roots of both genotypes treated with Cd. GR and GST showed different behavior than APX in tomato genotypes. While GR and GST were activated in both genotypes at T1, APX was activated firstly in the tolerant than in the sensitive. These results appears to indicate that Cd-induced stress increased the activity of some antioxidant enzymes, which may represent a secondary defensive mechanism against oxidative stress that are not as direct as primary defensive responses such as PCs production and vacuolar compartmentalization (Sanità di Toppi and Gabbrielli, 1999). GSH-conjugate formation is mediated by GST, an enzyme that contains active sites that bind GSH to cytotoxic compounds such as MDA (Blair, 2010). This may explain why we did not observe at T1 differences in MDA content of CR roots (Figure 3a), which might be quickly conjugated by GST (Figure 4e).

At the earlier stage of Cd exposure (T1) GSH increased while GSSG decreased in the tolerant roots which are in accordance to GR and GST results (Figures 4d and 4e). Since GR activity is responsible for maintaining GSH pools in the cells (Jozefczak *et al.*, 2012) converting GSSG into GSH, we believe that GR activity was the main responsible for the reduction of GSSG/GSH ratio at T3 in both genotypes. We observed an initial enhancement of the GSH pool in PR roots, which might allow the conjugation of oxidative stress metabolites. In accordance with Gill *et al.* (2013) GSH is an abundant compound in plants that directly scavenges ROS and may protect enzyme thiol groups. Under heavy metal stress it is well established in the literature that regulation of GSH biosynthesis undergoes with significant changes, such as increase of GSH oxidation and PCs production, resulting in a depletion of cellular GSH levels and consequently, the feedback inhibition is released (Jozefczak *et al.*, 2012). Cd exposure increased GSSG/GSH ratio in *A. thaliana*, which was accompanied by enhanced GR and APX activities (Semane *et al.*, 2007). At T2 a decrease of GSH content was observed only in roots of the sensitive genotype (Table 1),

which might be due to its utilization as substrate for GST enzyme. Another hypothesis suggests that a redox signal generated by a decreased GSH pool in the cells in combination with an altered GSSG/GSH ratio may increase the antioxidant capacity (Jozefczak *et al.*, 2014). Thus, for tomato roots we can suggest that the decreased GSH content could be due to increased antioxidant activity in tomato roots, especially of APX, GR and GST.

Summarizing, tomato genotypes differing in Cd-tolerance grown in media containing 35 μM CdCl_2 showed metal accumulation in roots and shoots tissues in both genotypes, but with increased Cd accumulation over time mainly in roots. Since PR (tolerant genotype) exhibited higher growth rate as compared to sensitive genotype, PR appears to have a more efficient mechanism to cope with Cd-induced stress. Regarding to antioxidant machinery, Cd induced decreased GSH content in roots whilst increased the activities of APX, GR and GST, which together may be the main players against oxidative stress in Pusa Ruby genotype exposed to Cd. Therefore, different tomato genotypes can employ distinct strategies to cope with Cd-induced challenges to the plant development.

References

- Akaike H. (1974) A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, **19**, 716-723.
- Alcântara B. K., Machemer-Noonan K., Silva F. G., Azevedo R. A. (2015) Dry Priming of Maize Seeds Reduces Aluminum Stress. *Plos One* doi: 10(12):e0145742.
- Alexieva V., Sergiev I., Mapelli S., Karanov E. (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell and Environment*, **24**, 1337-1344.
- Alves L. R., Monteiro C. C., Carvalho R. F., Ribeiro P. C., Tezotto T., Azevedo R. A., Grato P. L. (2017) Cadmium stress related to root-to-shoot communication depends on ethylene and auxin in tomato plants. *Environmental and Experimental Botany*, **134**, 102-115.
- Anderson M. E. (1985) Determination of glutathione and glutathione disulfide in biological samples. *Methods in Enzymology*, **113**, 548-555.
- Anjum N. A., Ahmad I., Mohmood I., Pacheco M., Duarte A. C., Pereira E., Umar S., Ahmad A., Khan N. A., Iqbal M., Prasad M. N. V. (2012) Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids-A review. *Environmental and Experimental Botany*, **75**, 307-324.
- Anjum S. A., Tanveer M., Hussain S., Bao M., Wang L., Khan I., Ullah E., Tung S. A., Samad R. A., Shahzad B. (2015) Cadmium toxicity in Maize (*Zea mays* L.): consequences on antioxidative systems, reactive oxygen species and cadmium accumulation. *Environmental Science and Pollution Research*, **22**, 17022-17030.
- Arasimowicz-Jelonek M., Floryszak-Wieczorek J., Gwozdz E. A. (2011) The message of nitric oxide in cadmium challenged plants. *Plant Science*, **181**, 612-620.
- Azevedo R. A., Alas R. M., Smith R. J., Lea P. J. (1998) Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and

- roots of wild-type and a catalase-deficient mutant of barley. *Physiologia Plantarum*, **104**, 280-292.
- Azevedo R. A., Gratão P. L., Monteiro C. C., Carvalho R. F. (2012) What is new in the research on cadmium-induced stress in plants? *Food and Energy Security*, **1**, 133-140.
- Ball L., Accotto G.-P., Bechtold U., Creissen G., Funck D., Jimenez A., Kular B., Leyland N., Mejia-Carranza J., Reynolds H., Karpinski S., Mullineaux P. M. (2004) Evidence for a Direct Link between Glutathione Biosynthesis and Stress Defense Gene Expression in Arabidopsis. *The Plant Cell*, **16**, 2448-2462.
- Bashri G., Prasad S. M. (2015) Indole acetic acid modulates changes in growth, chlorophyll a fluorescence and antioxidant potential of *Trigonella foenum-graecum* L. grown under cadmium stress. *Acta Physiologiae Plantarum*, **37**, 49.
- Bataglia O. C., Furlani A. M. C., Teixeira J. P. F., Furlani P. R., Gallo J. R. (1983) Método de análise química de plantas. *LAC - Boletim Técnico* **78**, 48p.
- Beauchamp C., Fridovich I. (1971) Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*, **44**, 276-287.
- Benavides M. P., Gallego S. M., Tomaro M. L. (2005) Cadmium toxicity in plants. *Brazilian Journal of Plant Physiology*, **17**, 21-34.
- Bianconi D., Pietrini F., Massacci A., Iannelli M. A. (2013) Uptake of Cadmium by *Lemna minor*, a (hyper?) accumulator plant involved in phytoremediation applications. In: 16th International Conference on Heavy Metals in the Environment (ICHMET), Sep 23-27 2013 Rome, ITALY. CEDEX A: E D P Sciences.
- Blair I. A. (2010) Analysis of endogenous glutathione-adducts and their metabolites. *Biomedical Chromatography*, **24**, 29-38.
- Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 7.
- CETESB (2016) Valores orientadores para solos e águas subterrâneas no estado de São Paulo. In. <http://www.cetesb.sp.gov.br/wp-content/uploads/sites/11/2014/12/DD-256-2016-E-Valores-Orientadores-Dioxinas-e-Furanos-2016-Intranet.pdf>.
- Constantine N. G., Ries S. K. (1977) Superoxide Dismutases: I. Occurrence in Higher Plants. *Plant Physiology*, **59**, 309-314.
- Cuypers A., Hendrix S., Amaral dos Reis R., De Smet S., Deckers J., Gielen H., Jozefczak M., Loix C., Vercampt H., Vangronsveld J., Keunen E. (2016) Hydrogen Peroxide, Signaling in Disguise during Metal Phytotoxicity. *Frontiers in Plant Science*, **7**, 470.
- Cuypers A., Plusquin M., Remans T., Jozefczak M., Keunen E., Gielen H., Opdenakker K., Nair A. R., Munters E., Artois T. J., Nawrot T., Vangronsveld J., Smeets K. (2010) Cadmium stress: an oxidative challenge. *BioMetals*, **23**, 927-940.
- Cuypers A., Vangronsveld J., Clijsters H. (2001) The redox status of plant cells (AsA and GSH) is sensitive to zinc imposed oxidative stress in roots and primary leaves of *Phaseolus vulgaris*. *Plant Physiology and Biochemistry*, **39**, 657-664.
- Dalcorso G., Farinati S., Furini A. (2010) Regulatory networks of cadmium stress in plants. *Plant signaling & behavior*, **5**, 663.
- Ding S. H., Lu Q. T., Zhang Y., Yang Z. P., Wen X. G., Zhang L. X., Lu C. M. (2009) Enhanced sensitivity to oxidative stress in transgenic tobacco plants with decreased glutathione reductase activity leads to a decrease in ascorbate pool and ascorbate redox state. *Plant Molecular Biology*, **69**, 577-592.
- Dixit V., Pandey V., Shyam R. (2001) Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). *Journal of Experimental Botany*, **52**, 1101-1109.

- Egan S. K., Bolger P. M., Carrington C. D. (2007) Update of USFDA's Total Diet Study food list and diets. *Journal of Exposure Science and Environmental Epidemiology*, **17**, 573-582.
- Gallego S. M., Pena L. B., Barcia R. A., Azpilicueta C. E., Lannone M. F., Rosales E. P., Zawoznik M. S., Groppa M. D., Benavides M. P. (2012) Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environmental and Experimental Botany*, **83**, 33-46.
- Ghelfi A., Gaziola S. A., Cia M. C., Chabregas M., Falco M. C., Kuser-Falcao P. R., Azevedo R. A. (2011) Cloning, expression, molecular modelling and docking analysis of glutathione transferase from *Saccharum officinarum*. *Annals of Applied Biology*, **159**, 267-280.
- Gill S. S., Anjum N. A., Hasanuzzaman M., Gill R., Trivedi D. K., Ahmad I., Pereira E., Tuteja N. (2013) Glutathione and glutathione reductase: A boon in disguise for plant abiotic stress defense operations. *Plant Physiology and Biochemistry*, **70**, 204-212.
- Gill S. S., Tuteja N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, **48**, 909-930.
- Gratão P. L., Monteiro C. C., Antunes A. M., Peres L. E. P., Azevedo R. A. (2008a) Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. *Annals of Applied Biology*, **153**, 321-333.
- Gratão P. L., Monteiro C. C., Rossi M. L., Martinelli A. P., Peres L. E. P., Medici L. O., Lea P. J., Azevedo R. A. (2009) Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium. *Environmental and Experimental Botany*, **67**, 387-394.
- Gratão P. L., Monteiro C. C., Tezotto T., Carvalho R. F., Alves L. R., Peters L. P., Azevedo R. A. (2015) Cadmium stress antioxidant responses and root-to-shoot communication in grafted tomato plants. *BioMetals*, **28**, 803-816.
- Gratão P. L., Polle A., Lea P. J., Azevedo R. A. (2005) Making the life of heavy metal-stressed plants a little easier. *Functional Plant Biology*, **32**, 481-494.
- Gratão P. L., Pompeu G. B., Capaldi F. R., Vitorello V. A., Lea P. J., Azevedo R. A. (2008b) Antioxidant response of *Nicotiana tabacum* cv. Bright Yellow 2 cells to cadmium and nickel stress. *Plant Cell, Tissue and Organ Culture*, **94**, 73.
- Habig W. H., Jakoby W. B. (1981) Assays for differentiation of glutathione S-transferases. *Methods Enzymol*, **77**, 398-405.
- Heath R. L., Packer L. (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, **125**, 11.
- Hediji H., Djebali W., Cabasson C., Maucourt M., Baldet P., Bertrand A., Zoghalmi L. B., Deborde C., Moing A., Brouquisse R., Chaibi W., Gallusci P. (2010) Effects of long-term cadmium exposure on growth and metabolomic profile of tomato plants. *Ecotoxicology and Environmental Safety*, **73**, 1965-1974.
- Hoagland D. R., Arnon D. I. (1950) The water-culture method for growing plants without soil. In: *California Agricultural Experiment Station*, p. 32. Berkeley.
- Hossain M. A., Piyatida P., da Silva J. A. T., Fujita M. (2012) Molecular Mechanism of Heavy Metal Toxicity and Tolerance in Plants: Central Role of Glutathione in Detoxification of Reactive Oxygen Species and Methylglyoxal and in Heavy Metal Chelation. *Journal of Botany*, **2012**, 37.
- Jozefczak M., Keunen E., Schat H., Blik M., Hernández L. E., Carleer R., Remans T., Bohler S., Vangronsveld J., Cuypers A. (2014) Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity vs antioxidant capacity. *Plant Physiology and Biochemistry*, **83**, 1-9.
- Jozefczak M., Remans T., Vangronsveld J., Cuypers A. (2012) Glutathione Is a Key Player in Metal-Induced Oxidative Stress Defenses. *International Journal of Molecular Sciences*, **13**, 3145-3175.

- Kandziora-Ciupa M., Ciepał R., Nadgórska-Socha A., Barczyk G. (2016) Accumulation of heavy metals and antioxidant responses in *Pinus sylvestris* L. needles in polluted and non-polluted sites. *Ecotoxicology*, **25**, 970-981.
- Kandziora-Ciupa M., Nadgórska-Socha A., Barczyk G., Ciepał R. (2017) Bioaccumulation of heavy metals and ecophysiological responses to heavy metal stress in selected populations of *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. *Ecotoxicology* doi: 10.1007/s10646-017-1825-0.
- Khan N. A., Singh S. S., Nazar R. (2007) Activities of antioxidative enzymes, sulphur assimilation, photosynthetic activity and growth of wheat (*Triticum aestivum*) cultivars differing in yield potential under cadmium stress. *Journal of Agronomy and Crop Science*, **193**, 435-444.
- Kraus T. E., Fletcher R. A., Evans R. C., Pauls K. P. (1995) Paclobutrazol enhances tolerance to increased levels of UV-B radiation in soybean (*Glycine max*) seedlings. *Canadian Journal of Botany*, **73**, 797-806.
- Kubo K., Watanabe Y., Matsunaka H., Seki M., Fujita M., Kawada N., Hatta K., Nakajima T. (2011) Differences in Cadmium Accumulation and Root Morphology in Seedlings of Japanese Wheat Varieties with Distinctive Grain Cadmium Concentration. *Plant Production Science*, **14**, 148-155.
- Kumar S., Asif M. H., Chakrabarty D., Tripathi R. D., Dubey R. S., Trivedi P. K. (2013) Expression of a rice Lambda class of glutathione S-transferase, OsGSTL2, in *Arabidopsis* provides tolerance to heavy metal and other abiotic stresses. *Journal of Hazardous Materials*, **248**, 228-237.
- Laemmli U. K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, **227**, 680-685.
- Lee D. H., Lee C. B. (2000) Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. *Plant Science*, **159**, 75-85.
- Li T. Q., Yang X. E., Lu L. L., Islam E., He Z. L. (2009) Effects of zinc and cadmium interactions on root morphology and metal translocation in a hyperaccumulating species under hydroponic conditions. *Journal of Hazardous Materials*, **169**, 734-741.
- Lombi E., Tearall K. L., Howarth J. R., Zhao F. J., Hawkesford M. J., McGrath S. P. (2002) Influence of iron status on cadmium and zinc uptake by different ecotypes of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology*, **128**, 1359-1367.
- Lu Z. W., Zhang Z., Su Y., Liu C. F., Shi G. R. (2013) Cultivar variation in morphological response of peanut roots to cadmium stress and its relation to cadmium accumulation. *Ecotoxicology and Environmental Safety*, **91**, 147-155.
- Lux A., Martinka M., Vaculik M., White P. J. (2011) Root responses to cadmium in the rhizosphere: a review. *Journal of Experimental Botany*, **62**, 21-37.
- López-Millán A.-F., Sagardoy R., Solanas M., Abadía A., Abadía J. (2009) Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics. *Environmental and Experimental Botany*, **65**, 376-385.
- Medici L. O., Azevedo R. A., Smith R. J., Lea P. J. (2004) The influence of nitrogen supply on antioxidant enzymes in plant roots. *Functional Plant Biology*, **31**, 1-9.
- Mendoza-Cozatl D. G., Jobe T. O., Hauser F., Schroeder J. I. (2011) Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. *Current Opinion in Plant Biology*, **14**, 554-562.
- Milner M. J., Kochian L. V. (2008) Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Annals of Botany*, **102**, 3-13.
- Monteiro C. C., Carvalho R. F., Gratao P. L., Carvalho G., Tezotto T., Medici L. O., Peres L. E. P., Azevedo R. A. (2011) Biochemical responses of the ethylene-insensitive Never ripe tomato mutant subjected to cadmium and sodium stresses. *Environmental and Experimental Botany*, **71**, 306-320.

- Nakano Y., Asada K. (1981) Hydrogen-peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology*, **22**, 867-880.
- Nogueirol R. C., Monteiro F. A., Gratao P. L., da Silva B. K. D., Azevedo R. A. (2016) Cadmium Application in Tomato: Nutritional Imbalance and Oxidative Stress. *Water Air and Soil Pollution*, **227**, 20.
- Nogueirol R. C., Monteiro F. A., Gratao P. L., Borgo L., Azevedo R. A. (2015) Tropical soils with high aluminum concentrations cause oxidative stress in two tomato genotypes. *Environmental Monitoring and Assessment*, **187**, 73.
- Pereira G. J. G., Molina S. M. G., Lea P. J., Azevedo R. A. (2002) Activity of antioxidant enzymes in response to cadmium in *Crotalaria juncea*. *Plant and Soil*, **239**, 123-132.
- Perez-Chaca M. V., Rodriguez-Serrano M., Molina A. S., Pedranzani H. E., Zirulnik F., Sandalio L. M., Romero-Puertas M. C. (2014) Cadmium induces two waves of reactive oxygen species in *Glycine max* (L.) roots. *Plant Cell and Environment*, **37**, 1672-1687.
- Piotto F. A., Tulmann-Neto A., Franco M. R., Boaretto L. F., Azevedo R. A. (2014) Rapid screening for selection of heavy metal-tolerant plants. *Crop Breeding and Applied Biotechnology*, **14**, 1-7.
- Pompeu G. B., Vilhena M. B., Gratao P. L., Carvalho R. F., Rossi M. L., Martinelli A. P., Azevedo R. A. (2017) Abscissic acid-deficient sit tomato mutant responses to cadmium-induced stress. *Protoplasma*, **254**, 771-783.
- R (2015) A language and environment for statistical computing. R Foundation for statistical Computing, Vienna. Available at <http://www.R-project.org/>. In.
- Sanità di Toppi L., Gabbrielli R. (1999) Response to cadmium in higher plants. *Environmental and Experimental Botany*, **41**, 105-130.
- Sebastian A., Prasad M. N. V. (2014) Red and blue lights induced oxidative stress tolerance promote cadmium rhizocomplexation in *Oryza sativa*. *Journal of Photochemistry and Photobiology B-Biology*, **137**, 135-143.
- Semane B., Cuypers A., Smeets K., Belleghem F. V., Horemans N., Schat H., Vangronsveld J. (2007) Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. *Physiologia Plantarum*, **129**, 519-528.
- Sharma S. S., Dietz K.-J. (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science*, **14**, 43-50.
- Smith I. K., Vierheller T. L., Thorne C. A. (1988) Assay of glutathione-reductase in crude tissue-homogenates using 5,5'-Dithiobis(2-Nitrobenzoic acid). *Analytical Biochemistry*, **175**, 408-413.
- Soares C., de Sousa A., Pinto A., Azenha M., Teixeira J., Azevedo R. A., Fidalgo F. (2016) Effect of 24-epibrassinolide on ROS content, antioxidant system, lipid peroxidation and Ni uptake in *Solanum nigrum* L. under Ni stress. *Environmental and Experimental Botany*, **122**, 115-125.
- Su Y., Liu J. L., Lu Z. W., Wang X. M., Zhang Z., Shi G. R. (2014) Effects of iron deficiency on subcellular distribution and chemical forms of cadmium in peanut roots in relation to its translocation. *Environmental and Experimental Botany*, **97**, 40-48.
- Tamas L., Mistrik I., Zelinova V. (2016) Cadmium activates both diphenyleiuronium- and rotenone-sensitive superoxide production in barley root tips. *Planta*, **244**, 1277-1287.
- Tausz M., Sircelj H., Grill D. (2004) The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? *Journal of Experimental Botany*, **55**, 1955-1962.
- Teklić T., Lončarić Z., Kovačević V., Singh B. R. (2013) Metallic trace elements in cereal grain – a review: how much metal do we eat? *Food and Energy Security*, **2**, 81-95.

- Vitória A. P., Lea P. J., Azevedo R. A. (2001) Antioxidant enzymes responses to cadmium in radish tissues. *Phytochemistry*, **57**, 701-710.
- Wang H., Wang T., Ahmad I. (2015) Involvement of phosphate supplies in different transcriptional regulation pathway of *Oryza sativa* L.'s antioxidative system in response to arsenite and cadmium stress. *Ecotoxicology*, **24**, 1259-1268.
- Weber M., Trampczynska A., Clemens S. (2006) Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd²⁺ - hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant, Cell & Environment*, **29**, 950-963.
- Wei S. H., Li Y. M., Zhan J., Wang S. S., Zhu J. G. (2012) Tolerant mechanisms of *Rorippa globosa* (Turcz.) Thell. hyperaccumulating Cd explored from root morphology. *Bioresource Technology*, **118**, 455-459.
- Xu J., Yin H., Li Y., Liu X. (2010) Nitric oxide is associated with long-term zinc tolerance in *Solanum nigrum*. *Plant Physiology*, **154**, 16.
- Yadav S. K. (2010) Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *South African Journal of Botany*, **76**, 167-179.
- Yannarelli G. G., Fernandez-Alvarez A. J., Santa-Cruz D. M., Tomaro M. L. (2007) Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. *Phytochemistry*, **68**, 505-512.
- Zhao F. J., Jiang R. F., Dunham S. J., McGrath S. P. (2006) Cadmium uptake, translocation and tolerance in the hyperaccumulator *Arabidopsis halleri*. *New Phytologist*, **172**, 646-654.

3. ROOT MORPHOLOGY AND NUTRIENT USE EFFICIENCY IN TOMATO UNDER CD-INDUCED STRESS: COMPARING CONTRASTING GENOTYPES FOR METAL-TOLERANCE

Abstract

Nutritional disorders are among various deleterious effects caused by heavy metals exposure in plants. In this study we focused on investigate the effect of cadmium (Cd) on root morphological parameters as well as nutrient absorption and nutrient use efficiency in tomato genotypes. For this, two contrasting genotypes, Pusa Ruby (PR) and Calabash Rouge (CR), previously characterized as tolerant and sensitive to Cd-induced stress, respectively were grown in hydroponics and exposed to Cd during seven days. The Cd-exposure revealed damages in root morphology, the Cd-exposure strongly limited root development in tomato genotypes and it was more drastic for CR plants. The nutrient use efficiency in PR was less affected when exposed to Cd than in CR, in fact, the tolerance mechanism of PR reduced the damages by Cd-induced stress in root morphology and consequently exhibited better nutrient absorption and use efficiency, compared to the sensitive genotype.

Keywords: Heavy metals; Cadmium; Root; Nutrient use efficiency; *Solanum lycopersicum*

3.1. Introduction

Heavy metals have become one of the main classes of abiotic stress agents for living organisms because of their increasing use in the developing areas of the industry and high bioaccumulation and toxicity (Nagajyoti *et al.*, 2010). Quite a few of the lower molecular weight heavy metals are essential minerals for plants, such as zinc (Zn), copper (Cu), manganese (Mn), molybdenum (Mo) and iron (Fe), but most of them are non-essential for biology (Lin and Aarts, 2012). Micronutrients belong to the most intensively studied metals in terms of their impact on plants being essential to plant growth, however, its excess can cause toxic effects (Sarwar *et al.*, 2010). Cadmium (Cd), on the other hand, is a non-essential and highly toxic element that competes with micronutrients for uptake and transport in plants (Hall and Williams, 2003), thus posing a threat to crop productivity and human health (Chmielowska-Bak *et al.*, 2014).

To survive in soils with high levels of heavy metals, plants present mechanisms to reduce the damages caused by the metal in cells (Hall, 2002; Gratão *et al.*, 2008a). The efficacy of these mechanisms in plants determines the tolerance level to metal-induced stress. Most of the Cd-tolerant plants are ion-excluders, limiting the metal accumulation and root-to-shoot transport (Gallego *et al.*, 2012), while others use the Cd sequestration in

metabolically inactive parts such as root cell walls and vacuoles for Cd detoxification (Wang *et al.*, 2015). According to Lux *et al.* (2011b), anatomical alterations during Cd uptake may have an effect on accumulation processes and vegetative growth of the plants when they are exposed to this metal. Some plant species can accumulate high concentrations of Cd in the shoots, being designated as hyperaccumulators (Milner and Kochian, 2008). In metal hyperaccumulator plant species, root length determines the capacity to acquire water and nutrients, and therefore metal uptake capacity is more strongly related to root length than root biomass production (Boot and Mensink, 1990; Wei *et al.*, 2012). According to Li (2009), root length, surface area and volume could serve as valuable parameters when describing and comparing root systems. Nonetheless, in the large majority of plant species, Cd is accumulated mainly in roots with only limited amounts being transported to the leaves (Pereira *et al.*, 2002). Moreover, recent studies with grafted plants revealed the crosstalk aspects of Cd-induced stress signaling and accumulation among plant organs (Gratão *et al.*, 2015), as well as signals mediated by the hormones ethylene and auxin (Alves *et al.*, 2017).

Roots play a significant role in water and nutrients acquisition from soil solution. However, heavy metal excess in the root medium can reduce root growth by disturbing its integrity and metabolism (Gratão *et al.*, 2008a; Gratão *et al.*, 2015). The deleterious effects by Cd-stress on root system morphology and metabolism may be different among species (Wei *et al.*, 2012; Vaněk *et al.*, 2016), ecotypes (Li *et al.*, 2009) or cultivars (Kubo *et al.*, 2011). The root reaction to Cd-induced stress in various plant species was recently reviewed (Lux *et al.*, 2011a). Inhibition of root growth and branching as a result of the inhibitory effect of Cd on cell division was observed in pea by Fusconi *et al.* (2006) and in peanut by Lu *et al.* (2013). Lunáčeková *et al.* (2003) described species-specific reactions and changes in root tissue organization and development after Cd exposure. Furthermore, the development of root apoplastic barriers was shown to limit Cd uptake and transport in *Merrillia plumbea* (Lux *et al.*, 2011b) and in maize (Vaculík *et al.*, 2009).

Mechanisms involving absorption and transport of Cd in plants are not fully understood (Clemens, 2001; Redjala *et al.*, 2009; Mendoza-Cozatl *et al.*, 2011). Interactions among Cd and nutrients absorption by roots indicate the presence of common transporters to explain the Cd acquisition and partitioning in plants (Redjala *et al.*, 2009). Nutritional disorders are among the various deleterious effects caused by heavy metals excess in plants (Hippler *et al.*, 2016). Excess of Cd in roots affecting the nutritional status is contradictory among plants species (Przedpelska-Wąsowicz *et al.*, 2012). Metal-induced stress disturbs root morphology and the integrity of cell walls and plasma membrane, causing cell leakage

and nutrients losses (Bielen *et al.*, 2013). Excess of Cd in the root medium also reduces the activity and the expression of genes encoding the plasma membrane H⁺-ATPase activity, which play a role in the absorption of nutrients by energizing transport and creating a proton gradient (H⁺) between the cytosol and apoplast (Rizzardo *et al.*, 2012; Mao *et al.*, 2014). In *Zea mays* seedlings grown with Cd excess in the root medium exhibited reduced absorption and accumulation of N in roots due to down-regulation of genes encoding N transporters and enzymes of the assimilation process (Rizzardo *et al.*, 2012). Detailed kinetic studies performed by Cataldo *et al.* (1983) demonstrated competitive inhibition of Cd uptake and transport with cationic micronutrients, such as Cu, Fe, Mn and Zn, indicating a common absorption site among them, hence, the chances of adverse effect of Cd on plant growth through alterations in micronutrient-mediated catalysis reactions are high (Sebastian and Prasad, 2016). However, in *Arabidopsis halleri*, a metal-tolerant specie, Przedpelska-Wąsowicz *et al.* (2012) suggested that nutrient uptake was not affected by absorption sites competition between Cd and nutrients by roots, but by an ability to maintain ion homeostasis under Cd-stress.

Recent studies are being targeted in order to select Cd-tolerant cultivars aiming to understand better the genetic, physiological and biochemical characteristics of these plants. Up to now crop-breeding programmes have not overtly bred for improving abiotic stress tolerance and the reasons for that are many, among them the incomplete understanding of the mechanisms of stress tolerance (Gilliam *et al.*, 2017), making this topic very attractive for research. Most of the information available about Cd responses in plants comes from studies with model plants like the Cd-hyperaccumulator *Thlaspi caerulescens* (Lombi *et al.*, 2002) and Cd-tolerant *Arabidopsis halleri* (Zhao *et al.*, 2006; Weber *et al.*, 2006). However, there is still a lack of information about how Cd disturbs root morphology and metabolism, especially for commercial crops, such as tomato, and how these damages can modify nutrient acquisition by root.

In a previous study, we confirmed that the tomato genotype Pusa Ruby exhibits higher tolerance for Cd-induced stress compared to Calabash Rouge, once the former exhibited higher activity of antioxidant enzymes such as APX, GR and GST in roots. Furthermore, due to lower accumulation of reactive oxygen species (ROS) in roots of the Cd-tolerant genotype compared to the sensitive, it would result in less damages to root growth and metabolism. Here, we hypothesized that Cd-tolerant tomato genotype is less prone to root damaging and nutritional disorders caused by Cd toxicity than the sensitive one. Therefore, in this work we focused on investigate the effect of Cd on root

morphological parameters as well as biomass partitioning and nutrients absorption and use efficiency in two contrasting tomato genotypes.

3.2 Methodology

3.2.1 Plant material, growth conditions and treatments

Two tomato genotypes with differential tolerance to Cd-induced stress were selected based on previous studies (Piotto *et al.*, 2014): *Solanum lycopersicum* cv. Pusa Ruby (PR) and *Solanum lycopersicum* cv. Calabash Rouge (CR) characterized as tolerant and sensitive to Cd stress, respectively. The seeds were sterilized with 5% hydrogen peroxide for 15 min and washed with double distilled-deionized water. The seeds were sown in trays with vermiculite substrate and maintained with Peters commercial solution (JR Peters Inc. 20-20-20 at 1 g L⁻¹) applied during 20 days in order to maintain seedling development. The seedlings were cultivated in a greenhouse under the following conditions: an average mean temperature of 27°C, 11.5 h/13 h (winter/summer) photoperiod, and 250-350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR irradiance, natural radiation reduced with a reflecting mesh (Aluminete-Polysack Industrias Ltda, Leme, Brazil). Twenty-day-old seedlings were removed from the trays and transferred to a hydroponic system (10-L trays) containing a 10% ionic strength solution (Hoagland and Arnon, 1950), pH 6.5 checked daily, and the total volume maintained at a constant level by using distilled-deionized water. Plants were maintained in hydroponics for 24 h as an adaptation period in order to mitigate stress generated by seedling transplanting, and also to increase nutrient concentration from 10 to 50% ionic strength. Afterwards, for stress induction, a 35 μM CdCl₂ treatment was used and plants were sampled in three times predetermined in preliminary experiments: 1, 4 and 7 days after Cd addition, designated as T1, T2 and T3, respectively. Moreover, T0 samples (day zero treatment) were collected representing the initial condition before applying the stress. Four biological replicates composed of three plants each were collected. The control was cultivated in trays without Cd application (-Cd) whilst Cd treatments were represented as +Cd.

3.2.2 Plant growth and root morphology

Plants were destructively harvested and separated into roots and shoots, dried at 55°C until reaching a constant weight. Relative growth rate was calculated according to Hunt *et al.* (2002). Furthermore, biomass partitioning was calculated by the equation [Biomass partitioning = (total plant dry weight $\times$ 100) / (shoot or root dry weight)].

Three biological replicates were sampled randomly for root length, volume and surface area measurements, using fresh tissue with the WinRhizo PRO 2009c image analyzer (Regent Instruments Inc., Québec City, QC, Canada) coupled to a professional Epson XL 10000 scanner (Japan) equipped with a light transparency unit (TPU), as described by Bouma *et al.* (2000).

3.2.3 Nutritional status and nutrient absorption and use efficiency

Dried plant materials were ground to pass a 200-mesh sieve and Cd and the nutrients concentration were determined by plasma emission spectrometry (ICP-OES, Perkin-Elmer 5100 PC, Norwalk, CT, USA) after nitro-perchloric digestion (Bataglia *et al.*, 1983). The accumulation of nutrients by plants was obtained from the product of dry weight (g per plant) and the nutrient concentration (g kg^{-1}) in the plant tissue.

The amount of nutrient absorbed per root (NAR) dry weight (DW) was calculated according to the equation $\text{NAR (mg g}^{-1} \text{ root DW)} = [\text{nutrient accumulation at T3 (mg)} - \text{nutrient accumulated at T0 (mg)}] / [\text{root DW at T3 (g)} - \text{root DW at T0 (g)}]$. The efficiency of nutrient absorbed by stressed plants (% of nutrient absorbed) was calculated by $[(\text{NAR in stressed plants} \times 100) / (\text{NAR in control plants})]$.

Nutrient use efficiency (NUE) was calculated according to Siddiqi and Glass (1981) by the equation $(\text{NUE, g}^2 \text{ mg}^{-1}) = (\text{total plant dry weight, g})^2 / (\text{total nutrient accumulation in the plant, mg})$.

3.2.4 Statistical analysis

All experiments were performed in completely random design. Data from all tables and figures represent averages from four biological replicates ($n = 4$). A two-way ANOVA was performed to evaluate the effect of tomato genotypes and Cd concentration on the evaluated variables, with a level of significance of $\alpha = 0.05$. When the interaction of upper levels and rootstock varieties (Genotype*Cd) was significant, means were compared using the Tukey test at 5% level of significance.

3.3 Results

3.3.1 Cd accumulation and impact on plant growth

To evaluate Cd accumulation in tissues of tomato genotypes over time, pairwise comparisons with respective controls were performed within each tissue type (roots and

shoots) of each genotype. Control plants that grew without metal (-Cd) addition in the nutrient solution showed only trace amounts of the metal in the plants (<5.0 mg per plant; data not shown). The treatment with 35 μM CdCl_2 (+Cd), after seven days, resulted in Cd accumulation in plants of up to 431 μg per plant in PR and 336 μg per plant in CR (Figure 6).

Figure 6. Cadmium (Cd) accumulation and partitioning to roots of tomato genotypes Pusa Ruby (PR) and Calabash Rouge (CR), seven days after Cd addition in the nutrient solution. The treatments are with Cd (+Cd) and without Cd (-Cd). Values represent the means of four biological replicates ($n = 4$) $\pm$ SD of each treatment (-Cd, nil Cd; +Cd, 35 μM Cd). Vertical lines represent standard error of the mean ($n = 4$); Genotypes: means followed by different uppercase letters are significantly different by Tukey test ($p < 0.05$).

The Cd accumulation in roots was also higher in PR than CR, whereas CR exhibited higher accumulation of Cd in shoots than PR (Figure 6). Regardless of plant tissue, the metal accumulation and partitioning were higher in roots compared to shoots for both genotypes (Figure 6). The tomato genotypes presented contrasting behavior related to Cd partitioning in plant tissues, in which the tolerant genotype (PR) exhibited 70% and 30% of total Cd partitioned to roots and shoots respectively, whilst sensitive genotype (CR) exhibited 53% to roots and 47% of Cd partitioned to shoots (Figure 6).

The presence of Cd in the nutrient solution inhibited the growth of tomato plants (Figure 7).

Figure 7. Relative growth rate and biomass partitioning between shoots and roots of two tomato genotypes Pusa Ruby (PR) and Calabash Rouge (CR), after 1 day (T1), 4 days (T2) and 7 days (T3) after Cd addition in the nutrient solution. The treatments are with Cd (+Cd) and without Cd (-Cd). Vertical lines represent standard error of the mean (n = 4); Genotypes: means followed by different uppercase letters are significantly different by Tukey test ($p < 0.05$). Cd treatments: means followed by different lowercase letters are significantly different by Tukey test ($p < 0.05$).

After 24 h of Cd-exposure (T1), the relative growth rate of the CR plants decreased (Figure 7) whereas at the same time PR showed a slight increase in growth, suggesting that these plants were initially indifferent to Cd. On the other hand, in the presence of Cd overtime a clear decrease in the plant growth rate was observed for both genotypes (Figure 7). When biomass partitioning is concerned, differences were detected after seven days of Cd exposure (T3) with contrasting effects between tissues, biomass increased in roots and decreased in shoots for both genotypes. Altogether, biomass partitioning for shoots were always higher than for roots in tomato genotypes (Figure 7).

3.3.2 Cd effect on root morphology parameters

In order to verify if there were differences in Cd uptake related to roots characteristics between contrasting genotypes to Cd-tolerance, root morphology analyses were performed. Root length, surface area and volume decreased in both genotypes when exposed to Cd, being more severe in the sensitive genotype (CR; Figure 8).

Figure 8. Morphology parameters of root length, surface area and volume of tomato genotypes Pusa Ruby (PR) and Calabash Rouge (CR), one day (T1), four days (T2) and seven days (T3) after Cd addition in the nutrient solution. The treatments are with Cd (+Cd) and without Cd (-Cd). Vertical lines represent standard error of the mean ($n = 4$); Genotypes: means followed by different uppercase letters are significantly different by Tukey test ($p < 0.05$). Cd treatments: means followed by different lowercase letters are significantly different by Tukey test ($p < 0.05$).

Root length was decreased by Cd in all time points and similar results were observed for surface area, which at T2 and T3 the sensitive genotype faced even more drastic reductions (Figure 8). Regarding root volume, it remained unaltered at T1 in both tomato genotypes and decreased at T2 and T3 by Cd-exposure in both genotypes. The Cd-exposure strongly limited root development and it was more drastic for CR plants (Figure 8, right panel).

3.3.3 Nutrients absorption and use efficiency changes under Cd-induced stress

After seven days, Cd exposure of the root system disturbed the nutrients taken up by roots and consequently the concentrations in shoots and roots of both tomato genotypes (Figure 9).

Figure 9. Nutrients concentration in tomato genotypes Pusa Ruby (PR) and Calabash Rouge (CR), seven days after Cd addition in the nutrient solution. The treatments are with Cd (+Cd) and without Cd (-Cd). Vertical lines represent standard error of the mean (n = 4); Genotypes: means followed by different uppercase letters are significantly different by Tukey test ($p < 0.05$). Cd treatments: means followed by different lowercase letters are significantly different by Tukey test ($p < 0.05$).

In shoots, plants exposed to Cd exhibited higher concentrations of P, K, S and B, but lower concentrations of Ca and Cu when compared to control plants. In roots, both genotypes

with Cd in the nutrient solution exhibited an increment in the concentrations of Ca, Cu, S and B, and reduction of P and Mn (Figure 9). Some particular effects in nutrient concentrations were observed among the genotypes when Cd was applied (Genotype*Cd interaction). Tolerant plants (PR) in the presence of Cd (+Cd) exhibited higher concentrations of K in roots, whereas CR genotype exhibited higher concentrations Mn in shoots and Mg in roots, both when compared to their respective controls (-Cd; Figure 9). A specific effect was observed for Fe, in which the PR genotype exhibited a 3.2-fold higher concentration of the micronutrient in the shoots whilst CR exhibited a 1.7-fold higher concentration in roots, both when compared to their respective controls plants (-Cd; Figure 9).

Besides the concentration of some nutrients increased after Cd was added in the nutrient solution (+Cd; Figure 9), nutrients accumulation reduced in both tomato genotypes when exposed to Cd (Table 2). Among them Ca and Mn were more affected by a reduction of >50% when compared to control plants (Table 2). P, K, Mg, and Zn had a reduction of 40-50% in their accumulation when exposed to Cd, whilst the accumulation of Fe and Cu were reduced by 30% (Table 2). On the other hand, the reduction of S accumulation in CR (1.2 mg per plant of S) exposed to Cd was higher than in PR (1.7 mg per plant of S) (Table 2).

Absorption of nutrients per root dry weight (NAR) decreased when plants were grown with Cd in the nutrient solution (Table 3). When the plants were grown without Cd addition in the nutrient solution, CR exhibited in general 3-fold higher NAR for all nutrients compared to PR (Table 3). However, with the addition of Cd in the nutrient solution, PR plants exhibited lower decrease in the absorption efficiency of stressed plants compared to CR (Table 3), except for B, Cu and Mn. Furthermore, PR plants exposed to Cd exhibited the same NAR for S and higher NAR for Fe when compared to control plants, which represented an absorption efficiency of the stressed plants of 101 % for S and 167% for Fe (Table 3).

Control plants (-Cd) of both genotypes exhibited similar nutrient use efficiency (NUE) for all nutrients evaluated, except for P, for which CR exhibited higher NUE than PR (Table 4). However, when Cd was added in the nutrient solution, NUE decreased for all nutrients in both tomato genotypes (Table 4). Furthermore, when exposed to Cd, the NUE for Ca, Mg, B, Cu, Fe, Mn, and Zn in the sensitive genotype were lower than verified for the tolerant one (Table 4).

Table 2. Nutrients accumulation in tomato genotypes Pusa Ruby (PR) and Calabash Rouge (CR), seven days after Cd addition in the nutrient solution. The treatments are with Cd (+Cd) and without Cd (-Cd).

Genotype	Cd treatments	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn
----- mg per plant -----						----- µg per plant -----					
Tolerant (PR)	-Cd	3.4±0.2 ¹ Ba ²	25±1.2 Aa	12.5±0.8 Aa	5.3±0.4 Aa	2.2±0.1 Aa	18±1.7 Aa	12.2±1.3 Aa	710±45 Aa	190±17 Aa	32±2.8 Aa
	+Cd	1.8±0.1 Ab	15±0.4 Ab	4.6±0.1 Ab	2.7±0.1 Bb	1.7±0.1 Aa	11±0.1 Ab	9.3±0.1 Ab	564±35 Ab	47±2 Ab	17±0.2 Ab
Sensitive (CR)	-Cd	4.2±0.4 Aa	25±2.1 Aa	14.8±1.4 Aa	5.7±0.8 Aa	2.5±0.3 Aa	19±1.8 Aa	11.8±1.4 Aa	756±79 Aa	191±9 Aa	34±3.0 Aa
	+Cd	1.6±0.1 Ab	10±0.4 Bb	4.0±0.1 Ab	3.2±0.1 Ab	1.2±0.1 Bb	12±0.4 Ab	6.7±0.1 Bb	557±9 Ab	43±1 Ab	15±0.4 Ab
F test³											
Genotype		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cd		***	***	***	***	***	***	***	*	***	***
Genotype*Cd		*	*	ns	*	*	ns	*	ns	ns	ns

¹Standard error of the mean (n = 4)

²Genotypes: means followed by different uppercase letters are significantly different by Tukey test ($p < 0.05$). Cd treatments: means followed by different lowercase letters are significantly different by Tukey test ($p < 0.05$).

³F test in the ANOVA of Genotype vs Cd for each parameter evaluated. ns: not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3. Nutrients absorption per root dry weight and absorption efficiency in tomato genotypes Pusa Ruby (PR) and Calabash Rouge (CR), seven days after Cd addition in the nutrient solution. The treatments are with Cd (+Cd) and without Cd (-Cd).

Genotype	Cd treatments	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn
Nutrient absorption per root dry weight											
----- mg g ⁻¹ root DW -----											
Tolerant (PR)	-Cd	29.1±0.3 Ba	248±6 Ba	129±11 Ba	27.2±3.3 Ba	20.3±2.9 Ba	0.17±0.01 Ba	0.08±0.01 Ba	1.74±0.21 Bb	0.85±0.08 Ba	0.21±0.01 Ba
	+Cd	15.8±2.3 Bb	144±12 Bb	54±5 Bb	11.6±4.9 Ab	20.6±3.6 Aa	0.10±0.02 Bb	0.03±0.01 Ab	2.92±0.74 Aa	0.31±0.09 Ab	0.07±0.02 Ab
Sensitive (CR)	-Cd	94.8±1.2 Aa	631±38 Aa	409±30 Aa	82.7±10.1 Aa	59.0±7.7 Aa	0.42±0.03 Aa	0.19±0.04 Aa	4.03±0.66 Aa	1.47±0.10 Aa	0.50±0.05 Aa
	+Cd	36.4±4.3 Ab	293±18 Ab	96±9 Ab	19.1±11.9 Ab	26.7±5.7 Ab	0.31±0.03 Ab	0.05±0.02 Ab	1.02±0.32 Bb	0.48±0.11 Ab	0.08±0.02 Ab
F test³											
Genotype		**	***	***	***	*	**	**	ns	***	***
Cd		***	***	***	**	ns	**	***	ns	**	**
Genotype*Cd		ns	ns	ns	**	**	ns	**	***	**	**
Absorption efficiency of stressed plants											
----- % of nutrient absorbed -----											
Tolerant (PR)	+Cd	54±6 A	58±5 A	42±9 A	43±9 A	101±12 A	63±7	34±7	167±15 A	37±5	33±3 A
Sensitive (CR)		38±7 B	46±4 B	23±3 B	23±4 B	45±10 B	74±10	23±5	25±4 B	32±6	16±5 B
F test³											
Genotype		*	*	**	**	***	ns	ns	***	ns	**

¹Standard error of the mean (n = 4)

²Genotypes: means followed by different uppercase letters are significantly different by Tukey test ($p < 0.05$). Cd treatments: means followed by different lowercase letters are significantly different by Tukey test ($p < 0.05$).

³F test in the ANOVA of Genotype vs Cd for each parameter evaluated. ns: not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 4. Nutrients use efficiency in tomato genotypes Pusa Ruby (PR) and Calabash Rouge (CR), seven days after Cd addition in the nutrient solution. The treatments are with Cd (+Cd) and without Cd (-Cd).

Genotype	Cd treatments	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn
----- g ² mg ⁻¹ -----											
Tolerant (PR)	-Cd	0.11±0.01 ¹ Aa ²	0.014±0.01 Aa	0.029±0.004 Aa	0.068±0.01 Aa	0.16±0.02 Aa	19.6±1.6 Aa	29.5±0.9 Aa	0.51±0.05 Aa	1.9±0.2 Aa	11.2±1.2 Aa
	+Cd	0.04±<0.01 Ab	0.005±<0.01 Ab	0.016±0.001 Ab	0.028±0.01 Ab	0.04±<0.01 Ab	6.5±0.3 Ab	8.1±0.3 Ab	0.13±0.01 Ab	1.6±0.3 Ab	4.4±0.2 Ab
Sensitive (CR)	-Cd	0.08±0.02 Ba	0.014±0.02 Aa	0.024±0.003 Aa	0.064±0.02 Aa	0.14±0.02 Aa	19.3±2.3 Aa	30.3±3.0 Aa	0.49±0.09 Aa	1.9±0.1 Aa	10.4±1.3 Aa
	+Cd	0.03±<0.01 Bb	0.004±<0.01 Ab	0.011±0.001 Bb	0.014±<0.01 Bb	0.04±0.01 Ab	3.5±0.4 Bb	6.4±0.6 Bb	0.08±0.01 Bb	1.0±0.1 Bb	2.9±0.3 Bb
F test³											
Genotype		*	ns	*	ns	ns	ns	ns	ns	ns	ns
Cd		***	***	***	***	***	***	***	***	***	***
Genotype*Cd		ns	ns	*	*	ns	*	*	*	*	*

¹Standard error of the mean (n = 4)

²Genotypes: means followed by different uppercase letters are significantly different by Tukey test ($p < 0.05$). Cd treatments: means followed by different lowercase letters are significantly different by Tukey test ($p < 0.05$).

³F test in the ANOVA of Genotype vs Cd for each parameter evaluated. ns: not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.4. Discussion

3.4.1. Cd accumulation and impact of stress on plant growth rate and biomass partitioning

PR and CR tomato genotypes were previously characterized as Cd-tolerant and Cd-sensitive genotypes, respectively (Piotto *et al.*, 2014). In the present work we aimed to characterize the same genotypes regarding the root morphology under Cd-induced stress to shed some light on possible mechanisms involved in nutrient use efficiency (NUE) related to Cd tolerance in tomato genotypes. It is well known that Cd accumulation in plants can vary among plant species and also among cultivars within species (Gratão *et al.*, 2008a). In our study, for both genotypes Cd was accumulated more in roots than in shoots (Figure 6) and similar results of Cd accumulation in roots have been reported in maize (Anjum *et al.*, 2015), rice (Hassan *et al.*, 2005), pea (Sandalio *et al.*, 2001; Dixit *et al.*, 2001), soybean (Perez-Chaca *et al.*, 2014) and tomato (López-Millán *et al.*, 2009) among a variety of plant species studied so far. The higher Cd allocation found in roots of the tolerant genotype (PR) compared to sensitive one explains a possible mechanism used by tolerant plants to protect shoots and photosynthetic organs against Cd dangerous effects. Moreover, probably PR genotype uses some other tolerance strategy not related to ion-excluder, once Cd entered the roots. In citrus plants exposed to Cu excess, the metal-accumulation in roots was described as mechanism to limit the metal transport and accumulation in the leaves (Hippler *et al.*, 2016). Moreover, tolerant plants develop extracellular barriers and can store Cd-chelates in vacuoles (Lux *et al.*, 2011a), a mechanism that may be employed by PR roots. Plants can adjust to variations in resource availabilities by variable partitioning to root and shoot growth (Hodge, 2009). Under Cd-induced stress, tomato genotypes exhibited most of the Cd partitioned to roots, especially in root tissue of the tolerant genotype (Figure 6). According to Lux *et al.* (2011a) metal dilution through the plant growth may influence shoot Cd concentration. The Cd sequestered either in the vacuole or expelled in the root apoplasts through facilitated membrane pumps also determines the root-to-shoot Cd translocation (Mohamed *et al.*, 2012). A high proportion of Cd in the soluble fraction as well as its chemical form could limit root-to-shoot translocation of Cd, resulting in low Cd levels in the shoots (Su *et al.*, 2014). Apparently effective root-to-shoot translocation of metals suggests a tolerance strategy that may be used by the PR genotype. The hypothesis which has attracted most attention suggests that high heavy metal concentrations in aerial tissues may function as a self-defence strategy evolved in hyperaccumulator species (Rascio and Navari-Izzo, 2011).

Growth differences were observed between tomato genotypes when treated with Cd (Figure 7). The sensitive genotype (CR) showed more severe symptoms (Figure 8) suggesting that intracellular detoxification or defense mechanisms maybe exist or be more efficient in the tolerant genotype PR, which showed no decrease in growth at the first time point, but the opposite (Figure 7), probably due to a hormetic effect previously reported under Cd-induced stress (Fornazier *et al.*, 2002; Guelfi *et al.*, 2003; Zhang *et al.*, 2009). In all time points the relative growth rate of tomato genotypes decreased in the presence of Cd (Figure 7). Cd is an element easily absorbed by plant roots and accordingly causes root cells die affecting the root development and plant growth (Nogueirol *et al.*, 2016). These findings are in accordance with Lu *et al.* (2013) who found root and shoot biomass of peanuts cultivars significantly affected after treatment with 20 μM CdCl₂. In our study we found similar growth rates for both genotypes in the absence of Cd (-Cd), whilst stressed plants had impaired growth induced by metal exposure (Figure 7). In heavy-metal sensitive plants, ROS-induced cellular damage causes cell death affecting plant growth and development (Sharma and Dietz, 2009). Analyzing the growth rate data obtained they agree with those reported by Piotto *et al.* (2014) about the sensitivity of CR genotype to Cd-induced stress.

Regarding biomass partitioning it was observed in all time points shoots higher than roots for both tomato genotypes (Figure 7). Laporte *et al.* (2015) detected in sunflower cultivars metal uptake more positively correlated with shoot biomass than with root biomass, whereas Garcia *et al.* (2006) also working with sunflower observed Cd accumulation mainly in the roots but with a major inhibition of biomass production. On the other hand, here tolerant genotype apparently showed increased in root biomass partitioning whilst the sensitive appears to allocate resources in shoot growth (Figure 7).

3.4.2. Cd affected root morphology

Apart from biomass accumulation, root architecture is also affected by metal stress in the root medium (Wei *et al.*, 2012). The morphological variables such as root length, surface area and volume decreased in both genotypes after Cd exposure (Figure 8). Initially, at the first time point (T1), the tolerant genotype roots appeared to be indifferent to Cd-induced stress, with no differences detected for any morphology parameter, which agrees with the slight increased growth exhibited (Figure 2, chapter 1), whereas the opposite occurred for the sensitive genotype showing that CR growth was immediately affected by Cd-induced stress (Figure 8). The application of Cd in hydroponic conditions significantly reduced the root length, surface area and

root tips of three pepper cultivars compared to the control (Huang *et al.*, 2015). Treatment with Cd significantly affected the root architecture and reduced the root length in rice plants (Kim *et al.*, 2014). Our results are in accordance with the findings reported by Lu *et al.* (2013) studying peanut who showed that Cd-exposure significantly decreased mainly root surface area and length, while the volume was unaffected. In tomato, volume was significantly affected by Cd only in sensitive roots (CR) after four days of Cd-exposure (Figure 8). However, since root absorption capacity is related to root length, the decreases of surface area and length suggest that capacity of resource acquisition in tomato genotypes was diminished. Cd toxicity restricted growth of rice cultivars as estimated in terms of root length and area (Yu *et al.*, 2006; Song *et al.*, 2015).

Heavy metal in the root medium increase the amount of ROS causing lipid peroxidation of membrane degradation (Gratão *et al.*, 2008b; Hippler *et al.*, 2016; Nogueirol *et al.*, 2016). Furthermore, Cd excess also affect a range of process related to root architecture, such as hormonal signals (Alves *et al.*, 2017; Pompeu *et al.*, 2017; Souza *et al.*, 2017) and expression of genes (Rizzardo *et al.*, 2012), such as NRT/PTR family, related to nitrate and hormones transporting through cell membrane and responsible for lateral root growth (Boursiac *et al.*, 2013).

Changes of root morphology and structure may also affect the uptake and xylem loading of Cd, affecting Cd partitioning in plant (Lux *et al.*, 2011a). In this study all morphological parameters of tomato roots decreased after Cd exposure. At T2 PR roots decreased 53% of surface area, 48% of volume and 62% of length, while CR roots faced even more drastic reductions in surface area about 73%, 70% of volume and 77% of length (Figure 8). It allows to affirm that Cd exposure strongly limited tomato root development and it was more drastic for CR genotype (Figure 8).

3.4.3. Nutritional status changes by Cd-induced stress

Nutritional status of both tomato genotypes was affected by Cd excess in the root medium (Figure 9). In this study, nutritional imbalances might have occur firstly by the reduced root growth and surface area (Figure 8), which allowed plants to take up water and nutrients (Pinto and Ferreira, 2015; Zhao *et al.*, 2017). Heavy metal excess also increases cell leakage and nutrient losses to the external solution (Dong *et al.*, 2006) as well as reduces the activity of plasma membrane H⁺-ATPase, which play a role in the nutrients absorption by energizing transport and creating a proton gradient (H⁺) between the cytosol and apoplast (Camacho-Cristóbal and González-Fontes, 2007; Siemianowski *et al.*, 2014). Furthermore, Cd competes for the same

absorption sites of cations (Hall, 2002), mainly for the metal-nutrients Cu, Fe, Mn and Zn (Figure 9 and Table 2). Previous reports showed Cd decreasing P, K, Mg and Ca uptake (Rivetta *et al.*, 1997; Benavides *et al.*, 2005; Lunáčková *et al.*, 2003). At cellular level Cd toxicity is known to cause alterations in nutrient homeostasis such as changes in Mg, K, Mn and Ca uptake rates (Greger *et al.*, 1991; Dong *et al.*, 2006). For Ca, Cd-excess was described to limit its absorption by competition for transporters in cell membrane and intracellular Ca-binding proteins (Nazar *et al.*, 2012; Rivetta *et al.*, 1997).

Specific responses on nutrient absorption and accumulation were observed between the two tomato genotypes used in this study. Nutritional status of PR was less affected than CR (Figure 9, Tables 2 and 3), probably due to the lower damages in the root morphology of the former (Figure 8). When grown with Cd in the nutrient solution, cell wall and plasma membrane integrities of CR root cells were likely more damaged. Besides Mg accumulation was higher in CR roots than in PR, and the former also exhibited lower accumulation of K, S and Mn (Table 2), which correspond to low control of membrane permeability in the CR genotype.

In this study, specific responses observed for S concentrations between the genotypes could be directly related to the tolerance mechanisms when exposed to Cd (Figure 9). Sulphur is related to the regulation of the activity of antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, catalase and glutathione reductase (GR) in plants under Cd stress (Bagheri *et al.*, 2016). In our case, S concentration was 45% higher in tolerant roots when compared to CR (Figure 4). Furthermore, S absorption by root dry weight in PR plants was the same when Cd was in the nutrient solution compared to control ones (Table 3). Sulphur availability limits the cysteine (Cys) content, a precursor compound of GSH, and plausible that GSH synthesis in plants can physiologically be regulated by the availability of both S and Cys (Gill *et al.*, 2013; Jozefczak *et al.*, 2012), suggesting that greater S availability may explain at least in part, the tolerant phenotype of PR roots under Cd-induced stress. Moreover, Cd may induce synthesis of phytochelatins (PCs), which are small Cys-rich peptides with high affinity for metals, forming complexes with metal (Cd-PC) that are rapidly compartmentalized into vacuoles (Vitória *et al.*, 2001; Yadav, 2010). However, more studies to confirm this hypothesis for CR and PR tomato genotypes under heavy metal excess in the root medium are necessary.

On the other hand, the increment of Fe concentration in the PR shoots and CR roots (Figure 9), when those plants were exposed to Cd, is likely a result of the homeostasis mechanism to avoid Cd transporting to shoots (Clemens, 2001). Plants exposed to heavy metal toxicity in the root medium likely exhibit an increment of Fe concentration in roots followed by a reduction in the shoots (Gratão *et al.*, 2015; Hippler *et al.*, 2016), such as observed for CR (Figure 9). It has

been suggested that the plant mechanism for metal homeostasis, which limits metal transporting to the shoots does not discriminate Fe, which result in Fe accumulation in roots. Iron immobilization in roots is likely a response of the plant homeostatic mechanism by increasing the amount of organic chelators in the cells, such as histidine, glutathione and nicotianamine (Kendziorrek *et al.*, 2014; López-Climent *et al.*, 2014; Andrés-Bordería *et al.*, 2017). In this study, we verified that PR plants exposed to Cd did not limit Fe transporting to shoots, as well as exhibited higher NAR for Fe than the control plants, with an efficiency of 167% of the nutrient absorbed by the stressed plants (Table 3). Furthermore, in PR plants, Cd likely did not compete with Fe for the uptake into root epidermal cells via *IRT1*, which shows broad substrate specificity towards divalent metals including Fe^{2+} , Zn^{2+} , Mn^{2+} , and Cd^{2+} (Mendoza-Cozatl *et al.*, 2014).

Micronutrients such as Zn, Fe and Mn play a vital role in mitigating Cd stress to plants. They could activate certain Cd avoidance and/or tolerance mechanisms in plants (Sarwar *et al.*, 2010; Barabasz *et al.*, 2016). Toxic heavy metals compete with the transport systems operating for micronutrient uptake, suggesting that Cd uptake in root cells thus appears to be an opportunistic event (Lin and Aarts, 2012), causing disorders in plant metabolism generated by replacement of elements such as Zn, Fe and Mn, which are essential cofactors of many enzymes. Mineral imbalances affect plant growth and can be a result from excessive Cd toxicity, affecting the rate of uptake and distribution of certain nutrients in plants (Nazar *et al.*, 2012), such as observed in this study for many nutrients (Tables 2 and 3).

Reducing nutrient absorption and accumulation in plants, as well as plant growth, Cd excess also reduced the nutrient use efficiency (NUE) of both tomato genotypes (Table 4). Nutrient use efficiency is the ability of a genotype/cultivar to acquire nutrients from the soil solution and/or to incorporate to utilize them in the plant biomass production (plant growth) or to produce seed, grain, fruits, forage and so one (Blair, 1993). Increasing NUE of crops is a key aspect in plant physiology with tremendous consequence concern around the world to increase the efficiency of fertilizers application, and consequently to improve crop yields and quality (Zotarelli *et al.*, 2009; Zambrosi *et al.*, 2011; Hippler *et al.*, 2015). Among the nutrients applied in crops more intensively, estimates of the overall efficiency of applied fertilizer have been reported to be about or lower than 50% for N, less than 10% for P, and about 40% for K (Baligar *et al.*, 2001). Here, we emphasize that soil contaminated with heavy metals, such as Cd, might result in a lower efficiency of nutrients supply. However, NUE in PR was less affected when exposed to Cd than CR (Table 4). In fact, the tolerance mechanism of PR reduced the damages by Cd-induced stress in root morphology and consequently exhibited better nutrient absorption and use efficiency, compared to the sensitive genotype.

References

- Alves L. R., Monteiro C. C., Carvalho R. F., Ribeiro P. C., Tezotto T., Azevedo R. A., Gratão P. L. (2017) Cadmium stress related to root-to-shoot communication depends on ethylene and auxin in tomato plants. *Environmental and Experimental Botany*, **134**, 102-115.
- Andrés-Bordería A., Andrés F., Garcia-Molina A., Perea-García A., Domingo C., Puig S., Peñarrubia L. (2017) Copper and ectopic expression of the Arabidopsis transport protein COPT1 alter iron homeostasis in rice (*Oryza sativa* L.). *Plant Molecular Biology*.
- Anjum S. A., Tanveer M., Hussain S., Bao M., Wang L., Khan I., Ullah E., Tung S. A., Samad R. A., Shahzad B. (2015) Cadmium toxicity in Maize (*Zea mays* L.): consequences on antioxidative systems, reactive oxygen species and cadmium accumulation. *Environmental Science and Pollution Research*, **22**, 17022-17030.
- Bagheri R., Ahmad J., Bashir H., Iqbal M., Qureshi M. I. (2016) Changes in rubisco, cysteine-rich proteins and antioxidant system of spinach (*Spinacia oleracea* L.) due to sulphur deficiency, cadmium stress and their combination. *Protoplasma*, 1-13.
- Baligar V. C., Fageria N. K., He Z. L. (2001) Nutrient use efficiency in plants. *Communications in Soil Science and Plant Analysis*, **32**, 921-950.
- Barabasz A., Klimecka M., Kendziorrek M., Weremczuk A., Ruszczynska A., Bulska E., Antosiewicz D. M. (2016) The ratio of Zn to Cd supply as a determinant of metal-homeostasis gene expression in tobacco and its modulation by overexpressing the metal exporter AtHMA4. *Journal of Experimental Botany*, **67**, 6201-6214.
- Bataglia O. C., Furlani A. M. C., Teixeira J. P. F., Furlani P. R., Gallo J. R. (1983) Método de análise química de plantas. *LAC - Boletim Técnico* **78**, 48p.
- Benavides M. P., Gallego S. M., Tomaro M. L. (2005) Cadmium toxicity in plants. *Brazilian Journal of Plant Physiology*, **17**, 21-34.
- Bielen A., Remans T., Vangronsveld J., Cuypers A. (2013) The Influence of Metal Stress on the Availability and Redox State of Ascorbate, and Possible Interference with Its Cellular Functions. *International Journal of Molecular Sciences*, **14**, 6382-6413.
- Blair G. (1993) Nutrient efficiency—what do we really mean. In: *Genetic Aspects of Plant Mineral Nutrition*, pp. 205–213. Dordrecht, The Netherlands: Kluwer Academic Publishers.
- Boot R. G. A., Mensink M. (1990) Size and morphology of root systems of perennial grasses from contrasting habitats as affected by nitrogen supply. *Plant and Soil*, **129**, 291-299.
- Bouma T. J., Nielsen K. L., Koutstaal B. (2000) Sample preparation and scanning protocol for computerised analysis of root length and diameter. *Plant and Soil*, **218**, 185-196.
- Boursiac Y., Lérans S., Corratgé-Faillie C., Gojon A., Krouk G., Lacombe B. (2013) ABA transport and transporters. *Trends in Plant Science*, **18**, 325-333.
- Camacho-Cristóbal J. J., González-Fontes A. (2007) Boron deficiency decreases plasmalemma H⁺-ATPase expression and nitrate uptake, and promotes ammonium assimilation into asparagine in tobacco roots. *Planta*, **226**, 443-451.
- Cataldo D. A., Garland T. R., Wildung R. E. (1983) Cadmium uptake kinetics in intact soybean plants. *Plant Physiology*, **73**, 844-848.
- Chmielowska-Bak J., Gzyl J., Rucinska-Sobkowiak R., Arasimowicz-Jelonek M., Deckert J. (2014) The new insights into cadmium sensing. *Frontiers in Plant Science*, **5**, 13.
- Clemens S. (2001) Molecular mechanisms of plant metal tolerance and homeostasis. *Planta*, **212**, 475-486.
- Dixit V., Pandey V., Shyam R. (2001) Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). *Journal of Experimental Botany*, **52**, 1101-1109.

- Dong J., Wu F. B., Zhang G. P. (2006) Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon esculentum*). *Chemosphere*, **64**, 1659-1666.
- Fornazier R. F., Ferreira R. R., Pereira G. J. G., Molina S. M. G., Smith R. J., Lea P. J., Azevedo R. A. (2002) Cadmium stress in sugar cane callus cultures: Effect on antioxidant enzymes. *Plant Cell, Tissue and Organ Culture*, **71**, 125-131.
- Fusconi A., Repetto O., Bona E., Massa N., Gallo C., Dumas-Gaudot E., Berta G. (2006) Effects of cadmium on meristem activity and nucleus ploidy in roots of *Pisum sativum* L. cv. Frisson seedlings. *Environmental and Experimental Botany*, **58**, 253-260.
- Gallego S. M., Pena L. B., Barcia R. A., Azpilicueta C. E., Lannone M. F., Rosales E. P., Zawoznik M. S., Groppa M. D., Benavides M. P. (2012) Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environmental and Experimental Botany*, **83**, 33-46.
- Garcia J. S., Grato P. L., Azevedo R. A., Arruda M. A. Z. (2006) Metal contamination effects on sunflower (*Helianthus annuus* L.) growth and protein expression in leaves during development. *Journal of agricultural and food chemistry*, **54**, 7.
- Gill S. S., Anjum N. A., Hasanuzzaman M., Gill R., Trivedi D. K., Ahmad I., Pereira E., Tuteja N. (2013) Glutathione and glutathione reductase: A boon in disguise for plant abiotic stress defense operations. *Plant Physiology and Biochemistry*, **70**, 204-212.
- Gillham M., Able J. A., Roy S. J. (2017) Translating knowledge about abiotic stress tolerance to breeding programmes. *The Plant Journal*, **90**, 898-917.
- Grato P. L., Monteiro C. C., Tezotto T., Carvalho R. F., Alves L. R., Peters L. P., Azevedo R. A. (2015) Cadmium stress antioxidant responses and root-to-shoot communication in grafted tomato plants. *BioMetals*, **28**, 803-816.
- Grato P. L., Monteiro C. C., Antunes A. M., Peres L. E. P., Azevedo R. A. (2008a) Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. *Annals of Applied Biology*, **153**, 321-333.
- Grato P. L., Pompeu G. B., Capaldi F. R., Vitorello V. A., Lea P. J., Azevedo R. A. (2008b) Antioxidant response of *Nicotiana tabacum* cv. Bright Yellow 2 cells to cadmium and nickel stress. *Plant Cell, Tissue and Organ Culture*, **94**, 73.
- Greger M., Brammer E., Lindberg S., Larsson G., Idestamalmquist J. (1991) Uptake and physiological-effects of cadmium in sugar-beet (*Beta vulgaris*) related to mineral provision. *Journal of Experimental Botany*, **42**, 729-737.
- Guelfi A., Azevedo R. A., Lea P. J., Molina S. M. G. (2003) Growth inhibition of the filamentous fungus *Aspergillus nidulans* by cadmium: an antioxidant enzyme approach. *The Journal of General and Applied Microbiology*, **49**, 63-73.
- Hall J. L. (2002) Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, **53**, 1-11.
- Hall J. L., Williams L. E. (2003) Transition metal transporters in plants. *Journal of Experimental Botany*, **54**, 2601-2613.
- Hassan M. J., Wang Z. Q., Zhang G. P. (2005) Sulfur alleviates growth inhibition and oxidative stress caused by cadmium toxicity in rice. *Journal of Plant Nutrition*, **28**, 1785-1800.
- Hippler F. W. R., Boaretto R. M., Quaggio J. A., Azevedo R. A., Mattos D. (2015) Towards soil management with Zn and Mn: estimates of fertilisation efficacy of Citrus trees. *Annals of Applied Biology*, **166**, 484-495.
- Hippler F. W. R., Cipriano D. O., Boaretto R. M., Quaggio J. A., Gaziola S. A., Azevedo R. A., Mattos-Jr D. (2016) Citrus rootstocks regulate the nutritional status and antioxidant system of trees under copper stress. *Environmental and Experimental Botany*, **130**, 42-52.
- Hoagland D. R., Arnon D. I. (1950) The water-culture method for growing plants without soil. In: *California Agricultural Experiment Station*, p. 32. Berkeley.
- Hodge A. (2009) Root decisions. *Plant, Cell & Environment*, **32**, 628-640.

- Huang B., Xin J., Dai H., Liu A., Zhou W., Yi Y., Liao K. (2015) Root morphological responses of three hot pepper cultivars to Cd exposure and their correlations with Cd accumulation. *Environmental Science and Pollution Research*, **22**, 1151-1159.
- Hunt R., Causton D. R., Shipley B., Askew A. P. (2002) A Modern Tool for Classical Plant Growth Analysis. *Annals of Botany*, **90**, 485-488.
- Jozefczak M., Remans T., Vangronsveld J., Cuypers A. (2012) Glutathione Is a Key Player in Metal-Induced Oxidative Stress Defenses. *International Journal of Molecular Sciences*, **13**, 3145-3175.
- Kendziorek M., Barabasz A., Rudzka J., Tracz K., Mills R. F., Williams L. E., Antosiewicz D. M. (2014) Approach to engineer tomato by expression of AtHMA4 to enhance Zn in the aerial parts. *Journal of Plant Physiology*, **171**, 1413-1422.
- Kim Y.-H., Khan A. L., Kim D.-H., Lee S.-Y., Kim K.-M., Waqas M., Jung H.-Y., Shin J.-H., Kim J.-G., Lee I.-J. (2014) Silicon mitigates heavy metal stress by regulating P-type heavy metal ATPases, *Oryza sativa* low silicon genes, and endogenous phytohormones. *BMC Plant Biology*, **14**, 13.
- Kubo K., Watanabe Y., Matsunaka H., Seki M., Fujita M., Kawada N., Hatta K., Nakajima T. (2011) Differences in Cadmium Accumulation and Root Morphology in Seedlings of Japanese Wheat Varieties with Distinctive Grain Cadmium Concentration. *Plant Production Science*, **14**, 148-155.
- Laporte M.-A., Sterckeman T., Dauguet S., Denaix L., Nguyen C. (2015) Variability in cadmium and zinc shoot concentration in 14 cultivars of sunflower (*Helianthus annuus* L.) as related to metal uptake and partitioning. *Environmental and Experimental Botany*, **109**, 45-53.
- Li T. Q., Yang X. E., Lu L. L., Islam E., He Z. L. (2009) Effects of zinc and cadmium interactions on root morphology and metal translocation in a hyperaccumulating species under hydroponic conditions. *Journal of Hazardous Materials*, **169**, 734-741.
- Lin Y.-F., Aarts M. G. M. (2012) The molecular mechanism of zinc and cadmium stress response in plants. *Cellular and Molecular Life Sciences*, **69**, 3187-3206.
- Lombi E., Tearall K. L., Howarth J. R., Zhao F. J., Hawkesford M. J., McGrath S. P. (2002) Influence of iron status on cadmium and zinc uptake by different ecotypes of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology*, **128**, 1359-1367.
- Lu Z. W., Zhang Z., Su Y., Liu C. F., Shi G. R. (2013) Cultivar variation in morphological response of peanut roots to cadmium stress and its relation to cadmium accumulation. *Ecotoxicology and Environmental Safety*, **91**, 147-155.
- Lunáčková L., Šottníková A., Masarovičová E., Lux A., Streško V. (2003) Comparison of Cadmium Effect on Willow and Poplar in Response to Different Cultivation Conditions. *Biologia Plantarum*, **47**, 403-411.
- Lux A., Martinka M., Vaculik M., White P. J. (2011a) Root responses to cadmium in the rhizosphere: a review. *Journal of Experimental Botany*, **62**, 21-37.
- Lux A., Vaculík M., Martinka M., Lišková D., Kulkarni M. G., Stirk W. A., Van Staden J. (2011b) Cadmium induces hypodermal periderm formation in the roots of the monocotyledonous medicinal plant *Merwillia plumbea*. *Annals of Botany*, **107**, 285-292.
- López-Climent M. F., Arbona V., Pérez-Clemente R. M., Zandalinas S. I., Gómez-Cadenas A. (2014) Effect of cadmium and calcium treatments on phytochelatin and glutathione levels in citrus plants. *Plant Biology*, **16**, 79-87.
- López-Millán A.-F., Sagardoy R., Solanas M., Abadía A., Abadía J. (2009) Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics. *Environmental and Experimental Botany*, **65**, 376-385.
- Mao Q. Q., Guan M. Y., Lu K. X., Du S. T., Fan S. K., Ye Y.-Q., Lin X. Y., Jin C. W. (2014) Inhibition of Nitrate Transporter 1.1-Controlled Nitrate Uptake Reduces Cadmium Uptake in *Arabidopsis*. *Plant Physiology*, **166**, 934.

- Mendoza-Cozatl D. G., Jobe T. O., Hauser F., Schroeder J. I. (2011) Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. *Current Opinion in Plant Biology*, **14**, 554-562.
- Mendoza-Cozatl D. G., Xie Q. Q., Akmakjian G. Z., Jobe T. O., Patel A., Stacey M. G., Song L. H., Demoin D. W., Jurisson S. S., Stacey G., Schroeder J. I. (2014) OPT3 Is a Component of the Iron-Signaling Network between Leaves and Roots and Misregulation of OPT3 Leads to an Over-Accumulation of Cadmium in Seeds. *Molecular Plant*, **7**, 1455-1469.
- Milner M. J., Kochian L. V. (2008) Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Annals of Botany*, **102**, 3-13.
- Mohamed A. A., Castagna A., Ranieri A., Sanita di Toppi L. (2012) Cadmium tolerance in *Brassica juncea* roots and shoots is affected by antioxidant status and phytochelatin biosynthesis. *Plant Physiology and Biochemistry*, **57**, 15-22.
- Nagajyoti P. C., Lee K. D., Sreekanth T. V. M. (2010) Heavy metals, occurrence and toxicity for plants: a review. *Environmental Chemistry Letters*, **8**, 199-216.
- Nazar R., Iqbal N., Masood A., Khan M. I. R., Syeed S., Khan N. A. (2012) Cadmium toxicity in plants and role of mineral nutrients in its alleviation.
- Nogueirol R. C., Monteiro F. A., Gratao P. L., da Silva B. K. D., Azevedo R. A. (2016) Cadmium Application in Tomato: Nutritional Imbalance and Oxidative Stress. *Water Air and Soil Pollution*, **227**, 20.
- Pereira G. J. G., Molina S. M. G., Lea P. J., Azevedo R. A. (2002) Activity of antioxidant enzymes in response to cadmium in *Crotalaria juncea*. *Plant and Soil*, **239**, 123-132.
- Perez-Chaca M. V., Rodriguez-Serrano M., Molina A. S., Pedranzani H. E., Zirulnik F., Sandalio L. M., Romero-Puertas M. C. (2014) Cadmium induces two waves of reactive oxygen species in *Glycine max* (L.) roots. *Plant Cell and Environment*, **37**, 1672-1687.
- Pinto E., Ferreira I. M. P. L. V. O. (2015) Cation transporters/channels in plants: Tools for nutrient biofortification. *Journal of Plant Physiology*, **179**, 64-82.
- Piotto F. A., Tulmann-Neto A., Franco M. R., Boaretto L. F., Azevedo R. A. (2014) Rapid screening for selection of heavy metal-tolerant plants. *Crop Breeding and Applied Biotechnology*, **14**, 1-7.
- Pompeu G. B., Vilhena M. B., Gratão P. L., Carvalho R. F., Rossi M. L., Martinelli A. P., Azevedo R. A. (2017) Absciscic acid-deficient sit tomato mutant responses to cadmium-induced stress. *Protoplasma*, **254**, 771-783.
- Przedpelska-Wąsowicz E., Polatajko A., Wierzbicka M. (2012) The Influence of Cadmium Stress on the Content of Mineral Nutrients and Metal-Binding Proteins in *Arabidopsis halleri*. *Water, Air, and Soil Pollution*, **223**, 5445-5458.
- Rascio N., Navari-Izzo F. (2011) Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? *Plant Science*, **180**, 169-181.
- Redjala T., Sterckeman T., Morel J. L. (2009) Cadmium uptake by roots: Contribution of apoplast and of high- and low-affinity membrane transport systems. *Environmental and Experimental Botany*, **67**, 235-242.
- Rivetta A., Negrini N., Cocucci M. (1997) Involvement of Ca^{2+} -calmodulin in Cd^{2+} toxicity during the early phases of radish (*Raphanus sativus* L.) seed germination. *Plant, Cell & Environment*, **20**, 600-608.
- Rizzardo C., Tomasi N., Monte R., Varanini Z., Nocito F. F., Cesco S., Pinton R. (2012) Cadmium inhibits the induction of high-affinity nitrate uptake in maize (*Zea mays* L.) roots. *Planta*, **236**, 1701-1712.
- Sandalio L. M., Dalurzo H. C., Gómez M., Romero-Puertas M. C., del Río L. A. (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of Experimental Botany*, **52**, 2115-2126.

- Sarwar N., Saifullah, Malhi S. S., Zia M. H., Naeem A., Bibi S., Farid G. (2010) Role of mineral nutrition in minimizing cadmium accumulation by plants. *Journal of the Science of Food and Agriculture*, **90**, 925-937.
- Sebastian A., Prasad M. N. V. (2016) Modulatory role of mineral nutrients on cadmium accumulation and stress tolerance in *Oryza sativa* L. seedlings. *Environmental Science and Pollution Research*, **23**, 1224-1233.
- Sharma S. S., Dietz K.-J. (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science*, **14**, 43-50.
- Siddiqi M. Y., Glass A. D. M. (1981) Utilization index: A modified approach to the estimation and comparison of nutrient utilization efficiency in plants. *Journal of Plant Nutrition*, **4**, 289-302.
- Siemianowski O., Barabasz A., Kendziorrek M., Ruszczyńska A., Bulska E., Williams L. E., Antosiewicz D. M. (2014) HMA4 expression in tobacco reduces Cd accumulation due to the induction of the apoplastic barrier. *Journal of Experimental Botany*, **65**, 1125-1139.
- Song W. E., Chen S. B., Liu J. F., Chen L., Song N. N., Li N., Liu B. (2015) Variation of Cd concentration in various rice cultivars and derivation of cadmium toxicity thresholds for paddy soil by species-sensitivity distribution. *Journal of Integrative Agriculture*, **14**, 1845-1854.
- Souza L. A., Monteiro C. C., Carvalho R. F., Gratao P. L., Azevedo R. A. (2017) Dealing with abiotic stresses: an integrative view of how phytohormones control abiotic stress-induced oxidative stress. *Theoretical and Experimental Plant Physiology*.
- Su Y., Liu J. L., Lu Z. W., Wang X. M., Zhang Z., Shi G. R. (2014) Effects of iron deficiency on subcellular distribution and chemical forms of cadmium in peanut roots in relation to its translocation. *Environmental and Experimental Botany*, **97**, 40-48.
- Vaculík M., Lux A., Luxová M., Tanimoto E., Lichtscheidl I. (2009) Silicon mitigates cadmium inhibitory effects in young maize plants. *Environmental and Experimental Botany*, **67**, 52-58.
- Vaněk T., Mot'ková K., Podlipná R. (2016) Accumulation of cadmium by halophytic and non-halophytic *Juncus* species. *Theoretical and Experimental Plant Physiology*, **28**, 415-423.
- Vitória A. P., Lea P. J., Azevedo R. A. (2001) Antioxidant enzymes responses to cadmium in radish tissues. *Phytochemistry*, **57**, 701-710.
- Wang H., Wang T., Ahmad I. (2015) Involvement of phosphate supplies in different transcriptional regulation pathway of *Oryza sativa* L.'s antioxidative system in response to arsenite and cadmium stress. *Ecotoxicology*, **24**, 1259-1268.
- Weber M., Trampczynska A., Clemens S. (2006) Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd²⁺ - hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant, Cell & Environment*, **29**, 950-963.
- Wei S. H., Li Y. M., Zhan J., Wang S. S., Zhu J. G. (2012) Tolerant mechanisms of *Rorippa globosa* (Turcz.) Thell. hyperaccumulating Cd explored from root morphology. *Bioresource Technology*, **118**, 455-459.
- Yadav S. K. (2010) Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *South African Journal of Botany*, **76**, 167-179.
- Yu H., Wang J. L., Fang W., Yuan J. G., Yang Z. Y. (2006) Cadmium accumulation in different rice cultivars and screening for pollution-safe cultivars of rice. *Science of the Total Environment*, **370**, 302-309.
- Zambrosi F. C. B., Mattos D., Syvertsen J. P. (2011) Plant growth, leaf photosynthesis, and nutrient-use efficiency of citrus rootstocks decrease with phosphite supply. *Journal of Plant Nutrition and Soil Science*, **174**, 487-495.
- Zhang Y., Shen G., Yu Y., Zhu H. (2009) The hormetic effect of cadmium on the activity of antioxidant enzymes in the earthworm *Eisenia fetida*. *Environmental Pollution*, **157**, 3064-3068.

- Zhao F. J., Jiang R. F., Dunham S. J., McGrath S. P. (2006) Cadmium uptake, translocation and tolerance in the hyperaccumulator *Arabidopsis halleri*. *New Phytologist*, **172**, 646-654.
- Zhao Y., Zhang S., Wen N., Zhang C., Wang J., Liu Z. (2017) Modeling uptake of cadmium from solution outside of root to cell wall of shoot in rice seedling. *Plant Growth Regulation*, **82**, 11-20.
- Zotarelli L., Dukes M. D., Scholberg J. M. S., Muñoz-Carpena R., Icerman J. (2009) Tomato nitrogen accumulation and fertilizer use efficiency on a sandy soil, as affected by nitrogen rate and irrigation scheduling. *Agricultural Water Management*, **96**, 1247-1258.

4. ROOT PROTEOMICS OF TWO TOMATO GENOTYPES WITH DIFFERENCES IN TOLERANCE TO CADMIUM-INDUCED STRESS

Abstract

While there are significant genotypic differences in cadmium (Cd) uptake and distribution in tomato cultivars, there is a gap of information about the molecular mechanisms involved in tolerance. In this study we presented a proteomic approach to examine root protein profile differences between two contrasting tomato genotypes, Pusa Ruby and Calabash Rouge, previously characterized as tolerant and sensitive to Cd-induced stress, respectively. A total of 380 proteins were classified as differentially accumulated proteins (DAP), 62 were shared between both genotypes, being 55 proteins increased in abundance and 29 decreased in tolerant genotype, whereas 165 increased and 193 decreased in abundance in the sensitive genotype when exposed to Cd. The great majority of DAP showed alterations in diverse pathways, including proteins involved in cell wall, stress response and redox activities. Cell wall related proteins such as chitinases, believed to act as second line defense components, showed contrasting responses in tomato genotypes, decreased accumulation in tolerant and increased in sensitive genotype. Tolerant roots in the presence of Cd decreased accumulation of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) while increased glutathione-S-transferase (GST) and thioredoxin (TRX). Among the changes in root proteome profile of the sensitive genotype it can be highlighted the increased abundance of GST and important decreases in SOD, monodehydroascorbate reductase (MDHAR) and TRX. Taken together, the present study suggests a more active involvement of GST, TRX and POD in Cd-induced stress response.

Keywords: Heavy metal stress; Proteome; Cadmium tolerance; Label-free, *Solanum lycopersicum*

4.1. Introduction

Cadmium (Cd) pollution is a worldwide major concern having among others deleterious effects on plants, affected food production and quality, apart from the risks to animal and human health caused by the consumption of contaminated products (Singh *et al.*, 2016). Among the heavy metals, Cd is one of the most toxic (Sanità di Toppi and Gabbrielli, 1999), so it is very important to reduce Cd accumulation on crops.

Due to the increase of agricultural areas contaminated by heavy metals, the problem of Cd toxicity worsens each year, but the number of plant species that have some tolerance degree is extremely restricted. The tolerance to Cd and detoxification process in plants are apparently organ-specific, with pathways involving organs such as the cell wall, plasma membrane, cytosol and vacuolar compartments (Hall, 2002). Most of Cd-tolerant plants are excluders, limiting metal accumulation and root-to-shoot transport of heavy metal (Gallego *et al.*, 2012). Other useful mechanism is related to Cd sequestration in metabolically inactive parts such as root cell walls, first structure of the plant cell to come in contact with heavy metals, or the vacuoles (Wei *et al.*,

2012). The cell wall can effectively absorb Cd, alleviating the toxic effects of this heavy metal (Parrotta *et al.*, 2015), so the cell wall-based protection mechanism is the only one of its kind used by plants to cope with the damage induced by Cd. Investigations are still needed to identify the mechanisms that allows plants to extract, transport, and sequester heavy metals for phytoremediation purposes (Farinati *et al.*, 2011). Most of the information available about Cd-tolerance in plants comes from studies with model plants such as *Arabidopsis thaliana* (Przedpelska-Wąsowicz *et al.*, 2012; Zhao *et al.*, 2006), *Thlaspi caerulescens* (Krämer *et al.*, 2000; Tuomainen *et al.*, 2006) and *Solanum nigrum* (Al Khateeb and Al-Qwasemeh, 2014; Soares *et al.*, 2016; Wei *et al.*, 2005) whereas there is a gap of information especially for important commercial crops such as tomato.

The identification of proteins involved in responses to heavy-metal stress is a fundamental step in understanding the molecular mechanisms of stress responses (Wang *et al.*, 2011). Such understanding could lead to the development of transgenic plants that have an enhanced tolerance to heavy-metal stress or to plants capable of being used in phytoremediation (removal of toxic elements from soils). In this context, proteomics is not only a powerful molecular tool for describing complete proteome, but also for comparative proteomes under different physiological conditions and between contrasting genotypes under the same conditions. Until now, several studies have been conducted to elucidate the response of model plants such as rice and *Arabidopsis spp.* under heavy metal stress at proteome level (Lee *et al.*, 2010; Semane *et al.*, 2010). However, there is a little overlap between the differentially expressed genes and accumulated proteins as revealed by a previous report (Semane *et al.*, 2010). Plants exposed to Cd presented variable responses at the proteome level. Approximately half of the upregulated proteins in rice roots exposed to Cd belonged to oxidative stress response and glutathione (GSH) metabolism (Lee *et al.*, 2010) while in poplar leaves, however, a decrease in some ROS scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT) and monodehydroascorbate (MDHAR) were also reported under Cd-induced stress (Kieffer *et al.*, 2009). Wang *et al.* (2011) described in wheat roots up-regulated proteins involved in heavy metal detoxification and antioxidant processes, for instance, glutathione-S-transferase (GST) activity was stimulated by Cd treatment. Although there is a large number of reports, many aspects of plant responses to Cd toxicity are still unknown and in general, little is known about stress-elicited changes in plants at the proteome level (Roth *et al.*, 2006). Moreover, researches mainly focus on the influences of Cd on the above-ground parts of the plants and little is known about Cd effects in root system, the plant organ directly exposed to soil and water pollutants (Chen *et al.*, 2003).

To cope with Cd stress, plants employ a range of cellular detoxification mechanisms (Gallego *et al.*, 2012). Thus, is a common sense in the literature that various genes are probably involved in Cd-response in plants and in this context identifying the proteins involved in Cd-response pathways is a fundamental step in understanding the molecular mechanisms of detoxification and accumulation of toxic Cd in plant cells (Ahsan *et al.*, 2012). Such works using proteomic approaches may help us to identify possible stress biomarkers, useful in phytoremediation and crop breeding purposes. Therefore, we evaluate the Cd-induced effects in roots, at a proteome level, of young tomato genotypes in order to identify candidate biomarkers related to Cd-tolerance aiming to deepen our understanding of the molecular basis of Cd responses in plants and ultimately to postulate potential mechanisms involved in Cd-tolerance.

4.2. Methodology

4.2.1. Plant growth, treatments and sample collection

Two tomato genotypes with differential tolerance to Cd-induced stress were selected based on previous studies (Piotto *et al.*, 2014): *Solanum lycopersicum* cv. Pusa Ruby (PR) and *Solanum lycopersicum* cv. Calabash Rouge (CR), characterized as tolerant and sensitive to Cd, respectively. The seeds were previously sterilized with 5% hydrogen peroxide for 15 min and washed with double distilled-deionized water and then were sown in trays with vermiculite substrate and maintained with Peters commercial solution (JR Peters Inc.; 20-20-20 at 1 g L⁻¹) applied during 20 days in order to maintain seedling development. The seedlings were cultivated in a greenhouse under following conditions: average mean temperature of 27°C, 11.5h/13h (winter/summer) photoperiod, and 250-350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR irradiance (natural radiation reduced with a reflecting mesh (Aluminete-Polysack Industrias Ltda, Leme, Brazil). Afterwards 20-day-old seedlings were removed from the trays and transferred to a hydroponic system (10-L trays) containing Hoagland solution at 10% ionic strength (Hoagland and Arnon, 1950), with pH = 6.5 checked daily and the total volume maintained at a constant level by using distilled-deionized water. Plants were maintained in hydroponics for 24 h as an adaptation period in order to mitigate stress generated by seedling transplanting, and also to increase nutrient concentration from 10 to 50% ionic strength. This procedure (gradual increase of salt concentration) was carried out in order to diminish plant stress due to an increased content of salts in solution. For stress induction, a 35 μM cadmium chloride (CdCl₂) solution was used and plants were sampled four days after Cd addition. Five biological replicates composed of three plants each (leaves and roots) were sampled in treatment. For control, five biological replicates composed of three plants

each were used without Cd application. Plants were destructively harvested and separated into roots and shoots, dried at 55°C until reaching a constant weight for growth measures. Fresh tissues (roots and shoots) were sampled and immediately frozen in liquid nitrogen and subsequently stored at -80°C for further analysis.

4.2.2. Protein extraction, quantification and digestion

Root tissues were ground in liquid nitrogen to a fine powder in a precooled mortar with pestle. Proteins were extracted as described by Hurkman and Tanaka (1986). Briefly, the powder (4g of fresh weight) was homogenized in 10 mL of extraction solution [0.9 M sucrose, 0.1 M Tris-HCl, 10 mM EDTA and 2% (v/v) β -mercaptoethanol] during 30 min shaking on ice. After that, 10 mL of Tris-buffered phenol (pH 8.0) were added and the mixture vortexed. Samples were incubated in a shaker for 30 min at 4°C and then centrifuged at 8,000 x g for 30 min at 4°C. The supernatant was transferred to a fresh tube and protein precipitation was carried out by adding five volumes of 0.1 M ammonium acetate prepared in methanol. Samples were placed at -20°C overnight for protein precipitation. Precipitates were collected after centrifugation at 8,000 x g for 30 min at 4°C and pellets washed twice with iced 80% acetone. Protein precipitates were resuspended in 300 μ L of resuspension buffer [7 M Urea, 0.4% (v/v) Triton X-100 and 50 mM DTT], and protein concentration was estimated using Bradford Protein Assay Kit (Bio-Rad) using BSA as standard (Bradford, 1976).

Protein precipitates were resuspended in 300 μ L of resuspension buffer [7 M Urea, 0.4 % (v/v) Triton X-100 and 50 mM DTT] and then aliquots of 200 μ g of the protein of each biological replicate were used for Filter Aided Sample Preparation (FASP), as described by Loziuk *et al.* (2015). Protein extracts were 2-fold diluted in buffer containing 100 mM DTT and incubated for 30 min at 56°C. After reduction, samples were alkylated for 1 h at 37°C, adding iodoacetamide to a final concentration of 200 mM. Denatured samples were transferred into a 0.5 mL 10 KDa molecular mass cutoff centrifugation filter (EMD Millipore Billerica, MA). Samples were washed 3x for 15 min at 14,000 x g at 20°C with buffer containing 8 M urea to remove detergents. Extra 3 washes for 15 min at 14,000 x g at 20°C, this time using trypsin digestion buffer containing 0.5 M urea and 10 mM CaCl₂, were performed. Protein digestion was conducted in the filter for 12 h at 37°C using a 1:50 enzyme:protein ratio of modified porcine trypsin. New collecting tube was used at this point. Digested peptides were eluted through centrifugation at 14,000 x g at 20°C for 15 min using 400 μ L of buffer containing 1% formic acid (v/v) and 0.001 % zwittergent 3-16 (Calbiochem, La Jolla, CA). Eluted peptides were frozen and stored at -80°C.

4.2.3. LC-MS/MS analysis

Frozen peptides samples were vacuum dried and reconstituted in 50 μ L of formic acid containing 0.001% zwittergent 3-16. Peptides samples were analyzed in duplicate using a Thermo Scientific EASY nLC II (Thermo Scientific, San Jose, CA) coupled to a quadrupole Orbitrap benchtop mass spectrometer (Q-Exactive, Thermo Scientific). A 4 μ L injection onto an analytical column, 75 cm x 15 cm (Eksigent Technologies, Dublin, CA) packed with Chrom XP C18-CL (3 μ m particle size, 120 Å pore size). A 240 min elution gradient utilizing a 5-30% B solution was performed at flow rate of 350 nL/min. Mobile phases A and B were composed of water/acetonitrile/formic acid (98/2/0.2% and 2/98/0.2%, respectively). Mass spectrometer parameters were previously optimized for proteomics (Andrews *et al.*, 2011) and implemented here for MS analysis as described by Loziuk *et al.* (2015).

For MS analysis, the peptides were analyzed in positive mode. MS spectra were acquired using data-dependent, top-12 MS/MS method using a resolving power of 70,000 for MS scans and 17,500 for MS/MS at $m/z = 200$. Automatic gain control (AGC) target for MS scans was set to 1E6 with a maximum ion injection time (IT) of 30 ms, and AGC target of 2E4 was used for MS/MS scans with a maximum injection time of 120 ms. The underfill ratio was set to 1%. The m/z range was set from 400 to 1600. Microscans were set to 1 for both MS and MS/MS scans. Charge state screening was enabled. Unassigned and 1+ charge states were rejected from MS/MS isolation and activation. A normalized collision energy of 27 was used with an isolation window of 2.0 m/z . Dynamic exclusion was set to 60s. The ambient ion used for lock mass was 445.120025 m/z . A capillary voltage of 2.0 kV was used, and the temperature was set to 250°C.

4.2.4. Protein identification

Database searches were performed using SEQUEST HT on Proteome Discoverer 2.1 (Thermo Scientific) platform against the *Solanum lycopersicum* database available at Phytozome website iTAG v2.3 (34,727 sequences). Search parameters were set as follows: oxidation of methionine was allowed as a variable modification and carbamidomethylation of cysteine as a static modification; enzyme, trypsin; number of allowed missed cleavages, 2; mass range, 200-2000; peptide tolerance 5 ppm; fragment ions tolerance, 0.02 Da. Percolator peptide validator was used at FDR < 1% based on peptide q-value. Proteins were considered as “present” if they were detected in at least four biological replicates and if were assigned by at least two different peptides.

4.2.5. Protein quantification and statistical analysis

Peptide-Spectrum Matches (PSMs) were divided by protein length defining a Spectral Abundance Factor (SAF). SAF values were then normalized against protein levels across different runs, according to Florens *et al.* (2006) producing the NSAF (normalized values). The NSAF values were multiplied by 10^6 in order to deal with integers and facilitate comparisons; NSAF were loaded into the Perseus software v.1.4.0.17 (available at <http://www.maxquant.org>) for data filtering, transformation and Student's test analysis. Briefly, NSAF values were log2 transformed in order to estimate the extent of differential protein abundance and missed values were replaced with a constant value of zero. Student's t-test was performed on Perseus for two sample comparison (p-value < 0.05, based on 2-tailed Student's t test with Benjamini-Hochberg correction for multiple testing at an FDR < 0.05). Proteins present in at least 3 biological replicates and not detected in other genotype or condition (control and Cd-treatment) were considered as exclusive.

4.2.6. Protein annotation and functional classification

The protein sequences identified in the experiment were blasted against UNIPROT (<http://www.uniprot.org/proteomes/>) and TAIR (<https://www.arabidopsis.org>) databases. The functional classification of differently accumulated proteins (DAP) was performed based on GO terms retrieval using David Annotation database tool (Huang *et al.*, 2007) and MapMan software version 3.5.1 (available at <https://mapman.gabipd.org>). The combined graph for biological process, molecular function and cellular component are presented at the third level of depth. The hierarchical clustering and heatmap were carried out using Perseus software loading the fold change data (control/treatment). For this, Euclidian distances were considered to build the similarity matrix (Cluster parameter: row header width = 300, column header height = 200).

A summary of all procedures can be found in Figure 10.

Figure 10. Proteomics workflow employed in the experiment of Cd-induced stress in roots of two tomato genotypes. Tolerant genotype Pusa Ruby (PR) and sensitive genotype Calabash Rouge (CR).

4.3. Results

4.3.1. Cd impact on tomato growth

A clear growth inhibition was observed both in roots and shoots after Cd exposure (Figure 11). Decreases in biomass accumulation in plants were detected in roots, 40% in tolerant genotype (PR) and 50% in sensitive genotype (CR) (Figure 11A). When shoot growth is concerned (Figure 11B) more pronounced effects were detected in CR genotype, which exhibited a 70% decrease whilst in PR the reduction was 60%.

Figure 11. Impact of Cd-induced stress on plant growth of tomato genotypes expressed as dry matter weight in A) Root and B) Shoot. Values represent the means of 5 replicates ($n = 5$), bars are standard deviation of the mean. Asterisk means significant alteration ($p < 0.05$) in relation to respective control. Tolerant genotype Pusa Ruby (PR) and sensitive genotype Calabash Rouge (CR).

4.3.2. Cd increased MDA and H₂O₂ contents in tolerant roots

Higher levels of malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) were observed in both genotypes when treated with Cd (Figure 12), however significant changes were only detected in the tolerant genotype, which in the presence of Cd increased MDA and H₂O₂ content (Figure 12A and 12B). No significant changes were observed in sensitive genotype.

Figure 12. Oxidative damage induced by Cd-exposure in tomato roots genotypes represented as A) Malondialdehyde (MDA, nmol g⁻¹ fresh weight) and B) Hydrogen peroxide (H₂O₂, μM g⁻¹ fresh weight). Values represent the means of 5 replicates ($n = 5$), bars are standard deviation of the mean. Asterisk means significant alteration ($p < 0.05$) in relation to respective control. Tolerant genotype Pusa Ruby (PR) and sensitive genotype Calabash Rouge (CR).

4.3.3. Large scale tomato roots proteome identification and quantification

A large scale quantitative proteomic approach was employed to determine alterations in the protein profile of roots of two genotypes of tomato differing in tolerance to Cd-induced stress. From all spectra collected for each biological replicate a total of 4,051 non-redundant proteins were detected in the whole experiment with an FDR lower than 1%. A total of 4,051 proteins were identified by LC-MS/MS in roots of tomato genotypes considering control and Cd-induced stress conditions (Figure 13A).

Figure 13. Proteins identified by LC-MS/MS in roots of tomato genotypes under control and Cd-induced stress. A) Proteins detected in each genotype. Overlapping numbers represent proteins detected in both genotypes. B) Number of proteins detected in each condition (control and stressed) within each genotype. Overlapped numbers represent proteins identified in both conditions. Tolerant genotype Pusa Ruby under control and stressed conditions PR and PRcd, respectively. Sensitive genotype Calabash Rouge under control and stressed conditions CR and CRcd, respectively.

From this total identified 85.83% of the proteins were shared between genotypes, being 8.61% proteins exclusively found in tolerant and 5.56% exclusively detected in sensitive genotype (Figure 13A). The tolerant genotype exhibited a slight higher number of identified proteins (3,826 proteins) compared with the sensitive genotype (3,701 proteins). When proteins detected in control and treated plants of tolerant genotype were compared, it was revealed that 3,151 proteins were present in both groups of plants and, 362 and 313 proteins were exclusively detected in control and Cd-treated plants, respectively (Figure 13B). The same comparison also revealed that in the sensitive genotype 2,910 proteins were detected in both types of plants and,

408 and 383 proteins were exclusively detected in control and Cd-treated plants, respectively (Figure 13B). The number of proteins exclusively detected in plants treated with Cd was much higher in CR than in PR (Figure 13B). This difference was also reflected in the numbers of differently accumulated proteins (DAP) detected between control and treated plants within each genotype.

The proteins sequences identified by LC-MS/MS in roots of tomato genotypes were contrasted with UNIPROT database as a reference for molecular mass and isoelectric point comparisons (Figure 14).

Figure 14. Distribution of molecular mass (A) and isoelectric point (pI) of proteins identified in tomato roots. Dark grey bars represent tomato proteome (4,051 proteins identified) and light grey bars represent total Uniprot database (23,392 proteins).

Concerning the molecular mass of identified proteins it was very similar with the reference, with almost 60% of proteins among 10-50 kDa and 25% of total identified proteins within the class 50-90 kDa (Figure 14A). Regarding to isoelectric point the range of classes are higher and the proteins were distributed evenly among them (Figure 14B), showing that there was no preference for any particular class.

A total of 380 proteins were classified as DAP, which 62 were shared between both genotypes (Figure 15). A total of 84 proteins were DAP in PR plants Cd-induced stress compared to control, being 55 of them increased in abundance and 29 decreased. In a similar comparison fashion, but showing higher numbers, the CR plants revealed 358 proteins detected as DAP, being 165 proteins increased in abundance and 193 decreased (Figure 15).

Figure 15. Venn diagram showing differentially accumulated proteins (DAP) detected in tomato roots under Cd-induced stress in each genotype. Overlapped numbers represent proteins identified in both genotypes. Numbers of proteins that increased and decreased abundance after Cd-exposure are represented inside dark grey and light grey arrows, respectively. Tolerant genotype Pusa Ruby (PR) and sensitive genotype Calabash Rouge (CR).

4.3.4. Functional classification and clustering analysis of DAP

To gain insight into the functional categories altered under Cd stress, DAP were linked to at least one GO term associated to biological process, molecular function and cellular component. An overall similar behavior in the GO distribution was observed for DAP in both genotypes (Figure 16).

Figure 16. Functional classification of differentially accumulated proteins (DAP) of tomato roots using DAVID Bioinformatics tool. DAVID analyses were based on Gene Ontology (GO) terms distribution: Biological process, Cell component and Molecular function. Tolerant genotype Pusa Ruby (PR, light grey bars) and sensitive genotype Calabash Rouge (CR, dark grey bars).

Within the biological process category, the cell wall organization or biogenesis, single-organism and metabolic cellular processes were the most representative classes in both genotypes (Figure 16). Regarding to cellular component category, cell part was overrepresented in both genotypes. Finally, oxidoreductase activity and ion binding were overrepresented within the molecular function category in both genotypes (Figure 16). Although an overall similar functional distribution between genotypes was observed, some differences can be highlighted between these plants. In the biological processes categorization, the response to biotic stimulus and response to stress were overrepresented in sensitive genotype (Figure 16). Among cellular component category, intracellular was most representative to the sensitive genotype whilst extracellular region and envelope were found as most representatives in the tolerant genotype (Figure 16B). Concerning molecular function, carbohydrate derivative binding and cofactor binding were overrepresented in the sensitive genotype while peroxidase activity was noteworthy in the tolerant plants (Figure 16).

Analysis of major clusters of DAP showed a clear enrichment related to peroxidase and glutathione-S-transferase (GST) in both genotypes, mainly in sensitive plants (Figure 17).

Figure 17. Major functional clusters of differentially accumulated proteins (DAP) enriched in tomato roots of both genotypes using DAVID Bioinformatics Resources. Tolerant genotype Pusa Ruby (PR, light grey bars) and sensitive genotype Calabash Rouge (CR, dark grey bars).

The sensitive genotype under Cd-conditions showed exclusive clusters including proteins involved in cytoskeleton, S-adenosylmethionine biosynthetic process, oxidoreductase activities, nitrite/sulphite reductases, chaperone and glycolysis. Furthermore, the heatmap revealed that DAP shared between tomato genotypes showed similar changes under Cd-induced stress (Figure 18).

Figure 18. Hierarchical clustering and heatmap analysis of differentially accumulated proteins (DAP) detected in tomato roots of both genotypes: tolerant Pusa Ruby (PR) and sensitive Calabash Rouge (CR). Fold change values were used to build the heatmap in Perseus software.

The great majority of these DAP showed alterations in protein abundance in diverse pathways (data not shown) including those involved in stress response, cell wall and redox activities (Tables 5 and 6). Tolerant roots in the presence of Cd decreased antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) while increased accumulation of glutathione-s-transferase (GST) and thioredoxin (TRX) (Table 5). Among the changes in root proteome profile of the sensitive genotype it can be highlighted the increased abundance GST, with important decreases in SOD, monodehydroascorbate reductase (MDHAR) and TRX (Table

6). Furthermore, peroxidases (POD) were shown altered in both genotypes after Cd exposure (Tables 5 and 6).

Table 5. Functional classification of proteins involved in cell wall, redox and stress response detected in tolerant genotype Pusa Ruby according to MapMan software. Red squares represent increased protein abundance or exclusively detected after Cd treatment. Blue squares represent reduced protein abundance after Cd treatment or exclusively detected in controls.

Accession	TAIR	Description	Log p-value	Exclusive	Fold change
Cell wall
Solyc04g040180.2.1	AT2G41380.1	S-adenosyl-L-methionine-dependent methyltransferases	7.43367		1.53815
Solyc08g061100.2.1	AT4G32410.1	Cellulose synthase	3.39495		-1.44344
Solyc11g008900.1.1	AT5G64740.1	Cellulose synthase	3.39495		-1.44344
Redox
Solyc01g067740.2.1	AT1G08830.2	Copper/zinc superoxide dismutase	2.71824		-0.774057
Solyc02g082760.2.1	AT4G35090.1	Catalase	2.74614		-0.28134
Solyc04g071600.2.1	AT2G47730.1	Glutathione S-transferase		yes
Solyc10g084400.1.1	AT5G02790.1	Glutathione S-transferase	6.08407		2.43785
Solyc07g056480.2.1	AT1G78380.1	Glutathione S-transferase	2.79341		2.57217
Solyc09g011590.2.1	AT3G09270.1	Glutathione S-transferase	5.44303		1.54529
Solyc12g056250.1.1	AT5G41210.1	Glutathione S-transferase	2.77563		0.489509
Solyc09g091840.2.1	AT3G24170.3	Glutathione-disulfide reductase	2.73657		0.41851
Solyc08g081010.2.1	AT4G23100.3	Glutamate-cysteine ligase	3.12387		1.15032
Solyc12g006380.1.1	AT1G06620.1	2-oxoglutarate (2OG) and Fe(II)-dependent	3.94028		7.05577
Solyc12g006380.1.1	AT1G06620.1	2-oxoglutarate (2OG) and Fe(II)-dependent	3.94028		7.05577
Solyc02g080640.2.1	AT4G21990.1	Thioredoxin superfamily	6.51603		2.32655
Stress response
Solyc00g072400.2.1	AT5G05340.1	Peroxidase superfamily protein	2.87797		-0.479179
Solyc00g072400.2.1	AT5G05340.1	Peroxidase superfamily protein	2.87797		-0.479179
Solyc00g072400.2.1	AT5G05340.1	Peroxidase superfamily protein	2.87797		-0.479179
Solyc08g075830.2.1	AT3G01190.1	Peroxidase superfamily protein	3.26239		-1.10259
Solyc04g071890.2.1	AT1G71695.1	Peroxidase superfamily protein	3.67982		0.332093
Solyc05g046020.2.1	AT1G05260.1	Peroxidase superfamily protein	2.77714		-0.434336

Solyc05g046020.2.1	AT1G05260.1	Peroxidase superfamily protein	2.77714		-0.434336
Solyc05g046020.2.1	AT1G05260.1	Peroxidase superfamily protein	2.77714		-0.434336
Solyc05g046020.2.1	AT1G05260.1	Peroxidase superfamily protein	2.77714		-0.434336
Solyc08g080670.1.1	AT4G11650.1	Osmotin	2.69419		0.248487
Solyc08g080670.1.1	AT4G11650.1	Osmotin	2.69419		0.248487
Solyc10g052880.1.1	AT1G33590.1	Leucine-rich repeat (LRR) family protein	4.06562		1.78429
Solyc01g097270.2.1	AT3G04720.1	Pathogenesis-related	2.8659		1.05153
Solyc08g078700.2.1	AT4G25200.1	Heat shock protein mitochondrion-localized	6.93255		2.35867
Solyc04g072020.2.1	AT3G23990.1	Heat shock protein		yes	

Table 6. Functional classification of proteins involved in cell wall, redox and stress response detected in sensitive genotype Calabash Rouge according to MapMan software. Red squares represent increased protein abundance or exclusively detected after Cd treatment. Blue squares represent reduced protein abundance after Cd treatment or exclusively detected in controls.

Accession	TAIR	Description	Log p-value	Exclusive	Fold change
Cell wall					
Solyc02g078670.2.1	AT1G26850.2	S-adenosyl-L-methionine-dependent methyltransferases		yes	
Solyc02g068430.2.1	AT2G36870.1	Xyloglucan endotransglucosylase/hydrolase		yes	
Solyc09g075120.2.1	AT2G47650.1	UDP-xylulose synthase	3.12215		-0.746021
Solyc11g066150.1.1	AT3G46440.2	UDP-xylulose synthase	2.88119		-1.03343
Solyc05g054590.2.1	AT2G28760.3	UDP-xylulose synthase	3.9227		-0.952419
Solyc04g011400.2.1	AT2G28760.3	UDP-xylulose synthase	2.13527		-0.440563
Solyc06g074670.2.1	AT1G08200.1	UDP-D-apiose/UDP-D-xylose synthase	3.82181		-0.546379
Solyc02g088690.2.1	AT3G29360.2	UDP-glucose 6-dehydrogenase family	2.13651		-0.816949
Solyc02g067080.2.1	AT5G15490.1	UDP-glucose 6-dehydrogenase family	2.32841		-0.545362
Solyc08g077910.2.1	AT4G17030.1	Expansin-like	2.51499		1.18386
Solyc11g072690.1.1	AT1G79500.4	Aldolase-type TIM barrel family	2.47208		-0.490226
Solyc04g005340.2.1	AT3G02230.1	Reversibly glycosylated polypeptide	2.03199		-0.303584
Solyc02g065740.2.1	AT3G02230.1	Reversibly glycosylated polypeptide	2.64182		-0.392983
Solyc05g012070.2.1	AT3G02230.1	Reversibly glycosylated polypeptide	1.99203		-0.284253
Solyc08g080140.2.1	AT1G63000.1	Nucleotide-rhamnose synthase/epimerase-reductase	2.22765		-0.441869
Solyc12g055930.1.1	AT4G10960.1	UDP-D-galactose 4-epimerase	4.03165		-1.21975
Solyc07g062130.2.1	AT1G78570.1	Rhamnose biosynthesis	3.25854		-0.854392
Solyc10g007480.2.1	AT1G78570.1	Rhamnose biosynthesis	2.84794		-0.51156
Solyc05g050130.2.1	AT5G24090.1	Chitinase	2.23596		4.51303
Solyc01g104950.2.1	AT5G64570.1	Beta-D-xylosidase	2.40608		-0.647301
Redox					
Solyc11g066390.1.1	AT2G28190.1	Copper/zinc superoxide dismutase	1.93099		-0.349326
Solyc06g048410.2.1	AT5G51100.1	Fe superoxide dismutase	2.20226		1.24065

Solyc04g071600.2.1	AT2G47730.1	Glutathione S-transferase		yes	
Solyc06g009020.2.1	AT2G47730.1	Glutathione S-transferase	2.51345		0.289784
Solyc09g074850.2.1	AT3G07940.1	Glutathione S-transferase		yes	
Solyc06g067870.2.1	AT1G17180.1	Glutathione S-transferase		yes	
Solyc07g056420.2.1	AT1G17180.1	Glutathione S-transferase	4.42624		2.95133
Solyc07g056470.2.1	AT1G78380.1	Glutathione S-transferase	4.5456		2.99939
Solyc09g011580.2.1	AT2G29420.1	Glutathione S-transferase	2.53854		1.03124
Solyc07g056500.2.1	AT1G04430.2	Glutathione S-transferase		yes	
Solyc09g011600.2.1	AT3G09270.1	Glutathione S-transferase	2.23671		1.19854
Solyc07g056480.2.1	AT1G78380.1	Glutathione S-transferase	3.73631		2.70823
Solyc07g056490.2.1	AT1G78380.1	Glutathione S-transferase	2.3136		1.31565
Solyc09g011520.2.1	AT3G09270.1	Glutathione S-transferase	3.32321		6.73616
Solyc12g056250.1.1	AT5G41210.1	Glutathione S-transferase	3.75101		0.479949
Solyc09g011590.2.1	AT3G09270.1	Glutathione S-transferase	4.12337		1.55058
Solyc10g084400.1.1	AT5G02790.1	Glutathione S-transferase	6.2159		2.14335
Solyc09g011630.2.1	AT3G09270.1	Glutathione S-transferase	3.69309		0.968991
Solyc02g068900.2.1	AT4G19880.1	Glutathione S-transferase	3.49817		0.926919
Solyc09g011540.2.1	AT3G09270.1	Glutathione S-transferase	2.35562		1.14823
Solyc10g082030.1.1	AT5G06290.1	2-cysteine peroxiredoxin	1.97758		0.486747
Solyc06g005260.2.1	AT5G40370.1	Glutaredoxin family protein	2.33265		0.622224
Solyc08g081010.2.1	AT4G23100.3	Glutamate-cysteine ligase	2.51312		0.761001
Solyc12g006380.1.1	AT1G06620.1	2-oxoglutarate (2OG) and Fe(II)-dependent	4.33727		7.85888
Solyc05g018520.2.1	AT5G20080.1	FAD/NAD(P)-binding oxidoreductase	2.19192		-0.430908
Solyc08g081530.2.1	AT1G63940.2	Monodehydroascorbate reductase	2.49411		-0.321089
Solyc09g009390.2.1	AT3G52880.1	Monodehydroascorbate reductase	3.08212		-0.339768
Solyc08g008670.2.1	AT2G45790.1	Phosphomannomutase	2.46347		-1.05317
Solyc05g056400.2.1	AT1G77510.1	PDI-like thioredoxin superfamily	2.635		0.349778
Solyc10g037980.1.1	AT5G42850.2	Thioredoxin superfamily protein	1.95787		-0.588132
Solyc11g012730.1.1	AT2G18990.1	Thioredoxin domain-containing	1.93205		-1.44038

Solyc02g089230.2.1	AT5G38900.1	Thioredoxin superfamily protein	4.44469		-2.60869
Solyc11g071280.1.1	AT3G17880.1	Thioredoxin-like disulfide reductase		yes	
Stress response					
Solyc08g062450.1.1	AT2G24290.1	HSP17.6II 17.6 kDa class II heat shock		yes	
Solyc08g062340.2.1	AT5G57410.1	HSP17.6II 17.6 kDa class II heat shock		yes	
Solyc06g076520.1.1	AT1G07400.1	HSP20-like chaperones superfamily protein	2.7412		0.748233
Solyc06g076560.1.1	AT1G07400.1	HSP20-like chaperones superfamily protein	2.90247		1.29333
Solyc06g076570.1.1	AT1G07400.1	HSP20-like chaperones superfamily protein	2.24339		1.08155
Solyc11g020040.1.1	AT5G49910.1	Heat shock protein 70 (Hsp 70) family	1.988		0.357884
Solyc06g036290.2.1	AT5G52640.1	Heat shock protein 70 (Hsp 70) family	2.83792		1.03554
Solyc12g043110.1.1	AT1G79920.1	Heat shock protein 70 (Hsp 70) family	2.20681		-0.175459
Solyc12g043120.1.1	AT1G79920.1	Heat shock protein 70 (Hsp 70) family	3.33864		-0.212166
Solyc08g078700.2.1	AT4G25200.1	Heat shock protein mitochondrion-localized	5.23904		3.33061
Solyc03g007890.2.1	AT5G52640.1	HSP81-1, ATHS83, HSP81.1, HSP83, ATHSP90.1	3.0541		1.21861
Solyc12g095800.1.1	AT1G21080.1	DNAJ heat shock N-terminal domain-containing	2.44787		-0.910323
Solyc08g080650.1.1	AT4G11650.1	Osmotin	2.32988		0.323913
Solyc08g080640.1.1	AT4G11650.1	Osmotin	3.58063		0.367908
Solyc08g080660.1.1	AT4G11650.1	Osmotin	2.21776		0.338039
Solyc02g087070.2.1	AT3G01420.1	Peroxidase	3.65829		5.71282
Solyc07g052550.1.1	AT1G05260.1	Peroxidase superfamily protein	3.77475		-0.578327
Solyc05g046030.2.1	AT1G05260.1	Peroxidase superfamily protein	2.10132		-0.407397
Solyc05g046020.2.1	AT1G05260.1	Peroxidase superfamily protein	2.83755		-0.493103
Solyc07g052530.2.1	AT1G05260.1	Peroxidase superfamily protein	4.71613		-0.676416
Solyc07g052540.2.1	AT1G05260.1	Peroxidase superfamily protein	4.24156		-0.667076
Solyc09g018590.1.1	AT5G05340.1	Peroxidase superfamily protein	1.98812		5.04556
Solyc04g080760.2.1	AT1G44970.1	Peroxidase superfamily protein	5.52402		1.59271
Solyc02g079500.2.1	AT5G05340.1	Peroxidase superfamily protein	2.30678		0.942402
Solyc10g076220.1.1	AT5G05340.1	Peroxidase superfamily protein	3.20381		0.77085
Solyc02g094180.2.1	AT4G37530.1	Peroxidase superfamily protein	2.28396		0.93945

Solyc05g050880.2.1	AT5G05340.1	Peroxidase superfamily protein	3.03235		1.23302
Solyc11g018800.1.1	AT1G14550.1	Peroxidase superfamily protein	2.19991		1.06904
Solyc10g076240.1.1	AT5G05340.1	Peroxidase superfamily protein	3.51033		1.0348
Solyc03g006700.2.1	AT5G05340.1	Peroxidase superfamily protein	3.69042		0.515784
Solyc07g047740.2.1	AT1G05260.1	Peroxidase superfamily protein	2.893		-1.20167
Solyc08g075830.2.1	AT3G01190.1	Peroxidase superfamily protein	3.64763		-1.37916
Solyc04g071890.2.1	AT1G71695.1	Peroxidase superfamily protein	3.28364		0.411396
Solyc00g072400.2.1	AT5G05340.1	Peroxidase superfamily protein	2.73174		-0.398857
Solyc01g105070.2.1	AT2G41480.1	Peroxidase superfamily protein	4.7414		0.711502
Solyc01g067860.2.1	AT2G39040.1	Peroxidase superfamily protein	1.93124		0.315159
Solyc03g080150.2.1	AT1G05260.1	Peroxidase superfamily protein	1.96124		0.505021
Solyc10g076210.1.1	AT5G05340.1	Peroxidase superfamily protein	3.50956		0.800713
Solyc10g086090.1.1	AT2G38870.1	Serine protease inhibitor, potato inhibitor	3.09357		0.691854
Solyc09g091000.2.1	AT1G24020.2	MLP-like protein	3.52004		1.22491
Solyc09g090990.2.1	AT1G24020.2	MLP-like protein	4.5043		1.05907
Solyc09g090980.2.1	AT1G24020.2	MLP-like protein	3.48046		0.75564
Solyc09g090970.2.1	AT1G24020.2	MLP-like protein	3.73183		1.18759
Solyc09g005400.2.1	AT1G70830.5	MLP-like protein	1.96626		-1.11161
Solyc01g097270.2.1	AT3G04720.1	Pathogenesis-related	3.10864		1.21253
Solyc01g106620.2.1	AT2G14580.1	Pathogenesis-related	2.1		0.406061
Solyc04g064880.2.1	AT1G78780.2	Pathogenesis-related	4.2716		3.14952

4.4. Discussion

4.4.1. Cd impaired tomato growth in both genotypes and increased MDA and H₂O₂ contents in tolerant roots

Roots are the site of impact under most natural conditions of toxic metal exposure. It was therefore decided to grow tomato plants hydroponically and to treat them via the addition of 35 μ M of CdCl₂ to the growth medium and then evaluate the Cd-induced effects after four days. The analysis was also focused on root tissue because the response is likely to be synchronized here. The Cd exposure reduced plant growth in both genotypes (Figure 11), being more drastic for the sensitive genotype, which was decreased by 50% and 70% in root and shoot growth, respectively, whilst the tolerant genotype was reduced by 40% and 60% in root and shoot growth, respectively (Figure 11A). Decreases in biomass accumulation after Cd exposure has been reported for the large majority of plant species studied so far (Gallego *et al.*, 2012) including maize (Anjum *et al.*, 2015), rice (Hassan *et al.*, 2005; Singh and Shah, 2014), pea (Dixit *et al.*, 2001; Sandalio *et al.*, 2001), soybean (Ahsan *et al.*, 2012; Perez-Chaca *et al.*, 2014) and tomato (Gratão *et al.*, 2008; Hedijsi *et al.*, 2010; López-Millán *et al.*, 2009; Nogueirol *et al.*, 2016). The sensitive genotype (CR) showed higher decrease in growth suggesting that intracellular detoxification or defense mechanisms maybe different from the tolerant genotype (PR). These findings are in accordance with Lu *et al.* (2013) who reported root and shoot biomass of peanuts cultivars decreased after treatment with 20 μ M CdCl₂. In this work similar growth rates for both genotypes were observed in the absence of Cd, whilst plants subjected to Cd exposure exhibited impaired growth induced by metal exposure (Figure 11).

The effect of Cd toxicity can be determined by evaluating MDA and H₂O₂ formed in plant tissues as products of lipid peroxidation and signaling, respectively. Our results revealed that Cd may be involved in increasing MDA and H₂O₂ levels in tolerant genotype (Figure 12A and 12B). According to Sharma and Dietz (2009b) heavy-metal tolerant genotypes normally produce higher levels of ROS compared to sensitive ones. This may be correlated with ROS scavenging systems such as the antioxidant enzymes activated in cells contaminated with Cd, protecting them against Cd-induced oxidative stress (Yannarelli *et al.*, 2007).

Most of the proteomic research performed so far on heavy metal toxicity revealed positive correlation between tolerance and increased abundance of scavenger proteins (Hossain and Komatsu, 2013). In the first chapter, we showed that ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione-S-transferase (GST) had increased activities in the

presence of Cd in roots of the tolerant genotype. Furthermore, once cellular ROS production is stimulated in response to metabolic imbalances imposed by abiotic stresses (Gill and Tuteja, 2010), it is possible to suggest that the tolerant genotype has a better system of signaling for defence response, compared to the sensitive one.

4.4.2. Root proteome profile of tomato genotypes under Cd-induced stress

Some reports have demonstrated that Cd-induced stress causes alterations in proteome profile of various plant species such as *Arabidopsis spp.* (Farinati et al., 2011; Roth et al., 2006; Semane et al., 2010), rice (Aina et al., 2007; Lee et al., 2010) and wheat (Wang et al., 2011), however no study have reported the effect of Cd on root proteome considering contrasting tomato genotypes for Cd-tolerance. The proteomic workflow presented here (Figure 10) was able to identify proteins distributed in all molecular mass classes, with almost 60% of all proteins among 10-50 kDa, exhibiting wide ranges of isoelectric point, mainly detected between 4-11 values (Figure 14). Therefore, protein detection evidenced an accurate system without the predilection of certain bands of molecular mass and isoelectric point.

4.4.3. Cd induced alterations in proteins related to cell wall

The proteins identified in tomato roots after Cd exposure are involved in a wide range comprising different functional categories (Figure 16). In a general overview, the proteome of the sensitive genotype was more responsive to excess of Cd, once the number of differentially accumulated proteins (DAP) detected in this genotype was higher than in the tolerant genotype (Figure 15). Interestingly there was contrasting cellular component response between tomato genotypes: sensitive roots showed strong alterations in proteins related to intracellular components while tolerant increased activities located in extracellular and envelope regions (Figure 16). Extracellular mechanisms are mainly implicated in avoidance of metal entry, whereas intracellular systems aim to reduce metal burden in the cytosol (Bellion et al., 2006). The moss *Scorpiurum circinatum* treated with different heavy metals, including Cd, immobilized the toxic ions in the cell walls, which is therefore used as the main detoxification site (Basile et al., 2012). Parrota et al. (2015) suggested that the cell wall acts as a barrier, preventing Cd from entering the cytoplasm where it is extremely toxic for plant cells. Furthermore, root exudates containing metal chelators enhance the uptake of certain metals (Hall, 2002). In plants when restraining Cd in roots, the metal is sequestered in the endoderm and the cortical regions of roots (Gall et al.,

2015). When this filter fails, Cd can penetrate the xylem vessels and translocate to the shoot, therefore, the specific composition of cell walls in the endoderm and cortex is critical (Akhter *et al.*, 2014). Here, the tolerant genotype decreased accumulation of cellulose synthase (EC 2.4.1.12) (Table 5). In rice plant, mutation in a cellulose synthase gene (*CESA9*) modified the structure of the secondary cell wall, which further reduced translocation of Cd within xylem and its accumulation inside the plant (Song *et al.*, 2013a). Since the cell wall of roots is considered as an effective barrier against penetration of Cd, a relationship can be established between higher accumulation of Cd in the roots vs lower accumulation in shoots (Figure 1, chapter 1). In tomato, Cd induced a dramatic decrease in the accumulation of proteins essential for cell wall maintenance in the sensitive genotype (Table 6), indicating that maybe Cd-tolerance goes through the capacity of roots to limit the diffusion of Cd, with intense remodeling of cell wall-located proteins.

Although specific studies have not been conducted to determine the impact of Cd exposure on the cell wall proteome and on the diversity of responses in closely related species, numerous cell wall localized proteins were identified in general proteome studies on the impact of heavy metals in plants, including general stress responsive proteins (Parrotta *et al.*, 2015). For instance, contrasting observations have been made about cell wall-localized chitinase in response to metal stress. Chitinases (E.C 3.2.2.14) are glycosyl hydrolases involved in defence response to biotic and abiotic stresses (Grover, 2012). In two flax cultivars with differential tolerance levels to Cd exposure, chitinase increased in both cultivars, but the protein accumulated at significantly higher levels in the tolerant cultivar (Hradilová *et al.*, 2010). On the other hand, in this study only the sensitive genotype increased the chitinase abundance in roots (Table 6). Previous report showed soybean roots specifically responding to different metal types and a polymorphism that coincides with sensitivity to metal toxicity (Mészáros *et al.*, 2014). In poplar plants chitinase activity levels correlate with sensitivity to Cd (Kieffer *et al.*, 2008). Under metal stress the chitinases are believed to act as second line defense components, possibly modify dynamics and permeability of the cell wall to metals, as well as affect metal binding and immobilization capability of the cell wall (Mészáros *et al.*, 2014).

4.4.4. Cd induced alterations in proteins related to redox balance

The great majority of DAP showed increased abundance in diverse pathways, including those involved in stress response and redox activities (Tables 5 and 6). Antioxidant enzymes are the key players in maintaining cellular redox status and stress induced plant tolerance (Gallego *et al.*, 2012; Gill and Tuteja, 2010; Sharma and Dietz, 2009a). Exposure to Cd decreased antioxidant

enzymes such as SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) in the tolerant genotype (Table 5) while alterations in SOD and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) were detected in the sensitive genotype (Table 6). Contradictory responses of SOD isoenzymes to Cd have been documented. Root proteome analysis of Cd-exposed *Brassica juncea* revealed increased of Fe-SOD but also decreased accumulation of Cu/Zn-SOD (Alvarez *et al.*, 2009). Accordingly, in tomato Cu/Zn-SOD decreased in both genotypes (Tables 5 and 6) and increased Fe-SOD in sensitive roots (Table 6). Although Cd may have a negative effect on important ROS-detoxifying enzymes, such as Cu/Zn SODs, probably by competing with Zn at the cofactor site, the plants still managed to restrict oxidative stress by upregulating different stress-coping proteins. Among them were several isoforms of peroxidases, their main function being the removal of hydrogen peroxide from chloroplasts and cytosol, once SOD isoenzymes are located in different compartments of the cells (Alscher *et al.*, 2002). The Cd-induced ROS accumulation may specifically induced Fe-SOD, once Cd may limit Cu and Zn uptake, thus limiting Cu/Zn-SOD production and inducing Fe-SOD accumulation (Alvarez *et al.*, 2009). The Cd-mediated repression of peroxidases and the shifts in SOD abundances indicate the possible accumulation of H₂O₂ in tolerant roots (Figure 12). In Cd-stressed poplar plants, the SOD activity decreased, while the enzymatic activity of CAT increased significantly in roots, suggesting that these plants trigger a response based on enhanced oxidase activity (Kieffer *et al.*, 2008).

CAT activity decreased after Cu exposure in *Arabidopsis thaliana* roots (Cuypers *et al.*, 2011). In the regulation of H₂O₂ content, CATs have a high reaction rate, but lower affinity to H₂O₂ as compared to APX, what explains our findings in tomato roots. In the first chapter we demonstrated that SOD and CAT activities were not changed in the presence of Cd (Figure 4), whereas the proteome analysis revealed CAT decreased activity in tolerant roots, suggesting the hypothesis of SOD and CAT independent mechanisms to detoxify the radicals superoxide and H₂O₂, respectively, in contaminated tomato cells. Important decrease of MDHAR abundance was observed in sensitive roots exposed to Cd (Table 6), which might indicate non-enzymatic disproportionation of monodehydroascorbate into ascorbate, which is essential for maintenance of balanced redox status (Hossain *et al.*, 2009).

Peroxidases (POD; E.C.1.11.1.7.) are important enzymes in oxidative stress response which participate in lignin biosynthesis, IAA degradation and converting H₂O₂ to water. Moreover, POD can use a wide range of electron donors such as ascorbate and guaiacol (Asada, 1992). Many POD enzymes were altered after Cd exposure in both tomato genotypes, with the majority decreasing accumulation mainly in the sensitive genotype (Tables 5 and 6). Decreased accumulation of POD were previously reported for poplar plants under Cd-induced stress

(Kieffer *et al.*, 2009). There are several POD isoenzymes in plants and each isoenzyme has variable amino acid sequences and it shows diverse expression profiles, suggesting their involvement in various physiological processes (Hiraga *et al.*, 2001).

Induction of GSH synthesis implies in higher metal binding capacity as well as enhanced cellular defense mechanism against oxidative stress (Jozefczak *et al.*, 2014). Since GSH is a precursor of phytochelatins (PCs) biosynthesis, the accumulation of GSH implies in higher metal binding capacity as well as enhanced cellular defense mechanism against oxidative stress (Verbruggen *et al.*, 2009). In a previous report enhanced expression of glutamine synthetase (GS) helped soybean cells to synthesize and accumulate more PCs to detoxify cytosolic Cd ions (Hossain *et al.*, 2012). Here, tomato sensitive genotype showed a decrease in GS accumulation in roots (Table 6), suggesting an important impact in nitrogen metabolism and ultimately the diminished capacity of these plants to synthesize PCs. Glutathione-S-transferases (GSTs) are involved in sulfur and GSH metabolism (Ghelfi *et al.*, 2011) and previously shown to be increased in various plant species that were exposed to Cd stress (Kieffer *et al.*, 2008; Zhao *et al.*, 2011). GSTs were markedly over accumulated in both tomato genotypes following Cd exposure (Tables 5 and 6) and it has been reported to be involved in the direct quenching of Cd ions, forming GSH-Cd complexes (Adamis *et al.*, 2004).

Thioredoxins (TRX), small proteins catalyzing thiol-disulfide interchange, are critical for redox regulation of protein function and signaling via thiol redox control (Gelhaye *et al.*, 2005). In tomato roots was detected increased thioredoxins accumulation in tolerant genotype, conversely it decreased in sensitive roots (Tables 5 and 6). Increased accumulation of TRX was also substantially higher in a Cu tolerant line of rice than in a sensitive one (Song *et al.*, 2013b) and a similar correlation was also reported for salt-resistant and salt-sensitive barley genotypes (Fatehi *et al.*, 2012). Protein thiols, such as TRX and glutaredoxins (GRX) are considered as protective and present in regulatory mechanisms in plants (Zagorchev *et al.*, 2013) and these proteins can form thiol bonds with heavy metals conferring ultimately tolerance (Kushwaha *et al.*, 2016).

The number and diversity of tolerance mechanisms to heavy metals found in plants might be larger than expected (Moussa and El-Gamal, 2010). In tomato genotypes, Cd-exposure altered many pathways in the plant metabolism suggesting a more active involvement of GST, TRX and POD in Cd tolerance.

References

- Adamis P. D. B., Gomes D. S., Pinto M. L. C. C., Panek A. D., Eleutherio E. C. A. (2004) The role of glutathione transferases in cadmium stress. *Toxicology Letters*, **154**, 81-88.
- Ahsan N., Nakamura T., Komatsu S. (2012) Differential responses of microsomal proteins and metabolites in two contrasting cadmium (Cd)-accumulating soybean cultivars under Cd stress. *Amino Acids*, **42**, 317-327.
- Aina R., Labra M., Fumagalli P., Vannini C., Marsoni M., Cucchi U., Bracale M., Sgorbati S., Citterio S. (2007) Thiol-peptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa* L. roots. *Environmental and Experimental Botany*, **59**, 381-392.
- Akhter M. F., Omelon C. R., Gordon R. A., Moser D., Macfie S. M. (2014) Localization and chemical speciation of cadmium in the roots of barley and lettuce. *Environmental and Experimental Botany*, **100**, 10-19.
- Al Khateeb W., Al-Qwasemeh H. (2014) Cadmium, copper and zinc toxicity effects on growth, proline content and genetic stability of *Solanum nigrum* L., a crop wild relative for tomato; comparative study. *Physiology and Molecular Biology of Plants*, **20**, 31-39.
- Alscher R. G., Erturk N., Heath L. S. (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany*, **53**, 1331-1341.
- Alvarez S., Berla B. M., Sheffield J., Cahoon R. E., Jez J. M., Hicks L. M. (2009) Comprehensive analysis of the *Brassica juncea* root proteome in response to cadmium exposure by complementary proteomic approaches. *Proteomics*, **9**, 2419-2431.
- Andrews G. L., Dean R. A., Hawkrige A. M., Muddiman D. C. (2011) Improving Proteome Coverage on a LTQ-Orbitrap Using Design of Experiments. *Journal of the American Society for Mass Spectrometry*, **22**, 773-783.
- Anjum S. A., Tanveer M., Hussain S., Bao M., Wang L., Khan I., Ullah E., Tung S. A., Samad R. A., Shahzad B. (2015) Cadmium toxicity in Maize (*Zea mays* L.): consequences on antioxidative systems, reactive oxygen species and cadmium accumulation. *Environmental Science and Pollution Research*, **22**, 17022-17030.
- Asada K. (1992) Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. *Physiologia Plantarum*, **85**, 235-241.
- Basile A., Sorbo S., Pisani T., Paoli L., Munzi S., Loppi S. (2012) Bioaccumulation and ultrastructural effects of Cd, Cu, Pb and Zn in the moss *Scorpiurum circinatum* (Brid.) Fleisch. & Loeske. *Environmental Pollution*, **166**, 208-211.
- Bellion M., Courbot M., Jacob C., Blaudez D., Chalot M. (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. *FEMS Microbiology Letters*, **254**, 173-181.
- Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 7.
- Chen Y. X., He Y. F., Luo Y. M., Yu Y. L., Lin Q., Wong M. H. (2003) Physiological mechanism of plant roots exposed to cadmium. *Chemosphere*, **50**, 789-793.
- Cuypers A., Karen S., Jos R., Kelly O., Els K., Tony R., Nele H., Nathalie V., Suzy V. S., Frank V. B., Yves G., Jan C., Jaco V. (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. *Journal of Plant Physiology*, **168**, 309-316.
- Dixit V., Pandey V., Shyam R. (2001) Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). *Journal of Experimental Botany*, **52**, 1101-1109.

- Farinati S., DalCorso G., Panigati M., Furini A. (2011) Interaction between selected bacterial strains and *Arabidopsis halleri* modulates shoot proteome and cadmium and zinc accumulation. *Journal of Experimental Botany*, **62**, 3433-3447.
- Fatehi F., Hosseinzadeh A., Alizadeh H., Brimavandi T., Struik P. C. (2012) The proteome response of salt-resistant and salt-sensitive barley genotypes to long-term salinity stress. *Molecular Biology Reports*, **39**, 6387-6397.
- Florens L., Carozza M. J., Swanson S. K., Fournier M., Coleman M. K., Workman J. L., Washburn M. P. (2006) Analyzing chromatin remodeling complexes using shotgun proteomics and normalized spectral abundance factors. *Methods*, **40**, 303-311.
- Gall H. L., Philippe F., Domon J.-M., Gillet F., Pelloux J., Rayon C. (2015) Cell Wall Metabolism in Response to Abiotic Stress. *Plants*, **4**, 112-166.
- Gallego S. M., Pena L. B., Barcia R. A., Azpilicueta C. E., Lannone M. F., Rosales E. P., Zawoznik M. S., Groppa M. D., Benavides M. P. (2012) Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environmental and Experimental Botany*, **83**, 33-46.
- Gelhaye E., Rouhier N., Navrot N., Jacquot J. P. (2005) The plant thioredoxin system. *Cellular and Molecular Life Sciences CMLS*, **62**, 24-35.
- Gill S. S., Tuteja N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, **48**, 909-930.
- Gratão P. L., Monteiro C. C., Antunes A. M., Peres L. E. P., Azevedo R. A. (2008) Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. *Annals of Applied Biology*, **153**, 321-333.
- Grover A. (2012) Plant Chitinases: Genetic Diversity and Physiological Roles. *Critical Reviews in Plant Sciences*, **31**, 57-73.
- Guelfi A., Azevedo R. A., Lea P. J., Molina S. M. G. (2003) Growth inhibition of the filamentous fungus *Aspergillus nidulans* by cadmium: an antioxidant enzyme approach. *The Journal of General and Applied Microbiology*, **49**, 63-73.
- Hall J. L. (2002) Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, **53**, 1-11.
- Hassan M. J., Wang Z. Q., Zhang G. P. (2005) Sulfur alleviates growth inhibition and oxidative stress caused by cadmium toxicity in rice. *Journal of Plant Nutrition*, **28**, 1785-1800.
- Hediji H., Djebali W., Cabasson C., Maucourt M., Baldet P., Bertrand A., Zoghalmi L. B., Deborde C., Moing A., Brouquisse R., Chaibi W., Gallusci P. (2010) Effects of long-term cadmium exposure on growth and metabolomic profile of tomato plants. *Ecotoxicology and Environmental Safety*, **73**, 1965-1974.
- Hiraga S., Sasaki K., Ito H., Ohashi Y., Matsui H. (2001) A Large Family of Class III Plant Peroxidases. *Plant and Cell Physiology*, **42**, 462-468.
- Hoagland D. R., Arnon D. I. (1950) The water-culture method for growing plants without soil. In: *California Agricultural Experiment Station*, p. 32. Berkeley.
- Hossain Z., Hajika M., Komatsu S. (2012) Comparative proteome analysis of high and low cadmium accumulating soybeans under cadmium stress. *Amino Acids*, **43**, 2393-2416.
- Hossain Z., Komatsu S. (2013) Contribution of proteomic studies towards understanding plant heavy metal stress response. *Frontiers in Plant Science*, **3**, 12.
- Hossain Z., López-Climent M. F., Arbona V., Pérez-Clemente R. M., Gómez-Cadenas A. (2009) Modulation of the antioxidant system in citrus under waterlogging and subsequent drainage. *Journal of Plant Physiology*, **166**, 1391-1404.
- Hradilová J., Řehulka P., Řehulková H., Vrbová M., Griga M., Brzobohatý B. (2010) Comparative analysis of proteomic changes in contrasting flax cultivars upon cadmium exposure. *Electrophoresis*, **31**, 421-431.

- Huang D. W., Sherman B. T., Tan Q., Collins J. R., Alvord W. G., Roayaei J., Stephens R., Baseler M. W., Lane H. C., Lempicki R. A. (2007) The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biology*, **8**, R183-R183.
- Hurkman W. J., Tanaka C. K. (1986) Solubilization of Plant Membrane Proteins for Analysis by Two-Dimensional Gel Electrophoresis. *Plant Physiology*, **81**, 802-806.
- Jozefczak M., Keunen E., Schat H., Blik M., Hernández L. E., Carleer R., Remans T., Bohler S., Vangronsveld J., Cuypers A. (2014) Differential response of Arabidopsis leaves and roots to cadmium: Glutathione-related chelating capacity vs antioxidant capacity. *Plant Physiology and Biochemistry*, **83**, 1-9.
- Kieffer P., Dommes J., Hoffmann L., Hausman J.-F., Renaut J. (2008) Quantitative changes in protein expression of cadmium-exposed poplar plants. *Proteomics*, **8**, 2514-2530.
- Kieffer P., Planchon S., Oufir M., Ziebel J., Dommes J., Hoffmann L., Hausman J.-F., Renaut J. (2009) Combining Proteomics and Metabolite Analyses To Unravel Cadmium Stress-Response in Poplar Leaves. *Journal of Proteome Research*, **8**, 400-417.
- Krämer U., Pickering I. J., Prince R. C., Raskin I., Salt D. E. (2000) Subcellular Localization and Speciation of Nickel in Hyperaccumulator and Non-Accumulator Thlaspi Species. *Plant Physiology*, **122**, 1343-1354.
- Kushwaha A., Rani R., Kumar S., Gautam A. (2016) Heavy metal detoxification and tolerance mechanisms in plants: Implications for phytoremediation. *Environmental Reviews*, **24**, 39-51.
- Lee K., Bae D. W., Kim S. H., Han H. J., Liu X., Park H. C., Lim C. O., Lee S. Y., Chung W. S. (2010) Comparative proteomic analysis of the short-term responses of rice roots and leaves to cadmium. *Journal of Plant Physiology*, **167**, 161-168.
- Loziuk P. L., Parker J., Li W., Lin C.-Y., Wang J. P., Li Q., Sederoff R. R., Chiang V. L., Muddiman D. C. (2015) Elucidation of Xylem-Specific Transcription Factors and Absolute Quantification of Enzymes Regulating Cellulose Biosynthesis in *Populus trichocarpa*. *Journal of Proteome Research*, **14**, 4158-4168.
- Lu Z. W., Zhang Z., Su Y., Liu C. F., Shi G. R. (2013) Cultivar variation in morphological response of peanut roots to cadmium stress and its relation to cadmium accumulation. *Ecotoxicology and Environmental Safety*, **91**, 147-155.
- López-Millán A.-F., Sagardoy R., Solanas M., Abadía A., Abadía J. (2009) Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics. *Environmental and Experimental Botany*, **65**, 376-385.
- Moussa H. R., El-Gamal S. M. (2010) Role of salicylic acid in regulation of cadmium toxicity in wheat (*Triticum aestivum* L.). *Journal of Plant Nutrition*, **33**, 1460-1471.
- Mészáros P., Rybanský L., Spieß N., Socha P., Kuna R., Libantová J., Moravčíková J., Pířelová B., Hauptvogel P., Matušíková I. (2014) Plant chitinase responses to different metal-type stresses reveal specificity. *Plant Cell Reports*, **33**, 1789-1799.
- Nogueiro R. C., Monteiro F. A., Gratao P. L., da Silva B. K. D., Azevedo R. A. (2016) Cadmium Application in Tomato: Nutritional Imbalance and Oxidative Stress. *Water Air and Soil Pollution*, **227**, 20.
- Parrotta L., Guerriero G., Sergeant K., Cai G., Hausman J.-F. (2015) Target or barrier? The cell wall of early- and later-diverging plants vs cadmium toxicity: differences in the response mechanisms. *Frontiers in Plant Science*, **6**, 133.
- Perez-Chaca M. V., Rodriguez-Serrano M., Molina A. S., Pedranzani H. E., Zirulnik F., Sandalio L. M., Romero-Puertas M. C. (2014) Cadmium induces two waves of reactive oxygen species in *Glycine max* (L.) roots. *Plant Cell and Environment*, **37**, 1672-1687.
- Piotto F. A., Tulmann-Neto A., Franco M. R., Boaretto L. F., Azevedo R. A. (2014) Rapid screening for selection of heavy metal-tolerant plants. *Crop Breeding and Applied Biotechnology*, **14**, 1-7.

- Przedpelska-Wąsowicz E., Polatajko A., Wierzbicka M. (2012) The Influence of Cadmium Stress on the Content of Mineral Nutrients and Metal-Binding Proteins in *Arabidopsis halleri*. *Water, Air, and Soil Pollution*, **223**, 5445-5458.
- Roth U., von Roepenack-Lahaye E., Clemens S. (2006) Proteome changes in *Arabidopsis thaliana* roots upon exposure to Cd²⁺. *Journal of Experimental Botany*, **57**, 4003-4013.
- Sandalio L. M., Dalurzo H. C., Gómez M., Romero-Puertas M. C., del Río L. A. (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of Experimental Botany*, **52**, 2115-2126.
- Sanità di Toppi L., Gabbriellini R. (1999) Response to cadmium in higher plants. *Environmental and Experimental Botany*, **41**, 105-130.
- Semane B., Dupae J., Cuyppers A., Noben J.-P., Tuomainen M., Tervahauta A., Kärenlampi S., Van Belleghem F., Smeets K., Vangronsveld J. (2010) Leaf proteome responses of *Arabidopsis thaliana* exposed to mild cadmium stress. *Journal of Plant Physiology*, **167**, 247-254.
- Sharma S. S., Dietz K.-J. (2009a) The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science*, **14**, 43-50.
- Sharma S. S., Dietz K. J. (2009b) The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci*, **14**.
- Singh I., Shah K. (2014) Evidences for structural basis of altered ascorbate peroxidase activity in cadmium-stressed rice plants exposed to jasmonate. *Biometals*, **27**, 247-263.
- Singh S., Singh A., Bashri G., Prasad S. M. (2016) Impact of Cd stress on cellular functioning and its amelioration by phytohormones: An overview on regulatory network. *Plant Growth Regulation*, **80**, 253-263.
- Soares C., de Sousa A., Pinto A., Azenha M., Teixeira J., Azevedo R. A., Fidalgo F. (2016) Effect of 24-epibrassinolide on ROS content, antioxidant system, lipid peroxidation and Ni uptake in *Solanum nigrum* L. under Ni stress. *Environmental and Experimental Botany*, **122**, 115-125.
- Song X.-Q., Liu L.-F., Jiang Y.-J., Zhang B.-C., Gao Y.-P., Liu X.-L., Lin Q.-S., Ling H.-Q., Zhou Y.-H. (2013a) Disruption of Secondary Wall Cellulose Biosynthesis Alters Cadmium Translocation and Tolerance in Rice Plants. *Molecular Plant*, **6**, 768-780.
- Song Y., Cui J., Zhang H., Wang G., Zhao F.-J., Shen Z. (2013b) Proteomic analysis of copper stress responses in the roots of two rice (*Oryza sativa* L.) varieties differing in Cu tolerance. *Plant & Soil*, **366**, 647-658.
- Tuomainen M. H., Nunan N., Lehesranta S. J., Tervahauta A. I., Hassinen V. H., Schat H., Koistinen K. M., Auriola S., McNicol J., Kärenlampi S. O. (2006) Multivariate analysis of protein profiles of metal hyperaccumulator *Thlaspi caerulescens* accessions. *PROTEOMICS*, **6**, 3696-3706.
- Verbruggen N., Hermans C., Schat H. (2009) Mechanisms to cope with arsenic or cadmium excess in plants. *Current Opinion in Plant Biology*, **12**, 364-372.
- Wang Y., Qian Y., Hu H., Xu Y., Zhang H. (2011) Comparative proteomic analysis of Cd-responsive proteins in wheat roots. *Acta Physiologiae Plantarum*, **33**, 349-357.
- Wei S. H., Li Y. M., Zhan J., Wang S. S., Zhu J. G. (2012) Tolerant mechanisms of *Rorippa globosa* (Turcz.) Thell. hyperaccumulating Cd explored from root morphology. *Bioresource Technology*, **118**, 455-459.
- Wei S. H., Zhou Q. X., Wang X., Zhang K. S., Guo G. L., Ma Q. Y. L. (2005) A newly-discovered Cd-hyperaccumulator *Solanum nigrum* L. *Chinese Science Bulletin*, **50**, 33-38.
- Yannarelli G. G., Fernandez-Alvarez A. J., Santa-Cruz D. M., Tomaro M. L. (2007) Glutathione reductase activity and isoforms in leaves and roots of wheat plants subjected to cadmium stress. *Phytochemistry*, **68**, 505-512.
- Zagorchev L., Seal C. E., Kranner I., Odjakova M. (2013) A Central Role for Thiols in Plant Tolerance to Abiotic Stress. *International Journal of Molecular Sciences*, **14**, 7405-7432.

- Zhao F. J., Jiang R. F., Dunham S. J., McGrath S. P. (2006) Cadmium uptake, translocation and tolerance in the hyperaccumulator *Arabidopsis halleri*. *New Phytologist*, **172**, 646-654.
- Zhao L., Sun Y.-L., Cui S.-X., Chen M., Yang H.-M., Liu H.-M., Chai T.-Y., Huang F. (2011) Cd-induced changes in leaf proteome of the hyperaccumulator plant *Phytolacca americana*. *Chemosphere*, **85**, 56-66.

5. FINAL CONSIDERATIONS

In this study it was determined global root responses of contrasting tomato genotypes to Cd-induced stress. In the first chapter, a temporal dynamic of Cd responses was shown. Tomato genotypes growing in media containing 35 μM CdCl_2 over seven days showed metal accumulation in roots and shoots tissues in both genotypes, but with increased Cd allocation over time mainly in roots. PR (tolerant) accumulated lower levels of Cd in the shoots, exhibiting higher growth rate and higher levels of MDA in roots compared to CR (sensitive). Therefore, the PR genotype appears to have a more efficient mechanism to cope with Cd-induced stress. Enzymatic analysis revealed that the presence of Cd altered GSH content in roots of both genotypes, whilst increased the activities of APX, GR and GST, which together may be the main players against oxidative stress in PR genotype exposed to Cd.

Following the characterization of tomato root systems against Cd challenge the second chapter brings the root morphology parameter analysis. In this chapter we tried to establish a relationship between the root morphology and nutrient uptake, finally showing Cd impact on nutrient use efficiency (NUE) of tomato plants. Regarding to morphology root length, surface area and volume decreased in both genotypes exposed to Cd, being more severe in the CR genotype. Tomato roots exposed to Cd showed NUE for many macro and micronutrients in the CR genotype lower than verified for the PR. These findings reinforce the explanation that the tolerant phenotype observed in PR plants could be due to a better nutrient management and minor damages in root morphology under Cd stress. Moreover, under Cd exposure, CR showed more severe symptoms such as decrease in growth, suggesting that intracellular detoxification or defense mechanisms maybe exist or be more efficient in the PR genotype.

Finally, the last chapter brings a large scale quantitative proteomic approach employed to determine alterations in the protein profile of tomato roots exposed to Cd. Tomato genotypes were grown in hydroponics and exposed to Cd over four days. The spectral counting revealed a total of 380 differentially accumulated proteins (DAP), which 62 were shared between both genotypes which showed similar alterations after Cd exposure. In general, CR genotype presented higher number of DAP compared to PR. DAP showed alterations in diverse pathways, including proteins involved in cell wall, stress response and redox activities. Cell wall related proteins such as chitinases, believed to act as second line defense components, showed contrasting responses in tomato genotypes, decreased accumulation in tolerant and increased in sensitive genotype. Regarding to antioxidant responses Cd-induced decreases in antioxidant enzymes such as SOD and CAT, as well as observed in the first chapter. Increased accumulation of GST and TRX were

detected in tolerant genotype. Among the changes in root proteome profile of sensitive genotype we can highlight the increased abundance of GST, thus important decreases in SOD, MDHAR and TRX were detected. Taken together, the present study sheds light on molecular mechanisms involved in Cd tolerance in tomato genotypes and suggests a more active involvement of GST, TRX and peroxidases in Cd-induced stress response.

Summarizing, the tolerant genotype PR accumulated lower levels of Cd in the shoots, exhibited higher growth rate and higher levels of MDA in roots compared to sensitive genotype CR. Also, an initial enhancement of the GSH pool was detected in tolerant roots, which might allow the conjugation of oxidative stress metabolites, together with increased activities of APX, GR and GST, in addition to the higher NUE due to minor damage in root morphology determined the tolerance to Cd in PR plants. Furthermore, protein profile of PR roots altered by Cd exposure reinforces the hypothesis of an SOD and CAT independent defense system active in these plants. In this context, takes place the POD isoenzymes, important in oxidative stress response and it were decreased in sensitive genotype. The next step is to analyze carefully the metabolic pathways altered by the metal, as well as exclusive proteins of tolerant genotype, in the attempt to establish a molecular model of response to Cd in tomato contrasting genotypes. The proteomics opened a new opportunity in the study of Cd-induced stress response providing some biomarkers worth to be exploited.