SÃO PAULO STATE UNIVERSITY - UNESP

CAMPUS OF JABOTICABAL

SELENIUM AND SULPHUR: MITIGATION IN PLANT STRESSES

Leonardo Warzea Lima

Biologist

2016

SÃO PAULO STATE UNIVERSITY - UNESP

CAMPUS OF JABOTICABAL

SELENIUM AND SULPHUR: MITIGATION IN PLANT STRESSES

Leonardo Warzea Lima

Advisor: Dr. Priscila Lupino Gratão

Co-Advisor: Dr. André R. dos Reis

Dissertation submitted to the College of Agricultural and Veterinary Sciences – UNESP, Campus of Jaboticabal, in partial fulfillment of the requirements for the degree of Master of Science in Agronomy (Crop production).

2016

Lima, Leonardo Warzea
L732s Selenium and Sulfur: Mitigation in plant stresses / Leonardo Warzea Lima. – – Jaboticabal, 2016
xi, 97 f. : il. ; 28 cm

Dissertação (mestrado) – Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, 2016
Orientador: Priscila Lupino Gratão
Co-orientador: André Rodrigues dos Reis
Banca examinadora: Tiago Tezzoto, Tiago Santana Balbuena
Bibliografia

1. Estresse oxidativo. 2. Cádmio. 3. Selênio. 4. Enxofre. I. Título. II. Jaboticabal-Faculdade de Ciências Agrárias e Veterinárias.

CDU 635.64:546.4

Ficha catalográfica elaborada pela Seção Técnica de Aquisição e Tratamento da Informação – Serviço Técnico de Biblioteca e Documentação - UNESP,Câmpus de Jaboticabal

unesp

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO: SELENIUM AND SULPHUR: MITIGATION IN PLANT STRESSES

AUTOR: LEONARDO WARZEA LIMA
ORIENTADORA: PRISCILA LUPINO GRATÃO

Aprovado como parte das exigências para obtenção do Título de Mestre em AGRONOMIA (PRODUÇÃO VEGETAL), pela Comissão Examinadora:

Profa. Dra. PRISCILA LUPINO GRATÃO
Departamento de Biologia Aplicada À Agropecuária / FCAV / UNESP - Jaboticabal

Prof. Dr. TIAGO TEZOTTO
Centro Universitário Octávio Bastos / UNIFEOB - São João da Boa Vista/SP

Pesquisador TIAGO SANTANA BALBUENA
Departamento de Tecnologia / FCAV / UNESP - Jaboticabal

Jaboticabal, 18 de maio de 2016.

AUTHOR'S CURRICULUM INFORMATION

Leonardo Warzea Lima was born on March 01, 1987 in São Paulo, Brazil. His parents Eliana Regina Warzea Lima and Wladimir Godoy Lima. In 2005 he finished the high school in São Paulo. In 2010 his career path was clear and started to study for his bachelor degree in Biology at the São Paulo State University (UNESP), campus of Jaboticabal, which provided a rich and solid background for his research career. Among all the experiences on different departments, the one year training at the Plant Physiology Laboratory in 2012, working on the area of abiotic stress, was a watershed experience. Under the supervision of the Dr. Priscila Lupino Gratão, he could be surrounded by the routine of a laboratory with basic training and the specific methodology used to measure the level of the oxidative stress on different vegetable tissues. At the same year, he started his Undergraduate Research Mentorship Program, when received a scholarship to develop the project called "Selenium antioxidant effects on fruits of mutant tomato in response to the exposure to Cadmium". After one year studying Selenium (Se) and the heavy metal contamination, the interest in using science to minimize vegetable stress and environmental impact of different pollutants started to bloom. In 2013 he got involved with different academic activities at the university, occupying the position of public relations at the Government of Students Union and the student's representative to the Board of the Department of Biology, furthermore he created the Biological Sciences Academic Journal called "Conexão (Connection)", which became a powerful way to disseminate information and ideas through interviews and articles among students. As a curious Biologist, passionate about plant physiology, Leonardo was approved in 2014 to continue his studies on a Master of Science degree program in Agronomy, at the same University, in order to prepare for the long term goal of pursuing a career of teaching and research. As a Master's student, he became more independent, creative and proactive, able to conduct the research on the analysis of the antioxidants effects of Se and also to look forward to work on related areas of research. In March 2016, Leonardo was approved to continue his studies on a PhD program in Botany at the Colorado State University, Fort Collins-USA, under the supervision of the Dr. Elizabeth A. H. Pilon-Smits.

“*Discovery consists of seeing what everybody has seen, and thinking what nobody has thought”.*

Albert von Szent-Györgyi

To my parents, Wladimir and Eliana, for the unconditional love and support.

SUMMARY

Pages

ABSTRACT 11
CHAPTER 1 – GENERAL CONSIDERATIONS 12
1. Introduction 12
2. Literature Review 13
2.1 Antioxidant defense mechanisms 13
2.2 Enzymatic defense mechanisms 13
2.2.1 Superoxide dismutase (SOD, EC 1.15.1.1) 13
2.2.2 Catalase (CAT, EC 1.11.1.6) 14
2.2.3 Glutathione (GSH) 17
2.3 Non-enzymatic defense mechanisms 19
2.4 Cadmium contamination and the induced stress 20
2.5 Selenium as a stress alleviation strategy in plants 22
3. References 23
CHAPTER 2 - SULFUR METABOLISM AND STRESS DEFENSE RESPONSES IN PLANTS 27
Abstract 27
Keywords 27
Introduction 27
Sulfur uptake and assimilation 29
Phytohormones in S assimilation 35
Sulfur oxidation states in the cell 37
Methionine biosynthesis 39
Sulfur compounds related to plant defense 41
Glutathione (GSH), metallothioneins (MTs) and phytochelatins (PCs) 45
Glucosinolates 46
Final Considerations 47
Acknowledgments 49
References 49
CHAPTER 3 - SELENIUM AND HEAVY METALS 65
1. Selenium: a powerful antioxidant 65
2. Antioxidant defense mechanisms 65
3. Role of selenium against heavy metal stress in plants 66

3.1 Cadmium (Cd) 66
3.2 Arsenic (As) 69
3.3 Lead (Pb) 70
3.4 Other heavy metals 70
4. Final considerations 71
5. References 72
CHAPTER 4 - SELENIUM ALLEVIATES CADMIUM TOXICITY IN TOMATO FRUITS 74
Abstract. 74
Keywords. 74
1. Introduction 75
2. Material and methods 76
2.1 Plant material and experimental treatments 76
2.2 Fruits dry weight 77
2.3 Fruits concentration of chlorophyll and carotenoids 77
2.4 Fruits proline concentration 78
2.5 Fruits Cd^{2+} and Se quantification and nutritional analyses 78
2.6 Cd^{2+} and Se Translocation index 79
2.7 Statistical analyses 79
3. Results and Discussion 80
3.1 Nutritional analyses. 80
3.2 Cd^{2+} and Se concentration and translocation index 83
3.3 Fruit dry weight 85
3.4 Chlorophyll and carotenoids concentration 87
3.5 Proline concentration 90
4. Conclusions 92
5. Acknowledgements 92
6. References 92

LIST OF FIGURES

Pages

Chapter 1 – General Considerations

Figure 1. Dismutation reaction of the $O_2^{-\bullet}$ by the SOD metalloenzyme…….....14
Figure 2. Haber-Weiss reaction……14
Figure 3. Chemical structure of Catalase (CAT)……15
Figure 4. Simplified scheme of the H_2O_2 degradation by CAT……16
Figure 5. Ascorbate-glutathione cycle (Halliwell-Asada)……..18
Figure 6. GSH and Phytochelatins synthesis in plants……19

Chapter 2 - Sulfur metabolism and stress defense responses in plants

Figure 1. Biosynthetic pathways for S-containing amino acids and their derivatives……..34
Figure 2. Sulfur-containing defense compounds……...38
Figure 3. Sulfur assimilation and its physiological functions……48

Chapter 3 - Selenium and heavy metals

Figure 1. Selenium effects against the oxidative stress……68

Chapter 4 - Selenium alleviates cadmium toxicity in tomato fruits

Figure 1. Fruits dry weight……86
Figure 2. Fruits pigments concentration……..89
Figure 3. Fruits proline concentration……..…91

LIST OF TABLES

Pages

Table 1. Nutrients concentration in fruits……82
Table 2. Selenium (Se) and Cadmium (Cd^{2+}) concentration in fruits, leaves, roots and translocation index TI)……..84

SELENIUM AND SULPHUR: MITIGATION IN PLANT STRESSES

ABSTRACT - Plants do not have specific defense mechanisms to counteract the diverse range of abiotic stresses and pollutants into the environment, and its survival depends on the flexibility and adaptability of its own natural defense mechanisms**.** Furthermore, the maintenance of cellular homeostasis depends on several interlinked and complex mechanisms, while the cellular defense system does not follow a specific pattern of action and may differ due to various factors such as plant species, exposure time to the stress, plant developmental stage, different organs and tissues analyzed. In the light of these considerations**,** this dissertation aimed to highlight and investigate the role of Sulfur and Selenium against different plant stresses, through the enzymatic and non-enzymatic plant responses and other related defense mechanisms. In the first chapter the author characterize the general biochemical mechanisms of the antioxidant cell defense, specifically the reactive oxygen species (EROs) formation and its chemical singularities and the induced oxidative stress, the enzymatic antioxidant defense system, specifically the superoxide dismutase (SOD) and Catalase (CAT) enzymes, the non-enzymatic mechanisms against the stress, including the Ascorbate-Glutathione cycle, the GSH (reduced glutathione), the phytochelatins and also proline formation. The plant nutritional status during the stress is crucial in order to maintain a proper defense response. In view of this, the chapter two is a published review about the participation of Sulfur (S) on the stress defense. This nutrient has a role in fundamental processes such as electron transport, structure, regulation and it is also associated with photosynthetic oxygen production, abiotic and biotic stress resistance and secondary metabolism. Moreover, few chemical elements are considered benefic to plants, while Selenium (Se) is the most relevant. In the chapter three the author describes the role of Se to detoxify the stress induced by heavy metal contamination, its powerful antioxidant characteristics and the improvement of the antioxidant enzymes activity and overall defense mechanisms. The chapter four consists of a scientific project conducted by the author. The aim of this study was to investigate whether Selenium, under the form of selenite (Na_2SeO_3), may avoid the uptake, translocation and concentration of Cadmium ($CdCl_2$), in different tomato tissues, indicating possible mechanisms to counteract the stress, as well as to analyze the fruits overall status through the nutritional analyses, dry weight, pigments and proline concentration. The results demonstrate that alleviating effect of Se in tomato under Cd contamination could be related to restriction of Cd^{2+} uptake and translocation, enhancing micronutrient concentration in fruits and, finally, enhancing fruit proline concentration.

Key-Words: Heavy metal, acclimation, stress adaptations, oxidative stress

CHAPTER 1 – GENERAL CONSIDERATIONS

1. Introduction

In order to facilitate the understanding and comprehension of the degenerative mechanisms triggered by different sources of abiotic stresses, the meaning of the word “stress” must be primarily defined under the vegetal perspective. Plants maintain a very intimate and necessary contact with the environment in which they are located, through the water and minerals absorption from the soil as well as the capture of the light energy from the sun, among other processes. Therefore, it is easy to assume that the environment exerts an important role to the physiology, anatomy and the plants biochemistry, influencing systemically their growth and development.

Consequently, any alterations of the adequate and specific environmental conditions for each vegetable species to develop accordingly and healthy can disrupt physical and chemical changes in the plant as a result of a primary reaction to the stress, such as water deficiency or excess of salts in the soil, which can cause the stomatal closure and an increased production of compatible osmolytes, like proline and glycine-betaine, for example (BANU, et al., 2009; SHEVYAKOVA et al., 2013). Such modifications are reversible and all plant metabolism regulates after the environmental conditions returns to the regular state. However, when the stress is strongly severe or persists for a long time an imbalance in the cellular redox state occurs, mainly due to the overproduction of reactive oxygen species (ROS) above the cellular antioxidant capacity, leading to the destruction of membrane lipids, proteins, nucleic acids and other cellular components, resulting in a secondary stress called oxidative (OPDENAKKER et al., 2012, IRFAN et al., 2013).

These ROS are unstable and partially reduced forms of the atmospheric oxygen (O_2), formed during the aerobic cellular metabolism in all cell organelles that have the electron transport chain (the oxygen acts as the final electron acceptor in the chain) or a highly oxidized metabolic rate, such as mitochondria and peroxisomes (CUYPERS, et al., 2010). These ROS results from the transfer of one, two or three electrons to the O_2 molecule, forming respectively the superoxide radical ($O_2^{-\bullet}$), the hydrogen peroxide (H_2O_2) or the hydroxyl

radical ($OH^{\bullet}$) and also by the O_2 excitation processes which generates the "Singlet" oxygen (SHIEBER and CHANDEL, 2014).

The O_2 is a stable and not reactive molecule but, contrarily, these ROS are able to oxidize and denature many other molecules and structures due to the unpaired number of electrons in their final outer shell, leading to the cell destruction and thus to the death of tissues and organs such as roots, leaves, fruits or seeds. Consequently, the oxidative stress occurs when there is a serious imbalance between the ROS production and the antioxidant defense system, in any cellular compartment.

2. Literature Review

2.1 Antioxidant defense mechanisms

The cellular defense mechanisms corresponds to the enzymatic and non-enzymatic antioxidant responses capable to directly denature the ROS, such as the superoxide radical ($O_2^{-\bullet}$) or the hydrogen peroxide (H_2O_2), and also neutralize the stressor which is in excess in the cell environment (normally a metal ion), thus neutralizing the deleterious effects of the reactive processes.

2.2 Enzymatic defense mechanisms

2.2.1 Superoxide dismutase (SOD, EC 1.15.1.1)

The superoxide dismutases (SODs) are metalloenzymes that constitute the first enzymatic barrier against the induced oxidative stress, catalyzing the dismutation reaction of the $O_2^{-\bullet}$, forming O_2 (cell oxygen) and H_2O_2 (hydrogen peroxide) (SHIEBER and CHANDEL, 2014) (Figure 1). It is important to mention that the antioxidant process triggered by the SODs can be considered as the primary defense mechanism of the plant cells against the ROS, mainly because it interferes negatively and directly in the Haber-Weiss reaction (Figure 2), which produces the highly reactive and not destructible hydroxyl radical ($OH^{\bullet}$), from the combination of $O_2^{\bullet}$ and H_2O_2 (CUYPERS et al., 2010).

Figure 1. Dismutation reaction of the $O_2^{-\bullet}$, forming O_2 (cell oxygen) and H_2O_2 (hydrogen peroxide), by the superoxide dismutases (SODs) metalloenzymes.

Figure 2. Haber-Weiss reaction: hydroxyl radical (OH•) formation from the combination of $O_2^{\bullet}$ and H_2O_2.

These SODs enzymes are present in different plant tissues and are mainly found in chloroplasts, the mitochondrial matrix and the cytoplasm of cells. Moreover, three different isoforms of this metalloenzymes are known, differing by the metal ion present in the molecule active site and also by the action location in the cells, while the most abundant isoform in vegetables is the SOD which have copper (Cu) and zinc (Zn) in its active site (Cu / Zn - SODs), found mainly in the stroma of chloroplasts and also in the cytosol. Another important isoform are those SODs which have manganese (Mn) on the active site, present in the mitochondrial matrix. On the other hand, the SOD which have Fe (iron) in its active site are rarely found in plants, however this isoform can be associated with chloroplasts (KUMAR et al., 2014). These SOD isoforms are very similar when are compared among different plant species, so that the differences are associated with the isoform found and the concentration in the tissues.

2.2.2 Catalase (CAT, EC 1.11.1.6)

The hydrogen peroxide (H2O2) is an unstable and highly reactive molecule and its concentration generally becomes higher in the cellular environment during a stressful condition and also as a result of the enzymatic reaction of the SOD, which can induce the oxidative damage of other molecules and tissues. Consequently, the H_2O_2 can be quickly converted into H_2O (water) and O_2 (cell oxygen) by the specific action of enzymes such as catalase (CAT,

EC 1.11.1.6) and other peroxidases in different cellular compartments (ROYCHOUDHURY et al. 2012).

Catalase was the first antioxidant enzyme discovered and characterized, and basically consists of a polypeptide with an approximate weight of 70 kDa, arranged in a tetrameric molecule, so that each monomer contains a prosthetic heme group with a central atom of Fe, as shown in figure 3 (MHANDI et al., 2010).

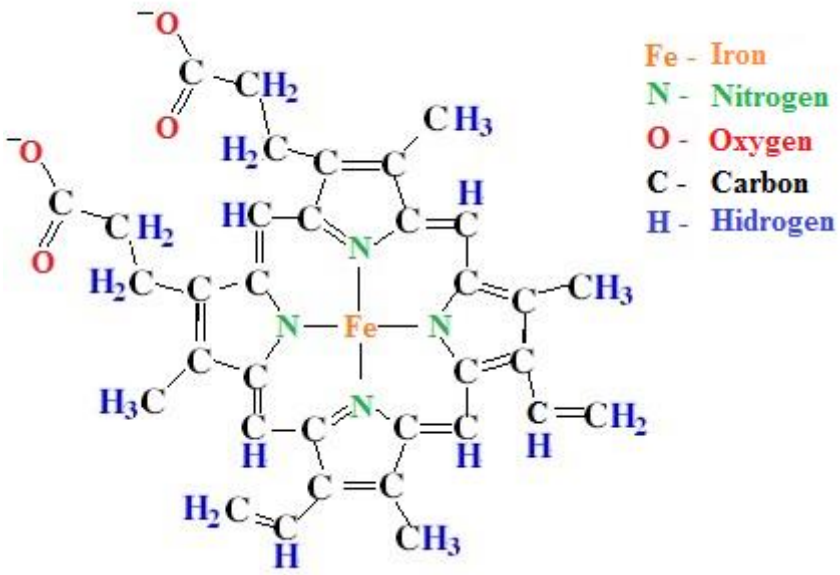

Figure 3. Chemical structure of Catalase (CAT).

This enzyme can be found in all living organisms and is the main route of H_2O_2 degradation in order to form H_2O and O_2, according to the reaction shown in Figure 4. The dismutation reaction occurs in four distinct steps, so that the O_2 molecule formed at the end of the reaction (step 4) is derived from a single molecule of H_2O_2, while the two H_2O molecules formed during the process (steps 2 and 4) are derived from the combination of the two H_2O_2 substrates (Figure 4).

Additionally, both CAT and other peroxidases primarily reduces the H_2O_2 molecule by breaking the O_2 binding site, forming a molecule of water and one intermediate molecule called compound I, where the central atom of Fe (from the CAT enzyme) covalently binds to the liberated oxygen after the H_2O_2 reduction (step 2). The CAT specific activity consists of the oxidation of a second H_2O_2 molecule to form O_2 and H_2O (step 4), while Fe is reduced again

to its original oxidation state and the process restarts (step 4 and 1). Finally, it is important to mention that CAT is unique among all the other enzymes responsible for the H_2O_2 degradation (peroxidases), mainly because it does not use any equivalent reducing agent, acting directly and efficiently during the entire process (Figure 4).

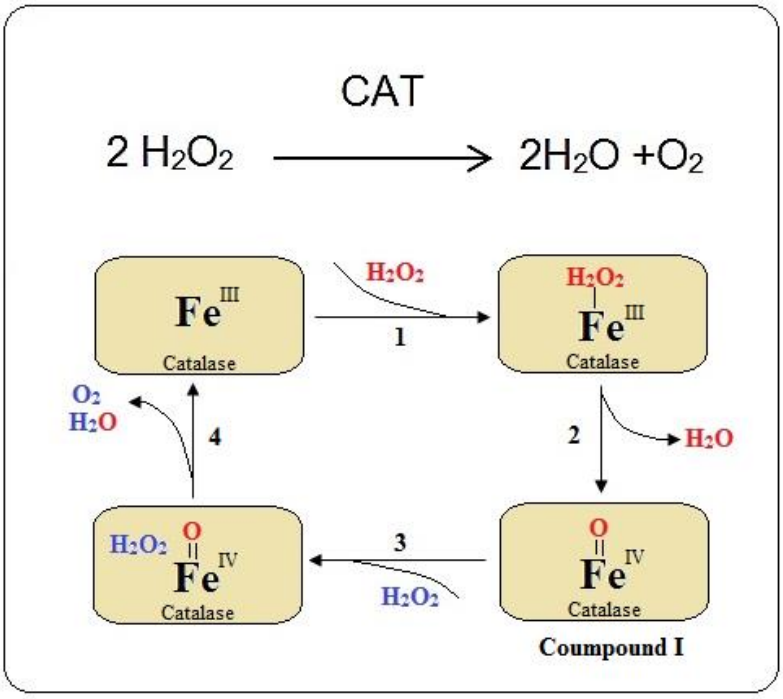

Figure 4. Simplified scheme of the H_2O_2 degradation catalyzed by the CAT enzyme, resulting in H_2O (water) and O_2 (oxygen) formation. Both H_2O_2 molecules used in the process as well as their products from their degradation (steps 2 and 4) are represented in the same color. Roman numbers represent the oxidation state of the Fe atom. Adapted from Mhamdi et al., 2010.

Furthermore, CAT has three different types of isoenzymes referred as CAT1, responsible for 80% of the total H_2O_2 degradation in the cell (specifically in peroxisomes), formed during the photorespiration process in chloroplasts. CAT2, mainly found in vascular tissues and CAT3, located in the mesophyll of leaves (MHAMDI et al., 2010). This enzyme is essential for the H_2O_2 denaturation process in plants that have higher rates of photorespiration, such as all the C3 plants.

Other enzymes are also responsible for the H_2O_2 degradation in the cell environment. Peroxidases are hemeproteins (have a heme group in the

molecule, characterized as a central iron atom in an organic porphyrin ring) responsible for the H_2O_2 molecule reduction concomitantly with the oxidation of a specific substrate, as the ascorbate peroxidase (APX, EC 1.11.1.11), glutathione peroxidase (GPX, EC 1.11.1.9) and guaiacol peroxidase (GPOX activity, EC 1.11.1.7), for example. These peroxidases participate in many essential metabolic processes such as the cell growth regulation, lignification, phenolic oxidation, defense against pathogens, antioxidant defense and protection against various stresses.

The guaiacol peroxidase (GPOX, EC 1.11.1.7) enzyme, for example, is also part of this group and presents basics and acids isoforms in plants. The acid isoform is directly related to the processes involved in the cell wall biosynthesis, including the lignin formation, while its basic isoform participates in the regulation of the AIA (indolylacetic acid, auxins) degradation. In vitro experiments the GPOX catalyze the hydrogen donors oxidation, in the absence of a specific substrate, however, in vivo, the detoxification can become the primary function of certain isoforms.

2.2.3 Glutathione (GSH)

Glutathione (tripeptide formed from the amino acids glutamate, cysteine and glycine) is a major metabolite in the defense system against ROS and its degenerative effects during the enzymatic antioxidant defense, participating along with the ascorbate peroxidase (APX) enzyme in the Halliwell-Asada cycle or ascorbate-glutathione cycle (Figure 5), being necessary to the proper functioning of this cycle. This metabolite can be found in the plant under the oxidized form (GSSG) or also the reduced form (GSH), which is important for non-enzymatic antioxidant defense (see section 2.3), so that the system always prioritize an increased concentration of GSH over the GSSG in the cellular environment.

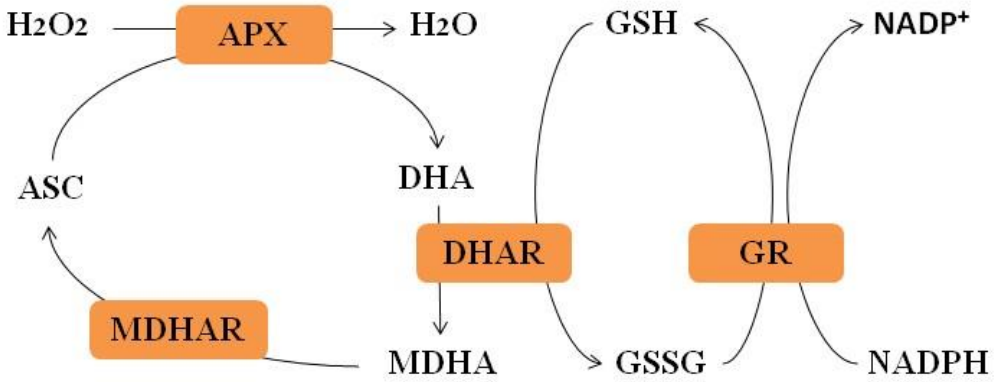

Figure 5. Ascorbate-Glutathione cycle (Halliwell-Asada). Enzymes: ascorbate peroxidase (APX, EC 1.11.1.11), dehydroascorbate reductase (DHAR, EC 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), glutathione reductase (GR, EC 1.6.4.2). Other compounds: hydrogen peroxide (H_2O_2), water (H_2O), ascorbate (ASC), dehydroascorbate (DHA), monodehydroascorbate (MDHA), glutathione (GSH), oxidized glutathione (GSSG). Adapted from Inzé and Montago (1995).

In this cycle, the antioxidant action occurs by the conversion of the H_2O_2 molecule into H_2O through the APX enzyme; however, the process is completely dependent upon the glutathione conversion cycle (Figure 5). The enzyme glutathione reductase (GR, EC 1.6.4.2) is responsible for the conversion of GSSG into GSH using as an electron donor the NADPH, while the formed GSH corresponds to the substrate used by the dehydroascorbate reductase (DHAR) enzyme in the ascorbate (ASC) formation pathway, which is used as a substrate for the APX enzyme antioxidant activity (Figure 5) (INZÉ and MONTAGO, 1995).

The glutathione reductase (GR) enzyme is almost of universal occurrence, being found in eukaryotes and in prokaryotes, from heterotrophic and photosynthetic bacteria to higher plants. This enzyme has the prosthetic group FAD (flavin adenine dinucleotide), responsible for catalyzing the electron transfer reaction from NADPH to GSSG, forming the GSH (VOET and VOET, 1995).

2.3 Non-enzymatic defense mechanisms

The non-enzymatic mechanisms for cellular detoxification against the ROS are important and act jointly with the enzymatic antioxidant system in order to maintain the cellular redox state. Participate in these processes the phytochelatins (PCs), proline, flavonoids, alkaloids and carotenoids, among others (FOYER and NOCTOR, 2012). The phytochelatins represent the most important route against the metals, semimetals and heavy metals contamination, since these molecules have the ability to complex and inactivate these compounds, storing them into vacuoles in the cell.

The glutathione (GSH) plays an essential role in both the enzymatic antioxidant defense against ROS and the PCs formation, and determining a pathway of action will result in the inactivation of the other corresponding via (ROYCHOUDHURY et al., 2012). The GSH synthesis process involves glutamate, glycine and cysteine, and the PCs are synthesized from the GSH already formed, as shown in Figure 6. The GSH formation consists of two reactions triggered by γGlutamate-cysteine ligase (EC 6.3 .2.2) and GSH synthase (EC 6.3.2.3) enzymes, while the conversion of GSH in phytochelatins occurs by the specific action of the glutathione-γGlutamyl-cysteinyl-transferase (EC 2.3.2.15) enzyme or PC synthase (Figure 6).

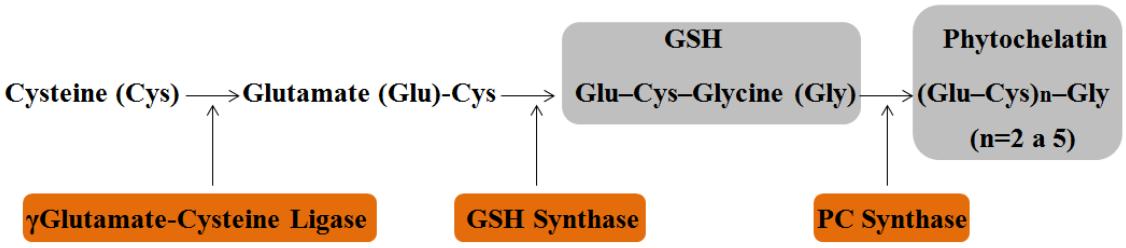

Figure 6. GSH and Phytochelatin synthesis in plants. Adapted from Inouhe, 2005.

The amino acid proline is part of the compatible osmolytes group, which is responsible for the cell osmotic adjustment maintenance during the water and salt stress and also by the excess of nutrients in the soil, with an important role in plant protection against other different abiotic stresses, such as the exposure

to low temperatures, soil acidity and the heavy metals exposure (SHEVYAKOVA et al., 2013).

Furthermore, different studies demonstrate that this osmolyte is able to actively clean the ROS from the cell during the oxidative stress, besides conferring protection and stabilization of some cellular structures such as membranes, proteins and also enzymes during severe stresses (BANU et al., 2009). It is important to note that the products resulting from the proline molecule catabolism, after the alleviation of the stress condition, will be used in the oxidative phosphorylation process in mitochondria, generating molecules of ATP (adenosine 5'-triphosphate), which is important in the cellular recovery processes (ASHRAF and FOOLAD, 2007).

Additionally, the proline synthesis in plants occurs in chloroplasts and also in the cytoplasm, with the glutamic acid as a precursor. This acid is converted to glutamate γ-semialdehyde acid (GSA) by the Δ1-pyrroline-5-carboxylate synthetase (P5CS) enzyme, which in turn is spontaneously converted to the Δ1-pyrroline-5-carboxylate (P5C), which it is then reduced to proline by the P5C reductase enzyme (VERBRUGGEN and HERMANS, 2008).

In summary, the non-enzymatic antioxidant system operates through different mechanisms responsible for the direct elimination of the stressor, cellular homeostasis regulation or even with molecules that acts directly as antioxidants, such as flavonoids, which inactivate the ROS, and alkaloids or carotenoids which are capable to donate H^+, which mitigates the negative effects of the oxidation processes caused by the reactive oxygen species.

2.4 Cadmium contamination and the induced stress

Industrial activities involved in metal smelting and the increasing in mining processes on a global scale can be considered as the major sources of environmental contamination. Heavy metals are among the main pollutants generated by these sources and represents a major threat to living organisms because of its high toxicity, persistence in the environment and bioaccumulation (LIU et al., 2010, 2011).

However, several other anthropogenic activities are responsible to release into the environment a large amount of these pollutants, which accumulates in

the soil and limit the plant productivity (ZHOU et al., 2013). According to Xu et al. (2013), the presence of heavy metals in agricultural soil, such as Cadmium (Cd), occurs as a consequence to the excessive use of phosphate fertilizers and pesticides, which allow its assimilation in crops and the consequent transfer through the food chain, representing a great danger to human and animal health.

Cadmium is the most toxic of all heavy metals. It is responsible to cause numerous health problems to humans (XU et al., 2013), because of its accumulation in different organs such as kidneys, liver and central nervous system, with a half-life that can reach fifteen to thirty years in the organism (GONÇALVES et al., 2012). Due to its strong global demand, approximately 30000 tons of Cd are released into the atmosphere every year, while 4000 to 14000 tons of this total amount comes only from industrial activities, such as the rechargeable batteries, alloys for welding, pigments and paints industry (ATSDR , 2012).

The presence of Cd in high concentration in the soil inhibits the plant growth (CHENN et al., 2011; IRFAN et al., 2013) causes a decrease in the chlorophyll and carotenoids content (LIU et al., 2010, 2011), affect the proper function of chloroplasts and the CO_2 fixation, induces the antioxidant enzymes inactivation and the ROS overproduction, leading to the lipid peroxidation process and the consequent oxidative stress (XU et al., 2013).

It is important to mention that Cd is not capable to induce the ROS overproduction directly but reacts with the Sulphur present in the thiol group in the cysteine and other different proteins, which results in a lower GSH concentration in the cell environment. This process results to a deflected defense system against the oxidative stress (CUYPERS et al., 2010). Furthermore, the increased concentration of iron (Fe) ions in the cellular environment, due to its replacement by Cd in different proteins, may also induce the production of OH^- radicals, which are highly reactive and resistant to cellular defense mechanisms, resulting in a severe oxidative stress (DORTA et al., 2003).

Great number of proteins and enzymes have metals in their active sites, the capacity of Cd to replace them due to chemical similarities molecules it is notable. Consequently, the antioxidant system metalloenzymes, such as the

SOD, can be inactivated by this replacement (GONÇALVES et al., 2008). According to Guimarães et al. (2008) CAT levels in peroxisomes are also drastically reduced in the presence of 50 mM of Cd due to its oxidation, which also impairs the oxidative stress management.

Leaf chlorosis is also a regular symptom of the Cd induced stress, which demonstrates that the cellular photosynthetic apparatus is severely affected by this heavy metal. The molecular structure of chlorophyll is characterized by the presence of a central Magnesium (Mg) atom and its replacement by Cd alters its stability, decreasing the absorption of the light energy and the consequent production of carbohydrates (CHENN, et al., 2011; XU et al., 2013). The leaf chlorosis appearance can also be explained by the decrease in the amount and replication of chloroplasts in the cell, deficiency of phosphorus (P), manganese (Mn) and a negative influence on the activity of the enzymes related to the production of chlorophyll (GUIMARÃES et al., 2008).

2.5 Selenium as a stress alleviation strategy in plants

Selenium (Se) is an essential micronutrient for humans and animals and exerts important roles in the organism, participating on the antioxidant defense system (CATANIA et al., 2009). The absence or inadequate ingestion of this nutrient in the diet can cause health problems related to malnutrition. In vegetables, Se in low concentrations exerts positive physiological effects, such as growth improvement, reduction of ROS concentration and lipid peroxidation, improves the accumulation of starch and sugars and provides a delayed senescence (XUE et al, 2001; FENG and WEI, 2012). Despite not been recognized as a nutrient, some studies demonstrate the beneficial effects to the plants, such as the increased antioxidant capacity after Se application (XUE et al., 2001; ZEMBALA et al, 2010) and also its ability to reduce the heavy metals availability while alleviate its toxic effects (MUÑOZ et al., 2007) (see chapters 3 and 4 for more detailed information regarding selenium and heavy metals).

This mineral can be found under different forms in the soil, such as selenite (SeO_3^{2-}), selenate (SeO_4^{2-}) and also under its organic molecules like SeCys (selenium atom bonded to a cysteine molecule) and SeMet (selenium

atom bonded to a methionine molecule), which are assimilated by the roots (NOWAK, 2013).

Furthermore, the presence of Se in the intracellular environment induces an increased levels and activity of different antioxidant enzymes, thereby regulating the concentration of ROS (see chapter 3). The glutathione peroxidase (GSH-Px) enzyme, for example, demonstrates an enhanced activity after the Se application, eliminating effectively the H_2O_2 and alleviating the oxidative stress (ZEMBALA et al, 2010; FENG and WEI, 2012). According to Feng and Wei (2012), Se can also stimulate the spontaneous dismutation of O_2^- into H_2O_2 radical, without the catalytic process exerted by the SOD enzyme. However, these authors report that excessive Se concentrations can lead to an imbalance in the levels of GSH, thiol radicals (-SH) and also the NADPH in the cell, which are important for the ROS elimination and stress defense mechanisms.

3. References

ASHRAF, M.; FOOLAD, M.R. Roles of glycine betaine and proline in improving plant abiotic stress resistance. **Environ. Exp. Bot**. v. 59, p. 206–216. 2007.

ATSDR; Public Health Statement, **Agency for Toxic Substances and Disease Registry**, 2012.

BANU, M.N.A.; HOQUE, M.A.; SUGIMOTO, M.W.; MATSUOKA, K.; NAKAMURA, Y.; SHIMOISHI Y.; MURATA, Y. Proline and glycinebetaine induce antioxidant defense gene expression and suppress cell death in cultured tobacco cells under salt stress. **J Plant Physiol**. v. 166, p. 146-156. 2009.

CATANIA, A. S.; BARROS, C. R.; FERREIRA S. R. G. Vitaminas e minerais com propriedades antioxidantes e risco cardiometabólico: Controvérsias e perspectivas, **Arq Bras Endocrinol Metab.** 2009.

CHENN, X.; WANG, J.; SHI, Y.; ZHAO, M.Q.; CHI, G. Y.I. Effects of cadmium on growth and photosynthetic activities in pakchoi and mustard. **Bot. Stud**. v. 52, p. 41-46, 2011.

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. Cadmium stress: an oxidative challenge. **Biometals.** v. 23, n. 5, p. 927-940. 2010.

DORTA, D. J.; LEITE, S.; DEMARCO, K. C.; PRADO, I. M. R.; RODRIGUES, T.; MINGATTO, F. E.; UYEMURA, S. A.; SANTOS D. A. C.; CURTI, C. A proposed sequence of events for cadmium-induced mitochondrial impairment. **J. Inorg. Biochem**. v. 97, p. 251–257, 2003.

FENG, R.W.; WEI, C.Y.; Antioxidative mechanisms on selenium accumulation in Pteris vittata L., a potential selenium phytoremediation plant. **Plant Soil and Environment**, v. 58, p. 105‑110, 2012.

FOYER, C. H.; NOCTOR, G. Managing the cellular redox hub in photosynthetic organisms. **Plant, Cell Environ**. v.35, n. 2, p. 199-201, 2012.

GONÇALVES, V.C.; MEURER, E.J.; TATSCH, F.R.P.; CARVALHO, S.A.; SANTOS NETO, O.A. Biodisponibilidade de cádmio em fertilizantes fosfatados. **Rev. Bras. Ciênc. Solo**, v. 32, p. 2871-2875, 2008.

GONÇALVES, V.C.; MEURER, E.J.; TATSCH, F.R.P.; CARVALHO, S.A.; SANTOS NETO, O.A. Behavior and brain enzymatic changes after long-term intoxication with cadmium salt or contaminated potatoes. **Food Chem. Toxicol.** v. 50, p. 3709–3718, 2012.

GUIMARÃES, M.A.; SILVA, D.J.H.; FONTES, P.C.R.; MATTEDI A.P. Produtividade e Sabor dos Frutos de Tomate do Grupo Salada em função de podas. **Biosci. J.** v. 24, p.32-38, 2008.

INOUHE, M. Phytochelatins. **Braz. J. Plant Physiol**. v. 17(1), p.65-78, 2005.

INZÉ, D.; Van MONTAGO, M. Oxidative Stress in Plants. **Curr. Opin. Biotech**. v.6, p.153-158, 1995.

IRFAN, M.; AHMAD, A.; HAYAT, S. Effect of cadmium on the growth and antioxidant enzymes in two varieties of *Brassica juncea*. **Saudi J. Biol. Sci**. 2013.

KUMAR, M.; SUGATHA, P.; HEBBAR, K.B. Superoxide Dismutase Isozymes and their Heat Stability in Coconut (*Cocosnucifera* L.)Leaves. **Ann. Biol. (Hissar)**, v. 30, p. 593-597, 2014.

LIU, X.Q., PENG, K.J., WANG, A.G., LIAN, C.L., SHEN, Z.G. Cadmium accumulation and distribution in populations of Phytolacca americana L. and the role of transpiration. **Chemosphere**, 78, 1136–1141. 2010.

LIU, C.F.; GUO, J.L.; CUI, Y.L.; LÜ, T.F.; ZHANG, X.H.; SHI, G. Reffects of cadmium and salicylic acid on growth, spectral reflectance and photosynthesis of castor bean seedlings. **Plant Soil**, v. 344, p. 131–141, 2011.

MHANDI, A.; QUEVAL, G.; CHAOUCH, S.; VANDERAUWERA, S.; VAN BREUSEGEM, F.; NOCTOR, G. Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. **J. Exp. Bot**. p. 1-24, 2010. DOI:10.1093/jxb/erq282

MUÑOS, A. H. S.; WROBEL, K.; CORONA, J. F. G. The protective effect of selenium inorganic forms against cadmium and silver toxicity in mycelia of *Pleurotus ostreatus*. **Mycol. Res.**, v. 5, p. 626-632. 2007.

OPDENAKKER, K.; REMANS, T.; KEUNEN, E.; VANGRONSVELD, J.; CUYPERS, A. Exposure of Arabidopsis thaliana to Cd or Cu excess leads to oxidative stress mediated alterations in MAPKinase transcript levels. **Environ. Exp. Bot.,** v. 83, p. 53-61, 2012.

ROYCHOUDHURY, A.; BASU, S.; SENGUPTA, D.N. Antioxidants and stress-related metabolites in the seedlings of two indica rice varieties exposed to cadmium chloride toxicity. **Acta Physiol. Plant**. v. 34, n. 3, p. 835-847, 2012.

SHEVYAKOVA, N.I.; MUSATENKI, L.I.; STETSENKO, L.A.; RAKITIN, V.Y.; VEDENICHEVA, N.P.; KUSNETSOV, V.V. Effect of ABA on the Contents of Proline, Polyamines, and Cytokinins in the Common Ice Plants under Salt Stress. **Russ. J. Plant Physiol**. v. 60, p. 741–748, 2013.

SHIEBER, M.; CHANDEL, N.S.. ROS Function in Redox Signaling and Oxidative stress. **Crr. Biol.** v.19,24(10), 2014. DOI: 10.1016/j.cub.2014.03.034.

VERBRUGGEN, N.; HERMANS, C. Proline accumulation in plants: a review. **Amino Acids.** v. 35, p. 753–759, 2008.

VOET, D.; VOET, J.G. Biochemistry, 2ª ed. New York, 1361p. 1995.

XU, D; CHEN, Z.; SUN, K.; YAN, D.; YANG, M.; ZHAO, Y. Effect of cadmium on the physiological parameters and the subcellular cadmium localization in the

potato (*Solanum tuberosum* L.). **Ecotox. Environ. Safety**, v. 97, p. 147-153, 2013.

XUE, T.L.; HARTIKAINEN, H.; PIIRONEN, V. Antioxidative and growth-promoting effect of selenium on senescing lettuce. **Plant Soil**, v. 237, p. 55–61. 2001.

ZEMBALA, M.; FILEK. M.; WALAS, S.; MROWIEC, H.; KORNÁS, A.; MISZALSKI, Z.; HATKAINEN, H. Effect of Selenium on macro and microelement distribution and physiological parameters of rape and wheat seedlings exposed to cadmium stress. **Plant and Soil**, v. 329, p. 457-468, 2010.

ZHOU, Y.; NING, X.; LIAO, X.;LIN, M; LIU, J.; WANG, J. Characterization and environmental risk assessment of heavy metals found in fly ashes from waste filterbag sobtained from a Chinese steelplant. **Ecotox. Environ. Safety**, v. 95, p. 130-136, 2013.

CHAPTER 2 - SULFUR METABOLISM AND STRESS DEFENSE RESPONSES IN PLANTS*

Abstract

Sulfur management is an important issue in crop plant nutrition. Sulfur has a role in fundamental processes such as electron transport, structure and regulation. It is also associated with photosynthetic oxygen production, abiotic and biotic stress resistance and secondary metabolism. Sulfate uptake, reductive assimilation and integration into cysteine and methionine are the central processes that direct oxidized and reduced forms of organically bound S into their various functions. Sulfur-containing defense compounds that are crucial for plant survival during biotic and abiotic stress include elemental sulfur, hydrogen sulfide, glutathione, phytochelatins, S-rich proteins and various secondary metabolites. Formation of these compounds in plants is closely related to the supply, demand, uptake and assimilation of S. This review will highlight the role of S during the stress response in plants and the relationship between S metabolism and primary S nutrition.

Keywords

Abiotic stress, Antioxidants, Oxidative stress, Plant nutrition, Sulfur uptake and metabolism

Introduction

Environmental variation triggers plant acclimation, adaptation or death. Natural and anthropogenic activities induce biotic and abiotic stresses during agricultural and forestry operations. Plant metabolism is often damaged by toxic compounds and hazardous chemicals present in soils and air (Su et al. 2014; Iannone et al. 2015). Thus, acclimation and adaptation processes are crucial to plant survival, and the identification and understanding of plant tolerance mechanisms are of major importance. Stress can be defined as any alteration in normal plant growth conditions (Boaretto et al. 2014). Some of these alterations

*Published. Journal: Tropical Plant Biology (2015) 8:60–73. DOI 10.1007/s12042-015-9152-1

are related to temperature, salinity, water supply, ozone, soil acidification and heavy metal toxicity, among others (Azevedo et al. 1998; Monteiro et al. 2011; Bulbovas et al. 2014; Nogueirol et al. 2015). Studies of plants have been conducted to evaluate the effects of these changes on growth and development. Plants possess very efficient defense pathways that allow the scavenging of reactive oxygen species (ROS), protecting the cells from oxidative damage (Gratão et al. 2005).

The primary function of regulatory mechanisms is to manage fluxes of sulfur (S) in response to developmental and environmental changing conditions. The goal for the plant is to optimize the use of available S to match the demands for growth and development, and resistance to stress (Hawkesford 2012). Sulfur assimilation starts from the uptake of external sulfate by the activity of sulfate transporter (SULTR) in roots. On the other hand, plants are able to use foliar absorbed H_2S as S source for growth, especially under conditions where the S to the roots is limited (Koralewska et al. 2008). Sulfate is activated by Adenosine-5´-triphosphate sulfurylase (ATPS, EC 2.7.7.4) and then catalyzed by Adenylyl-sulfate reductase (APR, EC 1.8.99.2) and sulfite reductase (EC 1.8.7.1) to produce sulfide. Regarding the primary metabolism in plants, nitrate and sulfate need to be reduced prior to their incorporation into various essential organic nitrogen (N) and S compounds. The uptake and assimilation of S and N are strongly interrelated, since the major proportion of the reduced N and S in plants is incorporated into amino acids and subsequently into proteins (Stulen and De Kok 2012). An important coordination with C/N metabolism occurs at the level of cysteine and methionine biosynthesis, with the cysteine synthase complex (serine acetyltransferase (SAT, EC 2.3.1.3) and O-acetylserine(thiol)lyase (OASTL, EC 4.2.99.8)) acting as both a sensor and a regulator, mediated by a reversible association/dissociation of the complex. SAT is active when associated with OASTL, but inactive when dissociated. As the dissociation is promoted by excess OAS, the complex effectively senses both OAS and S availability and self regulates further OAS production accordingly (Hawkesford 2012). Thus, OAS is a signal mediating between substrate availability and flux. OASTL, which is in excess, will always catalyze synthesis of cysteine given availability of OAS and sulfide. The formation of these compounds is closely related to the

supply, demand, uptake and assimilation of S in plants. In this review, we provide essential information about the role of these S-containing compounds, particularly with regard to abiotic stress acclimation and plant tolerance adaptations.

Sulfur uptake and assimilation

Most soils currently used for agricultural and forest crops are naturally low in fertility, and chemical fertilization should be implemented to provide the crop requirements for essential nutrients such as nitrogen (N), phosphorus (P), potassium (K) and S. S uptake is directly driven by demand. Inadequate S nutrition can cause the inefficient use of other nutrients, such as carbon (C) and N, leading to deficiencies and decreases in protein biosynthesis, chlorophyll content and eventually crop yield (Lunde et al. 2008; Mazid et al. 2011; Iqbal et al. 2013). On the other hand, environmental pollution from sulfur dioxide (SO_2), H_2S, sulfite (SO_3^{2-}) and sulfate (SO_4^{2-}) is a serious global problem and can be toxic to plants (Krischan et al. 2012).

S is a component of proteins, the amino acids cysteine (Cys) and methionine (Met), vitamins (biotin and thiamin), cofactors (Co-A and S-adenosyl methionine, SAM) and a range of secondary metabolites (Mazid et al. 2011). S is an essential macronutrient for living organisms and has multiple roles in plant development, including catalytic, regulatory and structural functions (such as in protein disulfide bonds, cellular membrane SO_4^{2-} esters and electron transport through Fe-S groups). S-containing compounds such as PCS and GSH also have a role in trace element homeostasis (Na and Salt 2011). S is an important substrate/reductant in reactions during abiotic stress processes; GSH, a major antioxidant in plant stress defense and the major non-protein S source in plants (Kopriva and Rennenberg 2004; Ghelfi et al. 2011; Rennenberg and Herschbach 2012; Seth et al. 2012) is present in all root and leaf cell compartments, with the exception of the apoplast in the absence of stress (Josefczak et al. 2012).

S is taken up from the soil solution predominantly as SO_4^{2-} in an energy-dependent process mediated by specific membrane-bound SO_4^{-} transporters (Buchner et al. 2004; Davidian and Kopriva 2010). Plants can also obtain

organic forms of S, such as S-containing amino acids, organic SO_4^{2-} and elemental S, from the soil solution. Although of less significance, S in the form of atmospheric SO_2 can be absorbed by plant leaves and fruits (Mazid et al. 2011), and atmospheric H_2S can be absorbed through leaf stomata (Riemenschneider et al. 2005).

The translocation of SO_4^- into plastids for assimilation, storage in vacuoles, and long-distance transport among organs requires specific transporters; the mechanism of plasma membrane transport is proton-coupled co-transport (Buchner et al. 2004). There are approximately 12 to 16 reported genes encoding SO_4^{2-} transporters (SULTR) in plant species. SULTR proteins can be classified according to their protein sequence similarities into SULTR 1 to 5 (for a review: Buchner et al. 2004; Davidian and Kopriva 2010). These transporters can move SO_4^{2-} into the plant when soils are deficient in S; SULTR1;1 (skilled in trace SO_4^{2-} uptake) and SULTR1;2 (major component) have been identified on root hairs and root epidermal and cortical cells of knockout mutants of *Arabidopsis* (Takahashi et al. 2011). From the structural perspective, SULTR are members of a family of membrane-bound solute transporters provided to 12 domains that cross the plasma membrane (Takahashi et al. 2012). SULTR2:1 is a low-affinity SULTR that appears to be involved in SO_4^{2-} translocation from roots to shoots. The initial uptake by the root epidermis and cortical cells depends on high-affinity transport, as well as on the displacement of SO_4^{2-} into tissues and/or organs, which can be very specialized. Low-affinity transporters (in *Arabidopsis*: AtSULTR2;1 and AtSULTR2;2) were shown to be more related to vascular transport of SO_4^{2-} and to the regulation of the cytoplasmic SO_4^{2-} concentration during accumulation in the vacuole (Buchner et al. 2004; Davidian and Kopriva 2010).

The coordination and the dynamics between the pathways of short- and long-distance transporters require specific signaling mechanisms to control and regulate a range of genes encoding specific proteins involved in S uptake, transport and assimilation (Davidian and Kopriva 2010). Expression of these transporters is regulated by internal and external sulfate signals and by the N, C and S reductive assimilation pathways, including phytohormones and variable metabolites (Gojon et al. 2009; Davidian and Kopriva 2010).

After uptake, SO_4^{2-} is assimilated into Cys, an amino acid at the intersection of primary metabolism, protein synthesis and the formation of low molecular weight S-containing defense compounds (Rausch and Wachter 2005; Gotor et al. 2014). Excess SO_4^{2-} transported to leaves is stored in vacuoles and constitutes a large S reserve for plant metabolism (Iqbal et al. 2013).

Cys synthesis is required for GSH biosynthesis and occurs in plastids, mitochondria and the cytosol. Activated SO_4^{2-} also forms 3′-phosphoadenosine 5′-phosphosulfate (PAPS, EC 1.8.99.2), the S-donor for sulfonation, sulfation or sulfuryl-transfer reactions; the reaction is catalyzed by sulfotransferases (STs) that play important roles in cell communication, plant development and defense (Negishi et al. 2001). In parallel with S assimilation, some 'dissimilatory' reactions, such as the release of H_2S from Cys 1 and 2, might contribute to defense processes (Rausch and Wachter 2005; Lisjak et al. 2011; for more information on H_2S see Lisjak et al. 2010 and Lisjak et al. 2013).

SO_4^{2-} assimilation is critical for providing reduced S for various cellular redox processes (for review: Jacob and Anwar 2008; Takahashi et al. 2011) and for the synthesis of GSH (Kopriva and Rennenberg 2004; Ghelfi et al. 2011; Seth et al. 2012). GSH has many distinct functions in plant cell metabolism, including controlling gene expression linked to the redox state of cells or subcellular compartments; being an important reducing cofactor of many enzymes related to ROS detoxification (for review: Noctor 2006; Foyer and Shigeoka 2011); and directly controlling the S assimilation pathway. Reduced forms of S decrease significantly during S uptake and assimilation (Kopriva 2006; Chan et al. 2013).

Many studies have emphasized the importance of N in S assimilation and plant stress defenses (Kopriva and Rennemberg 2004; Siddiqui et al. 2008; 2012; Salvagiotti et al. 2009; Carfagna et al. 2011). SO_4^{2-} assimilation declines under nitrate (NO_3^-) deficiency, and the capacity to reduce NO_3^- and the activity of nitrate reductase (NR, EC 1.6.6.1-3) are diminished in plants that are starved for SO_4^{2-} (Kopriva and Rennenberg 2004; De Bona et al. 2011). In tobacco plants, for example, SO_4^{2-} uptake by the roots was drastically reduced when NR was inactivated (Kruse et al. 2007; Siddiqui et al. 2012). Moreover, in N-starved plants, the activities of enzymes responsible for S assimilation and the mRNA levels associated with related genes decreased, but the addition of two distinct

N sources ($NO_3^-$and ammonium, NH_4^+) quickly restored the enzymatic function (Koprivova et al. 2000).

In cereal plant species, increases in S fertilization can enhance the efficiency of N uptake and use because S is a constituent of some enzymes involved in N metabolism (Salvagiotti et al. 2009; De Bona et al. 2011). Wheat plants exposed to distinct N and S levels revealed an important relationship between N and S. When N was less limiting, N uptake was high at the highest S concentration. This increase in uptake was more directly correlated with recovery efficiency than with internal use efficiency (Salvagiotti et al. 2009). Nonetheless, it has been demonstrated that adenosine 5' phosphosulfate reductase (APR, EC 1.8.99.2), the key enzyme for SO_4^{2-} assimilation, is regulated by carbohydrates (Lewandowka and Sirko 2008; Chan et al. 2013). The availability of Cys is another crucial factor in GSH synthesis, but an adequate supply of glutamate and glycine is also important (Kopriva and Rennenberg 2004).

The activity and expression of SO_4^{2-} transporters and APR in plants are modulated by their S status and the demand for growth (Koralewska et al. 2008). A key enzyme of plant S metabolism, O-acetylserine(thiol) lyase (OAS-TL, EC 2.5.1.47, also named cysteine synthase), catalyzes the formation of Cys from the sulfide ion (S^{2-}) and O-acetylserine, as illustrated in Fig. 1 (Youssefian et al. 2001). Cys biosynthesis can be regarded as the exclusive function of S reduction in plants and is a key limiting step in the production of GSH and in tolerance to biotic and abiotic stresses (Youssefian et al. 2001; Mera et al. 2014). OAS-TL plays a key role in the synthesis of Cys and GSH, which are required for regulation of plant responses in response to oxidative stress (Youssefian et al. 2001; Gotor et al. 2014).

Studies of barley plants demonstrated that N or S deficiency altered GSH levels in leaves. In N- and S-starved plants, GSH levels doubled, and the Cys concentration was shown to increase by 50% (Carfagna et al. 2011). In *Brassica juncea*, N and S enhanced the activity of adenosine triphosphate-sulfurylase (ATP-S, EC 2.7.7.4), a key enzyme in the S assimilation pathway, which activates SO_4^{2-} via an ATP-dependent reaction. In response to an environmental N deficit, the addition of NO_3^- or NH_4^+ rapidly improved ATP-S and OASTL function (Siddiqui et al. 2012). N addition positively affected OASTL

activity in plant roots, and a precise sequence of N metabolism and S assimilation is necessary to provide the N precursors for Cys biosynthesis (Carfagna et al. 2011). Thus, S assimilation is significantly related to assimilation of NO_3^- and C (Yoshimoto et al. 2007). Some transcriptional factors responsible for SO_4^{2-} uptake and assimilation have been identified, demonstrating a relationship between mRNA levels (to APR and ATP-S), protein biosynthesis and enzyme activity (Koprivova et al 2000; Hesse et al. 2003; Davidian and Kopriva 2010).

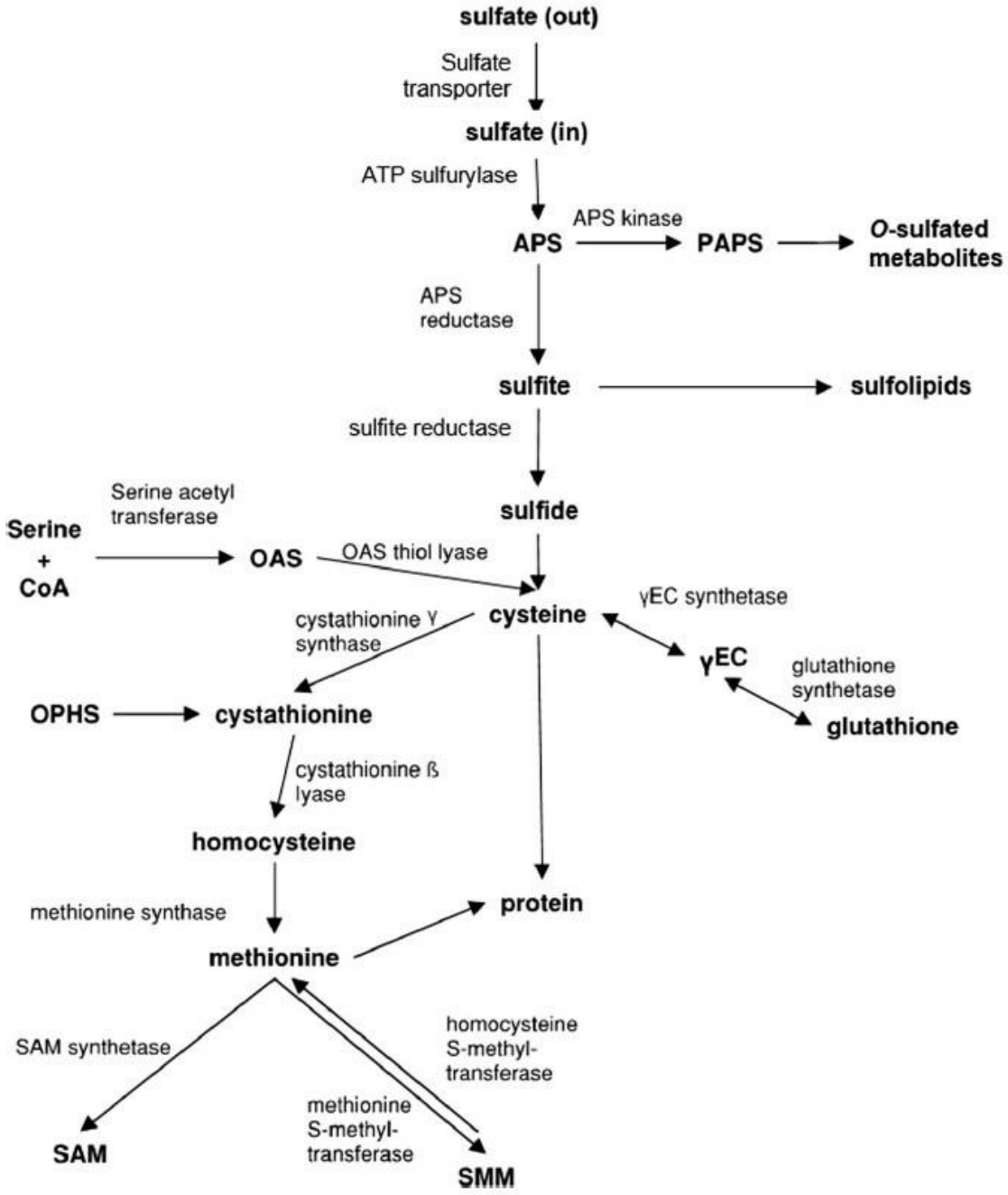

Figure 1. Biosynthetic pathways for S-containing amino acids and their derivatives. A key enzyme of plant S metabolism, OAS-TL, also named cysteine synthase, catalyzes the formation of Cys from the sulfide ion (S^{2-}) and O-acetylserine. APS: adenosine-5'-phosphosulfate; PAPS: 3'-phosphoadenosine-5'-phosphosulfate; γ-EC: γ-glutamyl-cysteine; OAS: O-acetylserine; CoA: acetyl coenzyme A; SAM: S-adenosylmethionine (S-AdoMet); SMM: S-methylmethionine (modified from Hawkesford 2005; Koprivova and Kopriva 2014).

Phytohormones in S assimilation

Some studies have reviewed the importance of the relationship between S assimilation and phytohormones (Maruyama-Nakashita et al. 2004; Maruyama-Nakashita et al. 2005; Kopriva 2006; Khan et al. 2013). Phytohormones are essential for plant acclimation and adaptation to environmental changes (Peleg and Blumwald 2011). The signaling pathway of phytohormones is linked to efficient nutrient use, plant defense pathways and plant developmental processes and metabolism (Fatma et al. 2012). Phytohormones such as cytokinins (CK), gibberellins (GA), auxins (AU), ethylene (ET), jasmonates (JA) and salicylic acid (SA) can interact with mineral nutrients under both normal and stress conditions, playing an essential role in salt stress control and affecting plant growth recovery, cell division, germination and seed production, even when applied exogenously (Fatma et al. 2012).

CK is known to be related to the N cycle and metabolism, being involved in N and P assimilation. Therefore, it seems that CK has a general role in the assimilation of nutrients, including S (Kopriva 2006).

The expression of indole-3-acetic-acid-amido synthetase (IAA-amido synthetase, EC 6.3.2.-) in rice seedlings was correlated with an increase in expression of LEA (late embryogenesis abundant) genes, which have been shown to promote drought stress tolerance (Zhang et al. 2009). The expression of many other genes related to auxin synthesis and to enzyme biosynthesis, transporters and activity can also be regulated by ET, whilst auxin seems to affect ET biosynthesis (Peleg and Blumwald 2011). ET has an important role in improving N use efficiency, photosynthetic rates and plant growth in N-optimal and N-deficient *Brassica juncea* plants (Khan et al. 2008). ET signaling increases with SA and/or JA, resulting in expression of a wide range of genes related to plant defense.

GA enhances the effects of salt stress in soybean, perhaps by regulating the availability of other phytohormones. Abscisic acid (ABA) reduced Na^+ and Cl^- content and, consequently, the Na^+/K^+ ratio and increased the Ca^{2+}, K^+, soluble sugar and proline contents in rice crops (Khorshidi et al. 2009; Iqbal et al. 2013). Phytohormones can alleviate the effects of salt stress and improve

plant tolerance by influencing proline metabolism. N and/or Ca^{2+} accumulation are altered, and a link between Ca^{2+} signaling and SA content improves proline content (Du et al. 2009; Al-Whaibi et al. 2012). Proline accumulation seems to be regulated by ABA-dependent and ABA-independent pathways (Iqbal et al. 2013).

ABA appears to be the phytohormone that responds most rapidly to plant stress. ABA synthesis and expression of ABA-inducible genes cause stomatal closure in plants under drought stress (Peleg and Blumwald 2011). Many genes associated with ABA biosynthesis and ABA receptors were identified in *Arabidopsis* (Brocard-Gifford et al. 2004) and maize (Peleg and Blumwald 2011).

Plants exposed to SA and ABA exhibited higher GSH concentrations and glutathione reductase activity (GR, EC 1.8.1.7, also named glutathione-disulfide reductase, GSR) (Kopriva 2006; Nazar et al. 2011; Pál et al. 2014). This result confirms the relationship among GSH content, S assimilation and stress defense. ABA is related to environmental stress adaptation and SA plays a key role in plant stress tolerance, expression of genes that encode chaperones, heat-shock proteins and antioxidants, and genes related to secondary metabolites (Kopriva 2006; Pál et al. 2014; Peleg and Blumwald 2011; for a review on the role of phytohormones in stress tolerance, see Carvalho et al. 2011). ABA increases the levels of mRNA encoding cytosolic OAS-TL, a key enzyme in S assimilation and metabolism (Kopriva 2006).

The increased demand for GSH can be met by activation of pathways involved in S assimilation and Cys biosynthesis. Microarray analyses have indicated that the messenger ribonucleic acid (mRNA) transcript level of OAS-TL was upregulated in response to zinc stress in *A. thaliana* (Becher et al. 2004) and was constitutively elevated in its metal-tolerant relative, *A. halleri* (Weber et al. 2004).

Many studies address the production and influence of GSH and Cys in relieving biotic and abiotic stress in plants, such as in the protective response to oxidative stress resulting from various factors (Ruiz and Blumwald 2002; Rahoui et al. 2014; Zhang et al. 2014). Under abiotic stress, GSH demand increases (to promote stress tolerance), activating enzymes in the S assimilation pathway. Enzymatic activity, genetic manipulation of enzymes involved in S assimilation

and external S supply can lead to abiotic stress tolerance in plants (Rennemberg et al. 2007; Nazar et al. 2011).

Cys content and GSH content and biosynthesis at the transcriptional level are regulated by JA (known to participate in the transduction of stress response) and methyl jasmonate (MeJA) (Shan and Liang 2010; Gfeller et al. 2011). Treatment of *Arabidopsis* with MeJa increased the mRNA levels corresponding to many genes involved in S assimilation and GSH synthesis, without affecting the content of S metabolites or of mRNA levels associated with SO_4^{2-} transporters (Kopriva 2006). These signaling compounds also increased the accumulation of mRNA associated with genes that are involved in S metabolism (Takahashi et al. 2011) and associated with S deficiency. This suggests that JA has a signaling role for inducing S assimilation under S deficiency (Sasaki-Sekimoto et al. 2005; Srivastava et al. 2013).

Several limitations of the suggested stress response have to be emphasized because of the importance of the GSH system with respect to other components of the photoprotective and antioxidative defense systems. The roles of JA, SA, GA and ABA in regulating S assimilation enzymes are crucial for acquired abiotic stress tolerance in plants.

Sulfur oxidation states in the cell

Under stressful conditions, the induction or increase in S compounds related to plant defense is also crucial for detoxification of excessive ROS (Noctor et al. 2012), resulting in diverse modes of action for S-containing secondary metabolites (Fig. 2). In the cell, S can be found in several oxidation states mediated by different enzyme families. For example, the sulfotransferase protein family (SOT, EC 2.8.2) catalyzes the transfer of sulfonate molecules in the highest oxidation state to an appropriate hydroxyl group of many substrates using 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as the sulfuryl donor. The SOT also catalyzes the sulfonation of a wide range of compounds and produces SO_4^{2-} esters and conjugates (Klein and Papenbrock 2004).

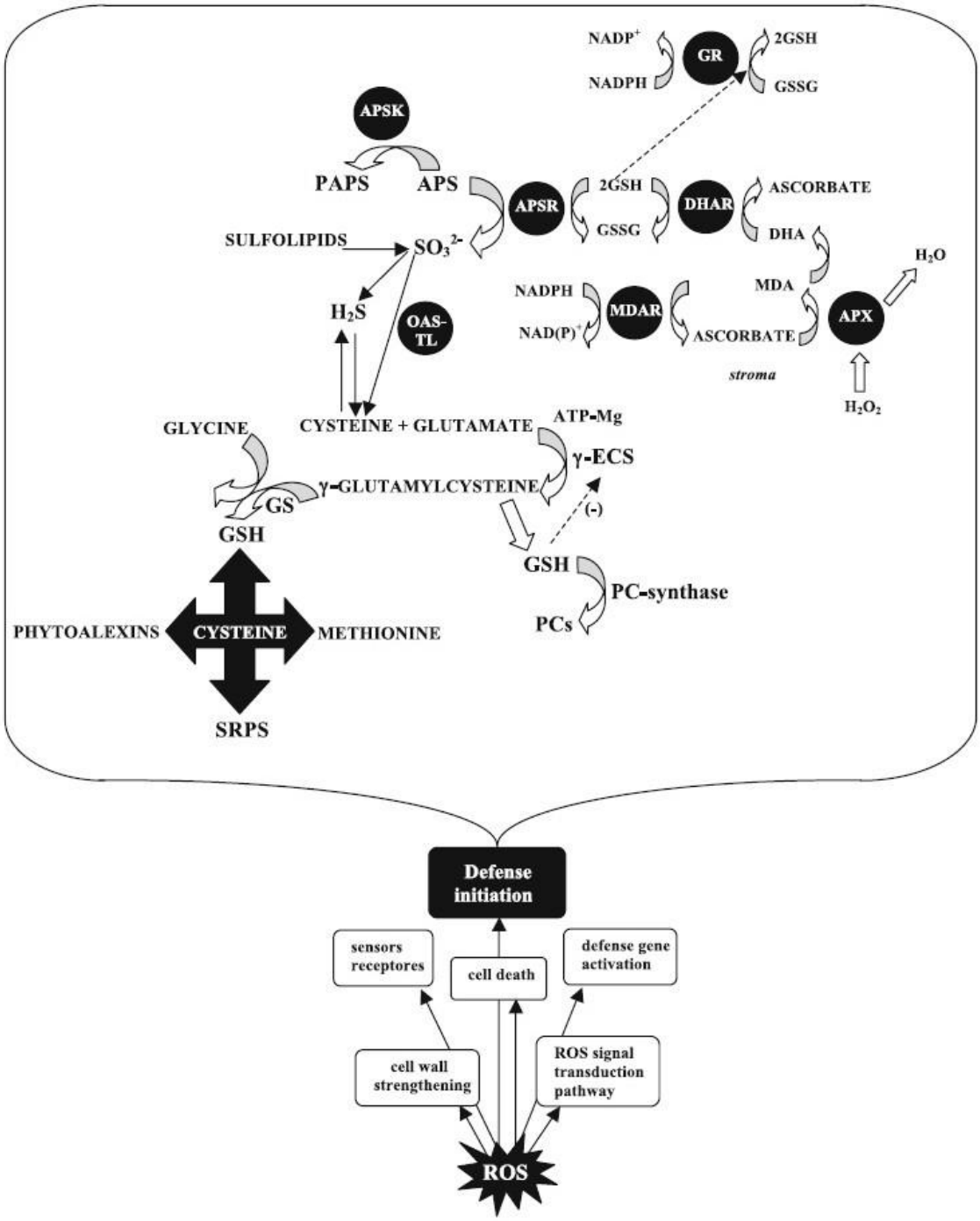

Figure 2. Sulfur-containing defense compounds. GR: Glutathione Reductase, APX: Ascorbate Peroxidase, GPX: Glutathione Peroxidase, MDHAR: Monodehydroascorbate Reductase, DHAR: Dehydroascorbate Reductase, APS: 5'-adenylylsulfate, PAP: 3'-phosphoadenylylsulfate, APSK: APS kinase, APSR: APS reductase, OAS-TL: O-acetylserine thiol lyase (modified from Rausch and Wachter 2005; Mendoza-Cózatl et al. 2005).

Cys is synthesized in the last stage of photosynthetic assimilation of sulfate in plant cells and is the first organic compound containing reduced S. Cys has essential roles in the function, structure and regulation of proteins, being the precursor of many important S-containing compounds involved in plant defense signaling and plant development (Gotor et al. 2014).

Macroarray analysis revealed an integrated signaling pathway in plant defense gene expression in *Arabidopsis*, with upregulation by MeJA of several genes related to S metabolism, GSH, Cys and Met biosynthesis and S-rich defense proteins involved in GS metabolism (Jost et al. 2005; Guo et al. 2013).

Methionine biosynthesis

Met synthesis links Cys biosynthesis to the aspartate-derived amino acid biosynthetic pathway (for review, see Hawkesford and Kok 2006; see also other papers published by the group of M. Hawkesford; for reviews on the aspartate pathway, see Azevedo et al. 1997; 2006). Biosynthesis of Met from Cys involves three enzymatic steps. O-phosphohomoserine (OPHS, EC 2.7.1.39) derived from the aspartate pathway is a common substrate for both threonine and Met synthesis, catalyzed by threonine synthase (TS, EC 4.2.3.1) and methionine synthase (MS, EC 2.1.1.13), respectively (Azevedo et al. 1997). Cystathionine γ-synthase (CgS, EC 2.5.1.48) catalyzes the synthesis of cystathionine from Cys and OPHS by trans-sulfuration (Hawkesford 2005). Cystathionine is then converted to homocysteine (a β-cleavage reaction) by cystathionine β-lyase (CbL, EC 4.4.1.8). Homocysteine is exported from chloroplasts and converted (by methylation) into Met through MS activity. The activity of CgS and TS will influence biosynthesis of Met and threonine, respectively. CgS activity almost certainly has a large effect on flux and is most likely feedback-regulated by Met or a derivative (Azevedo et al. 1997; 2006). Similarly, TS activity is regulated by S-adenosylmethionine (SAM, also known as S-AdoMet), which is a derivative of Met (Azevedo et al. 1997; Wang and Frey, 2007). These controls effectively maintain the Met pool within close constraints. Rather small gene families encode the proteins of this pathway (CgS: 2 genes, CbL: 1 gene, MS: 3 genes). Furthermore, Met is a gateway to

many other important S-containing metabolites, including S-methylmethionine (SMM), SAM and dimethylsulfonio-propionate (DMSP). SMM is a transportable derivative of Met. It can revert to Met by donating a methyl group to homocysteine in a reaction catalyzed by homocysteine S-methyltransferase (HMT, EC 2.1.1.10). Under some circumstances, SMM may be the major S constituent of the phloem sap, and it has a role in delivering S to sink tissues such as seeds (Hawkesford 2005).

SAM is one of the most important S-compounds in plant metabolism (Azevedo et al. 2006); it is involved in many processes and is the main methyl donor involved in transmethylation of proteins, nucleic acids, polysaccharides and fatty acids (Ma et al. 2003).

SAM is also a precursor of the polyamine (PA) synthetic pathway (spermidine/spermine biosynthesis pathway) and of nicotinamide biosynthesis (important for Fe nutrition in plants). SAM is known as the ‘activated Met form’ (Bürstenbinder and Sauter 2012), and up to 80% of the Met pool may be converted to SAM at the expense of adenosine triphosphate (ATP) utilization (Ravanel et al. 1998; Iqbal et al. 2013) by SAM synthetase (SAMS, EC 2.6.1.6, five genes in the family). Spermidine and spermine have multiple proposed roles, including stress response, pH regulation, DNA replication and senescence processes. Consumption of SAM may increase S demands to meet these needs, although ultimately Met is recycled. SAM is also the precursor for ET, a potent modulator of plant growth and development that is involved in stress signaling (Wang et al. 2002). The synthesis of ET from SAM is catalyzed by 1-aminocyclopropane-1-carboxylic acid synthase (ACCS, EC 4.4.1.14) and ACC oxidase (ACCO, EC 1.14.17.4). Met is not consumed in this reaction but is recycled, resulting in no net S demand. A side product of the final biosynthetic step for ET is cyanide, which is detoxified to β-cyanoalanine by β-cyanoalanine synthase (CAS, EC 4.4.1.9), an isoform of OAS-TL (Hatzfeld et al. 2000). DMSP is produced in high concentrations in many marine algae and in some higher plants, such as marsh grasses in the genus *Spartina*, sugar cane and *Wollastonia biflora*. It is synthesized in higher plants via SMM, but it is generally present in low concentrations in other plant species. Several roles have been proposed, including salt tolerance and herbivore deterrence.

SAM, in decarboxylated form and catalyzed by S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50), provides 5´-desoxy-(5´-),3-aminopropyl-(1), a methylsulfonic salt required for PA biosynthesis (Roy and Wu 2002; for a review in PA: Alcázar et al. 2010; Hussain et al. 2011; Bitrián et al. 2012). PA l metabolites are essential to plant survival and have been correlated with biotic and abiotic stress resistance in many plant species; studies have employed exogenous PA application and genetic manipulations of different plant species (Bitrián et al. 2013). Increased biosynthesis of putrescine and spermidine in transgenic tobacco plants that had human SAMDC inserted into their genomes resulted in greater resistance to salt, drought and biotic stress (Waie and Rajam 2003). Microarray, transcriptomic and proteomic studies have demonstrated the role of PA in signaling cascades that increase plant tolerance or resistance to biotic and abiotic stress (Hussain et al. 2011).

Sulfur compounds related to plant defense

Environmental stress usually affects plant cell homeostasis and development, increasing ROS production and leading to oxidative stress (Arruda and Azevedo 2009; Azevedo et al. 2011; Cia et al. 2012; Boaretto et al. 2014). Abiotic stress is a consequence of the effects of a wide variety of distinct external agents on plants, such as temperature (heat or chilling), water (drought or flooding), salinity, proton toxicity, heavy metals, overexposure to ultraviolet rays, ozone and others (Azevedo et al. 1998; Gratão et al. 2005; Monteiro et al. 2011; Bulbovas et al. 2014; Nogueirol et al. 2015). All living organisms have a series of pathways to combat environmental stress. In plant species, changes in photorespiration, enzymatic and non-enzymatic antioxidant pathways, regulation and responsive gene expression, and morphological and anatomical adaptations have been identified and investigated (Foyer and Shigeoka 2011). Excessive ROS generation has been considered a negative process for many years, but it is an essential component of signaling processes that prompt adjustments in gene expression and cellular structure in response to environmental changes (Shao et al. 2008; Foyer and Shigeoka 2011; Monteiro et al. 2011).

Plants can respond to abiotic stresses in a number of ways. These include, as a primary step, the induction of a network of signaling pathways and, at later stages, the response by specific proteins, metabolites and other compounds triggered by the signal transduction of the first step (Shulaev et al. 2008). Molecular analysis revealed that both short-term and long-term responses are important for understanding the progression of signaling events when the external and then the internal nutrient supply become depleted (Schachtman and Shin 2007). Similarly, it is critical to understand how experiments are designed because chronic and acute treatments with a stressor can produce completely different responses (Gratão et al. 2008). Furthermore, these distinct responses may improve the understanding or identification of the mechanisms involved in stress tolerance or of plants that are tolerant to the induced stressful condition. Signal transduction and detection networks that control plant responses to nutrient deprivation are not characterized for N and S to the extend it should considering how important these elements are. As already emphasized in this review, the S assimilation pathway is related to plant responses to abiotic stress and to defense mechanisms. It is a source of reduced S for many cellular processes and for synthesis of Cys, which is used in Met synthesis and/or incorporated into proteins or GSH (Siddiqui et al. 2012).

It is important to bear in mind that ROS is naturally produced by the cell metabolism. Oxidative stress occurs when the redox balance is disturbed and excess ROS induces a range of stress defense mechanisms (Gratão et al. 2005). ROS is an upstream mediator of nutrient signaling and increases rapidly after mineral nutrient deprivation, as indicated by Schachtman and Shin (2007). This is a major problem because the literature concerning the effect of an element/nutrient on a plant is extensive, but studies on the secondary responses and effects on the uptake and translocation of other essential elements are more limited. The resulting knowledge gap causes uncertainties regarding the full effects of the stressful conditions to which the plants were subjected. More integrated studies must be conducted.

Research on oxidative stress induced by heavy metals is increasing dramatically. The number of studies being published is astonishing, although the majority confirms known information. Nonetheless, it appears that the stress

induced by metals increases demand for reduced S, activating the expression of SO_4^{2-} transporters and enzymes of the assimilatory pathway (Hawkesford 2005; Hawkesford and Kok 2006; see also other papers published by the group of M. Hawkesford). Recent studies indicate that SO_4^{2-} transport in the plant vascular system, its assimilation in leaves and the recycling of S-containing compounds are related to drought stress signaling and response (Takahashi et al. 2011; Hawkesford 2012).

Plants are known to synthesize and release H_2S in a process catalyzed by L-cysteine desulfhydrase (LCD, E.C. 4.4.1.1) and involving conversion of L-Cys to H_2S, pyruvate and ammonia (García-Mata and Lamattina 2010). H_2S was formerly considered toxic to plant development, inducing excessive production of ROS. However, as understanding of its role in metabolic stress responses has increased, several studies have shown that its main function in plants is as a signaling molecule that controls physiological and biochemical processes (Jin et al. 2011; Li et al. 2012). For example, a study of spinach plants fumigated with H_2S gas demonstrated that approximately 40% of the H_2S was converted into GSH in plant leaves (Lisjak et al. 2011). H_2S had an essential role in alleviating the stress damage caused by aluminum chloride ($AlCl_3$) in germinating wheat seedlings, increasing esterase and amylase activity and maintaining low malondialdehyde (MDA) and H_2O_2 levels (Zhang et al. 2010). Pre-treatment with sodium hydrosulfide (NaHS, a H_2S donor) resulted in increased activity of guaiacol peroxidase (GPX, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6) and decreased aluminum (Al) uptake in Al pre-treated seeds of wheat; this confirmed H_2S as a signaling molecule in response to abiotic stress (Zhang et al. 2010). In *Vicia faba*, *A. thaliana* and *Impatiens walleriana,* H_2S also induced stomatal closure and ABA-dependent signaling, possibly through the regulation of ABC transporters in guard cells under drought, and enhanced tolerance of water stress (García-Mata and Lamattina 2010). Tobacco cells pretreated with NaHS also exhibited heat tolerance and regrowth after stress exposure (Li et al. 2012). *A. thaliana* exposed to the same pretreatment produced more H_2S, exhibited drought tolerance by limiting stomatal aperture and increased the production and expression of drought marker genes (Jin et al. 2011).

In plants, incorporation of O-acetylserine sulfhydrylase (OASS, EC 2.5.1.65) and serine acetyltransferase (SAT, EC 2.3.1.30) into the cysteine synthase (CysK, EC 2.5.1.47) complex plays a regulatory role in S assimilation and Cys biosynthesis (Francois et al. 2006). The molecular mechanisms for the coordination of S, nitrogen and carbon assimilation are not yet known in detail. O-acetylserine, a precursor of Cys, was proposed as the signal regulating SO_4^{2-} assimilation, but it is not likely to be the outgoing signal for N and C metabolism. In S-deprived plants, for example, the level of glucose, fructose and phosphoenolpyruvate decreased, while starch concentration increased (Lunde et al. 2008). The reduction in photosynthetic rate, increase in oxidation of the ferredoxin:thioredoxin system, changes in starch synthesis and degradation, and the lower use of carbohydrates as an energy source can explain the changes in carbohydrates content (Lunde et al. 2008).

Complementary deoxyribonucleic acid (cDNA) array analysis revealed that expression of genes involved in auxin synthesis is induced upon S-starvation, suggesting a possible role of phytohormones (Kopriva and Rennenberg 2004). Clearly, and despite significant progress in understanding the regulation of SO_4^{2-} assimilation and GSH synthesis, their coordination with N and C metabolism is not yet fully understood, and the several potential signal molecules identified are still far from being sufficiently explanatory (Kopriva and Rennenberg 2004).

Stressed plants usually exhibit decreased rates of cellular division and elongation and, consequently, reduced or inhibited growth. This response may not only be a means of preserving energy for the defense process but may also function as protection against hereditary damage (Li et al. 2014). The chloroplasts play a major role in modulating the plant response, being both sensitive to abiotic stress factors and a major site for S assimilation (Biswal et al. 2008). These organelles are also important for ROS production because of the interaction between electrons escape from the photosynthetic electron transport chain and molecular oxygen (Foyer and Noctor 2012). Chloroplasts can therefore coordinate the C, N and S metabolic pathways, providing essential precursors for the synthesis of S-containing compounds (Jamal et al. 2006; Biswal et al. 2008). Another interesting aspect is that Fe-deficiency can result in severe disruption to the thylakoid lamellae, with loss of grana, but such

damage to the photosynthetic apparatus can be diminished by S nutrition. Photosynthetic activity and sucrose synthase and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity are also closely related to S status (Muneer et al. 2014).

Glutathione (GSH), metallothioneins (MTs) and phytochelatins (PCs)

The stress response involves enzymatic antioxidant and other defense systems, including sulfur-containing compounds such as the essential macronutrient sulfur (S); glutathione (GSH: a S-containing thiol tripeptide, γ-L-glutamyl-L-cysteinyl-glycine); a class of phytochelatins [PCS: (g-Glu-Cys)n-Gly, n = 2 to 5 usually]; S-rich proteins; S-amino acids; hydrogen sulfide (H_2S); and a range of secondary metabolites (Gratão et al. 2012).

The GSH pool determines the degree of expression of genes linked to defense. It is controlled by many signaling pathways before and during stress, establishing a direct link between stress defense gene expression and GSH biosynthesis. Microarray, reverse transcription polymerase chain reaction (RT-PCR) and high performance liquid chromatography (HPLC) analyses of *Arabidopsis* plants exposed to cadmium (Cd) revealed that plants activate the S assimilation pathway by increasing transcription of specific genes that enhance the supply of GSH for PCS synthesis (Kawashima et al. 2011; Jobe et al. 2012). Moreover, roots and leaves have also been shown to exhibit distinct responses to Cd stress (Herbette et al. 2006; Gallego et al. 2012).

Metallothioneins are proteins with two structural domains (Cys-rich and metal-binding) involved in metal homeostasis and detoxification (Majic et al. 2008; Choppala et al. 2014; Gu et al. 2014). But in the case of metal stress, the action of PCS is essential. PCS are Cys-rich peptides related to GSH and most likely are synthesized in the same pathway (Qureshi et al. 2007; Zagorchev et al. 2013). Plants can inactivate the toxic effects of excessive ROS by intracellular chelation of the metallic ion by GSH and/or PCS in the cytosol. Depending on the plant species, these complexes can be transported into the vacuole by a specific metal-requiring enzyme (Pál et al. 2006; Yadav 2010; Hossain and Komatsu 2013). Increased GS can be considered a sulfate reserve for PCS synthesis, however the identity of the S-compounds that are reduced

when GS increases and the impact of metals on their metabolism are unknown, despite the hypothesis that GS provides an additional S source under metal stress condition (Ernst et al. 2008; Bell and Wagstaff 2014). Additionally, a common response by metal-stressed plants may be the activation of the ascorbate–glutathione cycle, either for the removal of H_2O_2 or to ensure the availability of GSH for the synthesis of these metal-binding proteins (Vitória et al. 2001; Jozefczak et al. 2012).

Glucosinolates

Glucosinolates are amino acid-derived secondary metabolites consisting of a thioglucose moiety, a sulfonated aldoxime, and a side chain derived from either aliphatic or aromatic amino acids (Halkier and Gershenzon 2006). When plant tissues are disrupted the glucosinolates are hydrolyzed by the highly active plant enzyme myrosinase, a thiolglucosidase. The cleavage of the glucose thioester linkage produces an unstable intermediate that rearranges into biologically active thiocyanates, isothiocyanates, nitriles and oxalidine-2-thiones, depending on reaction conditions and presence of additional proteins (Hell and Kruse 2006). These products are chemically very reactive and may interfere with proteins and free amino acids. They are generally caustic and potentially toxic, hence their antimicrobial activities in plant defence. Although the glucosinolates also occur in a number of other plant families, the economic importance of oilseed rape, mustard, and the cabbage subspecies raised further interest in the biochemistry and molecular biology of glucosinolates biosynthesis and degradation (Hell and Kruse 2006).

In the reductive S assimilation pathway, the activities of enzymes in the pathway are influenced by S supply, with some (*in vitro*) evidence that ATPS and APR can form a complex to by-pass a branch point in the S assimilation pathway catalyzed by adenosine-5´-phosphosulfate kinase (APSK). APSK forms 3´-phosphoadenosine 5´-phosphosulfate (PAPS), an important substrate for the formation of the secondary S-containing metabolites including glucosinolates, and is therefore a significant enzyme in members of *Brassicaceae* (Leung et al. 2006). Four APSK genes have been cloned from *Arabidopsis*, for example, APSK1, APSK2, and APSK4, all localized in the

plastid, while APSK3 is a cytoplasmic isoform (Mugford et al. 2009). The use of mutants has shown that APSK1 is able to produce sufficient PAPS to maintain normal plant growth (Mugford et al. 2010), while disruption of APSK1 and APSK2 expression reduces the biosynthesis of glucosinolates (Mugford et al. 2009) demonstrating that the expression of APSK genes are strongly linked to the biosynthesis of glucosinolates. However, in non-glucosinolate accumulating species, including for example onion, the secondary (APSK-mediated) pathway must also operate to generate important pools of sulfate esters.

In order to elucidate the synthesis and degradation of glucosinolates in plants, (Mugford et al. 2009) observed highly significant alterations in the levels of glucosinolates and their desulfo-precursors in *A. thaliana* apk1, apk2, apk3, apk4 and wild-type mutants. The levels of each individual glucosinolates were reduced in the leaves of the mutant so that total glucosinolate levels reached only 15% of that in wild-type mutant. The reduction was accompanied by a massive increase in desulfo-precursors, which reached a ten-fold higher concentration than the mature glucosinolates in wild-type leaves. A similar reduction in glucosinolate levels was detected in the seeds of the mutant plants, however, the sulfo-precursors did not accumulate in the seeds. These results confirm that glucosinolates are not synthetized in seeds but are transported in the mature sulfate form (Magrath and Mithen 1993).

Final Considerations

S is an essential chemical nutrient for plant growth and survival. S-containing defense compounds play significant roles in plant metabolism, stress response, cellular acclimation and adaptation (Fig. 3). They have gained much attention worldwide as biochemical genetics, plant physiology and breeding have been integrated to produce stress-tolerant plants and to identify preferential tolerance mechanisms. The development of transgenic plants with multi-stress tolerance should be the focus of research in the near future because stress factors rarely occur singly. Yet, the use of natural occurring or induced mutants cannot be left.

Our knowledge of the effects of transgenic S-containing compounds is still limited, partially because of the complex regulatory mechanisms described

previously. Significant advances in metabolic analyses with the “omics” techniques can improve identification of non-target gene products, enabling simultaneous consideration of gene expression, enzyme activity and metabolites.

In conclusion, this literature review is expected to improve our understanding of the essential mechanisms involved in oxidative stress in plants and the induction of S-containing compounds. The increased understanding may aid researchers in overcoming problems that occur in contaminated environments.

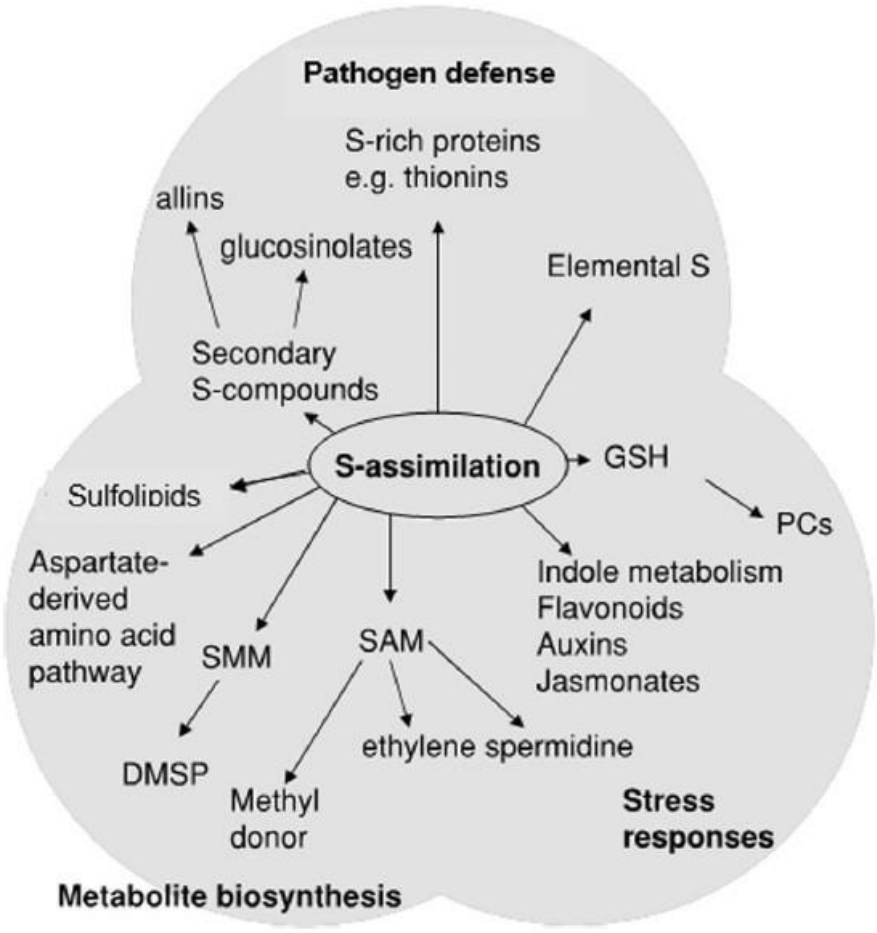

Figure 3. Sulfur assimilation is linked to multiple metabolic pathways responsible for a diverse range of physiological functions. Three major areas include primary metabolite biosynthesis, stress responses and pathogen defense. GSH: glutathione; PCS: phytochelatins; SAM: S-adenosylmethionine (S-AdoMet); SMM: S-methylmethionine; DMSP: dimethylsulfonioproprionate (Modified from Hawkesford 2005).

Acknowledgments

We would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP – Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - Brazil) for continuous financial support over the years for our projects on this and related subjects.

References

Alcázar R, Altabella T, Marco F, Bortolotti C, Reymond M, Koncz C, Carrasco P, Tiburcio AF (2010) Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. Planta 231:1237-1249

Al-Whaibi MH, Siddiqui MH, Basalah MO (2012) Salicylic acid and calcium induced protection of wheat against salinity. Protoplasma 249:769-778

Arruda MAZ, Azevedo RA (2009) Metallomics and chemical speciation: towards a better understanding of metal-induced stress in plants. Ann App Biol 155:301-307

Azevedo RA, Alas RM, Smith RJ, Lea PJ (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. Physiol Plant 104:280–292

Azevedo RA, Arruda P, Turner WL, Lea PJ (1997) The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. Phytochemistry 46:395-419

Azevedo RA, Carvalho RF, Cia MC, Gratão PL (2011) Sugarcane under pressure: an overview of biochemical and physiological studies of abiotic stress. Trop Plant Biol 4:42-51

Azevedo RA, Lancien M, Lea PJ (2006) The aspartic acid metabolic pathway, an exciting and essential pathway in plants. Amino Acids 30:143-162

Becher M, Talke IN, Krall L, Kramer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J 37:251-268

Bell L, Wagstaff C (2014) Glucosinolates, myrosinase hydrolysis products, and flavonols found in rocket (*Eruca sativa* and *Diplotaxis tenuifolia*). J Agric Food Chem 62:4481-4492

Biswal B, Raval MK, Biswal UC, Joshi P (2008) Response of photosynthetic organelles to abiotic stress: modulation by sulphur metabolism IN: Khan A, Singh S, Umar S (eds.) Sulphur Assimilation and Abiotic Stress in Plants. Springer-Verlag, Berlin Heidelberg pp167-191

Bitrián M, Zarza X, Altabella T, Tiburcio AF, Alcázar R (2012) Polyamines under abiotic stress: metabolic crossroads and hormonal crosstalks in plants. Metabolites 2:516-528

Boaretto LF, Carvalho G, Borgo L, Creste S, Landell MGA, Mazzafera P, Azevedo RA (2014) Water stress reveals differential antioxidant responses of tolerant and non-tolerant sugarcane genotypes. Plant Physiol Biochem 74:166-175

Brocard-Gifford I, Lynch TJ, Garcia ME, Malhotra B, Finkelstein RR (2004) The *Arabidopsis thaliana* ABSICISIC ACID-INSENSITIVE8 encodes a novel protein mediating absicisic acid and sugar responses essential for growth. Plant Cell 16:406-421

Buchner P, Takahashi H, Hawkesford M (2004) Plant sulphate transporters: co-ordination of uptake, intracellular and long distance transport. J Exp Bot 55:1765-1773

Bulbovas P, Souza SR, Esposito JBN, Moraes RM, Alves ES, Domingos M, Azevedo RA (2014) Assessment of the ozone tolerance of two soybean cultivars (*Glycine max* cv. Sambaíba and Tracajá) cultivated in Amazonian areas. Environ Sci Pollut Res 21:10514-10524

Bürstenbinder K, Sauter M (2012) Early events in the ethylene biosynthetic pathway – regulation of the pools of methionine and *S*-adenosylmethionine. Ann Plant Rev 44:19-52

Carfagna S, Vona V, Di Martino V, Esposito S, Rigano C (2011) Nitrogen assimilation and cysteine biosynthesis in barley: evidence for root sulphur assimilation upon recovery from N deprivation. Environ Exp Bot 71:18-24

Carvalho RF, Campos ML, Azevedo RA (2011) The role of phytochrome in stress tolerance. J Integr Plant Biol 53:920-929

Chan KX, Wirtz M, Phua SY, Estavillo GM, Pogson BJ (2013) Balancing metabolites in drought: the sulfur assimilation conundrum. Trends Plant Sci 18:18-29

Cherest H, Davidian JC, Thomas D, Benes V, Ansorge W, Surdin-Kerjan Y (1997) Molecular characterization of two high affinity sulfate transporters in *Saccharomyces cerevisiae*. Genetics 145:627-635

Choppala G, Saifullah Bolan, N, Bibi S, Igbal M, Rengel Z, Kunhikrishnan A, Ashwath N (2014) Cellular mechanisms in higher plants governing tolerance to cadmium toxicity. Crit Rev Plant Sci 33:374-391

Cia MC, Guimarães ACR, Medici LO, Chabregas SM, Azevedo RA (2012) Antioxidant responses to water deficit by drought-tolerant and - sensitive sugarcane varieties. Ann Appl Biol 161:313-324

Davidian JC, Kopriva S (2010) Regulation of sulphate uptake and assimilation – the same or not the same? Mol Plant Adv 3:314-325

De Bona FD, Fedoseyenko D, von Wirén N, Monteiro FA (2011) Nitrogen utilization by sulfur-deficient barley plants depends on the nitrogen form. Environ Exp Bot 74:237-244

Du L, Ali GS, Simons KA, Hou J, Yang T, Reddy AS, Poovaiah BW (2009) Ca(2+)/calmodulin regulates salicylic-acid-mediated plant immunity. Nature 457:1154-1158

Ernst WHO, Krauss GJ, Verkleij JAC, Wesenberg D (2008) Interaction of heavy metals with the sulphur metabolism in angiosperms from an ecological point of view. Plant Cell Environ 31:123-143

Fatma M, Khan MIR, Masood A, Khan NA (2013) Coordinate changes in assimilatory sulfate reduction are correlated to salt tolerance: involvement of phytohormones. Ann Rev Res Biol 3:267-295

Foyer CH, Noctor G (2012) Managing the cellular redox hub in photosynthetic organisms. Plant Cell Environ 35:199-201

Foyer CH, Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 155:93-100

Francois JA, Kumaran S, Jez JM (2006) Structural basis for interaction of O-acetylserine sulfhydrylase and serine acetyltransferase in the Arabidopsis cysteine synthase complex. Plant Cell 18:3647-3655

Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP (2012) Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. Environ Exp Bot 83:33-46

García-Mata C, Lamattina L (2010) Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. New Phytol 188:977-984

Gfeller A, Baerenfaller K, Loscos J, Chételat A, Baginsky S, Farmer EE (2011) Jasmonate controls polypeptide patterning in undamaged tissue in wounded Arabidopsis leaves. Plant Physiol 156:1797-1807

Ghelfi A, Gaziola SA, Cia MC, Chabregas SM, Falco MC, Kuser-Falcão PR, Azevedo RA (2011) Cloning, expression, molecular modelling and docking analysis of glutathione transferase from *Saccharum officinarum*. Ann App Biol 159:267-280

Gojon A, Nacry P, Davidian JC (2009) Root uptake regulation: a central process for NPS homeostasis in plants. Curr Op Plant Biol 12:328-338.

Gotor C, Laureano-Marin AM, Moreno I, Aroca A, Garcia I, Romero LC (2014) Signaling in plant cytosol: cysteine or sulfide? Amino Acids 14:1-10

Gratão PL, Monteiro CC, Antunes AM, Peres LEP, Azevedo RA (2008) Acquired tolerance of tomato (*Lycopersicon esculentum* cv. Micro-Tom) plants to cadmium-induced stress. Ann Appl Biol 153:321-333

Gratão PL, Monteiro CC, Carvalho RF, Tezotto T, Piotto FA, Peres LEP, Azevedo RA (2012) Biochemical dissecation of diageotropica and Never ripe tomato mutants to Cd-stressful conditions. Plant Physiol Biochem 56:79-96

Gratão PL, Polle A, Lea PJ, Azevedo RA (2005) Making the life of heavy-metal stressed plants a little easier. Funct Plant Biol 32:481-494

Gu CS, Liu LQ, Zhao YH, Deng YM, Zhu XD, Huang SZ (2014) Overexpression of *Iris. lactea* var. Chinensis metallothionein IIMT2a enhances cadmium tolerance in *Arabidopsis thaliana*. Ecotoxicol Environ Saf 105:22-28

Guo RF, Shen WS, Qian HM, Zhang M, Liu LH, Wang QM (2013) Jasmonic acid and glucose synergistically modulate the accumulation of glucosinolates in *Arabidopsis thaliana.* J Exp Bot 64:5707-5719

Halkier B, Geshenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol 57:303-333

Hatzfeld Y, Maruyama A, Schmidt A, Noji M, Ishizawa K, Saito K (2000) β-Cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis. Plant Physiol 123:1163-1171

Hawkesford MJ (2005) Sulphur. In: Broadley MR, White PJ (Eds) Plant Nutritional Genomics, 1st edn. Dordrecht, The Netherlands

Hawkesford MJ, De Kok LJ (2006) Managing sulphur metabolism in plants. Plant Cell Environ 29:382-395.

Hawkesford MJ (2012) Sulfate uptake and assimilation – whole plant regulation. Proceed Int Plant Sulfur Workshop 1:11-24

Hell R, Kruse C (2006) Sulfur in biotic interactions of plants. In: Sulfur in plant: an ecological perspective (EDs) Hawkesford MJ, De Kok JP. Springer, 264p.

Herbette S, Taconnat L, Hugouvieux V, Piette L, Magniette MLM, Cuine S, Aurov P, Richaud P, Forestier C, Bourguignon J, Renou JP, Vavasseur A, Leonhardt N (2006) Genome-wide transcriptome profiling of the early cadmium response of Arabidopsis roots and shoots. Biochimie 88:1754-1768

Hesse H, Trachsel N, Suter M, Kopriva S, von Ballooms P, Rennemberg H, Brunold C (2003) Effect of glucose on assimilatory sulfate reduction in roots of *Arabidopsis thaliana*. J Exp Bot 54:1701-1709

Hossain Z, Komatsu (2013) Contribution of proteomic studies towards understanding plant heavy metal stress responses. Frontiers in Plant Sci 3:1-12

Iannone MF, Groppa MD, Brnavides MP (2015). Cadmium induces different biochemical responses in wild type and catalase-deficient-tobacco plants. Environ Exp Bot 109: 201-211

Iqbal N, Masood A, Iqbal M, Khan R, Asgher M, Fatma M, Khan NA (2013) Cross-talk between sulfur assimilation and ethylene signaling in plants. Plant Signal Behav 8:104-112

Jacob C, Anwar A (2008) The chemistry behind redox regulation with focus on sulphur redox systems. Physiol Plant 133: 469-480

Jamal A, Fazli IS, Ahmad S, Kim KT, Oh DG, Abdin MZ (2006) Effect of sulfur on nitrate reductase and ATP sulfurylase activities in groundnut (*Arachis hypogea* L.). J Plant Biol 49:513-517

Jin Z, Shen J, Qiao Z, Yang G, Wang R, Pei Y (2011) Hydrogen sulfide improves drought resistance in *Arabidopsis thaliana.* Biochem Biophys Res Commun 44:481-486

Jobe TO, Sung DY, Akmakijan G, Pham A, Komives EA, Mendoza-Cózatil DG, Schroeder JI (2012) Feedback inhibition by thiols outranks glutathione depletion: a luciferase-based screen reveals glutathione-deficient γ-ECS and glutathione synthetase mutants impaired in cadmium-induced sulfate assimilation. Plant J 70:783-795

Josefczak M, Remans T, Vangronsveld J, Cuypers A (2012) Glutathione is a key player in metal-induced oxidative stress defences. Int J Mol Sci 13:3145-3175

Jost R, Altschmied L, Bloem E, Bogs J, Gershenzon J, Hahnel U, Hansch R, Hartmann T, Kopriva S, Kruse C, Mendel RR, Papenbrock J, Reichel M, Rennenberg H, Schnug E, Schmidt A, Textor S, Tokuhita J, Wachter A, Wirtz M, Rausch T, Hell R (2005) Expression profiling of metabolic genes in response to methyl jasmonate reveals regulation of genes of primary and secondary sulphur-related pathways in *Arabidopsis thaliana*. Photosynthesis Res 86:491-508

Kawashima CG, Matthewman CA, Huang S, Lee BR, Yoshimoto N, Koprivova A, Rubio-Somoza I, Todesco M, Rathjen T, Saito K, Takahashi T, Koptiva S (2011) Interplay of SLIM1 and miR395 in the regulation of sulfate assimilation in Arabidopsis. Plant J 66:863-876

Khan NA, Mir, Nazar R, Singh S (2008) The application of ethephon (an ethylene releaser) increases growth, photosynthesis and nitrogen accumulation in mustard (*Brassica juncea* L.) under high nitrogen levels. Plant Biol 10:534-538

Khordishi MB, Yarnia M, Hassanpanah D (2009) Salinity effects on nutrients accumulation in alfafa shoots in hydroponic condition. J Food Agric Environ 7:787-790

Klein M, Papenbrock J (2004) The multi-protein family of Arabidopsis sulphotransferases and their relatives in other plant species. J Exp Bot 55:1809-1820

Kopriva S (2006) Regulation of sulfate assimilation in Arabidopsis and beyond. Ann Bot 97:479-495

Kopriva S, Rennenberg H (2004) Control of sulfate assimilation and glutathione synthesis: interaction with N and C metabolism. J Exp Bot 55:1831-1842

Koprivova A, Kopriva S (2014) Molecular mechanisms of regulation of sulphate assimilation: first steps on a long road. Front Plant Sci 5:1-11

Koprivova A, Suter M, Op den Camp R, Brunold C, Kopriva S (2000) Regulation of sulfate assimilation by nitrogen in *Arabidopsis*. Plant Physiol 122:737-746

Koralewska A, Stuiver CEE, Posthumus FS, Kopriva S, Hawkesford MJ, De Kok LJ (2008) Regulation of sulfate uptake, expression of the sulfate transporters Sultr1;1 and Sultr1;2, and APS reductase in Chinese cabbage (*Brassica pekinensis*) as affected by atmospheric H_2S nutrition and sulfate deprivation. Funct Plant Biol 35:318-327

Krischan J, Makaruk A, Harasek M (2012) Design and scale-up of an oxidative scrubbing process for the selective removal of hydrogensulfide from biogas. J Hazard Mat 215:49-56

Kruse J, Kopriva S, Hansch R, Krauss GJ, Mendel RR, Rennemberg H (2007) Interaction of sulfur and nitrogen nutrition in tobacco (*Nicotiana tabacum*) plants: significance of nitrogen source and root nitrate reductase. Plant Biol 9:638-646

Leung SCS, Smith D, Chen R, McCallum JA, McKenzie M, McManus MT (2006) Characterization of adenosine 5´-phospho-sulfate kinase (APSK) genes from higher plants. In: Sulfur metabolism in plants (EDs) De Kok LJ, Rennenber H, Hawkesford J, 67-70

Lewandowska M, Sirko A (2008) Recent advances in understanding plant response to sulphur-deficiency stress. Acta Biochim Pol 55:457–471

Li H, Yan SH, Zhao L, Tan JJ, Zhang Q, Gao F, Wang P, Hou HL, Li LJ (2014) Histone acetylation associated up-regulation of the cell wall related genes is involved in salt stress induced maize root swelling. BMC Plant Biol 105:1-14

Li ZG, Gong M, Xie H, Yang L, Li J (2012) Hydrogen Sulfide donor sodium hydrosulfide-induced heat tolerance in tobacco (*Nicotiana tabacum* L) suspension cultured cells and involvement of Ca^{2+} and calmodulin. Plant Sci 185-186:185-189

Lisjak M, Srivastava N, Teklic T, Civale L, Lewandowski K, Wilson I, Wood ME, Whiteman M, Hancock JT (2010) A novel hydrogen sulfide donor causes stomatal opening and reduces nitric oxide accumulation. Plant Physiol Biochem 48:931-935

Lisjak M, Teklic T, Wilson ID, Whiteman M, Hancock JT (2013) Hydrogen sulfide: environmental factor or signalling molecule? Plant Cell Environ 36:1607-1616

Lisjak M, Teklic T, Wilson ID, Wood ME, Whiteman M, Hancock JT (2011) Hydrogen sulfide effects on stomatal apertures. Plant Signal Behav 6:1444-1446

Lunde C, Zygadlo A, Simonsen HT, Nielsen PL, Blennow A, Haldrup A (2008) Sulphur starvation in rice: the effect on photosynthesis, carbohydrate metabolism, and oxidative stress protective pathways. Physiol Plant 133:508-521

Ma XL, Wang ZL, Qi YC, Zhao YX, Zhang H (2003) Isolation of *S*-adenosylmethionine synthase gene from *Suadea salsa* and its differential expression under NaCl stress. Acta Bot Sinica 45:1359-1365

Magrath R, Mithen R (1993) Maternal effects on the expression of individual aliphatic glucosinolates in seed and seedlings of Brassica napus. Plant Breed 111:249-252

Majic DB, Samardzic JT, Milisavljevic MD, Krstic AM, Maksimovic VR (2008) Two metallothionein gene family members in buckwheat: Expression analysis in flooding stress using Real Time RT-PCR technology. Arch Biol Sci 60:77-82

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in *Arabidopsis* roots. Plant J 42:305-314

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Yamaya T, Takahashi H (2004) Induction of SULTR1;1 sulfate transporter in Arabidopsis roots involves protein phosphorylation/dephosphorylation circuit for transcriptional regulation. Plant Cell Physiol 45:340-345

Mazid M, Khan TM, Mohammad F (2011) Response of crop plants under sulphur stress tolerance. J Stress Physiol Biochem 7:25-57

Mera R, Torres E, Abalde J (2014) Sulphate more than a nutrient protects the microalga *Chlamydomonas moewusii* from cadmium toxicity. Aquatic Toxicol 148:92-103

Monteiro CC, Carvalho RF, Gratão PL, Carvalho G, Tezotto T, Medici LO, Peres LEP, Azevedo RA (2011) Biochemical responses of the ethylene-insensitive *Never ripe* tomato mutant subjected to cadmium and sodium stresses. Environ Exp Bot 71:306-320

Mugford SG, Yoshimoto N, Reichelt M, Wirtz M, Hill L, Mugford ST, Nakazato Y, Noji M, Takahashi H, Kramell R, Gigolashvili T, Flugge UI, Wasternack C, Gershenzon J, Hell R, Saito K, Kopriva S (2009) Disruption of adenosine-5´-phosphosulfate kinase in Arabidopsis reduces levels of sulfate secondary metabolites. Plant Cell 21:910-927

Mugford SG, Mattewman CA, Hill L, Kopriva S (2010) Adenosine-5´-phosphosulfate kinase is essential for Arabidopsis viability. FEBS Lett 584:119-123

Muneer S, Lee BR, Kim KY, Park SH, Zhang Q, Kim TH (2014) Involvement of sulphur nutrition in modulating iron deficiency responses in photosynthetic organelles of oilseed rape (*Brassica napus* L.). Photosynthesis Res 119:319-329

Nazar R, Iqbal N, Syeed S, Khan NA (2011) Salicylic acid alleviates decreases in photosynthesis under salt stress by enhancing nitrogen and sulfur assimilation and antioxidant metabolism differentially in two mungbean cultivars. J Plant Physiol 168:807-815

Nazar R, Iqbal N, Masood A, Syeed S, Khan NA (2011) Understanding the significance of sulfur in improving salinity tolerance in plants. Environ Exp Bot 70:80-87

Negishi M, Pedersen LG, Petrotchenko E, Shevtsov S, Gorokhov A, Kakuta Y, Pedersen LC (2001) Structure and function of sulfotransferases. Arch Biochem Biophys 390:149-157

Noctor G (2006) Metabolic signaling in defence and stress: the central roles of soluble redox couples. Plant Cell Environ 29: 409-425

Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, Queval G, Foyer CH (2012) Glutathione in plants: an integrated overview. Plant Cell Environ 35:454-484

Nogueirol RC, Monteiro FA, Gratão PL, Borgo L, Azevedo RA (2015) Tropical soils with high aluminum concentrations cause oxidative stress in two tomato genotypes. Environ Monit Assess 187:73.

Pál M, Horváth E, Janda T, Páldi E, Szalai G (2006) Physiological changes and defence mechanisms induced by cadmium stress in maize. J Plant Nutr Soil Sci 169: 239-246

Pál M, Kovács V, Szalai G, Soós V, Ma X, Liu H, Mei H, Janda T (2014) Salicylic acid and abiotic stress responses in rice. J Agron Crop Sci 200:1-11

Peleg Z, Blumwald E (2011) Hormone balance and abiotic stress tolerance in crop plants. Curr Op Plant Biol 14:290-295

Qureshi MI, Qadir S, Zolla L (2007) Proteomics-based dissection of stress-responsive pathways in plants. J Plant Physiol 164:1239-1260

Rahoui S, Ben C, Chaoui A, Martinez Y, Yamchi A, Rickauer M, Gentzbittel L, Ferjani EE (2014) Oxidative injury and antioxidant genes regulation in cadmium-exposed radicles of six contrasted *Medicago truncatula* genotypes. Environ Sci Pollut Res 21:8070-8083

Rausch T, Watcher A (2005) Sulphur metabolism: a versatile platform for launching defence operations. Trends Plant Sci 10:503-509

Ravanel S, Gakiere B, Job D, Douce R (1998) The specific features of methionine biosynthesis and metabolism in plants. Proc Natl Acad Sci USA 95:7805-7812

Rennenberg H, Herschbach C (2012) Sulphur compounds in multiple compensation reactions of abiotic stress responses. Sulphur Metabolism in Plants. Proceedings of the International Plant Sulphur Workshop 1:203-215

Riemenschneider A, Wegele R, Schimidt A, Papenbrock J (2005) Isolation and characterization of a D-cysteine desulphydrase protein from *Arabidopsis thaliana*. FEBS J 272:1291-1304

Roy M, Wu R (2002) Overexpression of *S*-adenosylmethionine descarboxylase gene in rice increases polyamine level and enhances sodium chloride-stress tolerance. Plant Sci 163:987-992

Ruiz JM, Blumwald E (2002) Salinity-induced glutathione synthesis in *Brassica napus*. Planta 214:965-969

Salvagiotti F, Castellarín JM, Miralles DJ, Pedrol HM (2009) Sulfur fertilization improves nitrogen use efficiency in wheat by increasing nitrogen uptake. Field Crops Res 113:170-177

Sasaki-Sekimoto Y, Taki N, Obayashi T, Aono M, Matsumoto F, Sakurai N, Suzuki H, Hirai MY, Noji M, Saito K, Masuda T, Takamita K, Shibata D, Ohta H (2005) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in *Arabidopsis*. Plant J 44:653-668

Sauter M, Moffatt B, Saechao MC, Hell R, Wirtz M (2013) Methionine salvage and *S*-adenosylmethionine: essential links between sulphur, ethylene and polyamine biosynthesis. Biochem J 451:145-154

Schachtman DP, Shin R (2007) Nutrient Sensing and Signaling: NPKS. Ann Rev Plant Biol 58: 47-69

Seth CS, Remans T, Keunen E, Jozefczak M, Gielen H, Opdenakker K, Weyens N, Vangronsveld J, Cuypers A (2012) Phytoextraction of toxic metals: a central role for glutathione. Plant Cell Environ 35:334-346

Shan C, Liang Z (2010) Jasmonic acid regulates ascorbate and glutathione metabolism in *Agropyron cristatum* leaves under water stress. Plant Sci 178:130-139

Shao HB, Chu LY, Lu ZH, Kang CM (2008) Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. Int J Biol Sci 4:8-14

Shulaev V, Cortesa D, Millerb G, Mittlerb R (2008) Metabolomics for plant stress response. Physiol Plant 132:199–208

Siddiqui MH, Mohammad F, Khan MN, Khan MNA (2008) Cumulative effect of soil and foliar application of nitrogen, phosphorus and sulfur on growth, physico-biochemical parameters, yield attributes and fatty acid composition in oil of erucic acid-free rapeseed-mustard genotypes. J Plant Nutr 31:1284-1298

Siddiqui MH, Mohammad F, Masrooor M, Khan M, Al-Whaibi M (2012) Cumulative effects of nitrogen and sulfur on *Brassica juncea* L. genotypes under NaCl stress. Protoplasma 249-139-153

Srivastava S, Srivastava AK, Suprasanna P, D'Souza SF (2013) Identification and profiling of arsenic stress-induced microRNAs in *Brassica juncea*. J Exp Bot 64:303-315

Stulen I, De Kok LJ (2012) Foreword: Exploring interactions between sulfate and nitrate uptake at a whole plant level. Proceed Int Plant Sulfur Workshop 1:1-8

Su Y, Liu J, Lu Z, Wang X, Zhang Z, Shi G (2014) Effects of iron deficiency on subcellular distribution and chemical forms of cadmium in peanut roots in relation to its translocation. Environ Exp Bot 97:40-48

Takahashi H, Buchner P, Yoshimoto N, Hawkesford MJ, Shiu SH (2012) Evolutionary relationships and functional diversity of plant sulfate transporters. Front Plant Sci 2:1-9

Takahashi H, Kopriva S, Giordano M, Saito K, Hell R (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Ann Rev Plant Biol 62:157-184

Vitória AP, Lea PJ, Azevedo RA (2001) Antioxidant enzymes responses to cadmium in radish tissues. Phytochemistry 57:701-710

Waie B, Rajam MV (2003) Effect of increased polyamine biosynthesis on stress responses in transgenic tobacco by introduction of human S-adenosylmethionine gene. Plant Sci 164:727-734

Wang KLC, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. Plant Cell 14:S131-S151

Wang SC, Frey P (2007) S-adenosylmethionine as an oxidant: the radical SAM superfamily. Trends Biochem Sci 32:101-110

Weber M, Harada E, Vess C, von Roepenack-Lahaye E, Clemens S (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential hyperaccumulation factors. Plant J 37:269-281

Yadav SK (2010) Heavy metal toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. South African J Bot 76:167-179

Yoshimoto N, Inoue E, Watanabe-Takahashi A, Saito K, Takahashi H (2007) Posttranscriptional regulation of high affinity sulfate transporters in *Arabidopsis* by sulfur nutrition. Plant Physiol 145:377-388

Youssefian S, Nakamura M, Orudgev E, Kondo N (2001) Increased cysteine biosynthesis capacity of transgenic tobacco overexpressing an O-acetylserine(thiol) lyase modifies plant responses to oxidative stress. Plant Physiol 126:1001-1011

Zagorchev L, Seal CE, Kranner I, Odjakova M (2013) A central role for thiols in plant tolerance to abiotic stress. Int J Mol Sci 14:7405-7432

Zhang B, Pasini R, Dan H, Joshi N, Zhao YH, Leustek T, Zheng ZL (2014) Aberrant gene expression in the *Arabidopsis* SULTR1;2 mutants suggests a possible regulatory role for this sulphate transporter in response to sulphur nutrient status. Plant J 77:185-197

Zhang H, Tan ZQ, Hu LY, Wang SH, Luo JP, Jones RL (2010) Hydrogen sulfide alleviates aluminium toxicity in germinating wheat seedlings. J Integrative Plant Biol 52:556-567

Zhang SW, Li CH, Cao J, Zhang YC, Zhang SQ, Xia YF, Sun DY, Sun Y (2009) Altered architecture and enhanced drought tolerance in rice via the down-regulation of indole-3-acetic acid by TLD1/OsGH3.13 activation. Plant Physiol 151:1889-1901

APPENDIX

Abbreviations

ABA	Abscisic acid
ACCO	1-aminocyclopropane-1-carboxylic acid oxidase
ACCS	1-aminocyclopropane-1-carboxylic acid synthase
Al	Aluminum
$AlCl_3$	Aluminum chloride
APR	Adenosine 5' phosphosulfate reductase
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
ATP-S	Adenosine triphosphate-sulfurylase
AU	Auxins
C	Carbon
CAS	β-cyanoalanine synthase
CAT	Catalase
CbL	Cystathionine β-lyase
Cd	Cadmium
CgS	Cystathionine γ-synthase
cDNA	Complementary deoxyribonucleic acid
CK	Cytokinins
Cys	Cysteine
CysK	Cysteine synthase
ET	Ethylene
GA	Gibberellins
DMSP	Dimethylsulfonio-propionate
GLU	Glutamate or glutamic acid
GLY	Glycine
GPX	Guaiacol peroxidase
GR	Glutathione reductase
GS	Glucosinolates
GSH	Glutathione

H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HMT	Homocysteine S-methyltransferase
HPLC	High-performance liquid chromatography
JA	Jasmonates
K	Potassium
LCD	L-cysteine desulfhydrase
MDA	Malondialdehyde
MeJA	Methyl jasmonate
Met	Methionine
mRNA	Messenger ribonucleic acid
MS	Methionine synthase
MTs	Metallothioneins
N	Nitrogen
NaHS	Sodium hydrosulfide
NR	Nitrate reductase
OASS	O-acetylserine sulfhydrylase
OASTL	O-acetylserine(thiol) lyase
OPHS	O-phosphohomoserine
PA	Polyamines
PAPS	3’-phosphoadenosine 5’-phosphosulfate
PCS	Phytochelatins
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
S	Sulfur
S^{2-}	Sulfide
SA	Salicylic acid
SAM	S-adenosyl methionine
SAMDC	S-adenosylmethionine decarboxylase
SAMS	S-adenosyl methionine synthetase, SAM synthetase
SAT	Serine acetyltransferase
SLC13	Sodium/ SO_4^{2-} co-transporter
SLC26	SO_4^{2-}/anion exchanger in animals

SMM	S-methylmethionine
SO_2	Sulfur dioxide
SO_3^{2-}	Sulfite
SO_4^{2-}	Sulfate
SOD	Superoxide dismutase
SOT	Sulfotransferase protein family
STs	Sulfotransferases
SUL	Proton/ SO_4^{2-}co-transporter in yeast
SULTR	Proton/ SO_4^{2-}co-transporter in plants
TS	Threonine synthase

CHAPTER 3 - SELENIUM AND HEAVY METALS*

1. Selenium: a powerful antioxidant

Selenium (Se) is an important micronutrient for humans and animals, participating as a constituent atom of some antioxidant enzymes, including glutathione peroxidase (GPOX), which is involved in hydrogen peroxide (H_2O_2) detoxification inside cells (Fairweather-Tait, 2011). The absence or inadequate intake of this element in the diet can cause a number of Se-related health diseases (Mehdi et al., 2013).

In higher plants, selenium has not been recognized as an essential nutrient so far, but at suitable levels it can induce a variety of beneficial effects, such as enhanced photosynthesis, growth improvement, higher accumulation of starch and sugars and delayed senescence (Zembala et al., 2010; Feng and Wei, 2012). In addition, in plants that grow in the presence of heavy metals, selenium can stimulate the cell antioxidant capacity through the enhancement of the activity of antioxidant enzymes and the synthesis of non-enzymatic molecules, like glutathione (GSH) and phytochelatins (PCs), and may induce the spontaneous dismutation of the superoxide radical ($O_2^{-\bullet}$) into H_2O_2 (Feng et al., 2013). The lower concentration of reactive oxygen species (ROS) due to Se would lower the lipid peroxidation process caused by metal-induced oxidative stress (Feng and Wei, 2012) (Figure 1).

2. Antioxidant defense mechanisms

Reactive oxygen species are the unstable and partially reduced forms of the atmospheric oxygen (O_2), which shows a great capacity to oxidize other cell compounds. These molecules are formed from the transfer of one, two or three electrons to the O_2 molecule, thus forming the superoxide radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and also the "Singlet" oxygen (O_2^1), respectively, during the aerobic cellular metabolism in organelles like mitochondria and peroxisomes (Shieber and Chandel, 2014). The cellular defense responses are important to maintain low concentrations of ROS, and involve enzymatic and non-enzymatic antioxidant mechanisms. The superoxide dismutase (SOD) constitutes the first enzymatic barrier against the oxidative stress, catalyzing the dismutation reaction of $O_2^{-\bullet}$, in order to form O_2 and H_2O_2 (Shieber and Chandel, 2014). Subsequently, H_2O_2 can be quickly

*Further publication (2017). Book: "Selenium in plants: Molecular, Physiological, Ecological and Evolutionary Aspects". Chapter 20: Interactions of Se with other elements and organic nutraceuticals. Springer, USA.

converted into H_2O and O_2 by the action of specific enzymes such as catalase (CAT) and peroxidases (POX). The non-enzymatic molecules implied in ROS detoxification are also important to maintain the cellular redox state, and mainly include glutathione (GSH), phytochelatins (PCs), proline, flavonoids, alkaloids, carotenoids (Foyer and Noctor, 2012).

3. Role of selenium against heavy metal stress in plants

High concentrations of heavy metals in soils can lead, directly or indirectly, to plant death and losses in crop yields. The reason is that metals can cause an imbalance in the cellular redox status due to the metal-induced overproduction of ROS over the cell antioxidant capacity (Cuypers et al., 2010; Hasanuzzaman et al., 2012). The result of elevate levels of ROS is the damage to membrane phospholipids, proteins, nucleic acids and other cellular components. However, a number of studies has shown that Se can improve the enzymatic and non-enzymatic antioxidant systems of plants, thus enhancing their capacity to overcome the oxidative stress (Malik et al., 2012; Lin et al., 2012; Feng et al., 2013) (Figure 1).

3.1 Cadmium (Cd)

Cadmium (Cd) is one of the most toxic among heavy metals. This metal can be complexed with the organic fraction of soil, and be released as Cd^{2+}, which is easily assimilated by plants through membrane transporters involved to the uptake of some nutrients, like Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+} and Zn^{2+} (Qin, et al., 2013). The presence of high concentrations of Cd^{2+} in soil can cause a decrease of the plant capacity to accumulate these and other nutrients and molecules, such as chlorophyll, carotenoids and a broad spectrum of proteins, including antioxidant enzymes, that contain some of these nutrients in their structure (Cuypers et al., 2010; Hasanuzzaman et al., 2012). Indeed, due to high metal chemical similarity, Cd^{2+} can replace other metals in the active sites of antioxidant metalloenzymes, e.g. SOD and CAT, therefore causing its inactivation (Cuypers et al., 2010).

Furthermore, the free Cd^{2+} ions shows high affinity to the thiol groups (R-SH) present in different molecules, such as reduced glutathione (GSH), proteins and cysteine (Cuypers et al., 2010). Selenium can also be incorporated in cysteine by replacing sulfur (S) into the thiol group to form the analog Se-amino acid Se-cysteine (SeCys) (Malagoli

et al., 2015). Consequently, Cd^{2+} and Se may compete for the same binding site into the thiol group of cysteine. As a result, Cd uptake, translocation and accumulation in plants may be reduced (Lin et al., 2012).

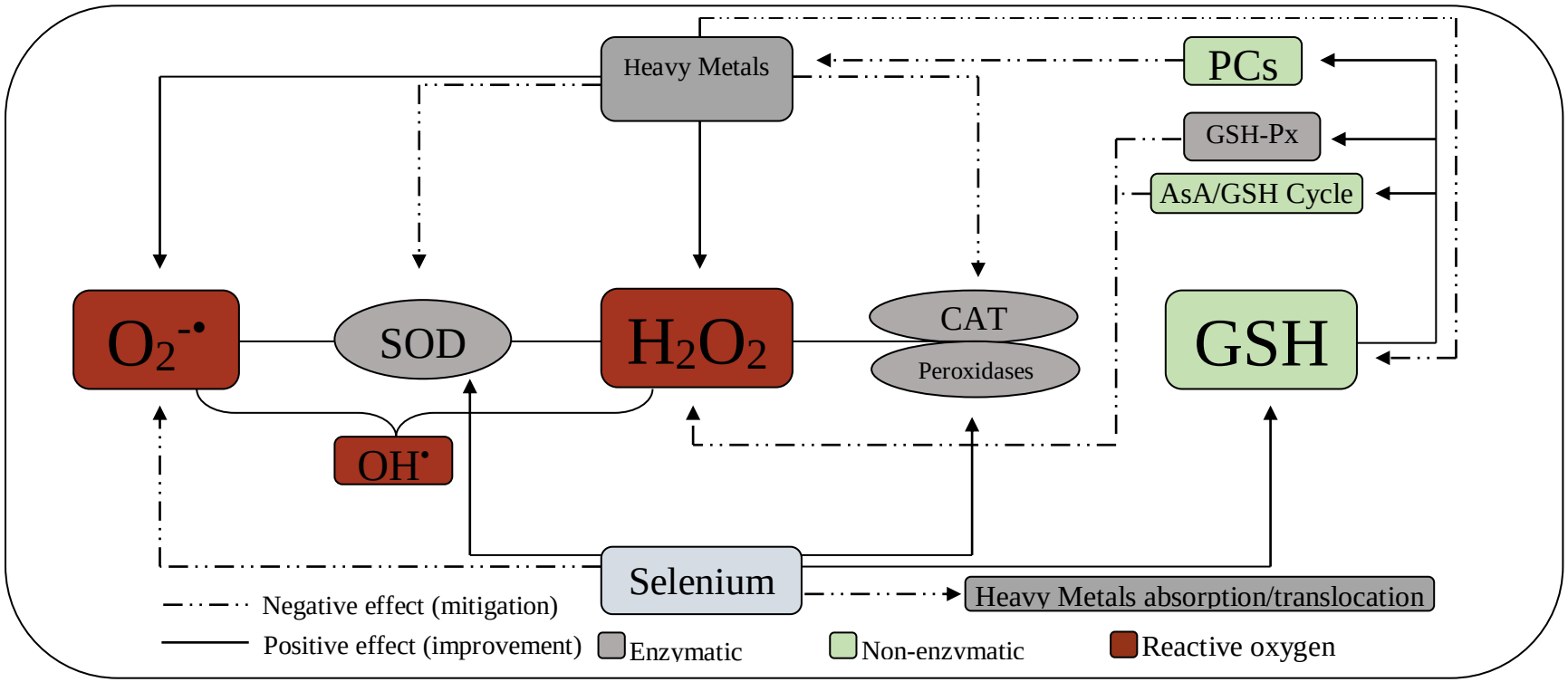

Figure 1. Selenium effects against the oxidative stress induced by the heavy metal contamination. AsA (Ascorbate), CAT (Catalase), GSH (reduced glutathione), GSH-Px (Glutathione peroxidase), PCs (Phytochelatins), SOD (Superoxide dismutase), Reactive Oxygen Species: superoxide radical ($O_2^{-\bullet}$); hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) formed from the combination of $O_2^{-\bullet}$ and H_2O_2 (Haber-Weiss reaction).

Recent studies reported the positive effect of Se on the activity of antioxidant enzymes in response to Cd stress. Lin et al. (2012) showed that the application of 3 μM Se to rice (*Oryza sativa*) plants can increase the activity of SOD, peroxidase or guaiacol peroxidase (POD/GPOX) enzymes in roots and leaves, respectively. Fifty μM Se were proved to improve the activity of CAT, GPOX, glutathione reductase (GR), ascorbate peroxidase (APX) and enzymes related to the ascorbate-glutathione cycle, like monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), as well as non enzymatic compounds of this cycle like GSH, especially in the oxidized form (GSSG), in rape (*Brassica napus*) (Hasanuzzaman et al., 2012). Moreover, 5 and 10 μM of Se increased the activity of CAT, APX and GR in leaves of sunflower (*Helianthus annuus*) (Saidi et al., 2014a), and concomitantly decreased ROS production, lipid peroxidation, oxidative stress, and recovered the membrane physicochemical characteristics.

3.2 Arsenic (As)

Arsenic (As) is a metalloid, which can be mainly found in the forms of arsenate (AsO43-) or arsenite (AsO_3) in soils and waters. As contamination in soils occurs mainly in response to anthropogenic activities, like the use of pesticides and sewage sludge in crop fields, as well as to other activities not directly related to agriculture, such as mining and metal melting. The detrimental effect triggered by this metal in plants is related to a reduction of growth and development caused by photosynthesis inhibition, inefficient nutrition and oxidative stress (Malik et al., 2012; Han et al., 2015).

Similar to Cd^{2+}, As bind to the sulfur presented in the T-SH group of GSH. Whether selenium is provided to plants, it can compete with As for the binding to the thiol group, thus actively reducing As absorption (Han et al., 2015). Likewise, 5 μM Se are reported to reduce As uptake in mungbean (*Phaseolus aureus* Roxb.), and alleviate oxidative stress by enhancing the activity of SOD, POD, APX enzymes and the synthesis of GSH and ascorbic acid (ASC) (Malik et al., 2012). Similar results were found in tobacco (*Nicotiana tabacum*) plants treated with 0.1 mgL^{-1} selenite (Han et al., 2015). Srivastava et al. (2009) found that 5 μM and 10 μM selenate decreased the lipid peroxidation process, likely because of higher production of the non-protein thiol GSH in *Pteris vittata* L.

3.3 Lead (Pb)

Lead is one of the most dangerous pollutants worldwide, where the main sources are fertilizers, pesticides, mining, metal melting, automobiles fumes and industrial waste or discharge. This heavy metal is considered carcinogenic to humans as it causes DNA damages and its synthesis inhibition, while in plants Pb can disrupt the membrane structure and permeability causing dehydration and decrease of the electron transportation in photosynthesis, this metal also binds to the thiol groups of amino acids, enzymes and proteins and, as a final result, induces the overproduction of ROS and oxidative stress (Mroczek-Zdyrska and Wojcik, 2012).

The Se beneficial effects against Pb in plants have been described by few authors recently and are directly related to the ROS scavenging in cells (Mroczek-Zdyrska and Wojcik, 2012; Yuan et al., 2013; Hu et al., 2014). For instance, 1.5 μM selenite concentration lowered the superoxide radical ($O_2^{-\bullet}$) production and concentration in the apical part of the root in *Vicia faba* L. *minor*, increased the activity of POD/GPOX enzymes and caused the content of thiol groups (T-SH) (Mroczek-Zdyrska and Wojcik, 2012). In addition, 1 μM selenite concentration improved the leaf biomass of coleus (*Coleus blumei* Benth.) and decreased the rate of lipid peroxidation, likely because of the higher GSH level in roots (Yuan et al., 2013). Hu et al. (2014) demonstrate that 0.5 mg kg^{-1} selenite could induce low Pb concentration in rice (*Oryza sativa*) tissues (shoot and husk).

3.4 Other heavy metals

Excess of some nutrients (metalloids) in plants can increase the production of ROS and cause decreased, denaturation and inactivation of antioxidant enzymes. Manganese (Mn) is an important microelement for plants, but at high concentration it can be toxic. The toxicity is related to photosynthesis suppression, membrane integrity disruption, lower protein metabolism and oxidative stress. As showed by Saidi et al. (2014b), 5 μM of selenate can effectively counteract the Mn detrimental effects in sunflower (Helianthus annuus) by improving the CAT, APX and GPOX activity.

On the contrary, chromium (Cr) has no biological function in plants and can be toxic at any concentration in soil, especially near areas with industrial activities. Se concentrations as high as 3 μM can improve SOD activity in rice roots alleviating the

toxic effects of Cr on growth, and increase H^+- ATPase activity, thus protecting the plants from Cr-induced oxidative stress (Cao et al., 2013).

Mercury (Hg) is also a harmful environmental pollutant, and soil contamination by this metal comes from mining, metal smelting, and industrial activities. Its presence in plants causes growth inhibition, oxidative stress, lipid peroxidation, deficient chlorophyll production and photosynthesis (Zhao et al., 2013). Selenite and selenate can improve growth in garlic (*Allium sativum*) and also avoid Hg absorption, translocation and accumulation in roots and leaves, when they are applied at doses higher than 1 mg L^{-1} (Zhao et al., 2013).

4. Final considerations

The maintenance of cellular homeostasis under heavy metals contamination depends on several interlinked and complex mechanisms. The antioxidant defense does not follow a specific pattern of action, even under Se application, and may differ due to various factors such as plant species, concentration, exposure time, nutrient concentration in soil, plant developmental stage, organs, form of Se and tissues analyzed. By this way, the plant defense against heavy metals and other different abiotic stresses is a dynamic and adaptive system, in which the Se is extensively investigated to improve the enzymatic and non-enzymatic antioxidant responses to counteract the heavy metal induced stress.

In this contest, Se can detoxify the oxidative stress directly, as a response to its powerful antioxidant characteristics, converting the superoxide radical ($O_2^{-\bullet}$) into hydrogen peroxide (H_2O_2). Indirectly, Se improves the antioxidant enzymes activities such as superoxide dismutase (SOD), catalase (CAT) and other peroxidases (POX), and the non-enzymatic antioxidant compounds concentration like the reduced glutathione (GSH), proline, flavonoids, alkaloids carotenoids and phytochelatins (PCs). Se is also expected to actively reduce the heavy metal absorption and translocation, mainly as a response to the production of PCs and competition for the same binding sites into the cell environment. All this processes, as a final analysis, provides better maintenance of the ROS production and concentration in cells and, as a consequence, decrease the induced oxidative stress and avoid the destruction of the cell membranes and structures.

5. References

Cao, F., Wang, N., Zhang, M., Dai, H., Dawood, M., Zhang, G., Wu, F. (2013). Comparative study of alleviating effects of GSH, Se and Zn under combined contamination of cadmium and chromium in rice (*Oryza sativa*). Biometals, v. 26, p. 297-308.

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, v. 23, n. 5, p. 927-940.

Feng, R.W., Wei, C.Y. (2012). Antioxidative mechanisms on selenium accumulation in *Pteris vittata* L., a potential selenium phytoremediation plant. Plant Soil Environ., v. 58, p. 105‑110.

Fairweather-Tait, S.J., Bao, Y., Broadley, M.R., Collings, R., Ford, D., Hesketh, J.E., Hurst, R. Selenium in Human Health and Disease. Antioxid Redox Signal. v. 14(7), p. 1337-1383.

Feng, R., Wei, C., Tu, S. (2013). The roles of selenium in protecting plants against abiotic stresses. Environ. Exp. Bot. v. 87, p. 58-68.

Foyer, C. H., Noctor, G. (2012). Managing the cellular redox hub in photosynthetic organisms. Plant, Cell Environ., v.35, n. 2, p. 199-201.

Han, D., Xiong, S., Tu, S., Liu, J., Chen, C. (2015). Interactive effects of selenium and arsenic on growth, antioxidant system, arsenic and selenium species of *Nicotiana tabacum* L. Environ. Exp. Bot. v.117, p. 12-19.

Hasanuzzaman, M., Hossain, M.A., Fujita, M. (2012). Exogenous Selenium Pretreatment Protects Rapeseed Seedlings from Cadmium-Induced Oxidative Stress by Upregulating Antioxidant Defense and Methylglyoxal Detoxification Systems. Biol. Trace. Elem. Res., DOI 10.1007/s12011-012-9419-4.

Hu, Y., Norton, G.J., Duan, G., Huang, Y., Liu, Y. (2014). Effect of selenium fertilization on the accumulation of cadmium and lead in rice plants. Plant Soil, v. 384, p. 131-140.

Lin, L., Zhou, W., Dai, H., Cao, F., Zhang, G., Wu, F. (2012). Selenium reduces cadmium uptake and mitigates cadmium toxicity in rice. J. Hazard. Mater., 235-236, 343-351.

Malagoli, M., Schiavon, M., dall'Acqua, S., Pilon-Smits, E.A.H., 2015. Effects of selenium biofortification on crop nutritional quality. Front. Plant. Sci., 6 (280), 1-5.

Malik, J.A., Goel, S., Kaur, N., Sharma, S., Singh, I., Nayyar, H. (2012). Selenium antagonises the toxic effects of arsenic on mungbean (*Phaseolus aureus* Roxb.) plants by restricting its uptake and enhancing the antioxidative and detoxification mechanisms. Environ. Exp. Bot., v 77, p. 242-248.

Mehdi, Y., Hornick, J.L., Istasse, L., Dufrasne, I. (2013). Selenium in the Environment, Metabolism and Involvement in Body Functions. Molecules, v. 18(3), p. 3292-3311.

Mroczek-Zdyrska, M., Wojcik, M. (2012). The Influence of Selenium on Root Growth and Oxidative Stress Induced by Lead in *Vicia faba* L. minor Plants. Biol. Trace. Elem. Res. v.147, p. 320-328.

Qin, Q., Li, X., Wu, H., Zhang, Y., Feng, Q., Tai, P. (2013). Characterization of cadmium (108Cd) distribution and accumulation in *Tagetes erecta* L. seedlings: Effect of split-root and of remove-xylem/phloem. Chemosphere, 93, 2284-2288.

Saidi, I., Chtouron, Y., Djebali, W. (2014a). Selenium alleviates cadmium toxicity by preventing oxidative stress in sunflower (*Helianthus annuus*) seedlings. J. Plant Physiol., v. 171(5), p. 85-91.

Saidi, I., Nawel, N., Djebali, W. (2014b) Role of selenium in preventing manganese toxicity in sunflower (Helianthus annuus) seedling. S. Afr. J. Bot., v. 94, p. 88-94.

Shieber, M., Chandel, N.S. (2014). ROS Function in Redox Signaling and Oxidative stress. Crr. Biol. v. 24(10), p. R453-R462.

Yuan, J., Hu, M., Zhou, Z. (2013). Selenium Treatment Mitigates the Effect of Lead Exposure in Coleus Blumei Benth. Environ. Anal. Toxicol., v. 3(6), http://dx.doi.org/10.4172/2161-0525.1000191.

Zembala, M., Filek. M., Walas, S., Mrowiec, H., Kornás, A., Miszalski, Z., Hatkainen, H. (2010). Effect of Selenium on macro and microelement distribution and physiological parameters of rape and wheat seedlings exposed to cadmium stress. Plant and Soil, v. 329, p. 457-468.

Zhao, J., Gao, Y., Li, Y.F., Hu, Y., Peng, X., Dong, Y., Li, B., Chen, C., Chai, Z. (2013). Selenium inhibits the phytotoxicity of mercury in garlic (*Allium sativum*). Environ. Res., v. 125, p.75-81.

CHAPTER 4 - SELENIUM ALLEVIATES CADMIUM TOXICITY IN TOMATO FRUITS*

Abstract. Selenium (Se) is a beneficial element for plants and essential to mammals, being increasingly used as a fertilizer. Cadmium (Cd) is toxic to crops and consumers. In order to identify the possible effect of Se in alleviating tomato stress from Cd^{2+} toxicity, the fruits dry weight, chlorophyll, carotenoids, proline concentration, macronutrients, micronutrients, Cd^{2+} and Se concentration, as well as $Cd^{2+}$and Se translocation index were analyzed in two different tomato genotypes, Micro-Tom (MT) and the *high pigment*-1 (*hp1*). Tomato plants were submitted to Cd^{2+} (0 and 0.5 mM $CdCl_2$) and Se (0 and 50 µM Na_2SeO_3), individually applied or in combination. Fruit growth was not affected by Se application neither when applied alone or with Cd^{2+}, nor were pigment concentrations in fruits of both genotypes. Contrarily, fruit proline concentration increased in genotype *hp1* when Se was applied alone and in the MT genotype when Se was applied with Cd^{2+}. Fruit micronutrient concentration were negatively affected by Cd^{2+} and by the mixture of Se and Cd^{2+}, while the Se-alone treatment enhanced the concentration of Mn and Zn in *hp1*and Fe in MT. Importantly, Se demonstrated a strong capability to reduce Cd^{2+} concentration in the roots of both genotypes and Cd^{2+} translocation to above ground parts only in the MT genotype. Thus, the alleviating effect of Se on Cd^{2+} stress in tomato could be related to restriction of Cd^{2+} uptake and translocation, enhancing micronutrient concentration in fruits and, finally, enhancing fruit proline concentration. Selenium fertilization may therefore be considered a promising way to alleviate Cd toxicity in tomato.

Keywords. Tomato, Abiotic stress, *High-pigment* 1, Micro-Tom, Heavy metal.

*Submitted (15/12/2015). Journal: Ecotoxicology and Environmental Safety

1. Introduction

Among the numerous sources of abiotic stress, the heavy metal contamination in agricultural soil is a worldwide concern for crop production (Gonzales et al., 2015), while the metal mining, smelting and subsequent industrial processes are important sources of this environmental pollution. The presence of heavy metals, specifically in cultivated soil, represent a great concern due to the high toxicity and persistence in the environment (Liu et al., 2011) and may be related to the excessive use of phosphate fertilizers, which lead to the metal uptake by crops and consequent food chain bioaccumulation, representing a major threat to living organisms (Xu et al., 2013).

Cadmium (Cd) is one of the most toxic among the heavy metals. It can be complexed with the organic fraction of the soil, and be released as Cd^{2+} (Gallego et al., 2012), which is easily assimilated by plants through the same membrane transporters involved to the uptake of nutrients like Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+} and Zn^{2+} (Qin et al., 2013), leading to a nutrient deficiency, while its presence in soil can result in a commercial rejection of certain cultivars and may become dangerous to human health, especially if it is accumulated in tissues assimilated into the food chain, leading to considerable losses in plant productivity and hazardous health effects to humans (Gallego et al. 2012; Qin et al. 2013).

Several studies have demonstrated beneficial effects of supplying plants with low Selenium (Se) concentrations, which appear to enhance photosynthesis (Wang et al., 2012), cause a lower lipid peroxidation and improve the restoration of the membrane and overall structure of chloroplasts (Feng et al., 2013), stimulate the cellular antioxidant systems (Djanaguiraman et al., 2010; Walaa et al., 2010; Feng et al., 2013) and also improve the Proline concentration (Hawrylak-Nowak, 2009; Walaa et al. 2010), which plays an important role in cellular osmotic regulation and plant protection

against different abiotic stresses, therefore its quantification is used as a plant stress gauge (Shevyakova et al., 2013). However, the specific effect of Se to the Cd uptake, translocation and concentration in tomato plants, in order to avoid the Cd bioaccumulation in the food chain, have not been elucidated.

Tomato (*Lycopersicum esculentum* Mill.) cv. Micro-Tom (MT) and *high pigment*-1 (*hp1*) genotypes are considered excellent models for biological studies by virtue of their short life cycle and reduced size. The *hp1* genotype is characterized by high concentrations of antioxidants such as carotenoids (particularly lycopene), flavonoids, vitamin C and chlorophyll which improves the antioxidant defense (Calvenzani et al., 2010; Kilamb et al., 2013),which could help provide a better understanding of the interplay between Se and antioxidants in relation to Cd stress in tomato plants.

The aim of this study was to investigate whether selenite (Na_2SeO_3, henceforth referred to as Se, may avoid the uptake, translocation and concentration of Cd ($CdCl_2$), henceforth referred to as Cd^{2+}, in different tomato tissues as well as to analyze the fruits overall status through the nutritional analyses, dry weight, pigments and proline concentration.

2. Material and methods

2.1 Plant material and experimental treatments

Seeds of tomato (*Lycopersicum esculentum* Mill.) cv. Micro-Tom (MT) and the genotype *high pigment*-1 (*hp1*), were cultivated in separate boxes containing a mixture of 1:1 (by volume) of commercial pot mix (Plantmax HT-Eucatex®, Brazil) and medium size vermiculite, supplemented with 1 $g.L^{-1}$of 10:10:10 NPK and 4 $g.L^{-1}$ of lime and maintained in a greenhouse. After the first true leaves appeared, seedlings were transplanted to 1 L Leonard pots (Gratão et al., 2012) (1 seedling per pot) filled with

sand and polystyrene (4:3) and supplied with Hoagland's nutrient solution (Hoagland and Arnon, 1950). Subsequently, twenty-one-day old plants were selected and further grown in the same solution spiked with either 0 mM (control) or 0.5 mM $CdCl_2$ (cadmium chloride, CAS: 10108-64-2) and 0 µM or 50 µM Na_2SeO_3 (anhydrous sodium selenite, CAS: 10102-18-8) applied either individually or simultaneously. The Hoagland solution, with or without $CdCl_2$ and $Na_2SeO_3$was changed weekly and the total volume was maintained at a constant level by using distilled water. The experiment was designed to have the following treatments: T1: Control; T2: Se 50 µM (MT, *hp1*); T3: Cd 0.5 mM (MT, *hp1*); T4: Cd 0.5 mM + Se 50 µM (MT, *hp1*). After a period of 95 days post germination, corresponding to 74 days of exposure to $CdCl_2$ and/or Na_2SeO_3, samples of fruits were harvested, rinsed and frozen immediately in liquid N_2, and stored at -80ºC for further analyses.

2.2 Fruits dry weight

Samples of fruits (total fruit weight per plant) were placed in a thermal convection laboratory oven (Fanem® SP, BR – model 330) at 70ºC for a week. Later, these fruits were ground in an electric mill (Marconi®, model 048) and the dry measurements were performed on an analytical balance (Denver instruments company®, model AA-200) accurate to 1.10^{-18} mg. Fruit production was quantified as biomass (g).

2.3 Fruits concentration of chlorophyll and carotenoids

Samples of fruit pulp were subjected to spectrophotometric pigment quantification in triplicate. Pigment extraction was carried out in 80% acetone and the extract was filtered through filter paper using a vacuum pump. Measurement of pigments was performed in a spectrophotometer (Beckman®, model DU-640) at the following wavelengths: chlorophyll-*a* in 663 nm, chlorophyll-*b* in 647 nm and

carotenoids (carotene[c] + xanthophyll [x]) in 470 nm. The total chlorophyll (chl) and carotenoids (car) contents were calculated as described by Lichtenthaler (1987):

Chl *a* = 12.25 x A663 – 2.79 x A647 Eq. (A.1)

Chl *b* = 21.50 x A647 – 5.10 x A663Eq. (A.2)

Chl *a*+*b* = 7.15 x A663 + 18.71 x A647 Eq. (A.3)

Car c+x = (1000 A470 – 1.82 Chl*a* – 85.02 Chl*b*)/198Eq. (A.4)

Pigment concentration was expressed in micrograms of pigment per gram of tissue fresh weight (µg g^{-1} FW).

2.4 Fruits proline concentration

Free proline concentration in fruits was measured as described by Gratão et al. (2012). Samples (0.5 g) of fruit pulp were extracted in 3% sulphosalicylic acid. The homogenate was centrifuged at 10.000 g for 15 minutes at 4ºC, and 2 mL of the supernatant was held for 1 hour in boiling water by adding 2 mL ninhydrin acid (C_9H_6O4 – CAS: 485-47-2) and 2mL of glacial acetic acid(CH_3CO_2H – CAS: 64-19-7), to which cold toluene (4 mL) was added. The absorbance was read at 520 nm and proline concentration calculated as mmol g^{-1} FW against a proline standard curve.

2.5 Fruits Cd^{2+} and Se quantification and nutritional analyses

Quantitative Cd^{2+}, Se and nutrient analysis was carried out using energy dispersive X-ray fluorescence spectrometry (EDXRF) as described by Tezzoto et al. (2013). Samples of fruits were dried at 70ºC for 7 days, and 0.2 g DW of fine powder, obtained following grinding with mortar and pestle, were microwave digested with 2 mL of 70% HNO_3, 2mL of H_2O_2 and 2 mL of Milli-Q water (18.2 MΩ cm a 25ºC) at a controlled pressure of 2 MPa, concentrated acids on a digestion block heated gradually to 203 ºC. Biological samples with increased concentrations of Cd^{2+}, Se and nutrients

were used to establish standard calibration curves. The samples were irradiated in triplicate for 300s using a Shimadzu® EDX 720 system (São Paulo, Brazil). The samples were irradiated using an Rh X-ray tube operated at 15 kV (Na to Sc) and 50 kV (Ti to U) applied on the rhodium tube. The detection was carried out using the Si (Li) detector cooled with liquid nitrogen. For all studies, matrices with 2048 dependent variables (columns) were constructed (energy values), and the independent variable was the Cd^{2+}, Se or nutrient concentration. The modeling and prediction tools for date exploration were performed with the use of the chemometrics package pirouette, version 3.11 (Infometrix®, Bothell, WA, USA).

2.6 Cd^{2+} and Se Translocation index

The Cd^{2+} and Se translocation index (TI, %) was calculated by dividing their concentration in the shoot (fruits, leaves) by their total plant concentration (root, leaves, fruits), and multiplying the quotient by 100.

2.7 Statistical analyses

The experimental design was randomized with five replication pots, being the results expressed as mean and standard error of mean (± SD) of three independent replicates of each extract for dry weight, pigments, proline concentration, Cd^{2+}, Se and nutrients concentration analyses. The statistical analysis was performed using the Assistat software version 7.7 (Silva and Azevedo, 2009). ANOVA followed by a multiple comparison between means by the Tukey test for each character, at a 0.05 level of significance was performed.

3. Results and Discussion

3.1 Nutritional analyses.

The application of Cd^{2+} caused a decrease in zinc (Zn) and iron (Fe) concentration in the MT fruits, while the *hp1* genotype contained less manganese (Mn) and Zn in the same situation compared to the control plants (Table 1). Furthermore, Se applied alone induced a decrease in Zn in the MT fruits, while an increase of Fe was noted (Table 1). The same treatment also induced an increase of Mn and Zn in *hp1* fruits, but, contrarily, the Fe concentration in fruits of this genotype was lower compared to the control (Table 1). When Se was supplied together with Cd^{2+}, the concentration of Fe and Zn decreased in fruits for both genotypes (Table 1), while Mn decreased only in *hp1* (Table 1) compared to the control plants.

Some reports have shown the synergetic effect of Cd^{2+} by stimulating the absorption of specific nutrients, whereas the concentration of Fe, for example, increased after Cd^{2+} contamination (Kumar et al., 2014). In our study, the application of 0.5 mM of $CdCl_2$ caused an increase in potassium (K) and Fe concentration specifically in the *hp1* fruits (Table 1), which could indicate that the *hp1* suffered less membrane damage, from lipid peroxidation or other damaging processes, which could stimulate mineral transportation during the oxidative stress caused by the heavy metal exposure. In contrast, the Zn concentration decreased in fruits of both genotypes when 0.5 mM of $CdCl_2$ was applied, the Fe concentration decreased specifically in the MT fruits and Mn concentration decreased in *hp1* under the same condition, in comparison to the control plants (Table 1).

The negative effect on nutrients concentration in response to the Cd^{2+} stress scan be related to various deleterious mechanisms, due to increased lipid peroxidation, decreased plasmalema fluidity, and, as a consequence, harm to the entire cell integrity,

which can directly reduce absorption and translocation of nutrients (Zembala et al., 2010; Kumar et al., 2014). Furthermore, the form Cd^{2+}, usually concentrated in the organic fraction of the soil, can actively compete with the uptake of those nutrients that have the same valence number, such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+}(Qin, et al., 2013; Kumar et al., 2014).

Combined supply of Cd^{2+} and Se resulted in a reduction in the Fe and Zn concentration in both genotypes and of Mn specifically in *hp1*, while no increases in nutrient absorption were observed (Table 1). However, the Se alone application increased the Mn and Zn concentration in the fruits of the *hp1* genotype and also Fe in the fruits of the MT, which could confer an efficient mechanism to alleviate the oxidative stress in this tissue, since this micronutrients are in the main active site of different isoforms of the superoxide dismutase enzyme (SOD, EC 1.15.1.1), the first enzymatic defense against the ROS in the cell environment (For review see Gratão et al., 2005).

Treatment		Macronutrients (g kg DW)					Micronutrients (mg kg DW)			
		Mg	**P**	**S**	**K**	**Ca**	**Mn**	**Fe**	**Cu**	**Zn**
MT	1	1.49 ± 0.14 a	3.81 ± 0.06 ab	2.14 ± 0.04 a	24.00 ± 1 bc	3.60 ± 0.10 a	33.20 ± 0.56 bc	88.00 ± 1 c	4.64 ± 0.33 a	25.30 ± 0.06 c
	2	1.47 ± 0.03 a	3.16 ± 0.08 ab	2.19 ± 0.10 a	25.20 ± 0.36 b	3.40 ± 0.40 a	36.00 ± 2 b	113.00 ± 1.76 b	5.72 ± 0.20 a	21.30 ± 0.38 de
	3	1.35 ± 0.11 a	3.52 ± 0.31 ab	2.60 ± 0.10 a	25.20 ± 0.95 b	3.70 ± 0.10 a	34.70 ± 0.16 b	43.70 ± 0.11 f	5.36 ± 0.16 a	18.10 ± 0.22 f
	4	1.25 ± 0.11 a	2.70 ± 0.51 b	2.09 ± 0.02 a	22.20 ± 0.10 c	3.10 ± 0.10 a	30.40 ± 0.20 c	41.90 ± 0.05 f	4.79 ± 0.20 a	19.60 ± ef
hp1	1	1.44 ± 0.07 a	3.61 ± 0.26 ab	2.44 ± 0.12 a	24.20 ± 1.15 bc	3.80 ± 0.17 a	33.60 ± 0.13 b	110.60 ± 0.46 b	5.24 ± 0.11 a	34.30 ± 1.09 b
	2	1.18 ± 0.08 a	4.69 ± 0.28 ab	2.36 ± 0.10 a	24.00 ± 1 bc	4.00 ± 0.87 a	46.00 ± 1 a	66.70 ± 0.58 d	5.70 ± 0.06 a	39.50 ± 0.52 a
	3	1.32 ± 0.02 a	5.81 ± 0.05 a	2.79 ± 0.38 a	29.60 ± 0.44 a	3.00 ± 0.50 a	17.70 ± 0.84 e	141.80 ± 0.96 a	4.26 ± 0.04 a	24.00 ± 1 cd
	4	1.27 ± 0.08 a	2.96 ± 0.21 b	2.05 ± 0.02 a	22.20 ± 0.17 c	3.60 ± 0.30 a	21.50 ± 0.47 d	54.60 ± 0.67 e	3.80 ± 0.29 a	18.60 ± 0.54 ef

Table 1. Nutrients concentration in fruits. 1. Control / 2. Na_2SeO_3 50 µM / 3. $CdCl_2$ 0.5 mM / 4. $CdCl_2$ 0.5 mM with Na_2SeO_3 50 µM. Values are the mean of three replicates. Lower case letters indicate statistically significant differences ($P < 0.05$).

3.2 Cd^{2+} and Se concentration and translocation index

The tomato genotypes (MT and *hp1*) showed differences in Cd^{2+}concentration in roots (Table 2), and also showed evidence of a Se effect to restrict Cd^{2+} translocation from root to shoot (Table 2). The Cd^{2+} concentration were much higher in roots when compared to the other tissues analyzed, for both genotypes. Furthermore, the roots of the MT genotype concentrated about 35% more Cd^{2+} compared to the *hp1* roots when exposed to the treatment in which the plants received only the heavy metal (Table 2). This difference between the genotypes could be directly related to plant growth, since the *hp1* fruits dry weight was much lower compared to MT under the Cd-alone treatment (Fig.1). However, the Cd^{2+} translocation index (TI) was considerably higher in the *hp1* genotype compared to the cv. MT (Table 2, 11.8% and 9% respectively) in the Cd^{2+} alone treatment, indicating that this heavy metal could be easily and readily translocated to the upper parts of the plant by the *hp1*compared to MT.

When Cd^{2+} was supplied simultaneously with Se, the heavy metal concentration in the root system and also in the leaves showed a very noticeable decrease for both genotypes compared to those treatments which received only Cd^{2+} (Table 2). This heavy metal can enter into root system through the cortical tissue, reaching the xylem through the apoplastic and symplastic pathway (Lux et al., 2011).

Additionally, Cd^{2+} could primarily be stored at the site of metal uptake, i.e. in the roots of most tomato varieties, which can be related to the restriction of translocation by the sequestration of Cd-chelates in vacuoles (Gallego et al., 2012), which can be improved by Se and ROS scavenging in the root cells (Clemens et al., 2013; Gratão et al., 2015). Likewise, when Se was applied simultaneously with the heavy metal, the

Cd^{2+} T.I. demonstrated to be much lower when compared to the Cd-alone treatment in the MT genotype, 7.9% and 9% respectively (Table 2).

Treatment			Cd^{2+} and Se concentration (mg kg^{-1} DW)			TI (%)
			Fruits	Leaves	Roots	
Cadmium	MT	Cd	30.2 ± 0.9 c	596.3 ± 0.5 a	6340.4 ± 0.7 a	9
	hp1	Cd	44.8 ± 0.8 a	503.9 ± 0.9 b	4092.08 ± 0.05 b	11.8
	MT	Cd + Se	9.4 ± 0.7 d	258.9 ± 0.9 d	3117.8 ± 0.3 c	7.9
	hp1	Cd + Se	41.8 ± 0.3 b	324.5 ± 0.6 c	2002.7 ± 0.9 d	15.4
Selenium	MT	Se	4.3 ± 0.7 a	2.8 ± 0.9 b	358.34 ± 0.6 a	1.9
	hp1	Se	5.4 ± 0.6 a	5.32 ± 0.4 a	210.44 ± 0.5 c	4.9
	MT	Cd + Se	0	0	310.9 ± 0.2 b	0
	hp1	Cd + Se	0	0	193.02 ± 0.05 d	0

Table 2. Selenium (Se) and Cadmium (Cd^{2+}) concentration in fruits, leaves, roots and translocation index (TI). Values are the mean of three replicates and standard deviation (±SD). Lower case letters indicate statistically significant differences ($P < 0.05$).

Furthermore, the free Cd^{2+} radical shows high affinity to the thiol groups (-SH) presented in different molecules such as the glutathione (GSH), proteins and also the amino acid cysteine (Cuypers et al., 2010). Additionally, Se can be assimilated by the sulfate pathway, due to its chemical similarity with S, which may cause the incorporation of Se into proteins, through the replacement of S by Se into the thiol group in the selenocysteine (SeCys) amino acid (for a review see Malagoli et al., 2015). Consequently, the competition for the same binding site into the amino acid cysteine and proteins, thiol group, by Cd^{2+} and Se, result in a lower uptake and translocation of the heavy metal (Lin et al., 2012).

The Se concentration data showed similarities with those from Cd^{2+}. The highest values were found in the roots compared to the other tissues analyzed for both genotypes (Table 2). Similarly, the MT genotype demonstrated almost 41% more Se in the root system compared to the *hp1* in the Se-alone treatment, 358 mg kg^{-1} DW vs. 210

mg kg^{-1} DW respectively (Table 2). Moreover, *hp1* showed a higher Se T.I. compared to MT, 4.9% and 1.9% respectively, which indicates that Se was readily translocated to the upper parts of the plant by the *hp1* compared to the MT genotype.

When Se and Cd^{2+} were applied simultaneously, Se concentrations decreased in the roots of both genotypes (Table 2). Interestingly, the presence of Se in the shoot was not detectable and the T.I. was zero in the same condition for both genotypes. As already stated, the competition for the same binding sites into amino acids and proteins can be related to the lower Se and also Cd^{2+} concentration in the root system. Other results in the literature have demonstrated that Se is not well translocated to shoot when applied in selenite form, maybe in response to its rapid assimilation into the organic forms of SeCys or SeMet and local incorporation into proteins in the root system (Malagoli et al., 2015).

3.3 Fruit dry weight

There were no significant differences in fruit biomass production between the treatments for either of the two tomato genotypes, Micro-Tom (MT) and *high pigment*-1 (*hp1*) (Fig. 1). Therefore, at this concentration and chemical form, Se did not positively or negatively affect fruit production. It is known that when supplied at low levels to the plant, Se can attenuate oxidative stress, enhance membrane stability, mainly in response to lower lipid peroxidation, and could also increase the concentration of starch and sugars (Pezzarosa et al., 2014; Qing et al., 2015). On the other hand, excess Se can induce reactive oxygen species (ROS) overproduction and thus oxidative stress, and can compete with S (sulfur) transport and metabolism, culminating in damage to plants and growth reduction (Capaldi et al., 2015).

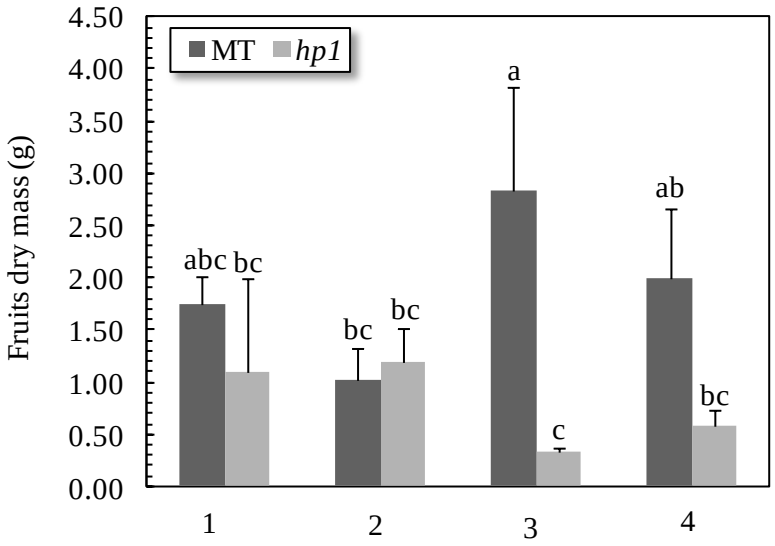

Fig. 1. Fruits dry weight. 1. Control / 2. Na_2SeO_3 50 µM / 3. $CdCl_2$ 0.5 mM / 4. $CdCl_2$ 0.5 mM with Na_2SeO_3 50 µM. Values are the mean of three replicates and standard deviation (±SD). Lower case letters above bars indicate statistically significant differences ($P < 0.05$).

The supply of 0.5 mM of $CdCl_2$ also did not reduce fruit dry weight of both genotypes as compared to the control plants (Fig. 1). Current reports have demonstrated the detrimental effects of Cd^{2+} on plant growth and development, mainly as a result of an imbalance of ROS production and the antioxidant system, reduction in water content, altered membrane permeability, lower photosynthesis and mineral deficiency (Clemens, et al., 2013; Irfan et al., 2014; Gratão et al., 2015).

The Se supply necessary to promote growth under an abiotic stress may differ depending on tissue type, plant species, stress type as well as Se speciation, concentration and application manner (for review: Feng et al., 2013). When Se was applied simultaneously with Cd, the fruit dry weight was also similar to the control plants for both genotypes (Fig. 1). Since $CdCl_2$ 0.5 mM alone did not significantly affect fruit production, the effect of Se on any negative effect of Cd on this parameter could not be tested.

3.4 Chlorophyll and carotenoids concentration

A quantification of carotenoids and chlorophyll in tomato fruit was carried out in order to investigate whether Se could protect fruit development and maturation during Cd^{2+} stress. Damaging effects of Cd^{2+} on photosynthesis and pigment concentration have been reported, predominantly through modifications of the chloroplast structure and the photosynthetic apparatus, as a response to the higher oxidative stress and lipid peroxidation (Gallego, et al., 2012). Additionally, the negative effect of Cd^{2+} specifically to the chlorophyll concentration can be a result of damages in pigments by ROS and a lower chlorophyll biosynthesis (Zhong et al., 2015).

Selenium (Na_2SeO_3) at the concentration of 50 μM did not significantly affect pigment concentration in fruits of both genotypes when it was applied alone or jointly with Cd^{2+}, as compared to the control plants (Fig. 2). There were in fact no significant differences in pigment concentrations between the treatments (Fig. 2) and thus Cd supplied at this concentration did not significantly affect pigment levels. However, it is noteworthy that Se-treated plants had slightly higher levels and Cd-treated plants slightly lower levels (NS) of various pigments, causing the chlorophyll-*a* concentration in fruits of the *hp1* genotype to be higher when Se was applied alone in comparison to *hp1* plants which received only Cd^{2+} (Fig. 2A, C). This effect cannot be considered a Se beneficial effect against Cd^{2+} stress in fruits due to the fact that the pigment concentration were similar in the Cd-treated plants and in plants where Se was applied jointly with Cd.

The *hp1* genotype demonstrates an exaggerated light responsiveness as well as a natural increased fruit pigmentation and vitamin C, which can confer an antioxidant capacity to deal with the increased radiation absorption, energy transformation and the oxidized environment in chloroplasts (Carvalho et al., 2011). Further analyzes on other

tissues of this genotype would help to elucidate the Se effect to pigments concentration and photosynthesis under the oxidative stress.

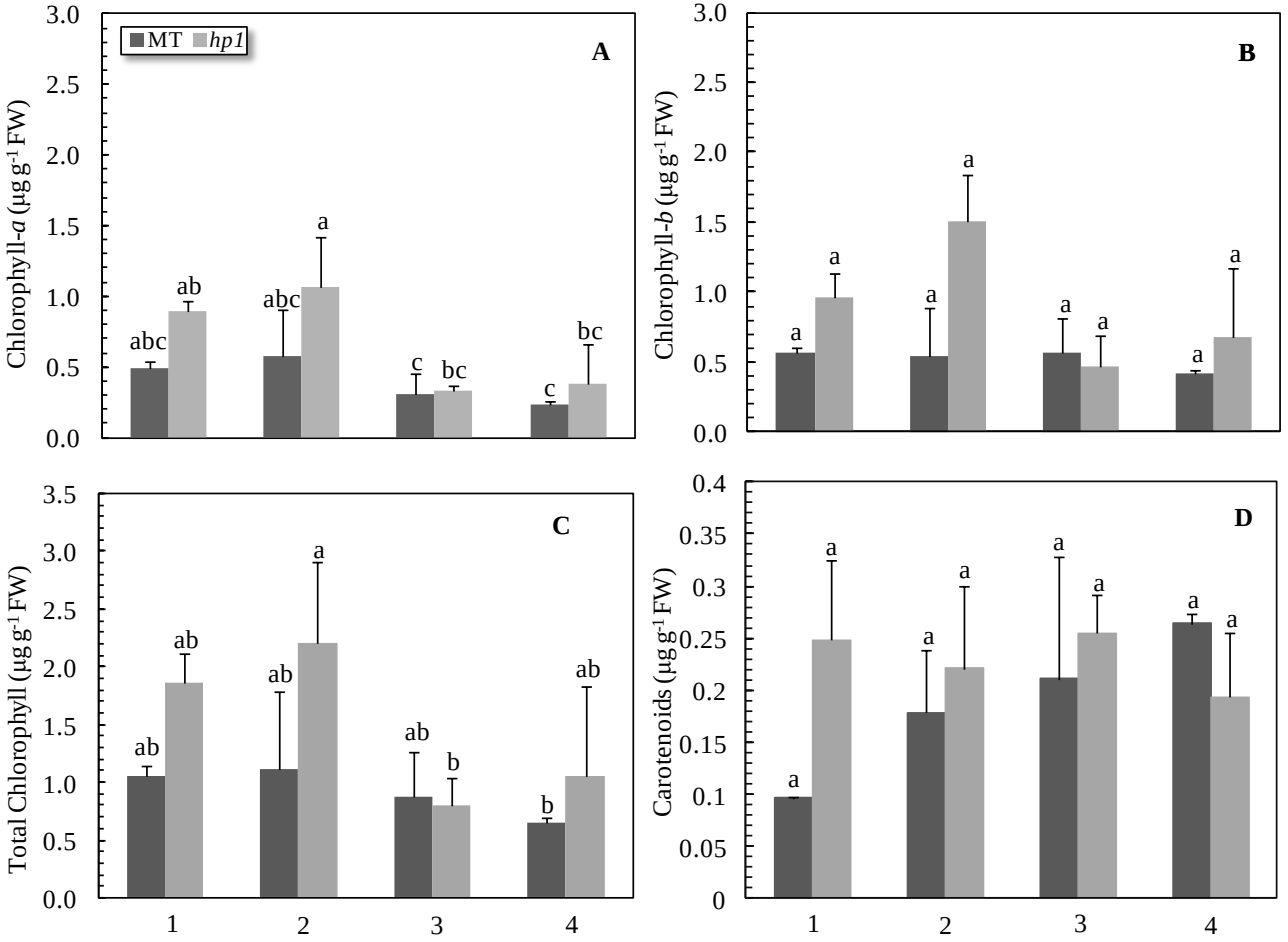

Fig. 2. Fruits pigments concentration. 1. Control / 2. Na_2SeO_3 50 µM / 3. $CdCl_2$ 0.5 mM / 4. $CdCl_2$ 0.5 mM with Na_2SeO_3 50 µM. Values are the mean of three replicates and standard deviation (±SD). Lower case letters above bars indicate statistically significant differences ($P < 0.05$).

3.5 Proline concentration

The proline concentration in control plants was similar for both genotypes (Fig. 3). Interestingly, when Se was applied alone, the proline concentration increased in the *hp1* fruits compared to the control (Fig. 3). The Cd-alone treatment, on the other hand, showed strongly decreased proline concentration in MT fruits in comparison to the control (Fig. 3). This result could be a response for a localized proline synthesis in the root instead of the shoot, related to the higher Cd^{2+}concentration in this tissue (Table 2). The simultaneous application of Se with Cd^{2+}increased the proline concentration for the MT genotype, compared to the Cd-alone treatment (Fig. 3). Proline accumulation in Se-supplied plants has been reported before. Walaa et al. (2010) observed that lipid peroxidation, induced by NaCl, was effectively minimized when the seedlings were pretreated with Se, showing an enhanced antioxidant activities and proline concentration. In addition, Hawrylak-Nowak (2009) found that supplementation of 5, 10 or 20 μM of selenite on cucumber seedlings improved the growth rate, photosynthetic pigments, proline concentration, and enhanced the antioxidant capacity of seedlings. However, the mechanisms related to the proline accumulation in Se-supplied plants have not been very well elucidated.

Proline concentration in a specific tissue under a stressful condition is widely varied among different plant species and is highly dependent on the type and the level of the stress whereupon the plant was submitted (Shevyakova et al., 2013). Proline overproduction can function as a mechanism to actively clear the cell ROS during oxidative stress, culminating in protection and stabilization of cell membranes and structures, proteins and enzymes during severe stresses (Ashraf and Foolad, 2007). Se application may induce proline synthesis through an improved activity of glutamyl kinase (GK EC 2.7.2.11), the first enzyme of the

proline biosynthetic pathway, and decreased activity of proline oxidase (PROX EC 15.5.2), responsible for the proline molecule denaturation (Khan et al., 2015).

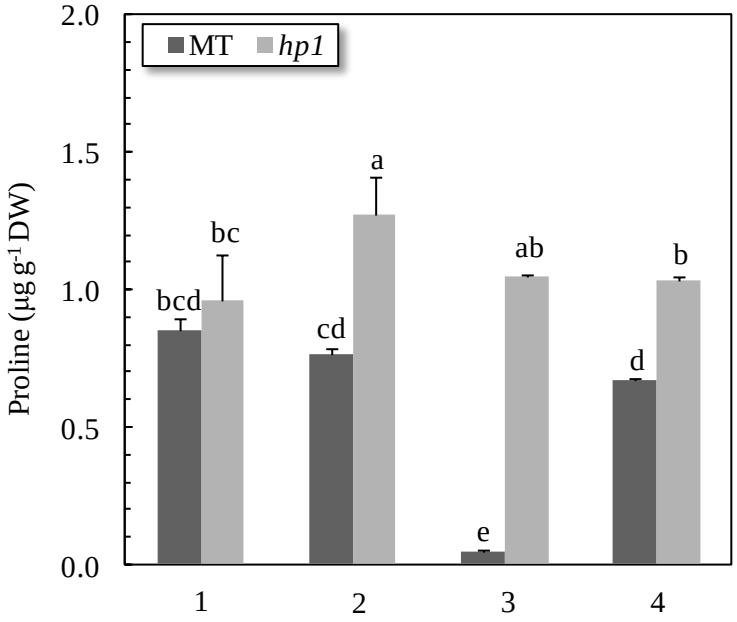

Fig. 3. Fruits proline concentration. 1. Control / 2. Na_2SeO_3 50 µM / 3. $CdCl_2$ 0.5 mM / 4. $CdCl_2$ 0.5 mM with Na_2SeO_3 50 µM. Values are the mean of three replicates and standard deviation (±SD). Lower case letters above bars indicate statistically significant differences ($P < 0.05$).

The products resulting from the catabolism of proline molecules, after attenuation of the stress condition, are used in the oxidative phosphorylation process in mitochondria in an effort to synthetize molecules of ATP (adenosine 5'-triphosphate), important upon the cell recovery processes (Ashraf and Foolad, 2007). In summary, the results demonstrate that 50 µM of Se increased the proline concentration in fruits of the *hp1*genotype, and also increased the proline concentration in MT fruits when it was applied simultaneously with Cd^{2+}, which could protect the tissue from the induced stress.

4. Conclusions

Overall, the data show an alleviating effect of 50 µM selenite against Cd toxicity in both tomato genotypes analyzed. The Se benefic effects included reduction in Cd uptake and translocation, particularly in the MT genotype, improvement of fruit Mn and Zn concentration in genotype *hp1* and Fe in the fruits of genotype MT and enhanced fruit proline concentration in both genotypes. This study suggests that Se fertilization may not only create nutritionally enhanced food but also be a means to alleviate Cd toxicity in tomato plants.

5. Acknowledgements

This study was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Grant nº. 445978/2014-7). We thank CAPES for the scholarship granted.

6. References

ATSDR., Agency for Toxic Substances and Disease Registry, 2012. Toxicological Profile for Cadmium, Public Health Statement, Cadmium CAS # 7440-43-9. U.S. Dept. Heal. Hum. Serv. Atlanta. GA.1, 1-430.

Ahmad, P., Jaleel, C.A., Salem, M.A., Nabi, G., Sharma, S., 2010. Roles of enzymatic and non-enzymatic antioxidants in plants during abiotic stress. Crit. Rev. Biotechnol. 30, 161-175.

Ashraf, M., Foolad, M.R., 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot. 59, 206-216.

Calvenzani, V., Martinelli, M., Lazzeri, V., Giuntini, D., Dall'Asta, D., Galaverna, G. Tonelli, C., Ranieri, A., Petroni, K., 2010. Response of wild-type and *high pigment-1* tomato fruit to UV-B depletion: flavonoid profiling and gene expression. Planta. 231, 755-765.

Capaldi, F.R., Gratão, P.L., Reis, A.R., Lima, L.W., Azevedo, R.A., 2015. Sulfur Metabolism and Stress Defense Responses in Plants. Trop. Plant Biol. DOI 10.1007/s12042-015-9152-1.

Carvalho, R.F., Aidar, S.T., Azevedo, R.A., Dodd, I.C., Peres, L.E.P., 2011.Enhanced transpiration rate in the *high pigment* 1 tomato mutant and its physiological significance, Plant Biol. 13, 546-550.

Clemens, S., Aarts, M.G.M., Thomine, S., Verbruggen, N., 2013. Plant science: the key to preventing slow cadmium poisoning. Trends Plant Sci. 18, 92-99.

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.

Djanaguiraman, M., Prasad, P.V.V., Seppanen, M., 2010. Selenium protects sorghum leaves from oxidative damage under high temperature stress by enhancing antioxidant defense system. Plant Physiol. Bioch. 48, 999-1007.

Feng, R., Wei, C., Tu, S., 2013.The roles of selenium in protecting plants against abiotic stresses. Environ. Exp. Bot. 87, 58– 68.

Gallego, S.M., Pena, P., Barcia, RA., Azpilicueta, C.E., Lannone, M.F., Maria, 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. Environ. Exp. Bot. 83, 33-46.

Gonzáles, A., Gil-Díaz, M., Lobo, M.C., 2015. Response of two barley cultivars to increasing concentrations of cadmium or chromium in soil during the growing period. Biol. Trace Elem. Res. 163, 235-243.

Gratão, P.L., Polle, A., Lea, P.J., Azevedo, R.A., 2005. Making the life of heavy metal-stressed plants a little easier. Funct. Plant Biol. 32, 481-494.

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 Physiol. Biochem. 56, 79-96.

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.

Hawrylak-Nowak, B., 2009. Beneficial effects of exogenous selenium in cucumber seedlings subjected to salt stress. Biol. Trace Elem. Res. 132, 259-269.

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Sta. 347, 1-32.

Irfan, M., Ahmad, A., Hayat, S., 2014. Effect of cadmium on the growth and antioxidant enzymes in two varieties of *Brassica juncea*. Saudi J. Biol. Sci. 21, 125-131.

Khan, M.I.R., Nazir, F., Asgher, M., Per, T.S., Khan, N.A., 2015. Selenium and sulfur influence ethylene formation and alleviate cadmium-induced oxidative stress by improving proline and glutathione production in wheat. J. Plant Physiol. 173, 9-18.

Kilamb, H.V., Kumar, R., Sharma, R., Sreelakshmi, Y., 2013. Chromoplast-specific carotenoid-associated protein appears to be important for enhanced accumulation of carotenoids in hp1 tomato fruits[1[C][W][OA].] Plant Physiol. 161, 1085-2101.

Kumar, A., Singh, R.P., Singh, P.K., Awasthi, S., Chakrabarty, D., Trivedi, P.K., Tripathi, R.D., 2014. Selenium ameliorates arsenic induced oxidative stress through modulation of antioxidant enzymes and thiols in rice (*Oryza sativa* L.). Ecotoxicology. 23, 1153–1163.

Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Method Enzymol. 148, 350-382.

Lin, L., Zhou, W., Dai, H., Cao, F., Zhang, G., Wu, F., 2012. Selenium reduces cadmium uptake and mitigates cadmium toxicity in rice. J. Hazard. Mater. 235-236, 343-351.

Liu, C.F., Guo, J.L., Cui, Y.L., Lü, T.F., Zhang, X.H., Shi, G. R., 2011. Effects of cadmium and salicylic acid on growth, spectral reflectance and photosynthesis of castor bean seedlings.Plant Soil.344, 131–141.

Lux, A., Martinka, M., Vaculík, M., White, PJ., 2011. Root responses to cadmium in the rhizosphere: a review. J. Exp. Bot. 62, 21-37.

Malagoli, M., Schiavon, M., dall'Acqua, S., Pilon-Smits, E.A.H., 2015. Effects of selenium biofortification on crop nutritional quality. Front. Plant. Sci. 6 (280), 1-5.

Mikkelsen, B.L., Jorgensen, R.B., Lingkjaer, M.F., 2015. Complex interplay of future climate levels of CO_2, ozone and temperature on susceptibility to fungal diseases in barley. Plant Pathol. 64, 319-327.

Nawaz, F., Ashraf, M.Y., Ahmad, R., Waraich, E.A., Shabbir, R.N., Bukhari, M.A., 2015. Supplemental selenium improves wheat grain yield and quality through alterations in biochemical processes under normal and water deficit conditions. Food Chem.175, 350-357.

Ning, C.L., Ding, N., Wu, G.L., Meng, H.J., Wang, Y.A., Wang, Q.H., 2013. Proteomics research on the effects of applying selenium to apple leaves on photosynthesis. Plant Physiol. Bioch. 70, 1-6.

Pezzarosa, B., Rosellini, I., Borghesi, E., Tonutti, P., Malorgio, F., 2014. Effects of Se-enrichment on yield, fruit composition and ripening of tomato (*Solanum lycopersicum*) plants grown in hydroponics. Sci. Hortic. 165, 106-110.

Praxedes, S. C., Ferreira, T. M., Filho, E. G.,2009. Acúmulo de prolina e aminoácidos em cultivares de feijão caupi com tolerância diferencial à salinidade. Caatinga. 22, 211-214.

Qin, Q., Li, X., Wu, H., Zhang, Y., Feng, Q., Tai, P., 2013. Characterization of cadmium (^{108}Cd) distribution and accumulation in *Tagetes erecta* L. seedlings: Effect of split-root and of remove-xylem/phloem. Chemosphere. 93, 2284-2288.

Qing, X., Zhao, X., Hu, C., Wang, P., Zhang, P., Zhang, X., Wang, P., Hanzhi, S., Jia, F., Qu, C., 2015. Selenium alleviates chromium toxicity by preventing oxidative stress in cabbage (*Brassica campestris* L. ssp. Pekinensis) leaves. Ecotox. Environ.Safe.114, 179-189.

Silva, F.A.S., Azevedo, C.A.V. de., 2009. Principal Components Analysis in the Software Assistat-Statistical Assistance. In: Proceedings of the 7th World Congress on Computers in Agriculture. Reno-NV-USA: American Society of Agricultural and Biological Engineers.

Shevyakova, N.I., Musatenko, L.I., Stetsenko, L.A., Rakitin, V.Y., Vedenicheva, N.P., Kusnetsov, V.V., 2013. Effect of ABA on the contents of proline, polyamines, and cytokinins in the common ice plants under salt stress. Russ. J. Plant Physl. 60, 741–748.

Singels, A., Jones, M., Marin, F., Ruane, A.C., Thorburn, P., 2014. Predicting climate change impacts on sugarcane production at sites in Australia, Brazil and South Africa using the Canegro model. Sugar Tech. 16, 347-355.

Tezzoto, T., Favarin, J.L., Neto, A.P., Gratão, P.L., Azevedo, R.A., Mazzafera, P., 2013. Simple procedure for nutrient analysis of coffee plant with energy dispersive x-ray fluorescence spectrometry (EDXRF). Sci. Agric.70, 263–267.

Wang, Y.D., Wang, X., Wong, Y.S., 2012. Proteomics analysis reveals multiple regulatory mechanisms in response to selenium in rice. J. Proteomics. 75, 1849-1866.

Walaa, A.E., Shatlah, M.A., Atteia, M.H., Sror, H.A.M., 2010. Selenium induces antioxidant defensive enzymes and promotes tolerance against salinity stress in cucumber seedling (*Cucumis sativus*). Arab Univ. J. Agric. Sci. 18, 65-76.

Xu, D., Chen, Z., Sun, K., Yan, D., Yang, M., Zhao, Y., 2013. Effect of cadmium on the physiological parameters and the subcellular cadmium localization in the potato (*Solanum tuberosum* L.). Ecotox. Environ. Safe. 97, 147-153.

Zembala, M., Filek. M., Walas, S., Mrowiec, H., Kornás, A., Miszalski, Z., Hartkainen, H., 2010. Effect of selenium on macro and microelement distribution and physiological parameters of rape and wheat seedlings exposed to cadmium stress. Plant Soil. 329, 457-468.

Zhong, Y., Chen, T., Zheng, W., Yang, Y., 2015. Selenium enhances antioxidant activity and photosynthesis in *Ulva fasciata*. J. Appl. Phycol. 27, 555-562.