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CÂMPUS DE JABOTICABAL

IMPACT OF HEAVY METAL IN AGRICULTURAL SOILS AND ROOT-TO-SHOOT STRESS COMMUNICATION

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TÍTULO DA DISSERTAÇÃO: IMPACT OF HEAVY METAL IN AGRICULTURAL SOILS AND ROOT-TO-SHOOT STRESS COMMUNICATION

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AUTHOR'S CURRICULUM INFORMATION

Leticia Rodrigues Alves was born in 1990 at Ribeirão Preto-SP- Brazil. She finished her course in Biological Sciences in 2013 at Federal University of Ouro Preto. During graduation, she was intern of Plant Physiology Laboratory and subsequently realized scientific initiation, under supervision of Alessandra Kozovits. Her interesting in plant physiology had increased, and during the eighth semester of graduation, she participated of Erasmus mundus, a Union European program to incentive education, which supported a scholarship at University of Porto- Portugal. In this new university, she studied specifics subjects associated to plant biology, such as plant physiology complementary, plant nutrition and plant development biology. Moreover, she participated of many scientific events and received award and honor during the XIV Brazilian Plant Physiology Congress. In 2014, she started to study mastering in crop production at UNESP / FCAV – Jaboticabal, under supervision of Dra. Priscila Lupino Gratão, in which place she studied antioxidant response of stress perception and signaling in plants. In this period, she participated of an international conference in Stockholm and of XV Brazilian Plant Physiology Congress. She was member of organized commission of “II Workshop of Abiotic Stress in cultivated plants”. In addition, she was co-adviser of two graduation students andauthor of two published papers and draft of others two.

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IMPACT OF HEAVY METAL IN AGRICULTURAL SOILS AND ROOT-TO-SHOOT STRESS COMMUNICATION

ABSTRACT

Heavy metal contamination is a worldwide concern and one of the most severe causes of abiotic stress in plants, triggering losses in crop production and contamination risks to human health. Cadmium concentration have been increased in environment due to several anthropogenic factors. Plants can uptake cadmium, which lead to a high production of reactive oxygen species and trigger an uncontrolled oxidation cascade in cells, resulting in damages to plant. In order to gain new information about this subject, we carried out a deep discussion about heavy metal contamination in agricultural soils, damages in cell homeostasis, antioxidant defense system and the impact to food consumers. Moreover, we studied root-to-shoot communication during stress condition and the specific responses of reactive oxygen species and hormones in this communication. For this propose, we used grafted hormonal mutants as a tool to investigate the modulation of antioxidant stress responses between organs.

Keywords: auxin, ethylene, hormonal mutants, oxidative stress.

IMPACTO DOS METAIS PESADOS EM SOLOS AGRÍCOLAS E A COMUNICAÇÃO DO ESTRESSE DA RAIZ PARA A PARTE AÉREA

RESUMO

A contaminação por metais pesados é um problema mundial e é uma das causas mais severas de estresse abiótico nas plantas, desencadeando perdas na produtividade e risco de contaminação para a saúde humana. A concentração de cádmio aumentou consideravelmente no meio ambiente devido a fatores antropogênicos. As plantas podem absorver o cádmio, levando a alta produção de espécies reativas de oxigênio, o que desencadeia cascatas de oxidação descontroladas, resultando em danos a planta. Com o objetivo de adicionar novas informações nesta área, foi realizada uma discussão sobre a contaminação dos solos agrícolas por metais pesados, os danos causados na homeostase celular, o sistema antioxidante de defesa e o impacto para os consumidores. Além disso, foi feito um estudo sobre a comunicação da raiz para a parte aérea durante uma condição de estresse, interconectando a resposta especifica das espécies reativas de oxigênio e a ação dos hormônios nesta comunicação. Pare este propósito, foi utilizado como ferramenta a enxertia e os mutantes hormonais para investigar a modulação da resposta antioxidante de defesa entre os órgãos.

Palavras-chave: auxina, etileno, mutantes hormonais, estresse oxidativo.

CHAPTER I – GENERAL CONSIDERATIONS

1.1. Introduction

Plants are continuously exposed to environmental stresses, which leads to losses in crop production due to changes in cell homeostasis. Cadmium (Cd) is a toxic heavy metal, which have being add in agricultural soil by industrial process, municipal waste and mainly agricultural practices. Cadmium can be uptake by plants and cause serious changes in structural, physiological and biochemical processes. There are an increase in reactive oxygen species (ROS) as a consequence of Cd contamination. Plants developed a complex defense systems including non-enzymatic and enzymatic mechanism to avoid oxidative stress and prevent an uncontrolled oxidation cascade.

In this dissertation, the first chapter consists of a literature review, which addresses important information from relevant articles to support our study. The chapter two, we carried out a review about the impact of agricultural practices, urban and industrial process in heavy metal contamination in soils. We focused in the most toxic heavy metals, such as lead, chromium, arsenic, zinc, cadmium, copper and nickel. Therefore, this review aims to compile important information to discuss on how heavy metals can arrive in agricultural soils, induce damages in cell metabolism, stimulate plant defense system, and can cause losses in crop production and the consequences for food security. This review was published in v.44, n.3, 2016 of Científica, a Journal of Agrarian Science (ISSN 1984-5529).

Although Cd leads to several damages to metabolism, the mechanisms of toxicity and tolerance is still unraveled. Current literature has shown a scarce number of studies of root-to-shoot stress communication and hormonal interaction, and the mechanisms involved in interaction of plant hormones with heavy metal-stress responses have not been well established.

Through the use of hormonal mutants, our research group have discussed the modulation of ROS signaling, as well as the cellular coordination of components, providing insights about the cross-talk between these pathways and phytohormones (GRATÃO et al, 2009, MONTEIRO et al., 2011; GRATÃO et al., 2012).

Moreover, the grafting technique can be used to improve the understanding about specific responses between plant root-to-shoot interactions during stresses. Yet, little is available in the literature when grafting has been used to study metals effect on plant development. Thus, we used grafting of Micro-Tom tomato plants to evaluate the crosstalk between antioxidant responses and signaling between plant organs (root-toshoot) during Cd stress (Gratão et al., 2015).

With these all relevant studies above mentioned, we build up the idea to work with hormonal mutants to study the role of hormones in root-to-shoot stress signaling and antioxidant defense mechanism during Cd-stressful condition.

In the third chapter, ongoing research in our lab investigated such aspects and also using grafting and tomato hormonal mutants which added an extra element more directly related to plant development to contribute in unraveling the mechanisms connecting signaling process and detoxification strategies, since hormones are directly involved in such a mechanism. For this purpose, throughout the use of the *Never* and *diageotropica* tomato mutants and grafting technique, the aim of this work was to understand or provide details about the modulation of oxidative stress by antioxidant systems and the role of auxin and ethylene during Cd-stressful condition.

1.2. Literature Review

1.3. The tomato as a model plant

Tomato (*Solanum lycopersicum*) is the second most globally highly produced vegetable crop after potato, with a production of 164 million tons, valued at ~$60 billion in 2013 (http://faostat.fao.org/). Tomato fruit are consumed worldwide for both fresh consumption and processing. Several studies have reported beneficial effects of tomato on human nutrition and health (ADALID et al. 2010; LIN et al. 2014). In addition, tomato is a widely adopted as an experimental model plant in different plant research fields, especially for those traits for which Arabidopsis is not a suitable option (CARVALHO et al., 2011). For instance, tomato have been used in genetics studies (WANG et al., 2013), evolution (MOYLE, 2008), reproductive biology (BEDINGER et al. 2011), genomics (KOBAYASHI et al., 2013; LIN et al. 2014), transcriptomics (MATAS et al. 2011), proteomics (YEATS et al. 2010) metabolomics (OSORIO et al. 2011) and stress physiology (GRATÃO et al., 2009, 2012, 2015; MONTEIRO et al., 2011; LUENGWILAI et al., 2013).

Micro Tom (MT) cultivar was originally created for use as an ornamental plant (SCOTT AND HARBAUGH, 1989), which simplified to cultivate tomatoes in smaller spaces and established as one of the most important models in genetic and physiology studies of cultivated plants (CAMPOS et al, 2010). The phenotype of Micro-Tom is the result of two key recessive mutations: dwarf (d) and miniature (mnt) (MEISSNER et al., 1997). Allelism tests also support that MT brings a mutation in gene D (LIMA et al., 2004). Moreover, Pnueli et al. (1998) suggested a mutation in SELF-PRUNING (SP) gene. These mutations triggered reduction in size. Its cycle is completed between 70 and 90 days, providing to study in a shorter period the complete life cycle, including flowering and fruits. Other advantage of its small size is allows us to realize grafting techniques (GRATÃO et al., 2015). MT Tomato has an additional advantage when used as model plant due to the great potential for the study of plant hormones. There are a large number of hormonal mutants available, most of them through the Tomato Genetics Resource Center, that exhibited changes in metabolism (synthesis and inactivation) and sensitivity to hormones (CAMPOS et al., 2009). Some of these mutants have been well characterized with respect to their physiology such as *Never ripe* (LANAHAN et al., 1994) and *diageotropica* (KELLY e BRADFORD, 1986).

1.4. Hormonal mutant *Never ripe* (*Nr)*

In tomato there are seven ethylene receptors (LeETR1-7), whereas tomato mutant *Never ripe* exhibit defect in LeETR3 (or Nr), which has a single amino acid change in the ethylene-binding region of NR, resulted lost in the capacity to respond to either endogenously generated or exogenously applied ethylene (LANAHAN et al., 1994). In the initial period of ripening, mutants *Nr* exhibit high NR gene expression, supporting the specific role of LeETR3 receptor during fruit ripining. Although *Nr* fruits maintained a very low ethylene sensitivity in ripening tomato fruit, exhibiting yellow to red colors (BARRY et al., 2005), no cell wall degradation and no increase syntheses of sugar is detected as a characteristic of fruit ripining (WILKINSON et al., 1995).

Fruit ripening is not a singular effect in *Nr* mutants, the reduced ethylene perception exhibit other affects in essential responses mediated by this hormone, including, triple response, leaf petiole epinasty, senescence of petals and flower abscission (LANAHAN et al., 1994). On the other hand, others receptors clearly was not affected in *Nr* mutants, once these plants were able to produce ethylene after pathogen attack indicating that the mutants are not impaired in ethylene biosynthesis (LANAHAN et al., 1994). The ethylene receptor and signalling mutants was extremely important and allow realize new researches for a more precise analysis of the physiological functions of ethylene.

1.5. Hormonal mutant *diageotropica* (*dgt*)

The *diageotropica* (*dgt*) tomato mutant (*Lycopersicon esculentum* Mill.), a spontaneously occurring, exhibit reduced sensitivity to the planthormone auxin (indole-3-acetic acid, IAA). Therefore, this mutant is characterized by several developmental and physiological defects, such as reduced gravitropic response, short space between internodes, lack of lateral roots, reduced vasculature, hyponasticleaves, reduced anthocyanin and chlorophyll content and weak apical dominance (ZOBEL, 1974; BALBI and LOMAX 2003). Interestingly, the *dgt* mutation seems to affect the expression of only a subset of auxin-regulated genes in a tissue and a specific period of development (BALBI and LOMAX 2003). Therefore, *dgt* mutation not affect auxin biosynthesis, on the other hand, it does affect a specific step in auxin perception or signaling (RETZER e LUSCHNIG, 2015) , once shoot apices of *dgt* seedlings exhibit normal levels of IAA (FUJINO *et al*., 1988) and normal rate of polar auxin transport (DANIEL *et al*., 1989). For this reason, *dgt* provides an interesting tool to study physiological processes mediated by auxin.

1.6. The role of auxin and ethylene under abiotic stresses

Plant hormones plays a fundamental role in the cellular responses and interact with redox signaling to control responses to abiotic stresses (BANKAJI et al., 2014; DAR et al., 2015). Nonetheless, mechanisms involved interaction of hormones with stress responses have been poorly understood (GRATÃO et al., 2012).

Ethylene mediates many complex process of plant growth, development and reproduction, including seed germination, root development, shoot and root growth, formation of adventitious roots, abscission of leaves and fruits, flowering, sex determination, and senescence of flowers and leaves. Moreover, this volatile molecule is considered a ‘stress hormone’ involved in multiple molecular and physiological plant processes, regulating various cellular defense responses (Schellingen et al., 2014; Van de Poel et al., 2015).

The biosynthesis of ethylene is well defined in higher plants and iniciated in the convertion of S-adenosyl-L-methionine (SAM) to ACC by the enzyme ACC synthase (EC 4.4.1.14, ACS). Then, ACC is converted to ethylene by the enzyme ACC oxidase (AC 1.4.3, ACO). Both ACS and ACO enzymes are encoded by a multigene family, each member are expressed in response to internal developmental and environmental conditions, such as wounding, flooding, drought, heavy metals and pathogen attack (YANG and HOFFMAN, 1984).

For instance, ethylene mediates the effects of ozone, leading to the suppression of stomatal closure during drought stresses (WILKINSON and DAVIES, 2009). Molecular genetics approaches and mutants provided evidence indicating that ethylene and NADPH oxidases act in sequence to control the responses of *Arabidopsis* to salinity stress, thought to confer salinity tolerance through improved Na+/K+ homeostasis (JIANG et al., 2013). Moreover, literature reports that ethylene production determines Cd stress alleviation by sulfur via regulatory interaction with antioxidant metabolism (ASGHER et al., 2014).

As well as ethylene, auxin hormone is related with stress signaling and defense responses (GEORGE et al., 2010), involving heavy metal resistence, accumulation in plants (FASSLER et al., 2010). Findings suggest that auxin is a component of the plant stress induced morphogenic response (POTTERS et al., 2007; 2009) which serves to avoid adverse effects of environmental stress (TOGNETTI et al., 2012). For instance, inhibition in root elongation and enhanced formation of lateral roots is observed in plants grown under contaminated soils. Exposure to aluminium inhibited cell division in the primary root meristem of *Zea mays*, whereas cell division was stimulated in the distal elongation zone (DONCHEVA et al., 2005), interesting aluminum stress is related to inhibition of basipetal IAA-transport (KOLLMEIER et al., 2000). In *Arabidopsis thaliana*, Cu inhibited root elongation, nonetheless, the number of lateral roots exhibited pronounced increase (PASTERNAK et al., 2005), other studies repost the patterning of lateral organ formation is directly related to auxin patterning and/or distribution (LAVERO et al., 2013). In above part of plants, excess ultraviolet -B cause increase in branches and smaller leaf area (ASHLEY et al., 2016), which can be easily related with the key role of auxin in shaping plant architecture. Moreover, auxin appears to promote stress tolerance by regulating the abundance of photosynthetic components and chloroplast structure (TOGNETTI *et al.*, 2012).

1.7. Cadmium

Cd pollution is considered one of the most severe environmental problems worldwide (LUX et al., 2011). Anthropogenic actions have been increased drastically Cd contamination. In the last five decades the worldwide release of Cd has extended 2.2 107 kg (COSTA et al., 2012). For instance, mining, industrial process, emissions from power stations and urban traffic, application of phosphate fertilizers in agricultural activities, wastewater, sewage sludge and atmospheric deposition may add 3-10 times more Cd to the atmosphere than natural sources, such as volcanic activity and forest fires (IRWIN, 1997). Cd affects morphologic, biochemical and physiologic aspects of plants development, by inhibiting photosynthesis and respiration, reducing water and nutrient uptake, altering gene and protein expression, inducing and inhibiting enzymes, enhancing accumulation of ROS and enhancing lipid peroxidation (SEMANE et al., 2010; GRATÃO et al., 2015).

Cd is mobile in soil and can move to deeper soil layers and ground water (SELVAM and WONG, 2009). Cd is an element with an insignificant biological demand (PRAPAGDEE et al., 2013). This heavy metal can accumulates in organisms and even low concentrations represents a huge carcinogenic risk. After absorbed, Cd has a long biological half-life (25-30 years), which results in chronic injuring to human healthy, such as the terrible "itaiitai disease" caused by the long-time ingestion of Cd-contaminated rice in Toyama-Japan (URAGUCHI and FUJIWARA, 2013).

Usually, most metals occur in soil in the forms that are not readily available for plant uptake. In soil solution, Cd occurs predominantly as Cd $^{2+}$ and also as ion complexes ($CdCl^+$, $CdOH^+$, $CdHCO^{3+}$, $CdCl_4$, $Cd(OH)_3$ and $Cd(OH)_4$, organic complexes, and Cd chelates (VERBRUGGEN et al., 2009). In acid soils, Cd forms minerals such as $CdCO_3$, CdO, $Cd(OH)_2$, and $Cd(PO_4)_2$. In alkaline soils, lowers Cd availability to plants due to $CdOH^+$ are bound more strongly to organic colloids and aluminum and iron oxides (PATORCZYK-PYTLIK and SPIAK, 2000). Furthermore, as alkalinity increases, Cd adsorption decreases due to the competitive adsorption of Ca^{2+} and Mg^{2+} ions (LAXEN, 1985).

It is well known that Cd is toxic for most plants even in low concentrations. The toxic concentrations of Cd varies among plant species, ecotypes, cultivars and plant tissues. In general, normal concentration of Cd in leaf tissue is 0.05-0.2 mg kg^1 dry mass (SOLÍS-DOMÍNGUEZ et al., 2007) and 3-30 mg kg^1 of Cd in plant tissue is enough to cause toxic effects (CHEN et al., 2011). The major visible symptoms of Cd-toxicity in plants are inhibited growth, chlorosis, necrosis until plant death. Cd affects the plant nutritional status probably by inhibiting the transporters/channels of other elements into the aerial part of the plant (SANDALIO et al., 2001), or by inhibiting the activities of enzymes related to element metabolisms (SANCHEZ-PARDO et al., 2013). Moreover, Cd cause an imbalance in the controlled cell redox state, trigging several biochemical pathways, including reactive oxygen species (ROS) generation, causing oxidative stress and, consequently, induction of antioxidant systems (GRATÃO et al., 2015; IANNONE et al., 2015).

1.8. Oxidative stress in plants

Oxidative stress is generate during biotic or abiotic stress condition as a consequence of a serious cell imbalance caused by reactive oxygen species (ROS) production more intense than antioxidants can detoxify, trigger drastic physiological challenges (GRATÃO et al., 2005). The production of ROS is an expected consequence of aerobic respiration under optimal growth condition. It is constantly formed in low concentration by several metabolic process in chloroplast, mitochondria, peroxisomes, plasma membrane, apoplast, and nucleus (GILL and TUTEJA 2010; SANDALIO et al. 2013). For instance, during the reduction of molecular oxygen O_2 to H_2O can be transferred one, two or three electrons to O_2, forming superoxide radicals ($O_2^{-\bullet}$), and the hydroxyl ($OH^{\bullet}$) and non-radical products like hydrogen peroxide (H_2O_2) and singlet oxygen ($_1O_2$) (DEMIDCHIK, 2015).

Although ROS are characterized to cause serious cell imbalance, recent papers report the importance of regulated ROS production as an intrinsic regulator of cell cycle progression (VIVANCOS et al., 2010). Probably there is a tenuous spatial and temporal regulation of ROS production and accumulation during plant development and stress response, but the crucial role of such responses remains poorly understood (XIA et al., 2015).

1.9. Defense mechanism against oxidative stress

Plants have numerous strategies to withstand oxidative stress (GILL and TUTEJA, 2010). The first line of defense is the activation and synthesis of enzymatic antioxidants and non-enzymatic compound to deal with the excessive production of ROS and its scavenging. There are many antioxidants enzymes, which show high affinity to specific ROS, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), guaicol peroxidase (GOPX), peroxidase (POX), glutathione- S-transferase (GST). Key antioxidants enzymes are discussed in detail bellow. On the other hand, non-enzymatic antioxidants are non-specific to different ROS, such as ascorbic acid

(AsA), glutathione(GSH), proline, polyamines, betaine, carotenes and flavonoids. Other substances can reduce catalytic activity of transition metals, such as metollothioneines, phytochelatines, pectins and other cell wall polysaccharides and structural proteins (ZAGORCHEV et al., 2013). Both antioxidant compounds and antioxidant enzymes function together to interrupt the uncontrolled cascade of oxidation and avoid cell damage (GRATÃO et al., 2005).

Other strategy adopted by cell under stress is the cell death programming, this mechanism probably protect living tissues from stresses. Cells under stressful condition quickly die by ROS-induced mechanisms (Demidchik et al., 2014). This step is common to provide a defense against infection or aggressive agents. Once stress condition is under control in plant metabolism, biosynthesis systems for reparation of damaged components is activate (YOSHIYAMA et al., 2013).

1.10. Superoxide dismutase

SOD (EC 1.15.1.1) can be considered the major antioxidant enzyme because participated of the first line of defense system against ROS toxicity. This enzyme catalyze the dismutation of $O_2{\bullet}^-$ to H_2O_2 and O_2 in all subcellular compartments including chloroplasts, mitochondria, nucleus, peroxisomes, cytoplasm and apoplasts (GILL et al., 2015). SOD is a metalloenzyme,first isolated from bovine blood (MANN and KEILIN, 1938). Initially, believed that SOD interacts with Cu-storage, past five decades, McCord and Fridovich (1988) were the first scientists to report the role of SOD in preparation of veterinary anti-inflammatory drug. SOD exhibit three well known isoforms, Mn-SOD is exclusive to mitochondria, Cu/Zn- SOD has been localized in cytosol, chloroplasts, and peroxisomes whilst Fe-SOD is found in peroxisomes, apoplast and mainly in chloroplasts (CORPAS et al. 2006). SOD isoforms (Cu/Zn-SOD, Mn-SOD, Fe-SOD) are nuclear coded and, when required, are transported to their organellar sites by means of NH_2-terminal targeting sequences (PAN et al. 2006).A recent finding describe a fourth group with Ni (Ni-SOD) presented in cyanobacteria, marine gammaproteobacteria and marine eukaryote (FINK and SCANDALIOS 2002).

1.11. Catalase

H_2O_2 is an highly reactive molecule and may be quickly converted into H_2O and O_2. CAT and other peroxidases realize this reaction in different cellular compartments, which is a vital part of antioxidant defense (GARG and MANCHANDA, 2009). CAT was the first antioxidant enzyme characterized, it consist in a tetrameric heme containing enzyme, with an approximate weight of 70 kDa(MHANDI et al., 2010). CAT occurs in peroxisomes, glyoxysomes, and similar organelles. This enzyme is an efficient ROS scavenger, exhibiting a highest turnover rate of reaction, which can dismutase about 6 million molecules of H_2O_2 per minute (GILL and TUTEJA, 2010). Moreover, CAT does not use any equivalent reducing agent, acting directly and efficiently during H_2O_2 conversion.

Most animals contain a single catalase gene, whereas angiosperm contain three catalase genes (CAT1, CAT2 and CAT3) (FRUGOLI et al., 1996). The CAT1 gene mostly expressed in seeds and pollen, CAT2 in roots, seeds and photosynthetic tissues, whereas CAT3 is found in vascular tissues and leaves (MCCLUNG, 1997). There was described six CAT isoforms in Arabidopsis (MICHAEL and MCCLUNG, 2002). Nonetheless, this number is not exactly among members of vegetal kingdom, some plants exhibited only two isoforms, such as barley (SKADSEN et al 1995) and peach (BAGNOLI et al., 2004).

1.12. Ascorbate peroxidase

Genomic and cDNA APX sequences were obtained from a great variety of plant species, proving that APX (E .C. 1.11.1.11) are widely distributed among higher plants. This enzyme belongs to a multigene family enzyme of heme peroxidases that reduce H_2O_2 with ascorbate as electron donor. APX are encoded by small gene families (PASSARDI et al., 2007) and their isoforms classification varies according to the specific subcellular localization, such as stromal APX (sAPX), thylakoid-bound APX (tAPX) in chloroplasts, mitochondria (mitAPX), and peroxisome membrane-bound APX (mAPX) and cytosolic APX (cAPX) (TEIXEIRA et al., 2006).

Tomato plants exhibit seven isoforms, three cytosolic, two peroxisomal and two chloroplastic (NAJAMI et al., 2008), whereas the plant model Arabidopsis was idenfied nine APX isoforms (CHEW et al., 2003). In another important model plant, rice, the APX gene group includes eight isoforms, being two cytosolic, two peroxisomal, two chloroplastic (stromal and thylakoid-bound) and two mitochondrial (TEIXEIRA et al., 2006).

Ascorbate-Glutathione cycle is an important ROS-scavenging system and APX play a crucial role in this cycle. Ascorbate (AsA) is oxidized to monodehydroascorbate (MDHA) that is then disproportionate into AsA and dehydroascorbate (DHA). Following reaction, the oxidized AsA is recycled to its reduced form wheremonodehydroascorbate reductase (MDHAR) activity is vital that performs NADPH-dependent regeneration of AsA from MDHA (ANJUM et al. 2014). In the absence of AsA, APX isoenzymes are unstable. Therefore, high level of endogenous AsA is required to maintain antioxidant system active and avoid oxidative damages (SHIGEOKA et al., 2002).

1.13. Gluthatione reductase

Glutathione reductase (GR) is a group of flavoenzymes and contains an essential disulfide group, where NADPH is required to reduce gluthatione oxidized (GSSG) to its reduced state (GSH) (GHISLA AND MASSEY, 1989). In most of the plants, GRforms a flavin adenine dinucleotide (FAD)-bound homodimer, with a molecular mass ranging from 100 to 150 kDa. GR issusceptibility to form tetramers and larger forms when is lack of thiols group. Although these larger forms show catalytic activity, GSH, a product of GR, maintains the enzyme in its dimeric form under cellular conditions (YOUSUF et al., 2012). There is three known GR isoforms in plants, such as cystolic, chloroplastic and mitochondrial. These isoforms of GR is stimulated by different environmental signals and exhibit diverse functions under adverse conditions (YOUSUF et al., 2012).

GR plays important role in ROS detoxification with GSH regeneration, which contributes to improve abiotic stress tolerance in plants (KAO, 2015). In AsA-GSH cycle, GR catalyzes the reduction of oxidized gluthatione (GSSG) back to its reduced form (GSH) with the additional oxidation of NADPH (ANJUM et al. 2012). The GSH formed will be used by GPX and dehydroascorbate reductase (DHAR). The GPX and DHAR convert H_2O_2 and dehydroascorbate (DHA) into H_2O and AsA, respectively (GILL et al., 2013). The maintenance of a proper GSH/GSSG ratio in plant cell has significant roles in stress signal transduction (PANG et al., 2010).

1.14. References

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CHAPTER II – HEAVY METALS IN AGRICULTURAL SOILS: FROM PLANTS TO OUR DAILY LIFE

2.1. Abstract

Heavy metal contamination in soils is a major environmental concern that affects large areas worldwide. Agricultural practices have been the main source of heavy metals in soil such as lead, chromium, arsenic, zinc, cadmium, copper and nickel. Plants can uptake this toxic metals and have evolved defense mechanisms to avoid or minimize damages. Exposure to toxic metals can intensify the production of reactive oxygen species (ROS), which are continuously produced in both unstressed and stressed plants cells. Some of the ROS are highly toxic and must be detoxified by cellular stress responses, if the plant is to survive and grow. Heavy metals contaminated plants cause losses in crop production and risks for human health. Thus, this review aims to compile some information about research work on concentration of heavy metals until they reach the food chain for transferring to crops.

Additional keywords: contamination, agriculture, reactive oxygen species

2.2. Introduction

Soils contaminated with heavy metals have become one of major environmental problems around the world (Gratão et al., 2015). The contamination may occur due the industrial expansion, mine tailing, combustion of fossil fuels, spillage of petrochemicals, disposal of high metal waste (eg. Batteries), atmospheric deposition and agricultural practices (Khan et al., 2008; Zang et al., 2010, Liu et al., 2016). As a result of which anthropogenic, such as fertilizers, biosolids, pesticides and wastewater, pollutants have overloaded the system and natural equilibrium has been disturbed. Agricultural practices may accumulate high levels of potentially heavy metals in soils, which may have significant consequences for the quality of plant health, soil biological processes and thus through biomagnifications enter the human body as well. Current literature has shown several definitions for heavy metal based on atomic number, chemical properties, density and toxicity. Heavy metals constitute an ill-defined group of inorganic chemical which exhibit metallic properties, including transition metals, metalloids, lanthanides, actinides and five times the specific gravity of water (Sharma, 2014). The most common heavy metals found in soils sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu) and nickel (Ni). Most metals may persist in soils for a long time after their introduction because they not undergo microbial or chemical degradation (Kirpichtchikova, 2006). Some of these heavy metals are micronutrients necessary for plant growth, although most of them are toxic.

There is a tenuous line about a micronutrient be toxic or benefit for plants health, they are delivered to the shoots often within minutes of absorption. Certain metals such as iron, manganese, molybdenum, copper, zinc, and nickel have demonstrated at least one specifically defined role in plant growth, so that plants fail to grow and reproduce normally in the absence of that element (Emamverdian et al., 2015). However, high concentrations of these metals can disrupt critical physiological processes and result in toxicity. Plants can uptake some elements without known biological functions and toxic at low concentrations, such as cadmium, arsenic, chromium, mercury and lead (Peralta-Videa et al., 2009). In order to cope with different heavy metals, plants possess defense systems to manage the metal-imposed oxidative challenge. However, up-regulation of these mechanisms and biomolecules may depend on plant species, the level of their metal tolerance, metal type and plant growth (Emamverdian, 2015). In this work, scattered literature was utilized to review the possible sources of heavy metal contamination by agricultural practices, their effects on plant metabolism and consequences to environment and risks to human health.

2.3. Sources of heavy metals in contaminated soils by agricultural practices

Heavy metals rates depend on the geological characteristics of soils and occur naturally in point sources in limited areas (Shubhra et al., 2015). Anthropogenic activities lead to the imbalance in geochemical cycles of metals, due to the disturbance and acceleration of nature geochemical cycle of metals (D'Amore et al., 2005). Furthermore, chemical compounds have been added in agriculture soils and most soils may accumulate amount of heavy metals capable to generate risks to plants, animals and human health. In order to obtain maximum yields, agricultural practices have been adopted through the uncontrolled use of inorganic fertilizers, biosolids from slugged and manures, wastewater and pesticides which can increase heavy metals content in agricultural soils and consequently can affect plant metabolism.

Inorganic Fertilizers: Macronutrients and micronutrients are essential to complete plant lifecycle. Generally, agricultural soils do not provide all nutrients, thus large quantities of fertilizers are regularly added to commercial farm soils to provide all nutrients for plant growth. However, the overuse or misuse of fertilizers in agriculture contributes to environmental deterioration and pollution, once the compounds used to produce fertilizers contain trace amounts of potentially toxic heavy metals (eg. Cd, Pb, As, Cu) (Zahra et al., 2010, Sakizadeh et al., 2016). Studies conducted by Wangstrand et al. (2007) have demonstrated that nitrogen fertilization increase Cd concentrations in soil and plants. Phosphate fertilizers are also a significant source for contaminate soils with pottentialy toxics elements such as Cd, F, Hg and Pb (Guo et al., 2006; Moraes, 2009) and As (Channa, 2015). Moreover, phosphate fertilizers are hazardous to rural community expose to this product, which may be causative factor of chronic kidney disease (Channa et al., 2015). Phosphate rock used for the manufacturing of fertilizers is mainly composed of phosphorous and minutely of many other relevant micronutrients for plants such as Mg, Mn, K, Na and Co. Nevertheless, it also contain environmental pollutants as Cd, Cu, Cr, Ni, Pb and Zn (Sabiha-Javied et al., 2009), which brings disadvantages to the soil equilibrium.

Maximum levels of toxic heavy metals allowed in fertilizers in several countries are summarized in Table 1.

Table 1-Maximum levels of toxic heavy metals allowed in fertilizers in several countries (1)

Country	As	Cd	Pb	Hg
	$mg\ kg^{-1}$			
OECD – Phosphates				
Switzerland, Finland	-	50	-	-
Sweden, Norway	-	100	-	-
Denmark	-	110	-	-
Belgium, Germany	-	210	-	-
Austria	-	275	-	-
United States – AAPFCO				
Phosphates(2)	13	10	61	1
With micronutrients(3)	112	83	463	6
Environmental Protection Agency – USA				
Texas State	41	39	300	17
Canada	75	20	500	-
California				
Phosphates(2)	2	4	20	-
With micronutrients(3)	13	12	140	-
Washington				
Phosphates(2)	13	10	61	1
With micronutrients(3)	112	83	463	6
Brazil – Proposed				
Phosphates(2)		0,75	37,5	-
Exclusive for micronutrients(3)		15; 150(5)	750; 7500(5)	-
Other mineral fertilizers		20	100	-
Brasil – Published				
Phosphates(2)	2	4	20	0,05
Exclusive for micronutrients(3)	500/4000(5)	15; 450(5)	750; 10000(5)	10

Other mineral fertilizers	250	57	1000	-
Australia				
Phosphates	-	300	-	5
Non phosphate	-	10	-	5
Macronutrients	-	-	100	5
Macronutrients + micronutrients(4)	-	-	500	5
Micronutrients(4)	-	50	2000	5
China	50	8	100	5
Japan – Phosphates	50	8	100	5

(1) Summarized by Malavolta and Moraes (2006); (2) mg per 1% P_2O_5; (3) mg per 1% micronutrient; (4) mg/kg P; (5) Maximum values based on total mass; OECD: Organization for Economic Co-operation and Development; AAPFCO: American Association of Plant Food Control Officials; – value not established.

The allowed amount of As, Cd, Pb and Hg in fertilizer ranging from As = 2 – 250 mg kg^{-1}, Cd = 8 – 300 mg kg^{-1}, Pb = 20 – 200 mg kg^{-1} and Hg = 0,05 – 5 mg kg^{-1}. These values are variable among the countries due they specific fertilizer law. Interestingly, Austria and Australia have the highest allowed Cd concentration in phosphate fertilizer. Cd occurs naturally in phosphate rocks (Roberts, 2014). The International Fertilizer Development Center (IFDC) summarized Cd content of phosphate rocks obtained from 35 sedimentary deposits in 20 countries (Van Kauwenberg, 2001; Roberts, 2014). Data for countries among the top world producers was summarized by Roberts (2014) and shown in Table 2.

It seems that concentration of Cd can vary widely between countries and within deposit in the same country. According to Roberts (2014), the overall average of Cd concentration for sedimentary deposits was 21 mg/kg with a range of less than 1 to 150 mg kg^{-1}. Compared to with non-phosphate containing rock, sedimentary phosphate rock deposits are about 69 times more enriched with Cd (Roberts, 2014). Data from 11 igneous phosphate rock deposits in 9 countries surveyed by IFDC showed an average Cd concentration of 2 mg kg^{-1} and were enriched 7.5 times crustal abundance (Roberts, 2014).

Table2 - Cadmium contentes (mg kg-1) of sedimentary and igneous phosphate rocks [1]

Country	Deposit	Average Cd	Range
Sedimentary Deposits		...............mg kg^{-1}.............	
China	Kaiyang	<2	-
Israel	Zin	31	20-40
	Undifferentiated	24	20-18
	Arad	14	12-17
	Oron	5	-
Jordan	El-Hasa	5	3-12
	Shidyia	6	-
Morroco	Undifferentiated	26	10-45
	Bou Craa	38	32-43
	Khouribga	15	3-27
	Youssoufia	23	4-51
Senegal	Taiba	87	60-115
Syria	Khneifiss	3	-
Togo		58	48-67
Tunisia		40	30-56
United States	Central Florida	9	3-20
	North Florida	6	3-10
	Idaho	92	40-150
	North Carolina	38	20-51
Other countries		13	<1-100
Overall Sedimentary Averages		21	<1-150
Igneous Deposits			
Brazil	Araxa	2	2-3
	Catalão	<2	-
South Africa	Phalaborwa	1	1-2
Russia	Kola	1	<1-2
Other countries		1	1-5
Overall Igneous Averages		2	<1-4

(1) Adapted from Van Kauwenbergh 2001; Roberts 2014.

The world production of phosphate fertilizer is approximately 85% from sedimentary deposits. Varying amounts of Cd in the phosphate rock move through the beneficiation and acidulation processes during fertilizer manufacture (Roberts, 2014). Amounts transferred depend on the manufacturing process. For ordinary (single) superphosphate (SSP) produced by reacting phosphate rock with sulphuric acid and triple super phosphate (TSP) produced by acidulation of the phosphate rock with phosphoric acid, all of the Cd in the phosphate rock is transferred to the SSP or TSP. According to Roberts (2014), depending on the phosphate rock source, SSP can contain from 2 to more the 40 mg kg^{-1} and TSP can have from less than 10 to over 100 mg/kg Cd content.

Moraes (2009) studied the transfer of heavy metals such as Cd, Cr, Ni and Pb to rice and lettuce plants using different source and levels of phosphate fertilizers. The concentration of Cd in grains increased with application of phosphate fertilizer as shown in Table 3. Although the toxic heavy metals added rates have been high, however, the metals concentration in the grains did not exceed the values espoused by the Coodex Alimentarius. The levels of heavy metals in grains ranged in mg kg^{-1}: Cd, 0.004 to 0.148; Cr <.006 to .062; Ni, 0.3 to 2.0, and Pb <0.002 to 0.039. According to Kabata-Pendias & Pendias (2000), the natural values for toxic heavy metals in lettuce leaves are Cd, 0.66 to 3.0 mg kg^{-1}; Cr <1.5 mg kg^{-1} and Pb, from 0.7 to 3.6 mg kg^{-1}. Lettuce plant isconsidered an accumulator of heavy metals, particularly Cd, which is toxic level in leaves 10-95 mg kg^{-1} (Malavolta, 2006).

Table 3 -Available phosphorus and cadmium in upland rice grains growing in Oxisol containing different phosphorus sources fertilizers.

Treatments	Phosphorus content		Applied$^{(1)}$	Cadmium contente		
	Rates	Soil		OA	DTPA	Grains
	—— mg dm^{-3}——		———— µg dm^{-3} ————			µg kg^{-1}
		Oxisol yellow-red				
Control	0	2	0	34	4	4
Togo phosphate rocks	50	16	14	32	6	9
	100	32	27	37	6	13
	200	57	55	44	8	24
	400	93	109	52	12	40
SFS Togo	50	21	7	41	18	40
	100	39	15	46	29	64
	200	74	29	55	36	74
	400	195	59	67	73	128
Mix pró-análise P	50	26	14	34	13	27
	100	77	27	41	23	46
	200	124	55	47	33	81

	400	281	109	55	60	139
LSD (Tukey 5%)	-	*7*	-	*12*	*8*	*35*
Oxisol						
Control	0	6	0	27	4	6
Togo phosphate rocks	50	11	14	23	5	6
	100	16	27	26	6	7
	200	26	55	25	8	16
	400	43	109	22	13	22
SFS Togo	50	16	7	23	14	22
	100	26	15	32	21	36
	200	71	29	40	49	78
	400	159	59	55	66	146
Mix pró-análise P	50	22	14	22	17	20
	100	72	27	21	21	28
	200	99	55	50	46	58
	400	249	109	70	75	148
LSD (Tukey 5%)	-	*6*	-	*27*	*35*	*48*
Correlations						
Cd applied	0.92^{***}	0.73^{***}	-	0.47^{***}	0.45^{***}	0.54^{***}
Cd organic acid	0.59^{***}	0.72^{***}	-	-	0.67^{***}	0.75^{***}
Cd DTPA	0.62^{***}	0.80^{***}	-	-	-	0.87^{***}
Cd content in grains	0.72^{***}	0.89^{***}	-	-	-	-

(1) Abbreviations: OA – Organic acids. The amount of Cd applied was calcuted based on Cd content in fertilizer sources multiplied by mass of each product necessary to achieve the levels of P; OA: Cd extracted in organic acid; DTPA: Cd extracted in diethylenetriaminepentaacetic acid; LSD: least significance difference; ***; **; * and ns: significant at 1%, 5%, 10% e non-significant, respectively. The chemical analysis were done in triplicates. Source: Moraes (2009).

In Brazil, according to ANVISA (1965), the maximum concentration of Cd in foods consumed in natura is 1 mg kg^{-1}. If consumed in crudely form, such as vegetables the result must be expressed in the fresh weight and in the case of rice grains, eaten after cooking, the result is expressed in dry mass. For vegetables consumed as leaf, Australian and New Zealand Food Authority (FSANZ, 2009) adopted the maximal levels for Cd as 0.1 mg kg^{-1} fresh weight. (approximately 3.3 mg kg^{-1} in the dry mass, whereas 97% of the fresh weight of lettuce is water). The Commission of the European Communities (CEC, 2001) and the Codex Alimentarius Commission (Codex, 2004) allow up to 0.2 mg of Cd per kg fresh weight. For rice grains, the critical values for Cd are raging from 0.2 to 0.4 mg kg^{-1} (CODEX, 2004) and for Pb it is approximately 0.19 mg kg^{-1} (Kabata-Pendias & Pendias, 2000). Chemical analyzes result for rice and lettuce produced in Brazil range from 0.002 to 0.55 mg kg^{-1} DW for lettuce and 0.001 to 0.060 mg kg^{-1} DW for rice grains (Masironi et al., 1977; Lenzi et al., 1990; Santos et al., 2004).

The evaluation of Cd, Ni and Pb only in the shoot part of young plants could have positive correlations with heavy metal concentrations in the edible parts of food, as has commonly been reported in studies conducted in Brazil (Gonçalves Junior & Pessoa, 2002; Armelin et al., 2008). However, evaluating the edible part, in this case grains, and compared with the legislation on maximum permissible levels of contaminants in foods is possible to evaluate and diagnose the potential entry of toxic metals in the food chain. Rodella (2005) reports that the risk assessment has been used as a tool to quantify the potential impact of contaminants via fertilizers supply in United States.

In addition, it must be considered that plants have mechanisms to prevent translocation of toxic heavy metals to grains (Kubota & Campen,1992). Genotypic variations in the accumulation of toxic heavy metal in the grains have been observed in several crops, including rice (Arao & Ae, 2003), being able to select plants with lower levels of heavy metals toxic in the edible part (Grant et al., 2008). The influence of factors such as the genotypic variation in the toxic heavy metal content in rice grains are summarized in Table 4.

To better understand the problem of heavy metals added to soil via fertilizers is fundamental importance the effort to set up long-term field experiments.

Table 4 - Yield, concentration and accumulation of Cd in young plants and grains of upland rice in response to Cd suply.

Cultivars(1)	Dry weight		Cd in young plants		Cd in grains	
	young plant	grains	Conc.	Accum.	Conc.	Accum.
	g plant^{-1}	g pot^{-1}	mg kg^{-1}	µg plant^{-1}	mg kg^{-1}	µg pot^{-1}
IAC 4440	2.1	24.0	5.8	12	1.76	42.3
PB 11	2.6	13.7	7.7	20	1.54	21.1
PB 05	2.7	21.8	8.0	21	1.53	33.5
BRSMG Relâmpago	2.5	11.5	9.1	23	1.23	14.1
BRSMG Caravera	2.1	15.9	7.6	16	1.21	19.2
BRSMG Curinga	2.4	17.3	4.0	10	1.04	18.0
Pérola	3.0	1.5	5.8	17	1.03	1.6
Bonança	1.9	13.0	8.7	16	0.95	12.3
Jaguari	3.3	1.7	3.9	12	0.93	1.6
Primavera	2.2	5.5	8.2	18	0.79	4.3
IAC 201	2.8	9.6	3.7	10	0.74	7.0
IAC 600	2.9	1.6	3.1	9	0.67	1.1
Gurani	2.1	11.9	4.7	10	0.65	7.8
Caiapó	2.3	9.3	3.1	7	0.61	5.7
Bico ganga	2.1	8.4	5.1	11	0.59	5.0
IAC 435	3.1	1.8	4.4	14	0.55	0.9
BRS Talento	1.6	12.9	4.6	7	0.53	6.9
Canastra	2.3	7.1	7.5	17	0.53	3.8
PB 01	2.9	16.0	8.4	24	0.53	8.5
Arroz preto	1.7	9.1	7.5	13	0.50	4.6
IAC 165	2.5	7.0	4.0	10	0.49	3.4
BRSMG Conai	2.2	13.2	5.2	11	0.48	6.3
Carajás	2.6	12.3	5.2	13	0.48	5.9

IAC 47	2.7	6.6	3.4	9	0.46	3.0
IAC 202	2.4	11.3	3.9	9	0.42	4.7
Pratão	2.6	1.7	5.2	14	0.38	0.3
Beira campo	1.8	13.4	5.7	10	0.36	4.8
Maravilha	2.2	6.4	5.2	11	0.32	2.1
IAC 25	2.5	9.2	3.5	9	0.32	2.9
Batatais	2.3	1.0	3.9	9	0.31	0.3
Dourado precoce	2.3	2.6	6.1	14	0.30	0.8
IAC 4	2.1	2.4	5.0	11	0.29	0.7
Cateto	2.9	2.4	4.7	13	0.28	0.7
IAC 1246	2.5	8.2	4.3	10	0.19	1.6
Cateto seda	2.3	6.9	6.9	16	0.17	1.2
LSD (Tukey 5%)	*1.2*	*7.9*	*2.6*	*9*	*0.13*	*7.2*
		Relationship				
Cd concentration in grains	$0,07^{NS}$	$0,56^{***}$	$0,39^{***}$	$0,41^{***}$	-	$0,85^{***}$

(1) Level of Cd applied in the pots before install the experiment was Cd = 1,0 mg dm^{-3}. Adapted from Moraes (2009).

Biosolids: The use of biosolids (eg. livestock manures, composts and sewage sludge) is an interesting organic fertilizers used in worldwide to improve soil quality and reclamation of degraded lands (Larney & Angers, 2012; Lu et al., 2012). Besides giving a destination for municipal wastes, biosolids is considered a nutrient source of organic carbon (Torri, 2014) and improves soil fertility (Scharenbroch et al, 2013, Tytla et al., 2016). However it is important to know the composition of materials used, once the quality of biosolids varies widely. An incorrect biosolids application in agricultural soils leads to accumulation of heavy metals such as As, Cd, Cr, Cu, Pb, Hg, Ni, Tl, Sb (Basta, 2005). Therefore, analysis about heavy metals contents should be strictly checked. Despite the increasing quantities of organic wastes, as biosolids, being applied to land, there are few studies about ecotoxicological risks of this practice (Domene et al., 2007). Application of biosolids onto forest land is often preferred to agricultural land because the biosolids can increase tree growth without the risk of contaminants entering the human food chain (Magesan & Wang, 2003).

However, the biosolids-derived heavy metals were strongly retained in the soil and the litter layer.

Wastewater: In development countries, wastewater is commonly used a source of irrigation water due to water scarcity for crop production (Scheierling et al., 2010). Moreover, the population growth and urbanization resulted in an increase of wastewater generation from domestic, commercial and industry sector and the reuse of wastewater in agriculture can reduce the potential for rivers and seas pollution (Qadir et al., 2007a; Asano et al., 2007). Some estimates have demonstrated that 20 million hectares in 50 countries are irrigated with urban wastewater (Scott et al., 2004) and about 80% of the generated wastewater in developing countries was used for the same matter (Cooper, 1991). The use of wastewater in agriculture also brings benefits to soil, providing nutrients and organic matter for a high crop production with low costs to farmers (Hanjra et al., 2012). On the other hand, there are wide risks to the environment in use of wastewater for irrigation (Qadir et al., 2007b), such as add pathogens in soil (Kazmia et al., 2008), saline salts (Li et al., 2009) and excess nutrients (Kalavrouziotis et al., 2008). Furthermore, there are great risks of soil contamination with toxic heavy metals (Murtaza et al., 2008), which affects food quality and human health (Hanjra & Qureshi, 2010; Cao et al., 2016). Untreated wastewater used for prolonged period in irrigation is one of the main sources of heavy metals pollution (WHO/FAO, 2007) resulting in heavy metal accumulation in a toxic level. In surface soil of farms irrigated with wastewater are common found heavy metals higher than the safe limits, such as Cd, Cr, Pb, Ni, Zn and Mn (Shameem et al, 2015). A research in Bangladesh indicated that even in irrigation with clean water the concentrations of toxic elements such as Cu, Fe, and Cd were above the safe limit set for the agricultural purpose (Jasim et al, 2010). The severity of the negative effects of wastewater irrigation depends on its source, composition and management (Drechsel et al., 2009). Public agencies must consider implementing of programs addressing known and potential effects to consumer and environment (Qadir, 2010), giving advice for farmers to be aware of the health risks associated with using wastewater for their crops (Keraita et al., 2010).

Pesticides: Pesticides have greatly improved agricultural and horticulture production world-wide for protecting plants against vector-borne diseases and pests. Nevertheless, the extensively fairly use of these products also bring disadvantages. Pesticides contain substantial concentration of metals, which contaminated soil with greatly exceeds background concentrations in long-term applications (Kelepertzis, 2014). For instance, elements such Cu, Hg, Mn, Pb and Zn was common compounds approved for use as insecticides and fungicides in UK in a recent past (Raymond, 2011). Several studies report that the application of pesticides may be a common source of Cu, Mn, Zn, Br, Sr and Ti in Gaza soils (Shomar, 2006), Cu, Cr and As in Australia and New Zealand soils (McLaughlin, 2000) and Cu, As, Pb and Zn in China topsoil (Tao et al., 2008) and Denmark (Jorgensen et al., 2005). The uncontrolled use of pesticides may result in environmental problem, mainly in contamination of agricultural soils with heavy metals. Thus, is important governmental agencies establish scientific rules about the correct application form to avoid damages to environmental and human health.

2.4 Effects of some heavy metals on plants

It was showed that plants can uptake heavy metals. Although, Zn, Ni and Cu are benefit in minutes concentration, few studies address about benefit levels to crop production. Poor et al. (2015) indicated that sorghum with application of 0.1 mu M $CuCl_2$ enhanced the maximal CO_2 assimilation rate and increased soluble sugar content in all plant tissues; however the transport of Fe and Mn was negatively affected. As well as, Zn applied at 6 mg kg(-1) was suitable dose for enhancing plant growth and quality of lily cut-flower (Shaheen et al., 2015).

Nonetheless, a huge number of studies reposts that low concentrations of these metals cause injury in any metabolism process such as seed germination and plant development. The redox metal can generate oxidative damages, which leads a high ROS production, resulting in cell homeostase imbalance (Flora, 2009, Emanverdian et al., 2015). Non-redox metals can also disrupt plant metabolism indirectly by inhibiting antioxidant enzymes inducing ROS production and decrease glutathione levels (Emanverdian et al., 2015). The Table 5 illustrates the available of some heavy metals in soil to plant uptake, accumulation and negative effects in plants.

Table 5 -Heavy metals in plant uptake and metabolism.

Heavy metal	***Form* available *toplant Uptake***	**Plant metabolism**	**Plant effects**	**References**
Cd	Cd^{2+}	non-essential element	• photosynthesis • water uptake, and nutrient uptake • necrotic cell death • uncontrolled cell proliferation • formation of reactive oxygen species	Ramos, 2002; Lee, 2003; Paralta-Videa, 2009; Gratão, 2015
As	As^{5+}	non-essential element	• metabolic processes of phosphate	Tripathi, 2007; Peralta-Videa, 2009
Pb	Pb^{2+} and lead-hydroxy complexes	non-essential element	• seed germination • plant growth • chlorophyll synthesis	Sharma & Dubey, 2005; Begonia et al., 2004;
Cr	Cr^{3+} Cr^{6+}	non-essential element	• seedling dry matter • development of stems and leaves during plant early growth stage • cell division • elongation of roots and shoot • metabolic disorders in seed germination	Singh et al., 2013; Oliveira, 2012; Nematshahi et al., 2012, Srivastava et al., 2011; Nath, 2008
Zn	Zn^{2+}	• component of special proteins known as zinc fingers that bind to DNA and RNA; • constituent of enzymes (oxidoreductases, transferases, Hydrolases) and ribosome.	• photosynthesis • genetic-related disorders • plant growth • DNA regulation and stabilization • Necrotic spotting	Broadley et al., 2007; Baran, 2013; Mirshekali et al., 2012; Ialelou et al, 2013; Todeschini et al, 2011.

Ni	Ni^{2+}	• key factor in activation of enzyme urease (nitrogen metabolism) • seed germination • iron uptake	• seed germination and seedling growth by hampering the activity of the enzymes (amylase and protease) • plant height and leaf area • Inhibition of lateral root formation and development • damages in photosynthetic apparatus • hampers mitotic cell division in root	Poonkothai & Vijayavathi, 2012; Bai & Wood, 2013; Khan et al., 2010; Sethy & Ghosh, 2013; Siddiqui et al., 2013
Cu	Cu^{2+}	• catalyzer of redox reaction in mitochondria,chloroplasts, and cytoplasm of cells • electron carrier during plant respiration	• root elongation and growth • lipid peroxidation • structural disturbance of thylakoid membranes • cell elongation • seedling growth • photosintesis	Doncheva & Stoyanova, 2007; Sharma & Dubey, 2010; Emamverdian et al, 2015

2.5 Strategies to defense plants against heavy metals

Plants may uptake essential and non-essential elements from soils in response to electrochemical potential gradient of the plasma membrane in the root cells or by diffusion of elements in the soil. The level of heavy metals accumulation differs between species and there are different defenses strategies to plant avoid heavy metal contamination. The first line of defense is exclude excess bioavailable metals at their roots by binding them to organic acids or storing them within vacuoles where they cannot interfere in important physiological processes (Hossain et al. 2012; Gall & Rajakaruna 2013; Sharma et al; 2016).

Other plants can accumulate or exclude heavy metals such as Cd by trichomes, improving the tolerance against this metal (Lavid et al., 2001; Lee et al., 2002; Hauser, 2014). A group of plants, such as Brassicaeae (Gall & Rajakaruna, 2013), tobacco (Choi et al., 2001), willows and poplars (Utmazian et al., 2007) are known as metal hyperaccumulators. These plants can tolerate and accumulate bioavailable metals in tissues and shows a high ability in metal uptake (van der Ent et al., 2013; Pollard et al., 2014). However, when these defenses is not enough, plants active other mechanism to deal with heavy metal uptake which will be explained below.

2.6 Antioxidant defense mechanism employed by plants against heavy metals stress

Heavy metal is a cause of abiotic stress in plant. In general, toxic elements are uptake by plants, altering cellular redox balance and induce oxidative stress (Yadav, 2010; Oksana, 2013, Arenas-Lago et al., 2016). The impact of oxidative stress on plant metabolism is intricate, causing a serious imbalance in cell homeostasis with damages in tissues and consequently losses in crop production (Sytar et al., 2013). Reactive oxygen species (ROS) are products of metabolic pathways/reactions occurred in various cellular organelles such as chloroplasts, mitochondria and peroxisomes (Anjum et al., 2010, 2011). The reduction of molecular oxygen results in generation of ROS such as singlet oxygen, superoxide radicals, hydroxyl radicals, hydrogen peroxide and peroxide radicals, which are commonly produced in low concentrations in plant metabolism (Demidchik, 2015). Indeed, disturbances in equilibrium between production and scavenging of ROS generated in presence of heavy metals may increase ROS production at intracellular levels, which can cause damage to proteins, lipids, carbohydrates and nucleic acids (Noctor, 2014; Gill & Tuteja, 2010). ROS, as a result of abiotic stress, is a major cause of losses in crop production in global scale (Mittler et al, 2002;). It is important for cell to avoid the high concentration of ROS thought the detoxification mechanisms to minimize eventual damages. Thereby, these defense systems are composed by nonenzymatic and enzymatic antioxidants thus interrupting the cascades of uncontroleed-oxidation by scavenging ROS. The antioxidant defense systems comprises antioxidant enzymes such as such as superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6), glutathione reductase (GR, E.C. 1.6.4.2), peroxidase (POD, E.C. 1.11.1.7), ascorbate peroxidase (APX, E.C. 1.11.1.11) and guaiacol peroxidases (GPX, E.C. 1.11.1.7) as illustrated in Figure 1, as well as non-enzimatic antioxidant such as ascorbic acid (AsA), glutathione (GSH), a-tocopherol, carotenoids and flavonoids.

(Gratao, 2005; Maleva et al., 2009; Shu et al., 2012; Kumar et al., 2012) and proline (Chen, 2005; Zarei, 2012).

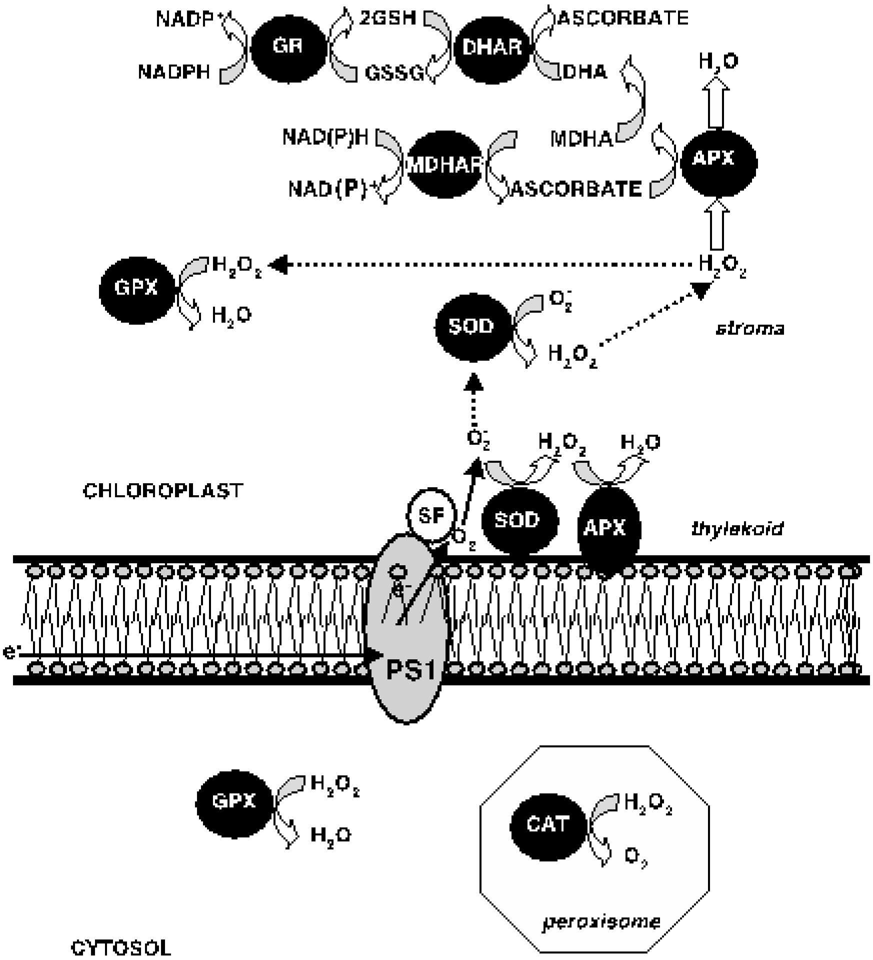

Figure 1-ROS-scavenging pathway. SF, stromal factor: PS1, photosystem I; GR, glutathione reductase; APX,ascorbate peroxidase, GPX, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; MDHAR, monodehydroascorbatereductase, DHAR, dehydroascorbatered. From Gratão et al., 2005.

2.7 Heavy metal in crop production and potential risks for human health

Food is the most common source of heavy metal for humans around the world, due to crops grown on heavy metal contaminated soils (Luo et al., 2009). Metals are bind with negative-charged particles of soil, once metals can detach, enter in the soil solution and become bioavailable to plants (Neilson & Rajakaruna, 2012).

Once uptake by plants, this metal may accumulate in plant tissues and prejudice food security. In populations which consuming regionally foods the contamination risks are higher. For example, subsistence farmers who live in soil contamination. In this case, the heavy metal in their diet is not diluted with food from other non-contaminated areas, while food source produced in contaminated and non-contaminated areas and heavy metal content predominate in low levels in developed cities (Chary et al., 2008). Agricultural practices add heavy metal in soil and recent studies indicate health risks in consumer cereals and vegetables cultivates in contaminated soils (Salehipour et al., 2015). For instance, sewage sludge increase the concentration of Cd, Cu, Pb and Zn more than the permissible levels allowed by EU standards and UK guidelines in maize and tsunga (Muchuweti, 2006).

Khan et al. (2008) reported about Cd, Pb and Cr contamination in vegetables cultivated with wastewater irrigation near industrialized areas with atmospheric pollution. High levels of metals (Cd, As, Pb and Mo) in some vegetables could be due to unnecessary use of fertilizers and wastewater (Ahamad et al., 2015). It was also informed that pharmacological medicinal plants cultivated with wastewater showed considerably higher metal contents compared with plants cultivated with clear water (Khan et al., 2015). Moreover, high concentrations of potentially toxic metals such as Cd, Pb and As was found in edible mushrooms in China (Liu et al., 2015).

Even rice shows high concentrations of Pb and As, which represents carcinogenic health risks (Omar, 2015, Ma et al., 2016). Products derived of plants also can transfer heavy metal to human body. For example, wine shows Zn and Cu concentrations higher than the toxicological safety limits due to vines receive antiparasitic treatments content heavy metals (Volpe, 2009). Moreover, cigarettes are huge source of heavy metals for humans, once tobacco plant retains heavy metals into the leaves (Agoroaei et al., 2014). Elevated heavy metals concentrations in vegetables may be transfer to food wed and pose serious health risks to humans. Metals (e.g., Cd, Pb and As) can cause mutagenesis and several diseases, as carcinogenesis, problems in the immune system, inhibit growth and fertility (Swarup & Dwivedi, 2002; Alonso et al., 2002; Mahajan et al., 2012). Considering that toxic heavy metals insert by food can cause adverse health effects in human, recent studies have recommended dietary supplements for people at risk of Cd and Pb exposure (Zhai, 2015). There are uncountable source of heavy metal though food chain, therefore is important people aware about risks and avoid soil contamination.

2.8 Future perspectives

There are massive pressures on agriculture to satisfy the food demand for world population and increase crop yields, In response to this demand, crop producers have adopted intensive agricultural practices which can contaminate environment with heavy metals. Therefore, is extremely necessary to formulate appropriate agricultural public policies at a world level to enhance the extension services and educate farmers about better management risks without having a negative effect on the food security and human health.

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CHAPTER III - GRAFTED TOMATO MUTANTS: AN APPROACH TO STUDY PLANT HORMONES AND ROOT-TO-SHOOT COMMUNICATION

3.1 Abstract

Stress perception and signaling pathways between plant organs is a complex mechanism and remain unanswered. Therefore, in order to further address the role of phytormones in modulation of stress perception from root-to-shoot signaling, we used ethylene-insensitive *Never ripe* (*Nr*) and auxin-insensitive *diageotropica* (*dgt*) tomato mutants associated with grafting technique. For this purpose, we analyzed lipid peroxidation, H_2O_2 content, chlorophyll content, proline, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione redutase (GR) activities in non-grafted and grafted tomato mutants submitted to cadmium (Cd). The results observed revealed different responses according with genotype, grafting combination and Cd application. The results showed that Cd accumulated mainly in roots and rootstocks. Nutrients uptake decreased in leaves of non-grafted plants, but differed among plant parts in all grafted plants. Proline and chlorophyll contents were higher in *dgt* plants. In the presence of Cd, scions of grafted plants increased antioxidant enzymes activities as a signaling from rootstocks. Nonetheless, it was possible to assign the involvement of ethylene and auxin on antioxidant responses, since *Nr* and *dgt* genotypes were less affected by Cd-stress. These results add new insights on relative importance of auxin and ethylene in regulating the modulation of antioxidant responses to stressful conditions.

Key words: Antioxidant enzymes, auxin, cadmium, ethylene, hormonal mutants, Micro-Tom, signaling, oxidative stress

3.2 Introduction

Cadmium (Cd) is the most dangerous toxic heavy metal to many organisms, which can enter in the environment from anthropogenic activities including herbicides, pesticides, chemical fertilizers, irrigation with contaminated water and pollutants from industrial processes (Hédiji *et al*., 2015; Zouari *et al*., 2016a). Although Cd is nonessential element, it can be easily taken up by plants causing morphological, structural, biochemical and physiological dysfunctions (Gratão *et al*., 2009; Fahad *et al*., 2015; Ahmad *et al*., 2016). Even in low concentrations, Cd can induce or stimulate an uncontrolled oxidation and cause alterations in cell homeostasis and electrolyte leakage, which trigger biochemical response against oxidative stress, inducing a wide range of antioxidant defense systems (Anjum *et al*., 2015; Zouari *et al*., 2016b).

The uncontrolled oxidation can be detoxified by a complex enzymatic and non enzymatic mechanism interacts in an attempt to minimize oxidative stress damages and maintain cell redox state (Zhang *et al*., 2014, Gratão *et al*., 2015). The primary step of defense at cellular level comprises antioxidant enzymes such as superoxide dismutase (SOD EC 1.15.1.1), which dismutates O^{-2} to H_2O_2 (Noctor and Foyer 1998). Subsequently, H_2O_2 may be detoxified to H_2O by ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, 1.11.1.6) and glutathione peroxidase (GPX, EC 1.11.1.9) (Roychoudhury *et al*., 2012; Hippler *et al*., 2015; Liu *et al*., 2015;; Khaliq *et al*. 2015a, b).

Moreover, non-enzymatic mechanisms may be also responsible for quenching excessively ROS, such as proline, flavonoids, carotenoids, ascorbate and glutathione (GSH) (Wu *et al*. 2007; Zouari *et al*., 2016a,b). The regeneration of GSH formed from oxidized glutathione (GSSG) is catalyzed by glutathione redutase (GR, EC 1.6.4.2) using NADPH as a reducing agent (Gratão *et al*., 2015;).

Plant-stress mechanisms may require the interplay between an interconnected network of cellular responses and signaling molecules to Cd-stressful conditions (Zouari*et al*., 2016a). For instance, plants hormones can also participate and interact with redox signaling to control responses to abiotic stresses (Sharma *et al*., 2010; Gratão *et al*., 2012; Bartoli *et al*., 2013; Bankaji *et al*., 2014; Baxter *et al*., 2014;). Nonetheless, mechanisms involving the interaction of hormones with stress responses have been poorly understood (Gratão *et al*., 2012), mainly the cross talk between root-to-shoot communication.

More specifically, ethylene can be considered a ‘stress hormone’ involved in multiple molecular and physiological plant processes, regulating various growth and cellular defense responses to toxic metals (Schellingen *et al*., 2014; Van de Poel *et al*., 2015), implying a synergistic effect between biosynthesis of ROS and ethylene (Djanaguiraman *et al*., 2009). Although the molecular relationship between ethylene biosynthesis and Cd stress has not been well established, ethylene may contribute to the regulation of the early Cd-induced oxidative challenge via the control of glutathione (GSH) content and expression of signaling genes (Schellingen *et al*., 2015). Therefore, there are many answers to enlighten about mechanisms of ethylene regulating plant responses to metal stress and its effects in plant sensitivity or tolerance (Asgher *et al*., 2015), since that ethylene insensitive mutants have demonstrated to be less sensitive to heavy metal-stressful conditions (Bueso *et al*., 2007, Gratão *et al*., 2012).

As well as ethylene, auxin hormone can also be related to stress signaling and defense responses (Tyburski *et al*., 2009; George *et al*., 2010), involving heavy metal tolerance and accumulation (Fassler *et al*., 2010). Current research has shown that auxin may induce morphogenic responses (Potters *et al*., 2007; Potters *et al*., 2009) which may avoid adverse effects of environmental stresses (Tognetti *et al*., 2012). In contrast, the suppression of auxin may improve stress tolerance by mediated growth suppression and reallocation of metabolic resources to resistance establishment (Park *et al*., 2007)

Although multiple stress responses are essential for plant survival to heavy metal-stress condition, the exact role of ethylene and auxin hormones in these responses has not been understood. Therefore, the use of hormonal mutants is a powerful tool to study the hormonal modulation, providing insights about the cross-talk between the ROS signaling and plant hormones during stressful conditions.

In tomato (*Solanum lycopersicum*), the ethylene *LeETR3* receptor is the correspondent to the mutation *Never ripe* (*Nr*) (Wilkinson *et al*., 1995), which do not respond to either endogenously generated or exogenously ethylene (Lanahan *et al*., 1994). Moreover, the *diageotropica* (*dgt*) tomato mutant exhibits a considerably reduced sensitivity to the hormone auxin (Kelly and Bradford, 1986), related to the *DIAGEOTROPICA (DGT)* gene, which encodes a component of a specific auxin-signaling pathway (Oh *et al*. 2006; Retzer *et al*.,2015). According to our prior studies with *Nr* and *dgt* tomato mutants (Monteiro *et al*., 2011; Gratão *et al*., 2012), it was possible to assign the involvement of ethylene and auxin on antioxidant mechanisms, since that the *dgt* might avoid stress imposed by Cd due to the fact that the known auxin-stimulated ethylene production be comprised in *dgt* plants. Whereas the *Nr* genotype was more affected by the Cd imposed stress by the fact that *Nr* retains a partial sensitivity to ethylene.

The plant vascular system can serve as a conduit for transport in long distance nutrients and signaling molecules inter-organs communication (Golecki *et al*., 1998; Spiegelman *et al*., 2013). Although grafting technique can be an elegant mechanism to demonstrate root-to-shoot as well as shoot-to-root comunication (Molnar *et al*., 2010), little is available in the literature when grafting has been used to study metals effect on plant development, in particular the antioxidant stress responses. In other our prior study, grafted tomato plants revealed distinct trends that clearly indicate signaling responses from the rootstocks, allowing sufficient time to activate defense mechanisms in shoot (Gratão *et al*., 2015).

Further analysis is obviously needed for a better understanding about the mechanisms connecting antioxidant responses, ROS and hormones and communication among plant organs. For this purpose, throughout the use of the *Nr* and *dgt* tomato mutants and grafting technique, the aim of this work was to understand or provide details about the modulation of oxidative stress by antioxidant systems and the role of auxin and ethylene during Cd-stressful condition.

3.3 Material and methods

3.3.1 Plant material and growth conditions

Seeds of tomato (*S. lycopersicum* L.) cultivar Micro-Tom (MT) and mutants *diageotropica* (*dgt*) and *Never ripe* (*Nr*) were sterilized with sodium hypochlorite solution (5%), rinsed three times and sown in boxes filled with a mixture of 1:1 (by volume) commercial pot mix (Bioplant ® HT Eucatex Brazil), vermiculite supplemented with 1 g L $^{-1}$ 10:10:10 NPK (Nitrogen-Phosphorus-Potassium) and 4 g

L $^{-1}$ lime ($MgCO_3$ + $CaCO_3$). The boxes were maintained in a greenhouse with an average mean temperature of 23,2 ºC, relative humidity 77,6%, 11.8 h photoperiod (autumn). When two true leaves were completely formed, two seedlings were transplanted to 0,350 L Leonard vases (Vincent, 1975) contained sterilized sand, polystyrene (4:3) and modified Hoagland´s nutrient solution (pH 5.6) (250 ml). After seedling establishment, only one plant was maintained per vase. Thirty five-old-plants further grown in the same solution with 0 mM or 1 mM $CdCl_2$ (Gratão *et al*., 2015), was changed weekly.

Forty-five-old-plants were grafted by cleft method and the plant was cut off about 3 cm above soil level; this part was used as rootstock and the portion of shoot was used as scion. The scion was inserted into the slit in the centre of the rootstock stem and the join parts were tied firmly with 5-cm-wide plastic film and a spring clip. When the grafting was completed, all seedlings were covered with transparent plastic bags and a shade net. The transparent plastic films were maintained during four days to ensure high relative humidity and the plastic binding films were moistened daily and removed when graft union was formed.. Germinating buds of rootstocks were also removed daily. After grafting, the exposure of Cd was blocked. The scion-rootstock established, apart from non-grafted plants, is presented in fig. 1.

After a period of 75 days post germination, corresponding to 45 days of exposure to $CdCl_2$ and 30 days of grafting, samples of roots and leaves (excluding petioles) were harvested, rinsed and immediately immersed in liquid N_2 and stored at -80ºC for further analysis.

3.3.2 Cd content and nutritional analysis

Samples of root and shoot (0.2 g DW) was milled and microwave digested with 2 mL 70 % HNO_3, 2 mL H_2O_2 and 2 mL Milli-Q water (18.2 MX cm a 25 ºC) at a controlled pressure of 2 MPa (Chilimba *et al*. 2011). The digested samples were diluted to 10 mL with Milli-Q water and stored. Cd content was determined by ICP-OES (Optima 8300, PerkinElmer). The contents of P, K, Ca, Mg, Cu, Fe, Mn and Zn were determined by X-ray fluorescence method (EDXRF) analysis, using samples prepared as loose powder, as described by Tezotto *et al*. (2013).

3.3.3 Chlorophyll determination

Fresh leaves (0,50 mg) were added in tube with 2 ml acetone (100%). After shook during 72 hour at 60g and 4ºC the sample was read spectrophotometrically in 470 nm to carotenoids, 645 nm and 662 nm to chlorophyll *a* and *b*, respectively**.** The chlorophyll and content was calculated as described by Lichtenthaler (1987).

3.3.4 H_2O_2 Content

The H_2O_2 content was estimated following the method of Alexieva *et al*. (2001). Plant tissues were homogenized in thiobarbituric acid (0,1%) and centrifuged at 10,000 g for 10 minutes. The supernatant was added in 100 mM potassium phosphate buffer (pH 7.50) and 1 M potassium iodide solution. This solution was incubed in ice for one hour, the absorbance was read at 390 nm, and H_2O_2 content was determined using a known H_2O_2 concentration curve as a standard.

3.3.5 Lipid peroxidation

Lipid peroxidation was measured by estimating the content of thiobarbituric acid reactive substances (TBARS) as describe by Heath and Packer (1968). Plant tissue was ground with 20% (w/v) polyvynilpyrrolidone (PVPP) and 0,1% trichloroacetic acid (TCA) After centrifugation at 11,000 g for 10 minutes, the supernatante was added in a solution of 20% TCA and 5% thiobarbituric acid (TBA) and was incubed in water bath at 95ºC for 30 minutes. The reaction was stopped in a cooled on ice bath during 10 minutes and centrifuged at 11.000 g for 10 minutes. The concentration of malondialdehyde (MDA) equivalents was determined by spetrophotometre with readings between 535 and 600 nm, datas was calculated using an extinction coefficient of 1.55 x 10^{-5} mol cm^{-1}, (Gratão *et al*. 2012).

3.3.6 Proline content

Proline content was determined spectrophotometrically as described by Bates (1973). Leaf samples were homogenized in 3 % sulphosalicylic acid. The mixture filtrate was reacted with 1 ml each of acid ninhdrin and glacial acetic acid and was held for 1 h in boiling water. The mixture was added toluene (4 mL) and the absorbance was measured at 520 nm and calculated as mmol g^{-1} fresh mass against standard proline.

3.3.7 Enzyme extraction and protein determination

Fresh leaf tissue was homogenized in a chilled mortar with a pestle with an extraction buffer containing 100 mM potassium phosphate buffer (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 5 % (w/v) insoluble polyvinylpolypyrrolidone (Boaretto *et al*. 2014) in volume/fresh mass (3:1). The homogenate was centrifuged at 10,000 g for 30 min, and the supernatant was stored at -80 ºC for prior assays to SOD, CAT, GR and APX enzymes activities. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

3.3.8 Superoxide dismutase assay

Samples of plant tissues were added in 50 mM potassium phosphate buffer (pH 7.8), metionine, nitro blue tetrazolium (NBT), ethylenediaminetetraacetic acid (EDTA) and riboflavine. The reaction occurred in a box with luminescent light (15 W) at 25 ºC, and the enzyme activity was read spectrophotometrically at 560 nm and expressed in U SOD/protein mg (Giannopolitis and Ries 1977).

Afterwards, SOD activity was carried out in gel as described by Rendón *et al*., (2013) to determine isoforms (Azevedo *et al*. 1998). Plant extract was submitted to non-denaturing-PAGE separation, the gel was rinsed in distilled-deionized water and maintained in the dark during 30 min in 50 mM potassium phosphate buffer (pH 7.8) containing 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium, 1 mM EDTA, and 0.3%

N,N,N´,N´-tetramethylethyllenediamine. The gels were rinsed with distilled–deionized water and then illuminated in water until the achromatic bands of SOD activity were visible on a purple-stained gel. SOD isoenzymes were distinguished and classified by their sensitivity to inhibition by 5 mM hydrogen peroxide (H_2O_2) or 2 mM potassium cyanide (Azevedo *et al*. 1998).

3.3.9 Catalase assay

Catalase activity was assayed spectrophotometrically at 25 ºC in a reaction mixture containing 1 mL of 100 mM potassium phosphate buffer (pH 7.5), containing 25 µL H_2O_2 (30 % solution). The activity was determined by monitoring the decomposition of H_2O_2 at 240 nm over 1 min according with Nogueirol *et al*. (2015) CAT activity was expressed as µmol min^{-1} mg^{-1} protein.

3.3.10 Ascorbate peroxidase assay

Ascorbate peroxidase activity was measured spectrophotometrically in a reaction containing plant extract, 80 mM potassium phosphate buffer (pH 7.0), 5 mM ascorbate, 1 mM EDTA and 1 mM H_2O_2 (Gratão *et al*. 2012). APX activity was essayed by monitoring the rate of ascorbate oxidation at 290 nm at 30 ºC. APX activity was expressed as µmol ascorbate min^{-1} mg^{-1} protein.

3.3.11 Glutatione reductase assay

Glutathione reductase was assayed spectrophotometrically at 30 ºC adopting the method of Carvalho *et al*. (2013) by monitoring the rate of reduction of GSSG. The essay mixture consisted of 100 mM potassium phosphate buffer (pH 7.5), containing 1 mM 5,500-dithiobis (2-nitrobenzoic acid), 1 mM GSSG and 0.1 mM NADPH read in absorbance at 412 nm over 1 min. GR activity was expressed as µmol min^{-1} mg^{-1} protein.

3.3.12 Statistical analysis

The experimental design was randomized with six plants from three replicate vase. The result of each plant sample was expressed as mean and standard error of mean (±SEM) of three independent replicates. The statistical analysis was performed using the Assistat software 7.7 Beta ® and a multiple comparison between means by the Duncan test followed an individual ANOVA for each character at a 0.05 level of significance.

3.4 Results

3.4.1 Plant growth and Cd content

Over 75 d after germination, all grafted plants exhibited dry mass reduction when compared with non-grafted plants. The grafting technique had a negative effect on growth, with decrease in rootstock and scion dry mass. In non-grafted plants, MT plants cultivated in $CdCl_2$ exhibited growth reduction of roots, whereas *Nr* plants showed growth reduction in leaves and *dgt* plants did not exhibited significante difference when compared to control plants (without $CdCl_2$) (Fig.2). In grafted plants, which had the rootstock cultivated to Cd before grafting, a small portion of Cd accumulate above parts, been pronounced in scions of grafted MT(-Cd)/*Nr*(+Cd) plants (Fig. 3). MT rootstock exhibited the highest Cd content. On the other hand, non-grafted mutants accumulated more Cd when compared to MT (Fig. 3)

3.4.2 Nutritional analysis

Macronutrients and micronutrients were uptake and portioned different among plants genotypes, Cd exposure and tissue analyzed (Table 1). In non-grafted plants following the Cd application, MT roots exhibited decrease in Ca concentration and P, Mg, K, Cu and Zn concentration decreased in MT scions. Rootstock of *Nr* decrease in Ca concentration, whilst Mg, S, Cu and Zn decrease in *Nr* Scion (Table 1). There was no differences in rootstock nutrients concentration of *dgt* plants, irrespective to Cd application. However, scions of *dgt* exhibited increase in S concentrations in the presence of Cd. In grafted plants it is possible to note peculiar differences in nutrients concentration pattern according with grafting combination and Cd application. For instance, scion of MT of grafted MT(-Cd)/MT(+Cd) plants did not exhibit differences in nutrient concentration when compared with scion control of grafted MT(-Cd)/MT(-Cd) plants. However, scion of grafted MT (-Cd)/MT(+Cd) plants exhibited low concentration of Cu when compared with scion of grafted MT (-Cd)/*dgt* (+Cd) plants. Moreover, scion of grafted MT (-Cd)/*Nr*(+Cd) plants decrease S concentration when compared with scion of grafted MT (-Cd)/MT(+Cd) plants. On the other hand, rootstock of *Nr* (-Cd)/*Nr*(+Cd) exhibited increase in Mg concentration when compared with rootstock of MT (-Cd)/*Nr* (+Cd), whilst Grafting MT (-Cd)/*Nr* (+Cd) and *Nr* (-Cd)/*Nr* (+Cd) exhibited increase in Mn accumule in rootstock. Moreover, rootstocks of grafted *dgt* (-Cd)/*dgt* (+Cd) plants exhibited increased P concentration when compared with rootstocks of grafted MT (-Cd)/*dgt* (+Cd) plants (Table 1).

3.4.3 Lipid peroxidation and H_2O_2 content

Lipid peroxidation, expressed as MDA content, was more pronounced in leaves for all genotypes of grafted and non-grafted plants when compared with roots (Fig. 4b). In the presence of Cd, non-grafted plants exhibited pronounced increase in MDA content in scions and rootstocks when compared to non-grafted control plants. The same pattern was observed among scions of all genotypes of grafted plants, following Cd application, which increased lipid peroxidation rates (Fig. 4b), being more pronounced in scions of grafted MT(-Cd)/MT(+Cd) and MT(-Cd)/*Nr*(+Cd) plants. The H_2O_2 content was higher in scions of grafted and non-grafted plants to $CdCl_2$ application (Fig. 4a), whereas rootstocks of *dgt* non-grafted plants seems to be less affected to Cd exposure.

3.4.4 Chlorophyll content

The chlorophyll content was different between genotypes and Cd exposure. It is clearly to note that chlorophyll content was less affected by Cd exposure in *dgt* plants (Fig. 5). Interesting, scions of MT plants grafted onto *dgt* rootstock exhibited high chlorophyll content when compared with scions of MT plants grafted onto MT or *Nr* rootstock (Fig. 5).

3.4.5 Proline content

Following treatment with $CdCl_2$, non-grafted plants exhibited higher proline content in both tissues, being more expressive in *dgt* mutant plants. In grafted plants, it is possible to note that proline content was lower in rootstock to Cd application. On the other hand, scions of grafted MT(-Cd)/MT(+Cd) and MT(-Cd)/*Nr*(+Cd) plants exhibited higher proline content when compared with grafting plants without Cd (Fig. 6).

3.4.6 Antioxidant enzymes activities

The analyzes of SOD (Fig. 7a), CAT (Fig. 7b), APX (Fig. 7c), and GR (Fig. 7d) revealed high levels of activities in roots and rootstocks of all genotypes and treatments when compared to scions. This data is in according with non-denaturing PAGE (Fig. 8). Although we did not carry out a densitometric analysis, it is possible to observe a pronounced SOD activity in roots. Three distinct SOD isoenzymes were detected characterized as Mn/SOD (SOD I and III), Fe/SOD (SOD II) and Cu/Zn – SOD (SOD IV) (Fig. 8). Changes were observed between tissue and Cd application. For instance, SOD I and III was more pronounced in roots of both grafted and non-grafted plants. Following Cd application, SOD IV decrease activity in leaves and roots of non-grafted and grafted plants (Fig. 8 lines 4-6, 12-16). SOD II was only observed in leaves and exhibited very low activity when compared to other isoenzyme (Fig. 8A). A light increase in SOD II activity was observed in leaves under Cd application (Fig. 8, line 4-6, 12-16). Although no differences was noted in SOD activity among genotypes in non-denaturing PAGE, we clearly observed this differences in SOD activity measured spectrophotometry (Fig. 7a). The presence of $CdCl_2$ also revealed higher SOD activity in leaves of non-grafted MT, *Nr* and *dgt* plants scion of grafted MT(-Cd)/MT(+Cd) and MT(-Cd)/*Nr*(+Cd) plants, whereas SOD activity was higher in all rootstocks with Cd application (Fig 7a).

The CAT (Fig. 7b.), APX (Fig. 7c) and GR (Fig. 7d) activities are crucial for detoxification H_2O_2 excess produced by SOD and other metabolic process. In this study, these enzymes revealed complex responses among non-grafted, grafted, genotypes and Cd application. Non-grafted plants exhibit increase in APX, CAT and GR activity following Cd application, whilst, scions of grafted plants without Cd exhibited increase in CAT (Fig. 7b) and APX (Fig. 7c) activities. When roots and rootstock were analyzed, an interesting tendency was observed. The application of Cd trigger differences in enzymes activities among genotypes of non-grafted and grafted plants (Fig. 7). Cd application in MT non-grafted increased CAT, APX and GR activities, whereas *Nr* decreased CAT activity (Fig. 7b). Grafted MT (-Cd)/*dgt* (+Cd) and Mt(-Cd)/*Nr* (+Cd) plants maintained low APX activity when compared with MT(-Cd)/MT (+Cd). On the other hand, MT(-Cd)/MT(+Cd) decreased GR (Fig. 7d) activity when compared with grafted MT/*dgt* (+Cd) and Mt/*Nr*(+Cd) plants.

3.5 Discussion

During plant development in environmental stresses, there is a complex mechanism in stress perception, signal transduction and antioxidant responses between root and shoot (Gratão *et al*. 2015) which seems to be modulated by auxin and ethylene (Togneti *et al*., 2012; Schellingen *et al*., 2015). Therefore, we used hormonal mutants and grafting technique to highlight the role of auxin and ethylene in stress perception in roots and signaling to shoot.

Cd toxicity have obvious negative effects on plant growth (Djebali *et al*., 2005; Lage-Pinto *et al*., 2008; Anjum *et al*., 2015) and can inhibit root drymass, root diameter and number of lateral root (Shafi *et al*., 2010). The inhibition of root and shoot growth was clearly observed in MT non-grafted plants exposed to $CdCl_2$ (Fig. 2). Grafting technique caused relevant decrease in plant growth in both stressed and non-stressed plants (Fig 2). Grafted plants suffer a number of processes during grafted formation, including adhesion of rootstock and scion, cell proliferation, connection of the vascular tissues and then resumption of growth (Melnyk *et al*., 2015). In Solanaceae plants are require 10–12 days for the establishment of a functional graft union and survival (Lin *et al*., 2016), thus, this might be the main reason of low biomass accumulation.Roots are the first organ to contact with Cd, therefore this tissue can accumulate high concentration of Cd among majority species (Hediiji *et al*., 2010; Gallego *et al*., 2012;). The data obtained in this work showed high Cd accumulation in roots and rootstocks, whose Cd content varied considerably among genotypes (Fig. 3). Cd-perception and translocation trigger signal transduction cascades and can be associated with hormones (Asgher *et al*., 2015). According to our prior ultrastructural study (Gratão et al. 2009), MT tomato plants stomata were closed, whereas the majority of stomata in *Nr* and *dgt* mutants maintained open during Cd-stressful condition, which could indicate that Cd accumulation was higher in non grafted mutants due to transpiration, based on the stomatal open. On the other hand, application of exogenous auxin also increase Cd uptake (Guo *et al*., 1999; Farroq *et al*., 2015).

Rootstock of grafted plants under Cd application (Fig. 3) exhibited a distinct trend when compared with Cd accumulated in non-grafted plants. Rootstocks of grafted hormonal mutants exhibited decrease in Cd accumulation when compared with MT rootstocks. The rootstocks had Cd exposure blocked at the moment of grafting, which can suggest that the inhibition of auxin and ethylene perception could contributed to increase Cd accumulated over time.

Cd has a high mobility and can move across plasma membranes in plants (Schwartz *et al*., 2003). Root developed mechanisms such as exodermis, endodermis and other extracellular barriers to reduce, but not inhibit, Cd translocation to upper parts (Lux *et al*., 2011), such as demonstrated by leaves of non-grafted and grafted plants (Fig. 3). According to our grafted study with the MT tomato line (Gratão *et al*. 2015), this differences could be related to positive relationship between stomatal closure and decrease in leaf conductance.

Macronutrients and micronutrients are involved in several metabolic and cellular functions, such as signal transduction and hormone perception (Hänsch and Mendel 2009). The uptake of essential nutrients and toxic elements depends of a genetic control through roots structure and uptake mechanisms at biochemical level and molecular signals from root-to-shoot. (Sarwar *et al*., 2010). There was a wide difference in pattern macronutrients and micronutrients content among genotypes, tissue, grafting combination and exposure (or not) to Cd as observed in table 1. Non-grafted plants exhibit subtle differences in nutritional analyses of macronutrients and micronutrients in roots. On the other hand, non-grafted plants under Cd stress exhibited pronounced changes in nutrients concentration of leaves.

In general, grafting technique did not affect nutrient-uptake and translocation. Rootstock of grafted MT(-Cd)/*Nr* (+Cd) and *Nr*(-Cd)/*Nr*(+Cd) plants exhibited an intense decrease Mn concentration (66% and 77% respectively), when compared with grafting control. In the presence of Cd, the uptake of polyvalent cations, as Mn, can decrease due to its interaction with essential nutrients (Gonçalves *et al*., 2009). Self-grafting combination with Cd application exhibited increase in S content in rootstock of self-grafted MT/MT and *dgt/dgt* plants, when compared with the same combination without Cd. It is well known that Cd may be detoxified in plants by chelation of metal ions with high-affinity phytochelatins, a family of S rich peptides, which indicates association of S containing groups as an important defensive response against Cd toxicity (Hall, 2002). Moreover, scion of grafted MT(-Cd)/MT (+Cd) plants increase S concentration when compared with grafted MT(-Cd)/*Nr* (+Cd) plants, suggesting that different rootstocks may exhibit dissimilar abilities in mineral uptake efficiencies (Tagliavini *et al*., 1993; Sarwar *et al*.,2010). Although theses rootstock above mentioned showed similar S content, the S content in MT scions was more pronounced in grafted MT(-Cd)/MT(+Cd) plants than MT(-Cd)/*Nr*(+Cd), which can suggest that translocation of S during Cd stress may be associate with ethylene signaling. On the other hand, rootstocks of grafted MT(-Cd)/*dgt* (+Cd) plants showed increased Fe content, whereas Mg content increase in rootstocks of grafted *Nr*(-Cd)/*Nr*(+Cd) plants. These elements participate to prevent oxidative stress binding of proteins and enzymes (Hänsch and Mendel 2009). Among all treatments, *dgt* plants seems to uptake and translocate less macronutrients and micronutrient when compared with *Nr* and MT. Auxin synthesis and transport contribute to establishment an auxin gradients for the initiation of lateral root in the pericycle (Fukaki and Tasaka, 2009). Tomato mutant *dgt* is relatively insentive to auxin, which trigger less lateral root formation and dry mass reduction (Fig. 2). This genotype may implicate in decrease in nutrients uptake, as we observed in our dates. Although ethylene-induced modifications of auxin synthesis and transport (Fukaki and Tasaka, 2009), the genotype *Nr* did not exhibit wide difference in nutrient uptake and dry mass root as exhibited by *dgt* plants. Although *dgt* plants exhibited low nutrients content, it was observed a pronounced Fe content in leaves of non-grafted plants and rootstocks of grafted MT(-Cd)/*dgt*(+Cd) plants.

A huge number of studies indicated that auxin and ethylene can modulate stress (Djanaguiraman *et al*., 2009Tyburski *et al*., 2009; Shishova and Lindberg 2010). Nonetheless, we observed that non-grafted *dgt* and *Nr* plants seems to be less affected to Cd-stressful conditions, which exhibited lowest MDA and H_2O_2 content in both tissue (Fig. 4a-b) even though accumulate more Cd when compared with MT genotype (Fig. 3). Therefore**,** auxin and ethylene suppression leads to less damage to cell homeostasis, once lipid peroxidation may triggers membrane leakage (Talukdar 2011; Piwowarczyk *et al*., 2016). Other studies is in according with our results, where the inhibition of auxin and ethylene perception can minimize Cd toxic effect through the increased membrane stability (Iakimova *et al*., 2008; Monteiro *et al*., 2011).

Grafted plants exposed to $CdCl_2$ in rootstock showed higher lipid peroxidation and H_2O_2 accumulation in scion (Fig. 4a-b), although exhibit low Cd concentration (Fig.3), indicating traffic of stress signal from rootstock to the scion. It is interesting to note that grafted MT(-Cd)/MT(+Cd) plants exhibit high lipid peroxidation, which indicates that the low sensitive to auxin and ethylene can regulate developmental responses under environmental stress. Moreover, MT scion exhibited changes in MDA content depending of rootstock combination. For instance, rootstock of grafted *Nr* (+Cd) and *dgt* (+Cd) plants trigger different response in MT scion when compared with MT(-Cd)/MT(+Cd), minimizing lipid peroxidation expressed in MDA content (Fig 4b).

The membrane selectivity and mechanisms of heavy metals-immobilization can avoid Cd uptake to the chloroplast (Siedlecka and Krupa 1999). Nonetheless, Cd exceeds these barriers and accumulated in chloroplasts, which exhibited changes in organelle shape and internal organization (Gratão *et al*., 2009), reducing their content per cell and per unit of leaf area (Baryla *et al*. 2001; Fagioni *et al*. 2009). Moreover, Cd toxicity decline chlorophyll content in diverse species (Muradoglu *et al*., 2015; Lysenko *et al*., 2015). These alterations in chloroplast of plants to Cd may occur due to an increase in ROS production in leaves (Fig. 4b). In our results, the chlorophyll content was lower in grafted and non-grafted plants to $CdCl_2$ application (Fig. 5). Chlorophyll content in non-grafted MT plants was more pronounced affected by Cd when compared with hormonal mutants plants. Probably, this result may relate to the high lipid peroxidation of the chloroplast membrane of MT plants measured as MDA content (Fig. 2a).

In contrast, chlorophyll content decreased more pronounced in scions of grafted MT(-Cd)/MT(+Cd) plants when compared to MT(-Cd)/*Nr*(+Cd) and MT(-Cd)/*dgt* (+Cd) plants (Fig. 5), indicating differences about how mutants and MT rootstocks may traffic information to MT scions. It is interesting to note that *dgt* plants exhibit high chlorophyll content when compared to MT and *Nr* genotypes as verified in grafted and non-grafted submitted to stress condition. This data can be associated with the size and number of starch grains of *dgt* genotype that are not reduced as occurs in MT and *Nr* starch grains when submitted to Cd application (Gratão *et al*., 2009). Moreover, *dgt* leaves exhibit dark green phenotype, due to a reduction in cell size, which causes an increased concentration of chloroplasts per leaf area (Koornneef *et al*., 1990) and can result in high chlorophyll content.

As a direct response of stress, plants enhance synthesis and reduced degradation of protective metabolites such proline, which contribute to stabilization of protein molecules and membranes (Seregin and Kozhevnikova, 2006). According to results, we observed high proline content in both tissue of non-grafted plants submitted to Cd, being more pronounced in *dgt* plants (Fig. 6). Although proline can be associated with scavenging ROS (Smirnoff and Cumbes, 1989), H_2O_2 content was high in plants submitted to Cd (Fig. 4b), indicating that proline was not enough to control the oxidation.

Scions of grafted MT(-Cd)/MT(+Cd) plants exhibited high proline content when compared to scions of grafted MT(-Cd)/*Nr* (+Cd) and MT(-Cd)/*dgt* (+Cd) plants, which suggest that the suppression of auxin and ethylene from rootstock can regulate proline accumulation in scion during stress condition (Fig. 6).

As a response of Cd-stressfull conditions, the antioxidant enzymes allow plants avoid or tolerate oxidative stress and survival to environmental adversities. In this study was select key antioxidant enzymes SOD, APX, CAT and GR based on several researches on Cd-stress responses in plants, which exhibited varied responses among genotypes, grafting combinations, tissues and Cd exposure.

Cd mediated enhanced SOD activity in a number of agricultural plants. SOD dismutes O_2^{-} to H_2O_2 and a high specific activity from this enzyme was observed in roots and rootstock under Cd application. Three SOD isoenzymes (SOD I, II, III and IV) were identified following non-denaturing PAGE analysis (Fig. 8) with a similar isoenzyme classification as reported by Gratão *et al.* (2012; 2015). The Mn/SOD (SOD I and III) and Cu-Zn/SOD (SOD IV) were highly active in both tissues, been more pronounced in roots and Fe/SOD (SOD II) was only present in leaves. Although upper parts of non-grafted *dgt* (+Cd), *Nr* (+Cd), grafted *dgt* (-Cd)/*dgt* (+Cd) and *Nr* (-Cd)/*Nr* (+Cd) plants exhibited less SOD activity (Fig. 7a) and H_2O_2 content (Fig. 4a) when compared with non-grafted MT(+Cd) and MT(-Cd)/MT (+Cd) plants, it is was clearly observed an increase in SOD II. This finding can indicate the involvement of auxin and ethylene signaling pathway. However, how these hormones could control SOD activity remain unknown. Scions of self-grafted MT(-Cd)/MT(+Cd) and MT(-Cd)/*Nr*(+Cd) plants exhibited similar SOD specific activities, suggesting that ethylene perception in rootstocks can not affect the SOD activity in scions.

MT, *Nr* and *dgt* non-grafted and grafted plants following Cd application exhibited lowest SOD IV activity in roots and leaves when compared with the same treatments without Cd (Fig. 8). By the other way, MT and *Nr* plants exhibited decrease in Zn and Cu concentrations in leaves (Table 1). This finding might be attributed to antioxidants responses, since Cu and Zn can act as cofactors of SOD (Gill *et al.,* 2015) Although no changes in Cu and Zn concentrations was observed in grafted combinations and non-grafted *dgt* plants under Cd stress, SOD IV (Cu/Zn-SOD isoenzyme) activity (Fig. 8) was affected by Cd application (Romero-Puertas *et al*., 2007).

Although roots and rootstocks have exhibited high activity of SOD I, III and IV bands, low H_2O_2 content was demonstrated (Fig 4a). However, H_2O_2 can also be produced through non-enzymatic process, such as signaling molecules (Van Breusegem *et al*., 2008) and during photorespiration (Fukuzumi, 2016), which may explain higher H_2O_2 content in leaves indicating a complex system of H_2O_2 scavenging during stressful condition.

H_2O_2 from superoxide dismutase subsequently can be reduced to H_2O by CAT and APX. Although low Cd accumulated in scion (Fig. 3), we observe increase in CAT and APX activity, when compared with scions without Cd application, indicating stress signaling from rootstock to scions of grafted plants under Cd stress. CAT activity was lower in scions of grafted *dgt* (+Cd)/*dgt*(-Cd) when compared with other genotypes. Thus, auxin suppression decrease scavenging of H_2O_2 by CAT, but *dgt* plants might exhibit other system independent-auxin to remove H_2O_2, due *dgt* plants exhibited low content of H_2O_2 in scion (Fig. 4a). The up-regulation of APX can increase the ability to overcome Cd stress (Wu *et al*., 2015). APX activity increase in scions of grafted plants under Cd stress can suggest that the trafficking of information from rootstock to upper parts is independent of hormones to active mechanisms to protect against Cd damages.

When other antioxidant enzymes were concerned, GR played an important role in keeping the metabolic balance between GSH and AsA contents and H_2O_2 degradation, which can be involved in heavy metal detoxification (Sharma and Dietz, 2009). Roots and rootstock exhibited higher GR activity when compared with leaves of non-grafted and grafted plants (Fig. 7d). *Nr* mutant accumulate more Cd in non-grafted plants (Fig. 3), which probably induced increase in GR and APX activities (7d-c). In non-grafted plants with Cd application, scion did not exhibit changes in GR activity as other enzymes, indicating that stress perception and signaling from rootstock was inefficient to active GR responses A study conducted by Jozefczak *et al*. (2014) indicated delay antioxidant responses in leaves as a consequence of Cd in roots.

On the other hand, rootstocks of grafted MT(-Cd)/MT (+Cd) plants exhibited lower GR activity when compared with other grafted plants with Cd application, suggesting that auxin and ethylene could regulate GR activity under Cd-stressful condition. Such findings suggest the possible interaction of stress signaling from root-to-shoot mediated by auxin and ethylene, which can be related to subsequent activation of antioxidant defenses in leaves, as observe with increase in enzyme activity in this tissue (Fig. 7a-d).

The *dgt* mutant might avoid stress imposed by Cd due to the fact that the known auxin-stimulated ethylene production be comprised in *dgt* plants, whereas the

Nr genotype can be more affected by the Cd imposed stress by the fact that *Nr* retains a partial sensitivity to ethylene (Gratão *et al*., 2009, Monteiro *et al*., 2011; Gratão *et al*., 2012). Therefore, stress signaling could be mediated by both auxin and ethylene, once MT scions exhibited different responses when a hormonal mutant was used as rootstock. Other studies in Arabidopsis suggesting involvement of early responses Cd stress and ethylene signaling (Schellingen *et al*., 2015). As well as auxin suppression signaling may enhance tolerance for both biotic and abiotic stresses, due to a reciprocity between auxin-dependent reprogramming of gene expression which trigger responses to stress (Navarro *et al*., 2006; Park *et al*., 2007; Wang *et al*., 2010).

In order to gain new insights on stress modulation root-to-shoot, the results obtained in this work allow us to indicate that as we observed a distinct trends that clearly indicate signaling responses from rootstock to scion, allowing enough time to stimuli defenses mechanisms in upper parts of the plant against oxidative stress caused by Cd. These responses of interactions between hormone and redox signaling pathways in the control of growth and cross-tolerance to stress can be used to manipulate heavy metal tolerance, since auxin and ethylene are directly involved in this mechanism. Further analysis arenecessary to a complete knowledge about mechanisms connecting antioxidant responses, ROS, stressful conditions and the signaling among plant organs. Such studies may contribute to explore various ways to improve crop productivity through the alleviation of Cd stress.

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3.8 Figures legends

Figure 1. Experimental design. (1-3) corresponding non-grafted plants with nutrient solution without Cd (-Cd), (4-6) non-grafted plants with 1 mM of $CdCl_2$ in nutrient solution (+Cd), (7-11) grafted plants with nutrient solution without Cd (-Cd) and (12-16) grafted plants with 1 mM of $CdCl_2$ in nutrient solution (+Cd) in rootstock before grafting.

Figure 2. Roots and leaves dry mass (g dry wt) of MT, *Nr* and *dgt* non grafted and grafted plants. Different uppercase letters on the top of the columns indicates non-grafted plants and lowercase indicates grafted plants with significantly different at P < 0.05 by Duncan test.

Figure 3. Cd accumulation (µg g^{-1} dry weight) of MT, *Nr* and *dgt* non grafted and grafted plants. Different uppercase letters on the top of the columns indicates non-grafted plants and lowercase indicates grafted plants with significantly different at P < 0.05 by Duncan test.

Table 1. Nutritional analysis of macronutrients (mg g^{-1}) and micronutrients (µg g^{-1}). Different uppercase letters on the top of the columns indicates non-grafted plants and lowercase indicates grafted plants with significantly different at P < 0.05 by Duncan test.

Figure 4. Content of **(a)** hydrogen peroxide (H_2O_2) (mmol g^{-1} fresh weight) and **(b)** lipid peroxidation measured as malondialdehyde (MDA) (mmol g^{-1} fresh weight) in

roots and leaves of MT, *Nr* and *dgt*. Different uppercase letters on the top of the columns indicates non-grafted plants and lowercase indicates grafted plants with significantly different at $P < 0.05$ by Duncan test.

Figure 5. Chlorophyll content measured in MT, *Nr* and *dgt* leaves 75 days post germination. Different uppercase letters on the top of the columns indicates non-grafted plants and lowercase indicates grafted plants with significantly different at $P < 0.05$ by Duncan test.

Figure 6. Proline content (mmol g^{-1} fresh weight) in roots and leaves of MT, *Nr* and *dgt* grown in 75 d period. Different uppercase letters on the top of the columns indicates non-grafted plants and lowercase indicates grafted plants with significantly different at $P < 0.05$ by Duncan test.

Figure 7. Antioxidant total enzyme activity. **(a)** SOD content (U/SOD mg of protein), **(b)** CAT specific activity (μmol min $^{-1}$ mg $^{-1}$), **(c)** APX specific activity (μmol min $^{-1}$ mg $^{-1}$) and **(d)** GR specific activity (μmol min $^{-1}$ mg $^{-1}$) in MT, *Nr* and *dgt* plants. Different uppercase letters on the top of the columns indicates non-grafted plants and lowercase indicates grafted plants with significantly different at $P < 0.05$ by Duncan test.

Figure 8. Superoxide dismutase (SOD) activity stained following non-denaturing polyacrylamide gel electrophoresis of **(a)** leaves and **(b)** roots isolated from non-grafted and grafted MT, *Nr* and *dgt* plants. The lanes listed are: (P) bovine SOD standard; (1) MT (-Cd), (2) *Nr* (-Cd), (3) *dgt* (-Cd), (4) MT (+Cd), (5) *Nr* (+Cd), (6) *dgt* (+Cd), (7) MT(-Cd)/MT(-Cd), (8) MT(-Cd)/*Nr* (-Cd), (9) MT(-Cd)/*dgt* (-Cd), (10) *Nr*(-Cd)/*Nr*(-Cd), (11) *dgt* (-Cd)/*dgt* (-Cd),), (12) MT(Cd)/MT(+Cd), (13) MT(-Cd)/*Nr* (+Cd), (14) MT(-Cd)/*dgt* (+Cd), (15) *Nr*(-Cd)/*Nr*(+Cd) and (16) *dgt* (-Cd)/*dgt* (+Cd) . The SOD isoforms are (I) Mn-SOD, (II) Fe-SOD; (III) Mn-SOD and (IV) Cu/Zn-SOD.

3.9 Highlights

- Root-to-shoot communication can involve specific responses of ROS and hormones
- Grafted hormonal mutants can be used as a tool to investigate the modulation of antioxidant stress responses inter organs.

Figure 1

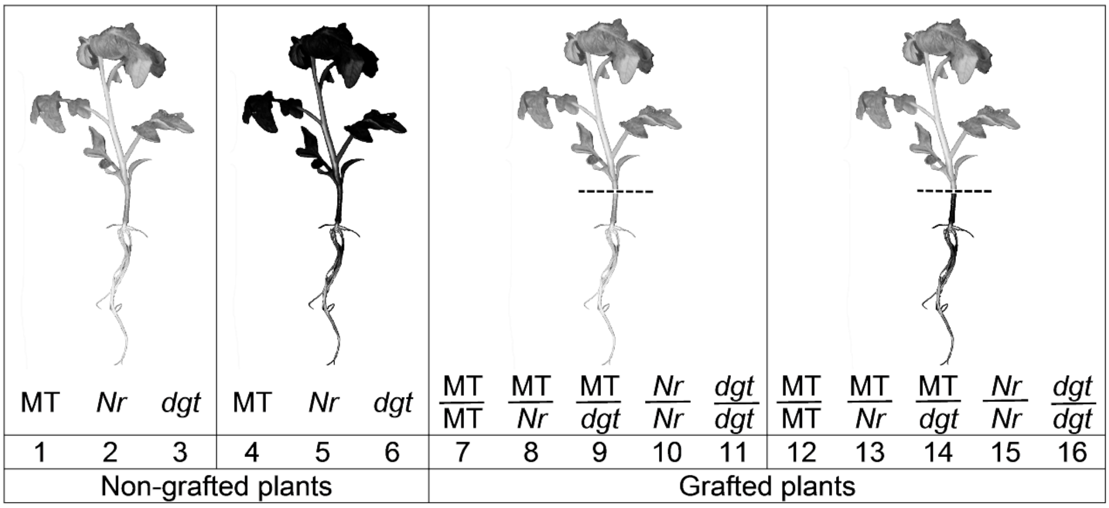


Figure 2

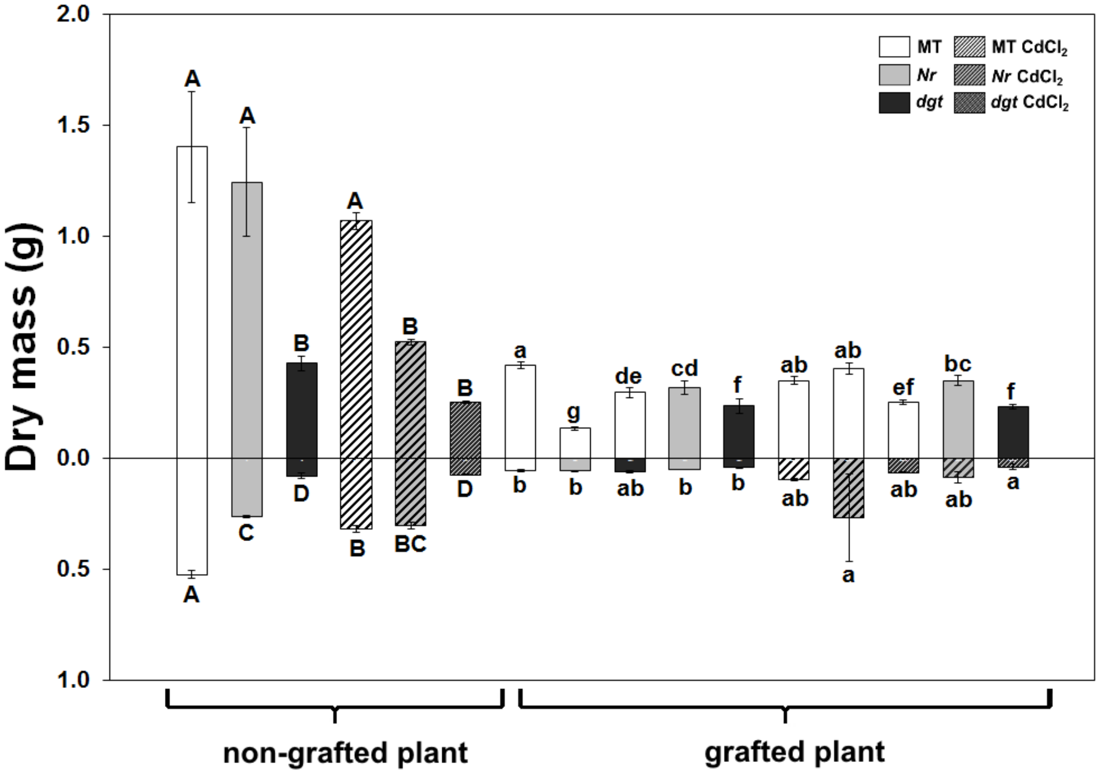

Dry mass (g)
2.0
1.5
1.0
0.5
0.0
0.5
1.0
MT
Nr
dgt
MT CdCl2
Nr CdCl2
dgt CdCl2
A
A
B
A
B
B
a
g
de
cd
f
ab
ab
ef
bc
f
A
C
D
B
BC
D
b
b
ab
b
b
ab
a
ab
ab
a
non-grafted plant
grafted plant

Figure 3

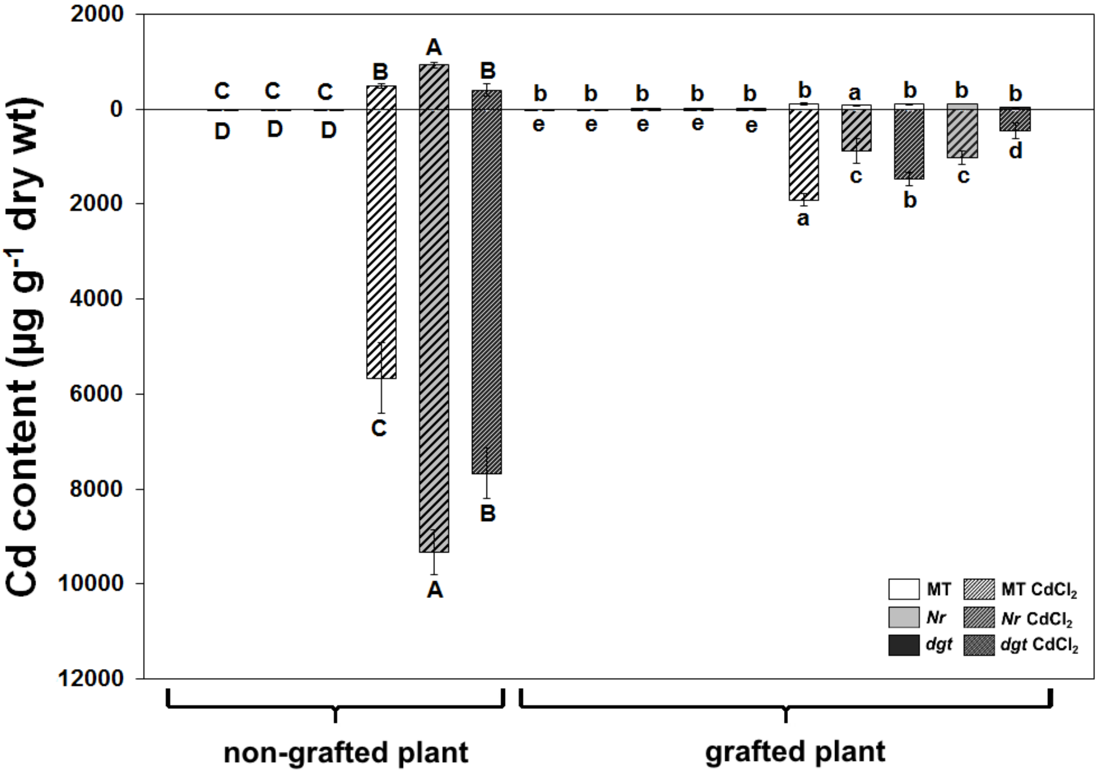

Cd content (µg g-1 dry wt)
2000
0
2000
4000
6000
8000
10000
12000
C C C B A B b b b b b b a b b b
D D D C A B e e e e e a c b c d
MT
Nr
dgt
MT CdCl2
Nr CdCl2
dgt CdCl2
non-grafted plant
grafted plant

Table 1

	Treatments		P	Ca	Mg	S	K	Cu	Fe	Mn	Zn
			mg g^{-1}					µg g^{-1}			
Non-grafted plant	MT	leaves	4.4 ± 0.5^{AB}	11.6 ± 0.3^{A}	3.4 ± 0.1^{A}	3.9 ± 0.2^{A}	18.2 ± 0.2^{A}	1.3 ± 0.1^{A}	75.7 ± 3.9^{B}	43.2 ± 4.3^{AB}	2.8 ± 0.3^{A}
	MT	root	2.0 ± 0.5^{A}	6.4 ± 1.0^{A}	3.9 ± 0.9^{AB}	3.0 ± 0.4^{A}	16.1 ± 1.4^{A}	3.0 ± 0.3^{A}	251.6 ± 23.5^{A}	189.0 ± 55.5^{A}	22.0 ± 18.4^{A}
	Nr	leaves	3.9 ± 0.4^{AB}	10.4 ± 0.4^{AB}	3.4 ± 0.1^{A}	3.4 ± 0.1^{AB}	17.8 ± 0.6^{A}	1.3 ± 0.1^{A}	75.9 ± 3.1^{B}	35.7 ± 4.1^{AB}	2.6 ± 0.3^{A}
	Nr	root	1.6 ± 0.3^{A}	6.1 ± 0.2^{A}	3.6 ± 0.4^{AB}	2.9 ± 0.3^{A}	11.1 ± 2.9^{AB}	3.4 ± 1.2^{A}	330.1 ± 23.5^{A}	257.6 ± 74.4^{A}	19.0 ± 10.8^{A}
	dgt	leaves	5.3 ± 0.2^{A}	11.1 ± 0.4^{A}	3.6 ± 0.1^{A}	3.3 ± 0.1^{B}	16.2 ± 0.4^{AB}	1.3 ± 0.0^{A}	106.1 ± 14.3^{A}	24.4 ± 1.1^{B}	1.4 ± 0.1^{B}
	dgt	root	1.5 ± 0.5^{A}	6.0 ± 0.6^{A}	3.6 ± 0.2^{AB}	2.5 ± 0.7^{A}	12.1 ± 1.6^{AB}	2.4 ± 1.1^{A}	394.8 ± 43.8^{A}	178.0 ± 47.0^{A}	32.2 ± 30.2^{A}
	MT (Cd)	leaves	2.8 ± 0.3^{C}	10.8 ± 0.4^{A}	3.1 ± 0.1^{B}	3.6 ± 0.1^{AB}	15.0 ± 0.8^{B}	1.0 ± 0.1^{B}	76.4 ± 6.6^{B}	33.3 ± 2.3^{AB}	1.0 ± 0.3^{B}
	MT (Cd)	root	1.3 ± 0.1^{A}	5.0 ± 0.4^{AB}	4.0 ± 0.2^{A}	3.1 ± 0.4^{A}	10.6 ± 0.4^{AB}	2.7 ± 0.4^{A}	360.9 ± 29.8^{A}	87.8 ± 24.5^{A}	1.9 ± 0.4^{A}
	Nr (Cd)	leaves	4.3 ± 0.3^{AB}	10.6 ± 0.4^{A}	2.7 ± 0.0^{C}	2.8 ± 0.2^{C}	16.6 ± 0.5^{AB}	1.1 ± 0.1^{B}	77.4 ± 4.6^{B}	73.3 ± 31.5^{A}	1.1 ± 0.2^{B}
	Nr (Cd)	root	0.8 ± 0.4^{A}	2.7 ± 1.4^{B}	1.9 ± 1.0^{B}	2.1 ± 1.1^{A}	6.8 ± 3.4^{B}	2.0 ± 1.0^{A}	249.5 ± 129.4^{A}	227.6 ± 195.6^{A}	1.4 ± 0.8^{A}
	dgt (Cd)	leaves	3.3 ± 0.6^{AB}	9.2 ± 0.5^{B}	2.4 ± 0.1^{D}	2.7 ± 0.2^{C}	16.6 ± 0.8^{AB}	1.3 ± 0.1^{A}	79.1 ± 13.3^{B}	29.7 ± 2.4^{AB}	1.5 ± 0.3^{B}
	dgt (Cd)	root	1.3 ± 0.1^{A}	4.7 ± 0.4^{AB}	2.9 ± 0.2^{AB}	3.0 ± 0.5^{A}	12.2 ± 0.6^{AB}	3.3 ± 0.1^{A}	226.2 ± 18.2^{A}	58.9 ± 8.7^{A}	1.8 ± 0.4^{A}
Grafted plant	MT	scion	4.1 ± 0.1^{ab}	11.5 ± 0.1^{ab}	3.5 ± 0.1^{ab}	3.4 ± 0.1^{ab}	18.5 ± 0.3^{bc}	1.2 ± 0.0^{b}	76.5 ± 7.8^{a}	38.4 ± 1.8^{ab}	2.7 ± 0.3^{a}
	MT	rootstock	1.4 ± 0.1^{bc}	7.0 ± 1.1^{a}	2.8 ± 0.6^{b}	1.7 ± 0.0^{cd}	14.0 ± 0.6^{bc}	2.3 ± 0.1^{b}	668.4 ± 168.7^{ab}	134.5 ± 38.9^{b}	8.3 ± 1.2^{a}
	MT	scion	4.1 ± 0.1^{ab}	10.1 ± 0.6^{ab}	3.0 ± 0.1^{ab}	3.0 ± 0.2^{bc}	20.5 ± 0.6^{ab}	1.6 ± 0.1^{a}	79.4 ± 9.0^{a}	36.5 ± 4.5^{ab}	2.9 ± 0.1^{a}
	Nr	rootstock	2.3 ± 0.3^{a}	5.8 ± 0.3^{ab}	2.6 ± 0.1^{b}	2.1 ± 0.0^{ab}	18.7 ± 0.2^{a}	3.1 ± 0.2^{ab}	379.7 ± 48.2^{b}	231.4 ± 21.2^{a}	20.0 ± 1.2^{a}
	MT	scion	4.0 ± 0.2^{ab}	12.0 ± 0.3^{a}	3.6 ± 0.3^{a}	3.8 ± 0.4^{a}	18.3 ± 0.5^{cd}	1.4 ± 0.1^{ab}	77.9 ± 5.9^{a}	20.4 ± 1.1^{cd}	2.1 ± 0.4^{ab}
	dgt	rootstock	1.4 ± 0.2^{bc}	4.7 ± 0.4^{bc}	3.8 ± 0.8^{b}	2.3 ± 0.3^{ab}	14.4 ± 1.7^{bc}	3.7 ± 1.1^{a}	267.8 ± 36.9^{b}	86.1 ± 14.3^{b}	3.2 ± 0.1^{a}
	Nr	scion	4.6 ± 0.6^{a}	9.9 ± 0.2^{bc}	2.9 ± 0.1^{b}	3.0 ± 0.2^{bc}	19.8 ± 0.8^{ab}	1.3 ± 0.1^{ab}	70.7 ± 5.2^{a}	44.0 ± 5.1^{a}	2.6 ± 0.2^{a}
	Nr	rootstock	1.8 ± 0.4^{ab}	5.4 ± 0.4^{ab}	3.0 ± 0.4^{b}	1.9 ± 0.2^{ab}	17.0 ± 0.4^{ab}	4.6 ± 2.3^{ab}	532.9 ± 146.7^{ab}	224.6 ± 72.0^{a}	60.1 ± 56.9^{a}
	dgt	scion	2.5 ± 0.6^{b}	8.9 ± 1.1^{c}	3.2 ± 0.4^{ab}	2.6 ± 0.3^{c}	15.8 ± 1.6^{e}	1.3 ± 0.0^{b}	67.4 ± 5.5^{a}	17.5 ± 1.9^{d}	1.6 ± 0.2^{b}
	dgt	rootstock	0.8 ± 0.1^{d}	4.1 ± 0.6^{bc}	3.4 ± 0.3^{b}	1.3 ± 0.3^{d}	10.6 ± 1.4^{d}	4.4 ± 2.4^{ab}	337.5 ± 6.7^{b}	89.4 ± 25.4^{b}	3.8 ± 0.9^{a}
	MT	scion	2.8 ± 1.4^{ab}	9.8 ± 0.5^{bc}	3.3 ± 0.1^{ab}	3.8 ± 0.1^{a}	20.9 ± 0.3^{ab}	1.2 ± 0.1^{b}	52.5 ± 22.2^{a}	30.7 ± 3.5^{bc}	2.0 ± 0.3^{ab}
	MT (Cd)	rootstock	1.1 ± 0.1^{cd}	4.4 ± 0.6^{bc}	3.0 ± 0.7^{b}	2.4 ± 0.2^{a}	14.3 ± 0.8^{bc}	2.1 ± 0.1^{b}	573.6 ± 45.3^{ab}	105.8 ± 12.9^{b}	3.7 ± 0.9^{a}
	MT	scion	4.1 ± 0.7^{ab}	10.6 ± 1.0^{ab}	3.3 ± 0.3^{ab}	3.1 ± 0.2^{bc}	20.5 ± 0.7^{ab}	1.4 ± 0.1^{ab}	70.3 ± 5.2^{a}	38.5 ± 6.5^{ab}	2.5 ± 0.6^{ab}
	Nr (Cd)	rootstock	1.8 ± 0.3^{ab}	4.8 ± 0.5^{bc}	4.4 ± 0.8^{b}	2.4 ± 0.2^{a}	16.2 ± 1.4^{ab}	3.1 ± 0.6^{ab}	385.7 ± 102.1^{b}	103.3 ± 22.8^{b}	49.6 ± 34.0^{a}
	MT	scion	4.2 ± 0.3^{ab}	10.2 ± 0.3^{ab}	3.6 ± 0.1^{a}	4.0 ± 0.2^{a}	21.4 ± 0.4^{a}	0.8 ± 0.3^{c}	69.8 ± 3.2^{a}	23.1 ± 1.9^{cd}	2.0 ± 0.3^{ab}
	dgt (Cd)	rootstock	1.2 ± 0.1^{bc}	3.8 ± 0.3^{c}	3.8 ± 0.1^{b}	2.0 ± 0.1^{ab}	11.5 ± 0.4^{cd}	3.1 ± 0.1^{ab}	835.1 ± 262.3^{a}	63.4 ± 0.9^{b}	2.5 ± 0.2^{a}
	Nr	scion	3.3 ± 0.3^{ab}	10.2 ± 0.1^{ab}	3.2 ± 0.2^{ab}	3.0 ± 0.1^{bc}	20.7 ± 0.5^{ab}	1.1 ± 0.0^{b}	61.3 ± 4.4^{a}	29.1 ± 1.0^{bc}	1.6 ± 0.1^{b}
	Nr (Cd)	rootstock	1.3 ± 0.1^{bc}	5.7 ± 0.3^{ab}	16.9 ± 10.3^{a}	2.4 ± 0.2^{a}	15.0 ± 1.5^{b}	3.1 ± 0.3^{ab}	505.6 ± 123.7^{ab}	50.8 ± 3.2^{b}	3.5 ± 1.3^{a}
	dgt	scion	2.7 ± 0.3^{ab}	9.6 ± 0.4^{bc}	3.3 ± 0.2^{ab}	3.0 ± 0.1^{bc}	17.9 ± 0.8^{de}	1.3 ± 0.1^{ab}	80.1 ± 9.4^{a}	20.8 ± 2.3^{cd}	1.6 ± 0.1^{b}
	dgt (Cd)	rootstock	0.9 ± 0.0^{d}	4.4 ± 0.3^{bc}	4.0 ± 0.4^{b}	1.8 ± 0.1^{bc}	9.4 ± 0.4^{d}	2.3 ± 0.2^{b}	478.6 ± 125.1^{ab}	61.7 ± 9.2^{b}	2.4 ± 0.5^{a}

Figure 4

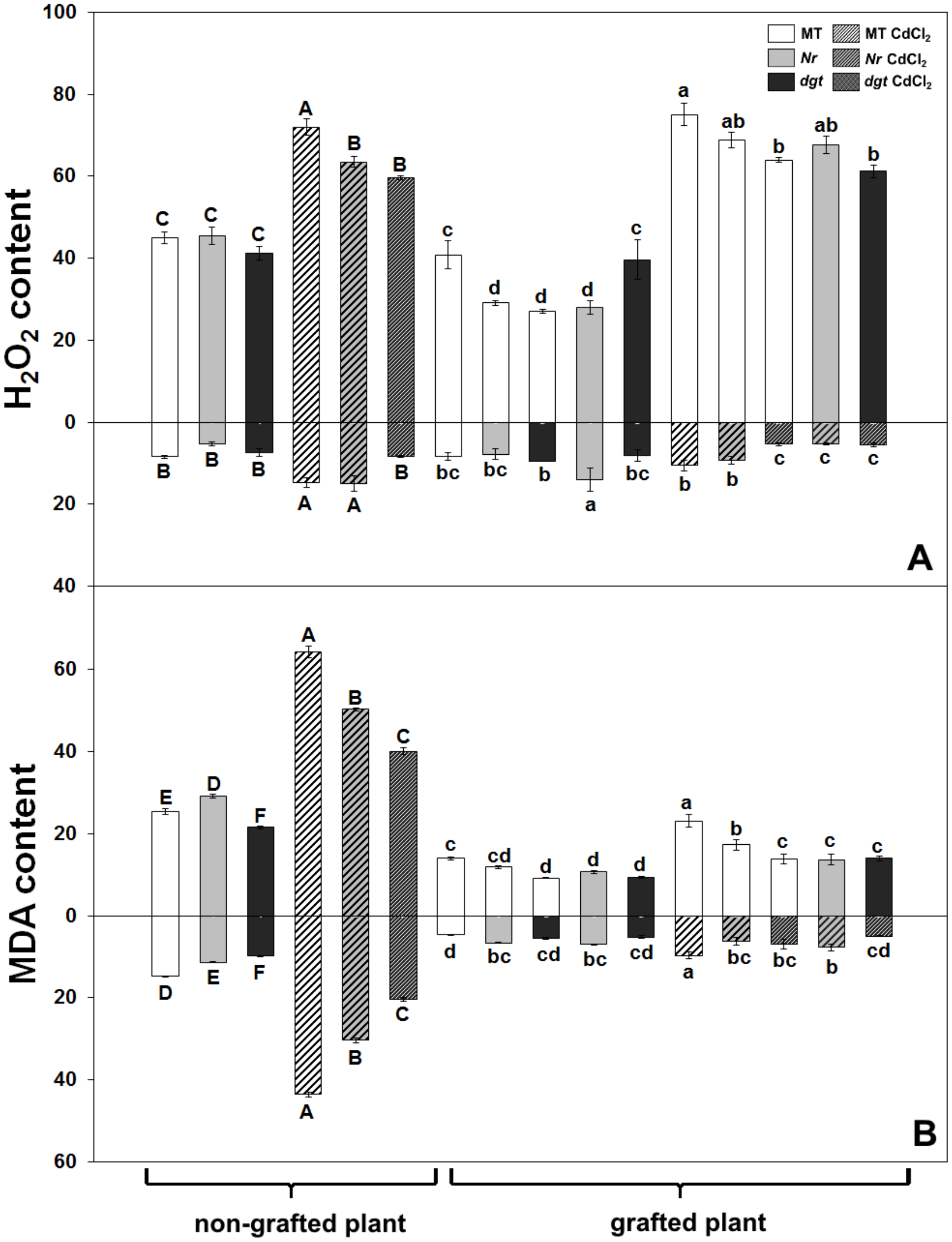

MT
Nr
dgt
MT CdCl$_2$
Nr CdCl$_2$
dgt CdCl$_2$
H_2O_2 content
MDA content
100
80
60
40
20
0
20
40
60
40
20
0
20
40
60
C C C A B B c d d d c a ab b ab b
B B B A A B bc bc b a bc b b c c c
A
E D F A B C c cd d d d a b c c c
D E F A B C d bc cd bc cd a bc bc b cd
B
non-grafted plant
grafted plant

Figure 5

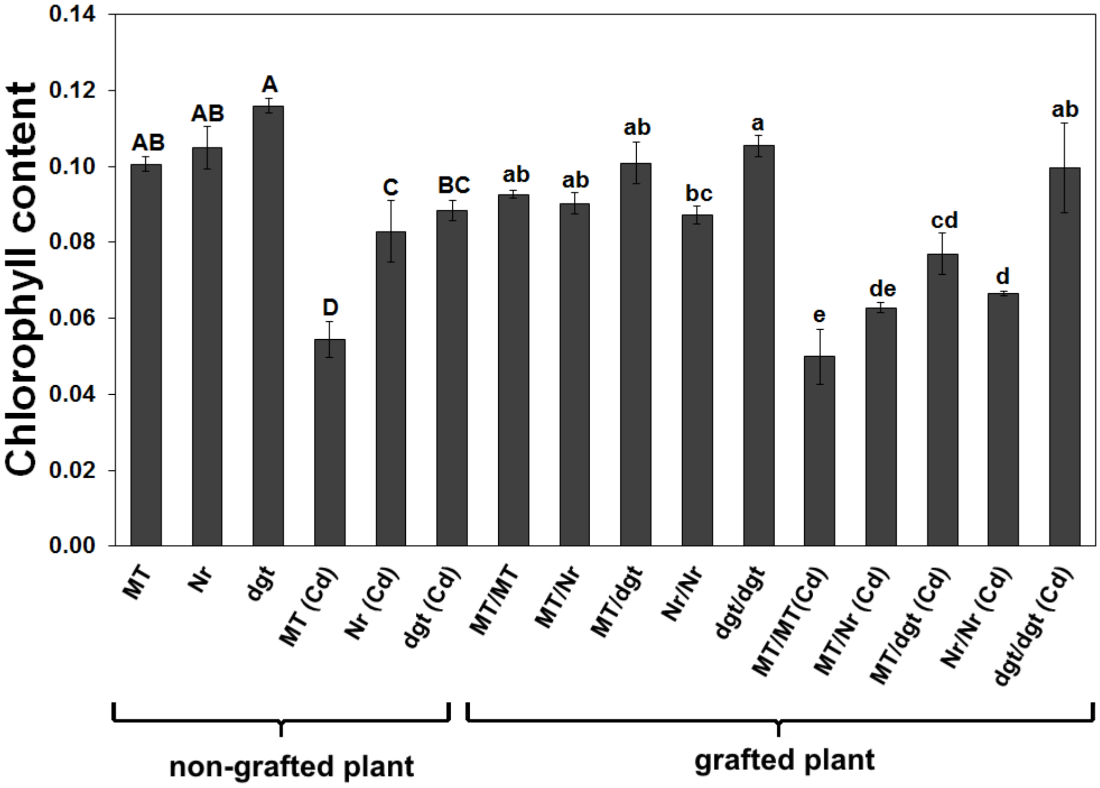

Chlorophyll content
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
AB
AB
A
D
C
BC
ab
ab
ab
bc
a
e
de
cd
d
ab
MT
Nr
dgt
MT (Cd)
Nr (Cd)
dgt (Cd)
MT/MT
MT/Nr
MT/dgt
Nr/Nr
dgt/dgt
MT/MT(Cd)
MT/Nr (Cd)
MT/dgt (Cd)
Nr/Nr (Cd)
dgt/dgt (Cd)
non-grafted plant
grafted plant

Figure 6

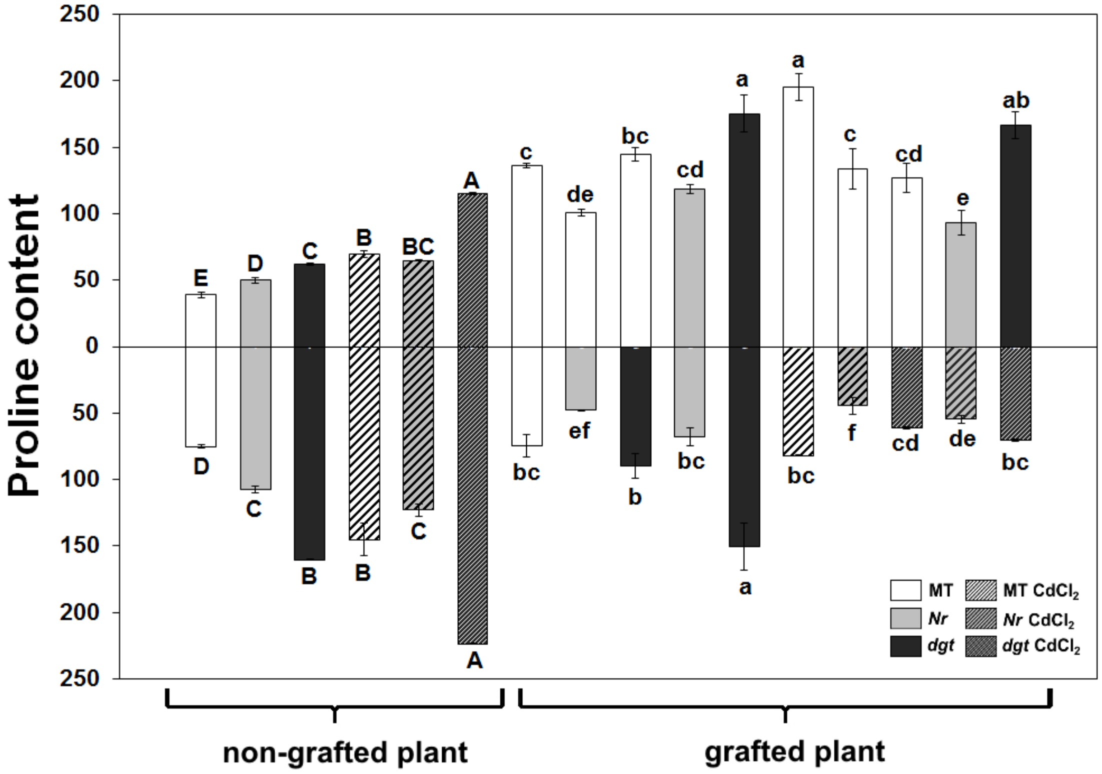

Proline content
250
200
150
100
50
0
50
100
150
200
250
E
D
C
B
BC
A
c
de
bc
cd
a
a
c
cd
e
ab
D
C
B
B
C
A
bc
ef
b
bc
a
bc
f
cd
de
bc
MT
Nr
dgt
MT $CdCl_2$
Nr $CdCl_2$
dgt $CdCl_2$
non-grafted plant
grafted plant

Figure 7

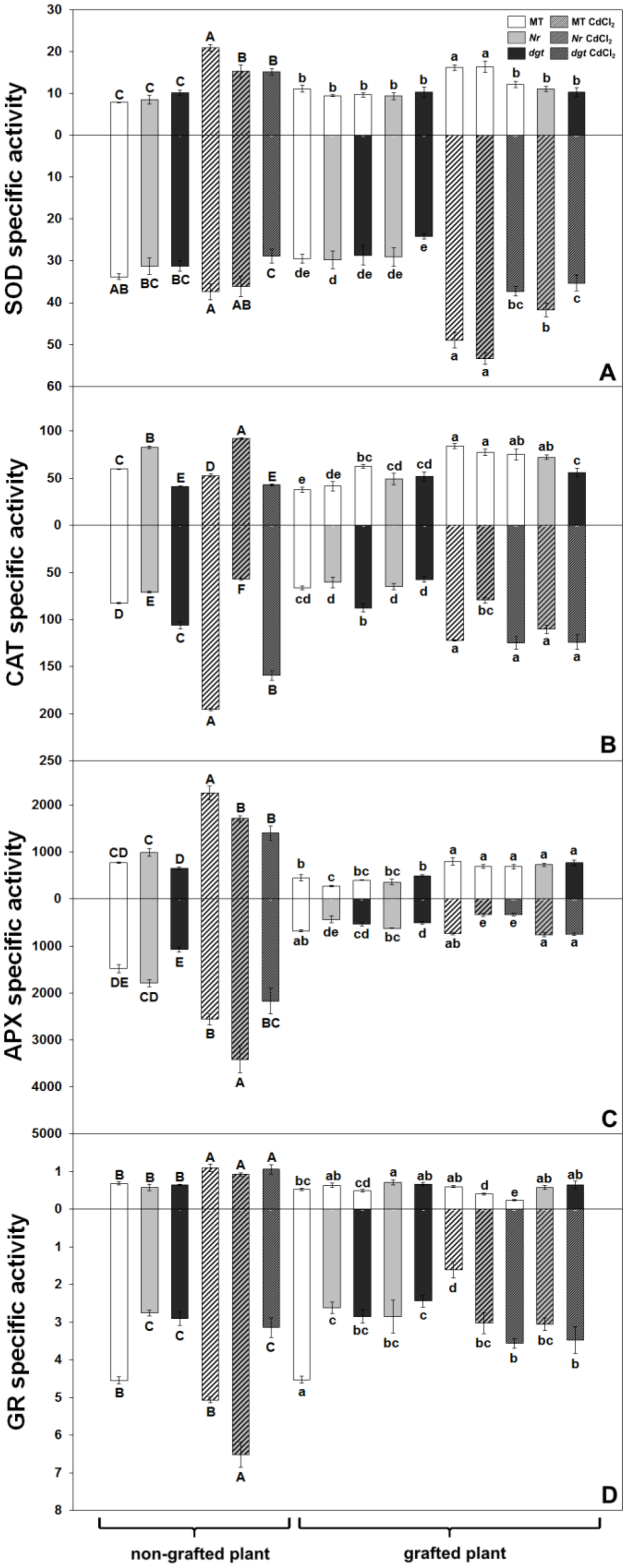
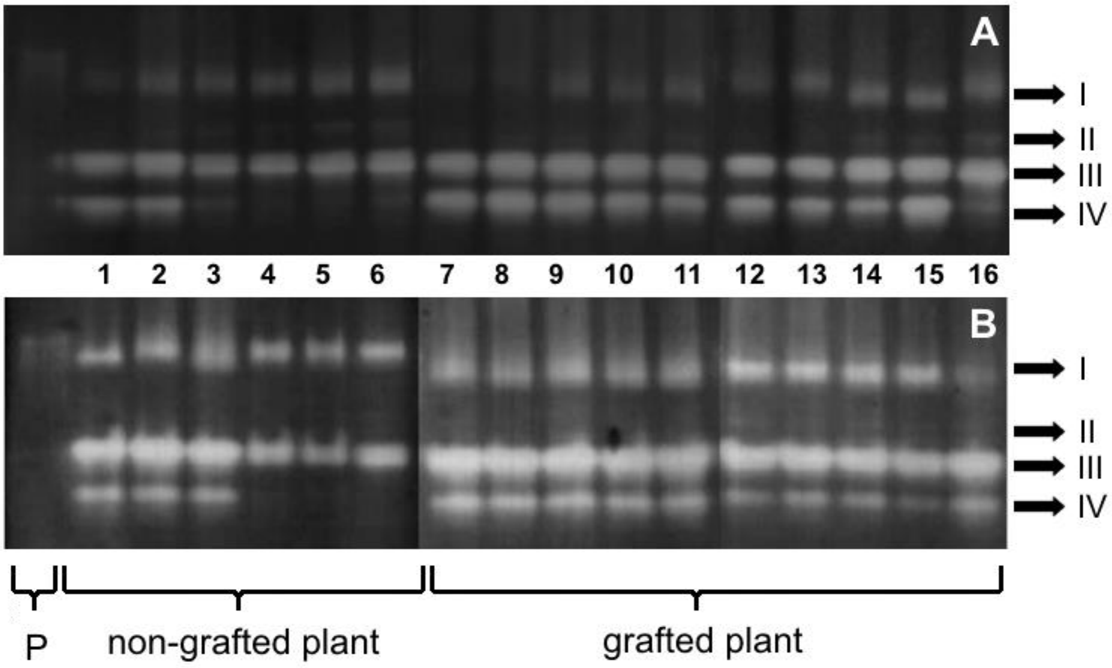

MT
Nr
dgt
MT CdCl₂
Nr CdCl₂
dgt CdCl₂
SOD specific activity
30
20
10
0
10
20
30
40
50
60
C C C A B B b b b b b a a b b b
AB BC BC A AB C de d de de e a a bc b c
A
CAT specific activity
100
50
0
50
100
150
200
250
C B E D A E e de bc cd cd a a ab ab c
D E C A F B cd d b d d a bc a a a
B
APX specific activity
2000
1000
0
1000
2000
3000
4000
5000
CD C D A B B b c bc bc b a a a a a
DE CD E B A BC ab de cd bc d ab e e a a
C
GR specific activity
1
0
1
2
3
4
5
6
7
8
B B B A A A bc ab cd a ab ab d e ab ab
B C C B A C a c bc bc c d bc b bc b
D
non-grafted plant
grafted plant

Figure 8


A
B
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
I
II
III
IV
P
non-grafted plant
grafted plant