UNIVERSIDADE ESTADUAL PAULISTA - UNESP CÂMPUS DE JABOTICABAL

BIOCHEMICAL AND STRUCTURAL ALTERATIONS INDUCED BY SELENIUM UNDER CADMIUM STRESS IN TOMATO PLANTS

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2019

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

2019

A474b

Alves, Leticia Rodrigues

Biochemical and structural alterations induced by selenium under cadmium stress in tomato plants / Leticia Rodrigues Alves. -- Jaboticabal, 2019

125 p.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal

Orientador: Priscila Lupino Gratão

1. plant physiology. 2. microscopy. 3. selenate. 4. selenite. 5. oxidative stress. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

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Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: BIOCHEMICAL AND STRUCTURAL ALTERATIONS INDUCED BY SELENIUM UNDER CADMIUM STRESS IN TOMATO PLANTS

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Jaboticabal, 02 de agosto de 2019

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. By the way, 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 2016, she concluded mastering in crop production at UNESP / FCAV – Jaboticabal, under supervision of Dra. Priscila Lupino Gratão, in which place she studied antioxidant responses in abiotic stress-conditions of stress perception and signalling in plants. In 2016, Leticia started Phd and she continued her studies in the same research line. In this period, she has ministered lectures and mini courses in universities and conferences, published five research articles and five chapters in books. Therefore, she was co-adviser of four graduation students.

DEDICATÓRIA

Dedico este trabalho a minha orientadora Profa. Dra. Priscila Lupino Gratão que nunca mediu esforços para garantir o melhor para minha formação acadêmica.

AGRADECIMENTOS

Á Deus por ter iluminado meu caminho até hoje;

A minha mãe Marta, pelo carinho e apoio incondicional durante todos esses anos de estudos, e ao meu pai Márcio, por nunca medir esforços para dar o melhor para nossa família.

Ao meu namorado Jonathan, pelo incentivo e companheirismo. Por me apoiar durante esta etapa, e sempre enxergar o melhor de mim.

A Prof. Dra. Priscila Lupino Gratão, por ser muito mais que minha orientadora, agradeço pelos ensinamentos, pelas oportunidades de aprimorar meu currículo, pela dedicação ao meu trabalho, e além de tudo uma grande amiga.

A Sonia (Soninha), por todas as histórias compartilhadas e apoio no dia a dia das análises laboratoriais. Você é extremamente importante para todos os alunos que passaram e passarão pelo laboratório de Fisiologia vegetal da UNESP/FCAV. Seria impossível fazer tudo que fiz sem você!

A todos os professores e funcionários do departamento de Biologia aplicada a agropecuária que de certa forma me acompanharam nesses anos;

Aos amigos do laboratório de Fisiologia Vegetal e aos colegas das disciplinas que fiz durante esses dois anos;

A UNESP/FCAV e ao programa de Produção Vegetal por possibilitar minha formação.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001. E pela Fundação de amparo a pesquisa do Estado de Sao Paulo (FAPESP- n° 2017/04787-6).

SUMARY

BIOCHEMICAL AND STRUCTURAL ALTERATIONS INDUCED BY SELENIUM UNDER CADMIUM STRESS IN TOMATO PLANTS

ABSTRACT

Cadmium (Cd) 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. This heavy metal increased in atmosphere due to human activities. Plants can uptake Cd, causing serious changes in structural, physiological and biochemical processes. Plants developed a complex defence systems including non-enzymatic and enzymatic mechanism to avoid oxidative stress and prevent an uncontrolled oxidation cascade. Some elements, such as selenium (Se), if used in adequate concentration, may induce an improvement in antioxidant system, growth and photosynthetic attributes. It is still unknown the mechanisms of Se in stress responses. The aim of this work was get new insights about the role of selenate and selenite-mediated detoxification strategies, including the evaluation of mineral nutrition, the activity of antioxidant enzymes and non enzymatic compounds, pigments, structural alterations and the role of Se in modulate ethylene, with the use of hormonal mutants as a tool. Our data indicates that Se is an interesting strategy to improve plant metabolism under normal or Cd stressful-condition. Selenium may induce enhancement in antioxidant defence metabolism, probably due to alterations in ethylene signalling. Moreover, under normal condition Se induce structural alterations in cells, which may contribute to plant development. Thus, the information available in this work is a fundamental step towards obtaining a better understanding about Se role in plant metabolism.

Key words: ethylene, microscopy, selenate, selenite, *Solanum lycopersicum,* oxidative stress

ALTERAÇÕES BIOQUÍMICAS E ESTRUTURAIS INDUZIDAS POR SELENIO SOB ESTRESSE POR CADMIO EM TOMATEIROS

RESUMO

As plantas estão expostas a adversidades no ambiente que as circundam, como a contaminação por cádmio (Cd). Este metal pesado tem aumentado na atmosfera devido a atividades humanas. As plantas podem absorver o Cd, causando sérias alterações estruturais, fisiológicas e bioquímicas. As plantas desenvolveram sistemas de defesa complexos, incluindo mecanismos não enzimáticos e enzimáticos para evitar uma cascata de oxidação descontrolada causada pelo estresse oxidativo. Alguns elementos, como o selênio (Se), se utilizados em concentração adequadas, podem induzir uma melhora no sistema antioxidante, no crescimento e nos atributos fotossintéticos. Ainda é pouco conhecido o papel do Se nas respostas das plantas ao estresse. O objetivo deste trabalho foi obter novas informações sobre o papel do selenato e selenito no sistema de desintoxicação das plantas, incluindo a avaliação da nutrição mineral, atividade de enzimas antioxidantes e conteúdo de compostos não enzimáticos, pigmentos, alterações estruturais e o papel do Se na modulação do etileno, com o uso de mutantes hormonais como ferramenta. Nossos dados indicam que o Se é uma estratégia interessante para melhorar o metabolismo da planta sob condições normais ou estressantes. O selênio pode induzir aumento da ação do metabolismo de defesa antioxidante, provavelmente devido a alterações na sinalização do etileno. Além disso, em condições normais, o Se induz alterações estruturais nas células, o que pode contribuir para o desenvolvimento das plantas. Assim, o Se é uma estratégia interessante a ser utilizada na agricultura para melhorar a produção agrícola.

Palavras chave: estresse oxidativo, etileno, microscopia, selenato, selenito, *Solanum lycopersicum*

CHAPTER I – GENERAL CONSIDERATIONS

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 been add in agricultural soil by industrial process, municipal waste and mainly agricultural practices. This metal can be uptake by plants and cause serious changes in structural, physiological and biochemical processes. As a consequence of Cd contamination, occurs an over production of reactive oxygen species (ROS), causing oxidative stress. Plants developed a complex defence systems including non-enzymatic and enzymatic mechanism to avoid oxidative stress and prevent an uncontrolled oxidation cascade. Some elements, such as selenium (Se), if used in adequate concentration, may induce an improvement in antioxidant system, growth and photosynthetic attributes.

In this Thesis, 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 study to define adequate Se concentration to tomato plants cv. Micro Tom under Cd stress. Although Se plays an important role in antioxidant metabolism under Cd stress, how this element induces positives responses remains unknown. 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; Gratão et al., 2015; Alves et al., 2017). Thus, in the third chapter we used information obtained in the second chapter to study the role of Se in modulate ethylene responses during Cd stress, using hormonal tomato mutant *epinastic* to add new insights about this mechanism. The data obtained in this work connecting Se-mediated signaling process and detoxification strategies, since hormones are directly involved in such a mechanism.

In the fourth chapter, we decided to investigated if positive responses of Se application could be related with structural changes in root and leaves under Cd

stress. The study of structural changes trigged by Se was possible due to a partnership with Laboratory of Hispatology and Structural Plant Biology – CENA - USP, which contribute to generate an inedited study. These data added an extra element more directly to this thesis related to the role of Se in plant development under normal or Cd stress conditions and contribute in unraveling the impact of Se application in agriculture.

2. Literature Review

2.1 The tomato as a model plant

Tomato (*Solanum lycopersicum* L.) is a dicotyledonous plant extensively produced and consumed around the world. Tomato fruit brings beneficial effects on human nutrition and health due to be a source of lycopene (Perveen et al. 2015). In addition, tomato is a widely adopted as an experimental model plant in different fields, especially for those 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; Luengwilai et al., 2013; Alves et al., 2017).

Micro Tom (MT) cultivar was originally developed for using 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 (mt) (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. 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).

Ethylene mutants is a powerful tool in genetic and biochemical studies to elucidate the ethylene modulation during stress (Monteiro et al., 2010). The *epinastic* tomato mutant exhibit high production of ethylene, but its gene function is unknown (Fujino et al., 1988). The use of this mutant is an interesting tool to investigate the interaction between Se and ethylene in modulating the defence responses of antioxidant system.

2.2 Cadmium

Cd pollution is considered one of the most severe environmental problems worldwide (Li et al., 2019). Anthropogenic activities increased drastically Cd contamination in the last five decades (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 (Alves et al., 2016; Li et al., 2019). It is well known that Cd is toxic for most plants even in low concentrations. The toxic concentrations of Cd can vary among plant species, ecotypes, cultivars and plant tissues. 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 and inhibiting or activing enzymes. 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 (Alves et al., 2017; Dubey et al.,2018; Li et al., 2019). Cd affects nutrient uptake 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).

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).

2.3 Oxidative stress in plants

Reactive oxygen species (ROS) are constantly produced in low concentration by several metabolic process in chloroplast, mitochondria, peroxisomes, plasma membrane, apoplast, and nucleus (Gill and Tuteja 2010; Sandalio et al. 2013). The production of ROS is an expected consequence of aerobic respiration under optimal growth condition. 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). However, during biotic or abiotic stress condition, a ROS overproduction may occur, being more intense than antioxidants could detoxify, causing oxidative stress (Zhou et al., 2019). The oxidative stress leads to a serious cell imbalance, causing direct effects to photosynthetic rate and growth. In fact, ROS must be detoxified to avoid damages to plant metabolism.

2.4 Defence mechanism against oxidative stress

Plants have numerous strategies to withstand oxidative stress (Gill and Tuteja, 2010). The first line of defence 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. The first defence line in ROS scavenging is composed by

superoxide dismutase (SOD - EC 1.15.1.1). This enzyme catalyze the dismutation of O_2•− 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). It is possible to find three well known SOD isoforms in plants. For instance, 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). A fourth isoform Ni-SOD can be found in cyanobacteria and marine eukaryote (Fink and Scandalios, 2002).

H_2O_2 is an highly reactive molecule and may be quickly converted into H_2O and O_2. Catalase (CAT) and other peroxidases realize this reaction in different cellular compartments, which is a vital part of antioxidant defense (Garg and Manchanda, 2009). 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.

Ascorbate peroxidase (APX - E.C. 1.11.1.11) and glutathione reductase (GR - E.C. 1.8.1.7) are other important antioxidant enzymes widely distributed among higher plants. Both enzymes have crucial role in Ascorbate-Glutathione cycle, that is an important ROS-scavenging system, which contributes to improve abiotic stress tolerance in plants (Kao, 2015, Zechmann., 2018). APX converts H_2O_2 to H_2O by oxidation of Ascorbate (AsA) to monodehydroascorbate (MDHA) that is then disproportionate into AsA and dehydroascorbate (DHA). Following reaction, the oxidized AsA is recycled to its reduced form where monodehydroascorbate reductase (MDHAR) activity is vital that performs NADPH-dependent regeneration of AsA from MDHA (Anjum et al. 2014). Glutathione reductase (GR) is required to reduce gluthatione oxidized (GSSG) to its reduced state (GSH) (Ghisla and Massey, 1989). By this way, GSH formed can be used by glutathione peroxidase (GPX) and dehydroascorbate reductase (DHAR). The GPX (E.C. 1.11.1.9) 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).

Non enzymatic antioxidants are not specific to different ROS like enzymes, but this mechanism is equally important to ROS scavenging. The main enzymatic antioxidants are known as AsA, GSH, proline, polyamines, betaine, carotenes and flavonoids. Both antioxidant compounds and antioxidant enzymes can avoid the uncontrolled cascade of oxidation and cell damages (Gratão et al., 2015).

2.5 Selenium in angiosperms

Although Se is not considered an essential nutrient to plants, it can be uptake mainly in selenate and selenite form. Selenate is chemically similar to sulphur (S) and may be uptake via S transport system, and then it is highly transported to above parts of plants (Shinmachi et al. 2010). Whereas, selenite enters in plants involving an active process because it reduces with the application of metabolic inhibitors (Li et al. 2008). Selenite is easily converted into some organic Se species, such as selenocystine, Se-methyl-selenocysteine, selenomethionine, and selenomethionine Se-oxide. This form is mostly accumulated in roots and are rarely transported to shoots (Wang et al., 2019).

Selenium exhibit both positive and negative effects to plant growth and development. In high concentrations, Se can inhibit plant growth and cause chlorosis in younger leaves (Schiavon and Pilon-Smits 2017). This effect is a consequence of oxidative stress caused due to nonspecific replacement of S with Se in proteins, causing more ubiquitinated proteins and damaging normal structure and function of proteins (Van Hoewyk, 2013). On the other hand, low concentrations can be beneficial to plants and can promote plant growth and improve photosynthesis (Gupta and Gupta 2017). Several studies reinforce that Se application, in low concentrations, may upregulate plant pathways involved in stress responses and enhance plant tolerance (Feng et al. 2013). For instance, Se application improve enzymes and non-enzymatic compounds of antioxidant system in rice under drought stress (Andrade et al., 2018), high temperature in sorghum (Djanaguiraman et al., 2018) and Cd toxicity in rice (Feng et al., 2013). However, how Se modulates these positive responses in antioxidant system still unknown.

2.6 Interplay between Selenium and ethylene during Cadmium stress

Plant hormones play a fundamental role in the cellular responses and interact with redox signalling to control responses to abiotic stresses (Bankaji et al., 2014; Dar et al., 2015). Ethylene can mediate many complex process of plant growth, development and reproduction. Moreover, this volatile molecule is considered a 'stress hormone' involved in multiple molecular and physiological plant processes, regulating various cellular defence responses (Schellingen et al., 2014; Van de Poel et al., 2015).

The biosynthesis of ethylene is well defined in higher plants and initiated in the conversion 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 is expressed in response to internal developmental and environmental conditions, such as wounding, flooding, drought, heavy metals and pathogen attack (Yang and Hoffman, 1984; Asgher et al., 2014). Moreover, recent study indicates that the inhibition of ethylene reverted the positive responses of Se (Khan et al., 2015). Thus, the ethylene induction resulting from Se application positive regulates proline and GSH biosynthesis under Cd stress and contribute to alleviate adverse effects of Cd stress on photosynthesis and growth (Khan et al., 2015). Nonetheless, mechanisms involved interaction of Se and ethylene with stress responses have been poorly understood.

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CHAPTER II - Mechanisms of cadmium-stress avoidance by selenium in tomato plants

[1]This chapter was submitted to Ecotoxicology Journal and we are waiting for evaluation.

Abstract

Cadmium (Cd) is probably the most damaging metal to plant species; with a long biological half-life, it can be uptake by plants, disturbing the cell balance and triggering several metabolic pathways. Selenium (Se) improves plant defence systems against stressful conditions, but the biochemical antioxidant responses to Cd stress in tomato plants is poorly understood. To further address the relationship of Cd-stress responses with Se mineral uptake, Cd and Se concentration, proline content, MDA and H_2O_2 production, and the activity of SOD, APX, CAT and GR enzymes were analysed in Micro-Tom (MT) plants submitted to 0.5 mM Cd. The results revealed different responses according to Se combination and Cd application. For instance, roots and leaves of MT plants treated with Se exhibited an increase in dry mass and nutritional status, reduced proline content and increased APX and GR activities when compared with plants with no Se application. Plants submitted to 0.5 mM Cd, irrespective of Se exposure, exhibited decreased proline, MDA and H_2O_2 content and increased SOD, CAT and GR activities. Selenium may improve tolerance against Cd, which stimulates MT plants to accumulate more Cd in their tissues, resulting in less oxidative damage to the cell. The results suggest that Se application is an efficient management technique to alleviate the deleterious effects of Cd-stress, enhancing the nutritional value and activity of ROS-scavenging enzymes in tomato plants.

Keywords: abiotic stress, antioxidant metabolism, selenium, *Solanum lycopersicum.*

1. Introduction

Cadmium (Cd) is a heavy metal that is toxic to living organisms. Due to bioaccumulation and its increasing use in industry, cadmium presents an increasing hazard (Alves et al. 2016, Edelstein et al. 2018). Plants can easily uptake this heavy metal, which triggers drastic alteration in tissues and metabolic pathways (Ma et al. 2017, Zhu et al. 2018). Even at low concentrations, Cd can modify root morphology, limiting the root-to-shoot transport of nutrients (Lux et al. 2011) and decrease nutrient uptake because Cd competes with and can replace the uptake of calcium, copper, iron, manganese and zinc (Rabêlo et al. 2018). Moreover, Cd provokes uncontrolled oxidation that disrupt cell balance and cause electrolyte leakage, which activates biochemical responses by favouring the production of reactive oxygen species (ROS), disrupting the plant defence systems (Alves et al. 2017).

Plants are able to detoxify excessive oxidation caused by Cd by complex enzymatic and non-enzymatic mechanisms that protect plant cells against oxidative damage and restore the cell redox balance (Gratão et al. 2015). The first defence response at the cellular level involves antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), which converts superoxide (O^{2-}) to hydrogen peroxide (H_2O_2) (Noctor and Foyer 2016). Next, H_2O_2 can be eliminated by ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9) (Noctor et al. 2018) and other peroxidases. Moreover, non-enzymatic mechanisms, such as proline, carotenoids, ascorbate and glutathione, are also involved in scavenging excess ROS in plant tissue (Zouari et al. 2016).

Plant-stress mechanisms may involve an interplay between interconnected networks of cellular responses and elements under Cd stress conditions that are not fully understood (Chmielowska-Bak et al. 2014). Exploring these mechanisms and developing strategies to reduce Cd accumulation and the damage it causes to plants is a fundamental issue. A recently studied strategy to decrease the oxidative damage and minimize the toxicity generated by Cd to plant tissues is the use of beneficial elements or substances to restore the photosynthetic capacity, improve the

antioxidant response and enhance crop production, such and selenium (Se) (Feng 2013, Alyemeni et al. 2018).

Application of Se at low concentrations promotes plant growth and alleviates the negative effects of Cd-induced stress by regulating ROS scavenger metabolism (Castillo-Godina et al. 2016; Alyemeni et al. 2018), abundant stress-responsive proteins (Sun et al. 2016) and delaying fruit senescence (Pezzarossa et al. 2014). Moreover, Se application can decrease Cd uptake in tomatoes (Abd et al. 2016) and translocation of Cd from root-to-shoot in rice plants (Wan et al. 2016). However, Se was not effective in preventing Cd uptake in rice with increased soil Cd concentrations (Huang et al. 2017). Therefore, further studies are needed to better understand the effects of Se on the biochemical mechanisms of Cd transport from root-to-shoot.

The miniature tomato cultivar Micro-Tom (MT) has been considered as a plant model based on growth at high plant density and production of viable fruits within a short life cycle (Meissner et al., 1997). Several studies have indicated the antioxidant potential of Se (Abd Allah et al. 2016, Castilo-Godina et al. 2016); however, there is a lack of information regarding the biochemical antioxidant responses to Cd stress in tomato plants. To better understand the role of different Se concentration on the modulation of Cd-stress responses in tomato plants, a pot experiment was performed using Micro-Tom under different concentrations of Cd and Se.

2. Material and methods

2.1 Plant material and growth conditions

Seeds of the tomato (*S. lycopersicum* L.) cultivar Micro-Tom (MT) were sterilized using a sodium hypochlorite solution (5%), rinsed three times and sown in boxes filled with a 1:1 (by volume) mixture of commercial pot mix (Plantmax HT Eucatex, Brazil) and vermiculite supplemented with 1 g L^{-1} 10:10:10 nitrogen-phosphorus-potassium and 4 g L^{-1} lime ($MgCO_3$ + $CaCO_3$). The boxes were maintained in a greenhouse. When two true leaves were completely formed, one seedling was transplanted per 0.350 L Leonard pot (Vincent 1975), a hydroponic cultivation system, where modified Hoagland´s nutrient solution (250 ml) rise up through cotton

strings by capillarity to hum the sterilized sand and polystyrene (4:3). Thirty six-day-old plants were grown in the same solution at four Se concentrations (0, 1, 5 and 10 μM of Na_2SeO_3) combined with or without Cd (0, 0.5 mM $CdCl_2$), which was changed every five days.

After a period of 75 days post germination, samples of roots and leaves were rinsed with distilled water and kept in paper bags and dried in a drying oven *(*60 ºC) until constant weight was achieved for dry mass determination and nutritional analysis. Samples of roots and leaves were harvested, rinsed and immersed in liquid N_2 and stored at -80 ºC for analysis of lipid peroxidation, H_2O_2 and proline concentration, enzyme extraction and protein determination.

2.2 Nutritional analysis, Cd and Se content

At a controlled pressure of 2 MPa, dry root and leaf samples (200.0 mg DW) were milled and microwave digested in 2 mL 70% HNO_3, 2 mL H_2O_2 and 2 mL Milli-Q water (18.2 MX cm a 25 ºC) (Chilimba et al. 2011). The digested samples were diluted to 10 mL with Milli-Q water and stored. The presence and concentration of Cd, Se, P, K, Ca, Mg, S, B, Cu, Fe, Mn and Zn were determined by radial view inductively coupled plasma optical emission spectrometry (ICP-OES JobinYvon, JY50P Longjumeau, France) equipped with a nebulization chamber. A certified reference material from the National Institute of Standards and Technology (NIST SRM 1567a Wheat flour, Gaithersburg, USA) was used for quality control in the analysis of acid digests by ICP OES. The following emission lines were used: Cd II 228.80; Se II 196.09; P I 213.618 nm; K I 769.897 nm; Ca I 422.673 nm; Mg I 280.270 nm; S I 181.972 nm; B I 249.773 nm; Cu I 324.754 nm; Fe II 259.940 nm; Mn II 259.373 nm; Zn II 231.865 nm and Ni II 231.604 nm.

2.3 Lipid peroxidation

Lipid peroxidation was assessed by analysing the content of thiobarbituric acid (TBA) reactive substances (TBARS) as described by Heath and Packer (1968). Fresh root and leaf mass (0.4 g) was ground with 20% (w/v) polyvinylpyrrolidone

(PVPP) and 0.1% trichloroacetic acid (TCA). After centrifugation at 11,000 xg for 10 min, the supernatant was added to a solution of 20% TCA and 5% TBA and incubated in a water bath at 95 ºC for 30 min. Samples were then placed in an ice bath for 10 min to stop the reaction and then centrifuged at 11,000 xg for 10 min. The concentration of malondialdehyde (MDA) equivalents was measured spectrophotometrically between 535 and 600 nm; data were calculated using an extinction coefficient of 1.55×10^{-5} mol^{-1} cm^{-1} (Gratão et al. 2012).

2.4 H_2O_2 content

Fresh plant tissue (0.4 g) was homogenized in trichloroacetic acid (0.1%) and centrifuged at 10,000 xg for 10 min. The supernatant was added to 100 mM potassium phosphate buffer (pH 7.50) and 1 M potassium iodide solution. This solution was incubated on ice for one hour, the absorbance was measured at 390 nm, and the H_2O_2 content was determined using a known H_2O_2 concentration curve as a standard following the method of Gratão et al. (2012).

2.5 Proline content

Fresh leaves and root samples (0.5 g) were homogenized in 3% sulphosalicylic acid and filtered. The mixture filtrate was added with 1 mL each of acid ninhydrin and glacial acetic acid and was placed in boiling water for 1 h. Toluene (4 mL) was added to the mixture, and the absorbance was measured spectrophotometrically at 520 nm and converted to mmol g^{-1} fresh weight against standard proline. The proline content was determined as described by Bates (1972).

2.6 Enzyme extraction and protein determination

Enzyme extraction was performed by Boaretto et al. 2014. Fresh leaves and roots (1.0 g) were homogenized in a chilled mortar with a pestle using an extraction buffer containing 100 mM potassium phosphate buffer (pH 7.5), 1 mM ethylenediamine tetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v)

insoluble polyvinylpolypyrrolidone in 3:1 and 2:1 volume/fresh weight ratio to leaves and roots, respectively. The homogenate was centrifuged at 10,000 xg for 30 min, and the supernatant was stored at -80 ºC for further determination of SOD, CAT, GR and APX activities. The protein concentration was assayed following the method of Bradford (1976) using bovine serum albumin as a standard.

2.7 Superoxide dismutase assay

Superoxide dismutase (SOD) activity was analysed by activity staining using non-denaturing PAGE (Azevedo et al. 1998). Leaf and root extracts were inserted to non-denaturing-PAGE separation. After that, the gels were rinsed in distilled-deionized water and kept in the dark for 30 min in 50 mM potassium phosphate buffer (pH 7.8) containing 0.05 mM riboflavin, 0.1 mM nitro blue tetrazolium, 1 mM EDTA, and 0.3% N,N,N´,N´-tetramethylethylenediamine. The gels were rinsed with deionized water and were exposed to artificial light under the water until the achromatic bands of SOD activity were visible on a purple-stained gel. SOD isoenzymes were differentiated by their sensitivity or inhibition to 5 mM hydrogen peroxide (H_2O_2) or 2 mM potassium cyanide (Azevedo et al. 1998).

2.8 Catalase assay

The activity of Catalase (CAT) was determined by checking the degradation of H_2O_2 at 240 nm over 1 min. Catalase activity (CAT) was measured spectrophotometrically at 25 ºC in a reaction mixture containing 1 mL of 100 mM potassium phosphate buffer (pH 7.5) and 25 µL H_2O_2 (30% solution) (Nogueirol et al. 2015). CAT activity was expressed as mmol min^{-1} mg^{-1} protein.

2.9 Ascorbate peroxidase assay

Ascorbate peroxidase (APX) was determined by monitoring the rate of ascorbate oxidation at 290 nm at 30 ºC and expressed as nmol ascorbate min^{-1} mg^{-1} protein. APX activity was assayed spectrophotometrically following Gratão et al.

(2012) in a reaction consisting of plant extract, 80 mM potassium phosphate buffer (pH 7.0) including 5 mM ascorbate, 1 mM EDTA, and 1 mM H_2O_2.

2.10 Glutathione reductase assay

The glutathione reductase (GR) assay mixture consisted of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 5, 5'-dithiobis (2-nitrobenzoic acid), 1 mM glutathione disulphide (GSSG) and 0.1 mM NADPH. The rate of decrease of GSSG was monitored by the intensification in absorbance at 412 nm over 1 min. GR activity was measured spectrophotometrically at 30 ºC and expressed as nmol min^{-1} mg^{-1} protein as described in Gratão et al. (2012).

2.11 Statistical analysis

The experimental design was randomized using six plants per treatment from three replicate pots in a factorial scheme of 4 x 2, following four Se concentrations and two Cd concentrations (presence or absence). The result of three independent replicates of each extract for plant growth, Cd concentration and nutritional analyses, TBARS and proline contents, CAT, APX and GR activities was expressed as the mean and standard error of the mean (±SEM). A multiple comparison between means by the Tukey test followed the application of an individual ANOVA for each characteristic at a 0.05 level of significance. The statistical analyses were performed using Agro Estat® software (Barbosa, 2015).

3. Results

3.1 Plant growth

Over 75 days post germination, plants with 1 and 5 µM Se concentrations exhibited a pronounced increase in dry mass without Cd exposure. Root dry mass exhibited an increase of 21% (1 µM Se) and 45% (5 µM Se), whereas leaf dry mass was enhanced by 30% (1 µM Se) and 31% (5 µM Se), when compared to control plants (without $CdCl_2$). With respect to Cd exposure, no significant results were observed for plant growth, irrespective of Se concentration (Fig. 1).

3.2 Cd and Se concentrations

Se concentration in MT roots and leaves was significantly increased with increasing Se concentrations. In the presence of $CdCl_2$, Se concentration decreased in roots when compared to plants without Cd application, with 51% for 1 µM Se, 73% for 5 µM Se and 40% for 10 µM Se, whereas in leaves, Se concentration decreased 23%, 56% and 77%, respectively (Table 1). When the Cd concentration was concerned, plants growing under 1 µM Se exhibited an increase of 121% in leaves (Table 1). Plants growing under 5 µM Se exhibited a pronounced decrease of 44% Cd concentration in roots, whereas leaves exhibited an increase of 38% in Cd concentration.

3.3 Nutritional analysis

Macronutrients and micronutrients were uptake and distributed differently depending on the Se concentration, Cd exposure treatment and the tissue analysed (Table 2). Roots of plants treated with Se, irrespective of Se concentration, only experienced decreased Ca concentrations. Phosphorous, K, Ca, Mg, Cu, Fe, Mn, Zn and Ni concentrations increased in leaves treated with Se, irrespective of Cd exposure (Table 2 -3). Cadmium reduced the concentrations of P, Ca, Mn and Ni in roots, while it increased the Fe concentration. However, Cd reduced the P concentration and increased the Fe and Mn concentrations in the leaves of tomato plants (Table 2-3). Plants following Cd and Se application exhibited an increase in the P and Mn concentrations in roots, whereas the K, Ca, Mg, S, Fe and Ni concentrations decreased in roots. However, this pattern of nutrient content in roots was modified by Cd exposure (Table 2-3).

3.4 Lipid peroxidation and H_2O_2 content

Lipid peroxidation, expressed as MDA content, exhibited interesting results, where Se application did not influence MDA content in either roots or leaves. In the presence of Cd, plants with 0 µM Se application exhibited a pronounced increase in

MDA content in leaves when compared with the highest Se concentration. Leaves with 1 μM Se exhibited a decrease of 57% in MDA content in the presence of Cd when compared with 0 μM Se. Roots did not exhibit significant changes in MDA content among treatments (Fig. 2A). The H_2O_2 content was higher in leaves with 1 μM Se, whereas leaves with 5 and 10 μM Se exhibited a decrease in H_2O_2 content. Leaves with 0 μM Se submitted to Cd exhibited an increase of 16% in H_2O_2 content when compared to control plants (0 mM Cd). When Se and Cd was applied, a significant reduction occurred in H_2O_2 content of leaves, mainly with 1 μM Se, (Fig. 2B).

3.5 Proline content

Proline content decreased in leaves and roots for all Se concentrations (Fig. 3). Following treatment with $CdCl_2$, leaves exhibited a decrease in proline content without Se application. On the other hand, leaves with 10 μM Se exhibited an increase of 17% in proline content.

3.6 Antioxidant enzyme activities

Three SOD isoenzymes were detected in leaves (Fig. 4A) and two in roots (Fig. 4B), which were characterized as Mn-SOD (SOD I), Fe-SOD (SOD II) and Cu/Zn-SOD (SOD III). Different changes were observed between the tissues analysed depending on the Se and Cd exposure treatment. For instance, the activity of SOD I exhibited the same pattern in leaves, irrespective of Se and Cd treatments, while leaves exhibited very low SOD III activity in the presence of Cd (Fig. 4A, line 5-8).

SOD III was affected by Cd exposure (Fig. 4A, lines 1-4) and was more pronounced in leaves with 0 mM Cd and 5 μM Se (line 3). In roots, SOD I was more pronounced in the presence of Cd (Fig. 4, line 5-8), although a slight increase was observed with Se application (line 6-8). Following Cd application, the activity of SOD II decreased in the roots (line 5-8), and increased with the presence of Se (line 6-8).

CAT (Fig. 5A), APX (Fig. 5B) and GR activities (Fig. 5C) are crucial for the detoxification of any excess H2O2 produced by SOD and/or by other metabolic processes. Leaves and roots treated with Se, irrespective of Se concentration, exhibited an increase in CAT activity following Cd application, being more pronounced for 10 μM Se concentration. When APX activity was concerned, leaves with 5 μM Se exhibited an increase of 34% in APX activity when compared with leaves without Se. Following Cd application, roots exhibited an increase in APX activity for all Se treatments, when compared with plants without Cd exposure. However, under Cd exposure, the application of 1, 5 or 10 μM Se decreased APX activity, when compared with plants under Cd without Se application.

The application of Se triggered differences in GR activity. Leaves with 5 μM Se exhibited increased GR activity by 67% compared with other Se concentrations without Cd exposure. Nevertheless, GR activity increased by 62% in leaves and 70% in roots following Cd application. Moreover, leaves and roots with 10 μM Se under Cd exposure exhibited strict increases of 65% and 84%, respectively, when compared with treatments with 0 μM Se under Cd exposure (Fig. 5C).

4. Discussion

Environmental stresses may drastically affected plant development, growth and yield due to changes in metabolism (Wang and Frei, 2011). Selenium application has been used in agriculture as an efficient strategy to improve plant response against stress and to avoid or minimize crop loss or damages (Kaur et al. 2016, Alyemeni et al. 2018). On the other hand, the beneficial effect of Se is dose-dependent, and higher concentrations in soil may damage plant development. Therefore, it is essential to optimize the concentration to be applied to each crop.

Several studies have indicated that Se can promote an increase in plant biomass, as observed in the present work (Lyons et al. 2009, Reis et al. 2018). The application of 1 μM and 5 μM Se efficiently promoted a gain in plant dry mass. For instance, roots exhibited increases in dry mass of 21% and 45%, respectively, while leaf dry mass increased by 30% and 31%, respectively (Fig. 1). These data demonstrate that adequate Se concertation results in improves growth. On the other

hand, 10 μM Se has no effect on plant growth, which could indicate that this concentration limits plant growth and can even be toxic to plant metabolism (Longchamp et al. 2015, Silva et al. 2018). It is well known that Cd damages plant growth (Ma et al. 2017, Zhu et al. 2018). The application of Cd resulted in a decrease in root dry mass when compared with control plants (Fig. 1). Although a number of studies indicate that Se treatment can reverse the reduction in growth caused by Cd in other crops, our treatments did not exhibit the same effect in MT plants.

The effect of Se on nutrient uptake is still controversial, but the beneficial effect of Se on nutrient uptake has been previously observed in many crops, such as cowpea (Silva et al. 2018), rice (Reis et al. 2017) and wheat (Yao et al. 2013). Several factors, such as the composition of the cell wall of the roots and biomass production, affect the uptake and transport of nutrients (Santos et al. 2017). Reis et al. (2018) reported increases in P, K, Cu and Zn concentrations in the leaves of rice plants supplied with Se. Moreover, cowpea under field conditions Silva et al. (2018) showed a decrease in P, K, and Cu concentration in leaves and an increase in Zn concentration in the leaves as a result of Se application. Se can act as a competitor with S for transporters involved in their uptake at the root plasma membrane (Zayed and Terry 1992), which can decrease S content.

The positive impact of Se on the nutritional status of plants under Cd stress can significantly contribute to growth because of its involvement in enzyme activation, signalling, and chlorophyll synthesis (Alyemeni et al. 2018). The interaction between Se and nutrients is a complex process, and sometimes, these elements may naturally present synergistic and antagonistic relations at the same time (Kabata-Pendias and Pendias, 2011).

Although Se is non-essential to plants, in the form of selenite it can be easily uptake via phosphate transporters and metabolized to organic forms in roots, such as selenomethionine, selenocysteine and several other unidentified Se species (Li et al. 2008). We found that the Se concentration in MT roots and leaves was significantly increased with increasing Se concentrations (Table 1). The Se concentration in plant tissue is dependent on the Se concentrations in soils, Se source and nutrient solution (Li et al. 2018). The Se concentration was higher in roots than in leaves. The Se accumulation observed in our data is due to the characteristics of selenite. A study

conducted by Lin et al. (2012) showed that selenite is poorly translocated to the above parts, which triggers a higher accumulation of Se in roots.

Cadmium toxicity clearly has negative effects on plant growth and can reduce root dry weight, root diameter and number of lateral roots (Rabêlo et al. 2018). In this study, we observed a reduction of 38% in root growth of 0 µM Se + 0.5 Cd application treatments when compared with control plants (without Cd) (Fig. 1). Cd can cause nutritional imbalances in plants due to i) competition of Cd^{2+} with nutrients using the same uptake sites (Clemens and Feng, 2016) and ii) modification in the translocation of nutrients (Nazar et al., 2012). Moreover, Cd exposure causes biochemical and structural alterations in plants that can result in changes in the nutritional status of the plant (Alves et al. 2017).

The reduced concentration of nutrients in the roots of plants submitted to Cd (Table 2-3) can be associated with reduced growth of these plants, as previously observed in other species (Ekvall and Greger, 2003, Rabêlo et al. 2018). In addition, our results showed that Cd increased the content of Fe in roots and leaves. The higher Fe content results from the formation of Fe plaques on the root surface of plants exposed to Cd due to the increase in the Fe^{2+} availability (Sebastian and Prasad, 2015). Exposure to Cd may increase the Fe availability in the rhizosphere and consequently the concentration of Fe in leaves, as observed in the present work. Apart from nutritional dynamics, Cd can also cause oxidative damage.

Plants have a range of potential mechanisms that may be involved in avoiding damage to plant metabolism caused by Cd. As shown in Table 1, plants accumulate more Cd in roots than in leaves, which may be a natural defence response of plants to Cd toxicity. Our data revealed that the application of 5 µM Se efficiently reduced Cd content by 44% in roots. Se is favourable to plant growth under Cd-stress conditions (Wu et al. 2017). Se was not effective in avoiding damage to plant growth caused by Cd (Fig. 1), but Se reduced MDA and H_2O_2 contents in plants exposed to Cd. The reduced growth of tomato plants exposed to Cd (Fig. 1) is a typical symptom of phytotoxicity in response to changes in nutrient uptake (Nazar et al. 2012). Cd and Se are bound to thiol groups of the amino acid cysteine, and it should be noted the competition for a shared binding site in proteins could explain the reduced Cd uptake and defence mechanisms against Cd toxicity (Schützendubel et al. 2001).

However, the application of 1 and 10 μM Se did not decrease the Cd concentration in roots. When the Cd content was measured in the above parts of the plants, we observed that plants under Cd exposure treated with 1 μM Se exhibited an increase of 121% in Cd content in leaves and of 70% in fruits (Table 1). Wan et al. (2016) revealed that Cd uptake kinetics result in selenite promoting Cd influx and increased Cd uptake in rice roots during short-term exposure, proving no competition in uptake between Se and Cd on the root surface. Furthermore, the results of Lin et al. (2012) indicate that Se markedly decreased Cd accumulation in the leaves of rice plants. It is still unknown how Se can decrease heavy metal translocation. Mounicou et al. (2006) indicated that Se- and Hg-containing protein complexes in roots are sufficiently stable to prevent the translocation of Hg to leaves. These findings suggest that Se ions are co-transported with Cd ions by the same protein carriers, causing an increase of Cd in the above parts of plants (Zembala et al. 2010, Mounicou et al. 2006). These findings could explain why 1 μM Se induced high Cd concentrations in leaves. In addition to Se altered the Cd content of plants, Cd interfered with Se concentrations in plant tissues. For instance, the presence of Cd caused a severe reduction of Se content in all plant tissues (Table 1). Cd is well known to induce the expression of sulphur-assimilating enzymes and sulphate uptake by roots (Yamaguchi et al. 2016), leading to increased Se uptake, mainly in the form of selenate, mediated by S transporters. However, in our work, plants exposed to Cd showed a decrease in plant growth, reduction in Se content and nutrient uptake. This fact may be due to the Se source. Selenite is easily transformed into organic forms in roots; these forms are more difficult to transport above to parts of the plant, and they compete with other ions in the cell and do not cooperate to reduce Cd in leaves.

Cadmium triggers an overproduction of ROS, causing oxidative stress in fundamental cellular structures such as lipids and proteins, which leads to an imbalance in plant metabolism (Gratão et al. 2015, Alves et al. 2017). A number of recent studies indicate that low concentrations of Se can modulate oxidative stress caused by Cd exposure (Abd et al. 2016, Alyemeni et al. 2018). For instance, plants with Cd exhibited an intense increase in lipid peroxidation when compared to plants with higher concentrations of Se under Cd stress (Fig. 2A). Furthermore, leaves with 1 μM Se reduced MDA content by 57% in the presence of Cd. The attenuation of Cd-

induced toxicity by Se may be attributed either to Se-triggered reactivation of membrane enzymes and subsequent restoration of metabolite transport or the competition of Se with Cd for some key specific binding sites, such as thiol groups of cysteine (Feng et al. 2013). Moreover, Se can induce ROS scarvenging though dismutation of superoxide anions to form H_2O_2 without the involvement of superoxide dismutase (Cartes et al. 2010) or selenocompounds can quench superoxide anions and hydroxyl radicals directly (Xue et al. 1993).

H_2O_2 content was high in leaves treated with 1 μM Se application; however, at higher concentrations, the H_2O_2 content decreased. The presence of Cd can produce an increase in H_2O_2 production of 16% in leaves with 0 μM Se application when compared with control plants. The application of Se efficiently decreased H_2O_2 production triggered by Cd exposure, mainly at 1 μM Se application (Fig. 2B). These data reinforce the conclusion that Se application leads to a reduction in ROS production, which contributes to the maintenance of the structural and functional integrity of membranes even under Cd stress (Alyemeni et al. 2018).

Plants increase the synthesis and reduce the degradation of proline, a protective metabolite which contribute to the stabilization of protein molecules and membranes, as a direct response against stress (Zouari et al. 2016). Under Cd exposure, there was a slight decrease in proline content in leaves not treated with Se, which could indicate that Cd stress was enough to enhance proline synthesis (Fig. 3). On the other hand, leaves with 10 μM Se exhibited an increase in proline content of 17%. It is well known that proline has a fundamental role in osmoregulation and ROS scavenging (Kaur et al. 2015). Se can increase proline content by inducing up-regulation proline-synthesizing enzymes concomitant with a reduction of catabolic enzymes (Abd et al. 2016, Alyemeni et al. 2018).

Antioxidant enzymes allow plants to avoid oxidative stress and to survive Cd stress conditions (Gratão et al. 2015). Strong evidence indicates that Se can improve the activity of antioxidant enzymes under normal and stress conditions (Abd et al. 2016, Alyemeni et al. 2018, Handa et al. 2018). The antioxidant enzymes SOD, APX, CAT and GR were selected based on their responses in a number of reports and the role of Se during Cd stress in plants. These enzymes exhibited varied responses among Se applications, Cd exposures and tissue types. SOD, which converts O_2 into

H_2O_2, has been increased in several plant species when exposed to Cd and Se (Alyemeni et al. 2018). Different isoenzymes contribute to cell protection against different toxic substances, such as Cd.

In this study, three SOD isoenzymes (SOD I, II and III) were identified following non-denaturing PAGE analysis (Fig. 4), with a similar isoenzyme classification as previously reported by Gratão et al. (2015) and Alves et al. (2017) for MT plants. Mn-SOD (SOD I), Fe-SOD (SOD II) and Cu/Zn-SOD (SOD III) isoenzymes were detected in leaves (Fig. 5A), and Mn-SOD (SOD I) and Fe-SOD (SOD II) were identified in roots (Fig. 4B). The activity of SOD I in leaves was not affected by any treatment. SOD I was more prevalent in roots in the presence of Cd (Fig. 4, line 5-8), but a slight increase occurred with the application of Se (line 6-8). It is well known that Cd exposure can induce upregulation of Mn-SOD at both transcript and activity levels (Rodríguez-Serrano et al. 2006), which supports a slight increase in Mn-SOD activity in roots under Cd stress conditions. Moreover, Se can contribute to an increase in its activity and a reduction in lipid peroxidation. SOD II decreased in leaves and roots due to Cd exposure (Fig. 4A-B, line 5-8), but the application of Se slightly increased its activity in roots (Fig. 4B, line 6-8). Cd can affect the expression and regulation of this isoenzyme (Romero-Puertas et al. 2007). Moreover, we can observe that the Fe content in roots and leaves under Cd exposure was lower when compared to plants without Cd exposure (Table 3). SOD III in leaves was completely inhibited by Cd presence; therefore, this isoform was visible only in 0 mM $CdCl_2$ treatments (Fig. 4A, lines 1-4). Plants treated with 5 µM Se exhibited an increase in Zn concentration (Table 3), which may improve Cu/Zn-SOD activity (SOD III) (line 3). Moreover, Se has synergistic effects on the transcription of the Cu/Zn-SOD isoform (Seppänen et al., 2003), which can provide extra strength to tomato plant defences to counteract Cd stress.

CAT (Fig. 5A), APX (Fig. 5B) and GR (Fig. 5C) activities are crucial for the detoxification of any excess H_2O_2 produced by SOD and/or by other metabolic processes. The current literature contains mixed results concerning changes in CAT activity with Se application. For instance, Alyemeni et al. (2018) observed that Se application increased CAT activity in tomatoes. However, a study conducted by Saidi et al. (2014) showed that only Se did not alter CAT activity in sunflowers. Our data

are in accordance with this finding, where the application of Se did not alter CAT activity in either the root or the leaves (Fig. 5A). When Cd was applied, an increase was observed in all treatments and was more pronounced at 10 μM Se. Similar results were observed by Lin et al. (2012) for rice, Khan et al. (2015) for *B. juncea* and Alyemeni et al., (2018) in tomato plants.

The glutathione-ascorbate cycle is a fundamental metabolic pathway that converts H_2O_2 to H_2O and O_2. This cycle involves antioxidant metabolites, such as ascorbate, glutathione and NADPH, and key enzymes, such as APX and GR. In the present study, leaves with 1 μM and 5 μM Se exhibited a significant increase in APX activity. Under Cd exposure, we observed an increase in APX activity in the roots in 1, 5 and 10 μM Se treatments, which may protect plant metabolism by nullifying the effects of ROS generated by Cd stress. Furthermore, Khan et al. (2015) demonstrated that selenium induced the upregulation of APX and GR in *B. juncea*, thus protecting photosynthetic electron transport from cadmium-induced oxidative damage via the maintenance of the NADP/NADPH ratio.

GR activity was more pronounced in leaves with 5 μM Se compared with other Se treatments without Cd exposure. Nevertheless, when Cd was applied, GR exhibited an increase of 62% in leaves and 70% in roots when compared with control plants. Plants treated with 10 μM Se under Cd stress exhibited a considerable increase in GR activity of 65% and 84%, respectively, in leaves and roots when compared with 0 μM Se application under Cd exposure (Fig. 5C). Wu et al. (2017) indicated that Se may play a role in enhancing the efficiency of GSH-AsA under Cd exposure, and the increased activity of GR by Se application may induce increased levels of GSH and AsA due to Se boosting GSH synthesis, which has a regulatory role in methylglyoxal detoxification and upregulation of GR (Wu et al. 2017). The different patterns of SOD, CAT, APX, and GR activities indicate that Se plays a fundamental role in optimizing the performance of the antioxidant system for alleviating Cd stress.

5. Conclusion

Oxidative stress triggered by Cd causes a serious cell imbalance between ROS production and antioxidant enzymes, which leads to intense physiological disorders in plants. The results clearly indicate distinct trends in plants treated with Se under Cd exposure. Thus, our findings demonstrate that Se application, mainly at 1 μM of Se concentration is an efficient management technique that can be used to alleviate deleterious effects of Cd-stress conditions and enhance Cd tolerance by improving the nutritional status, increased proline content and activity of ROS-scavenging enzymes in MT plants. However, the exactly way that Se promote these benefits in plants remains unknown. Thus, is necessary further studies exploring signalling molecules, hormones and other pathways to comprehend how Se acts in plants metabolism.

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7. Figures legends

Figure 1. Roots and leaves dry mass (g dry wt) in MT grown over 45-day period in the presence of 0 mM or 0.5 mM $CdCl_2$ and 1, 5 or 10 µM Se. Data above x-axis represent leaves and below x-axis, roots. Different uppercase letters on the top of the columns indicates the difference between - Cd or + Cd exposure and lowercase indicates the difference between Se concentrations with significantly different at $P < 0.05$ by Tukey test.

Figure 2. Content of **a** lipid peroxidation measured as malondialdehyde (MDA) (nmol g^{-1} fresh weight) and **b** hydrogen peroxide (H_2O_2) (µmol g^{-1} fresh weight) in roots and leaves of MT, plants grown over 45-day period in the presence of 0 mM or 0,5 mM $CdCl_2$ and 1, 5 or 10 µM Na_2SeO_3. Data above x-axis represent leaves and below x-axis, roots. Different uppercase letters on the top of the columns indicates the difference between - Cd or + Cd exposure and lowercase indicates the difference between Se concentrations with significantly different at $P < 0.05$ by Tukey test.

Figure 3. Proline content (µmol g^{-1} fresh weight) in roots and leaves of MT plants grown over 45-day period in the presence of 0 mM or 0,5mM$CdCl_2$ and 1, 5 or 10 µM Na_2SeO_3. Data above x-axis represent leaves and below x-axis, roots. Different uppercase letters on the top of the columns indicates the difference between - Cd or + Cd exposure and lowercase indicates the difference between Se concentrations with significantly different at $P < 0.05$ by Tukey test.

Figure 4. Superoxide dismutase (SOD) activity stained following non-denaturing polyacrylamide gel electrophoresis of leaves (**a)** and roots (**b**) isolated from non-grafted and grafted MT plants grown over 45-day period in the presence of 0 mM or 0,5mM$CdCl_2$. The lanes listed are: (S) bovine SOD standard; (1) MT (0 mM$CdCl_2$ + 0 µM Na_2SeO_3), (2) MT (0 mM $CdCl_2$ + 1 µM Na_2SeO_3), (3) MT (0 mM $CdCl_2$ + 5 µM Na_2SeO_3), (4) MT (0 mM $CdCl_2$ + 10 µM Na_2SeO_3), (5) MT (0,5 mM $CdCl_2$ + 0 µM Na_2SeO_3), (6) MT (0,5 mM $CdCl_2$ + 1 µM Na_2SeO_3), (7) MT (0,5 mM $CdCl_2$ + 5 µM

Na_2SeO_3), (8) MT (0,5 mM $CdCl_2$ + 10 µM Na_2SeO_3).The SOD isoforms are (I) Mn-SOD, (II) Fe-SOD and (III) Cu/Zn-SOD.

Figure 5. Antioxidant total enzyme activity. **a** CAT specific activity (mmol min^{-1} mg^{-1}), **b** APX specific activity (nmol min^{-1} mg^{-1}) and **c** GR specific activity (nmol min^{-1} mg^{-1}) in MT plants grown over 45-day period in the presence of 0 mM or 0,5 mM $CdCl_2$ and 1, 5 or 10 µM Na_2SeO_3. Data above x-axis represent leaves and below x-axis, roots. Different uppercase letters on the top of the columns indicates the difference between - Cd or + Cd exposure and lowercase indicates the difference between Se concentrations with significantly different at $P < 0.05$ by Tukey test

8. Figures

Figure 1

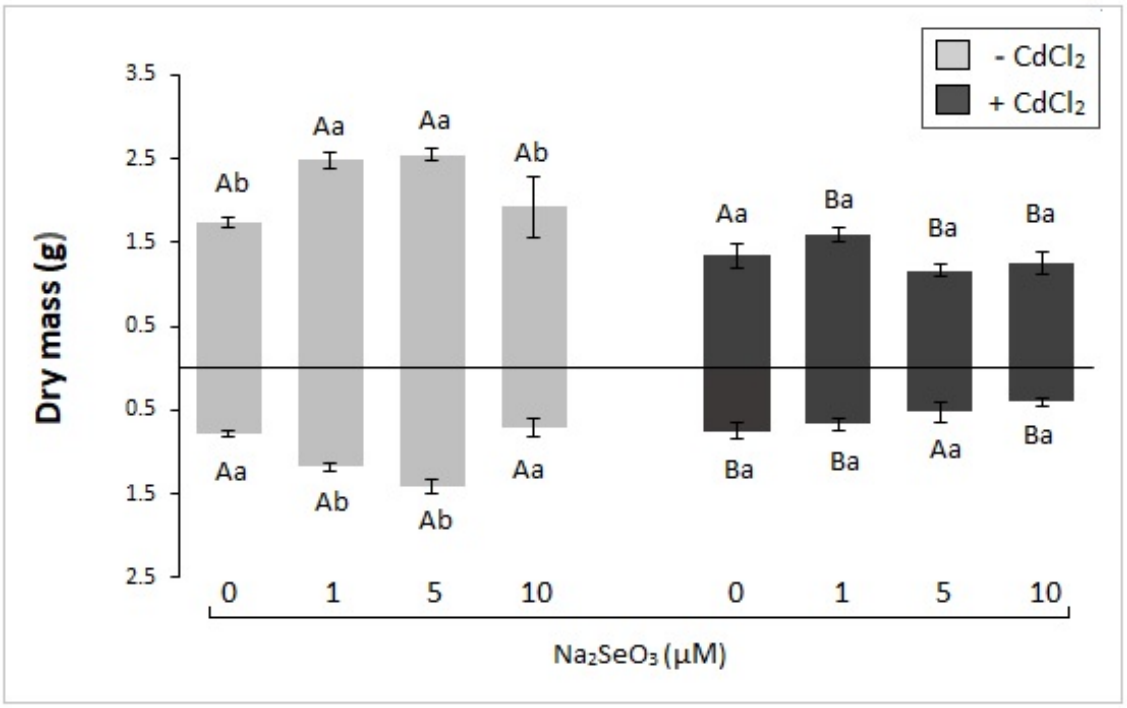


Figure 2

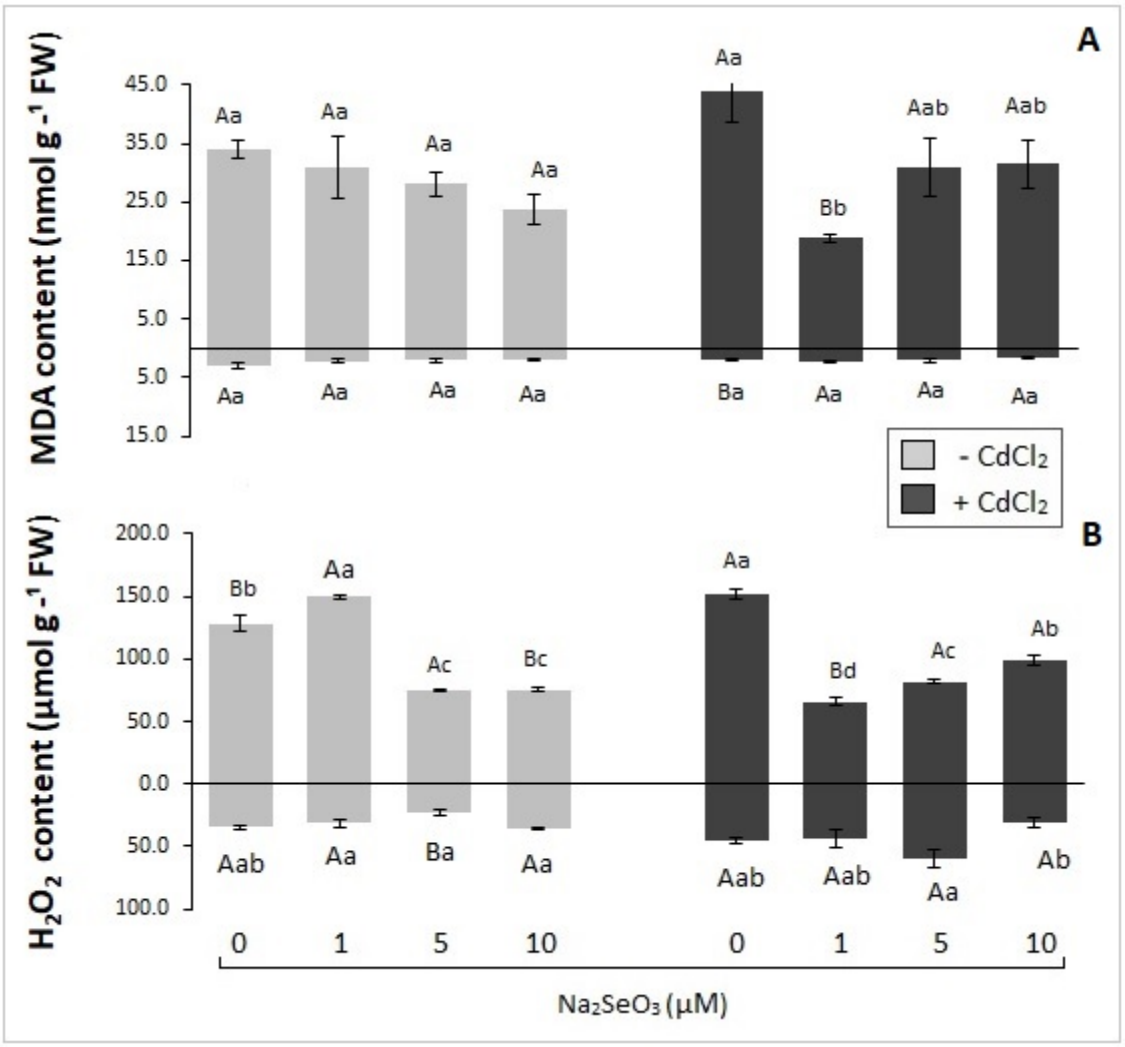

A
MDA content (nmol g-1 FW)
45.0
35.0
25.0
15.0
5.0
5.0
15.0
Aa Aa Aa Aa Aa Bb Aab Aab
Aa Aa Aa Aa Ba Aa Aa Aa
- CdCl2
+ CdCl2
B
H2O2 content (µmol g-1 FW)
200.0
150.0
100.0
50.0
0.0
50.0
100.0
Bb Aa Ac Bc Aa Bd Ac Ab
Aab Aa Ba Aa Aab Aab Aa Ab
0 1 5 10 0 1 5 10
Na2SeO3 (µM)

Figure 3

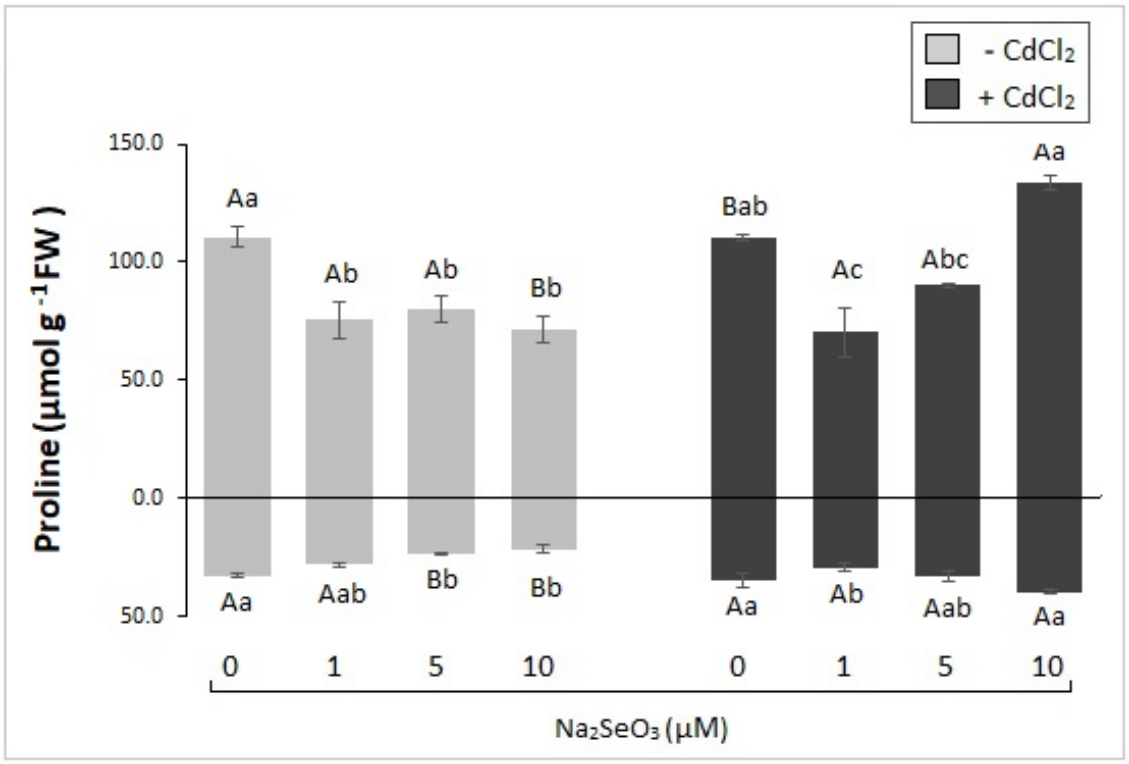

- CdCl2
+ CdCl2
Proline (µmol g-1FW)
150.0
100.0
50.0
0.0
50.0
Aa
Ab
Ab
Bb
Bab
Ac
Abc
Aa
Aa
Aab
Bb
Bb
Aa
Ab
Aab
Aa
0
1
5
10
0
1
5
10
Na2SeO3 (µM)

Figure 4

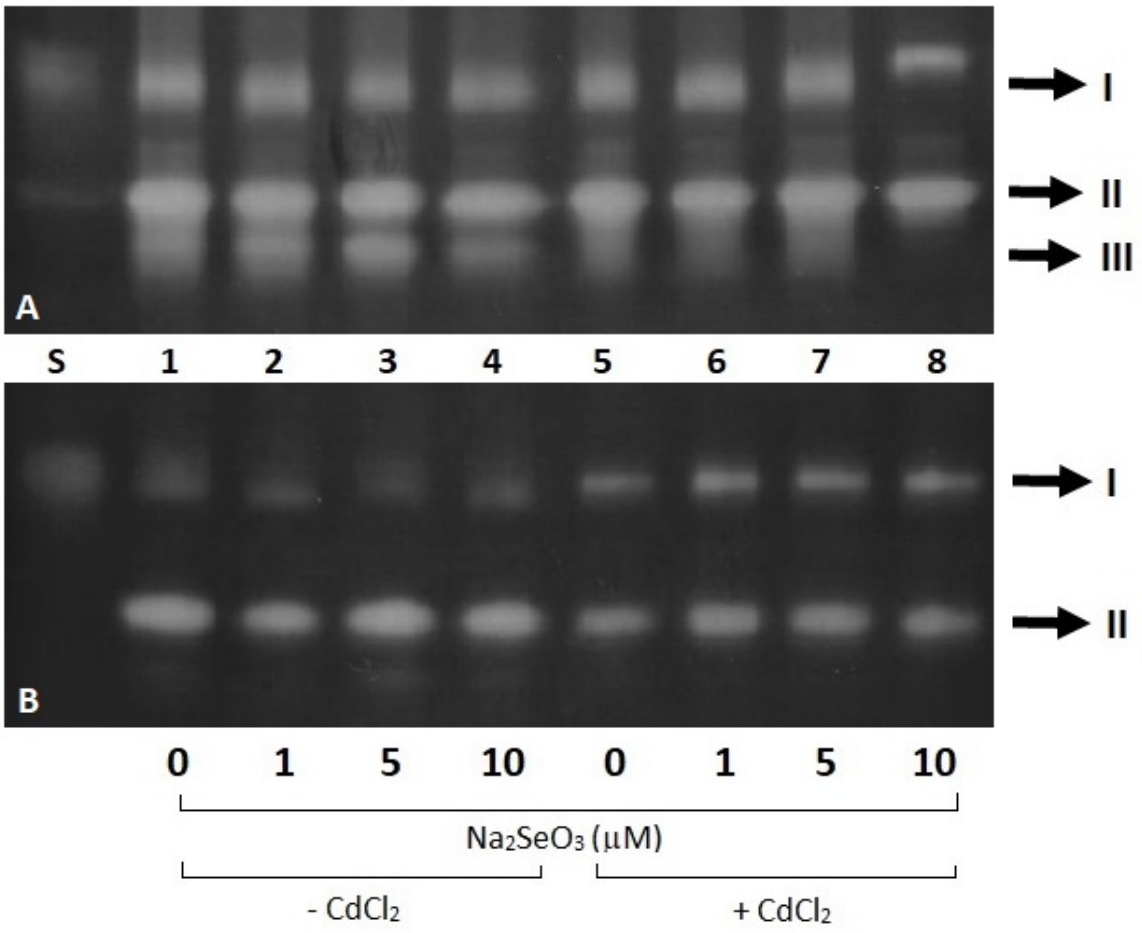
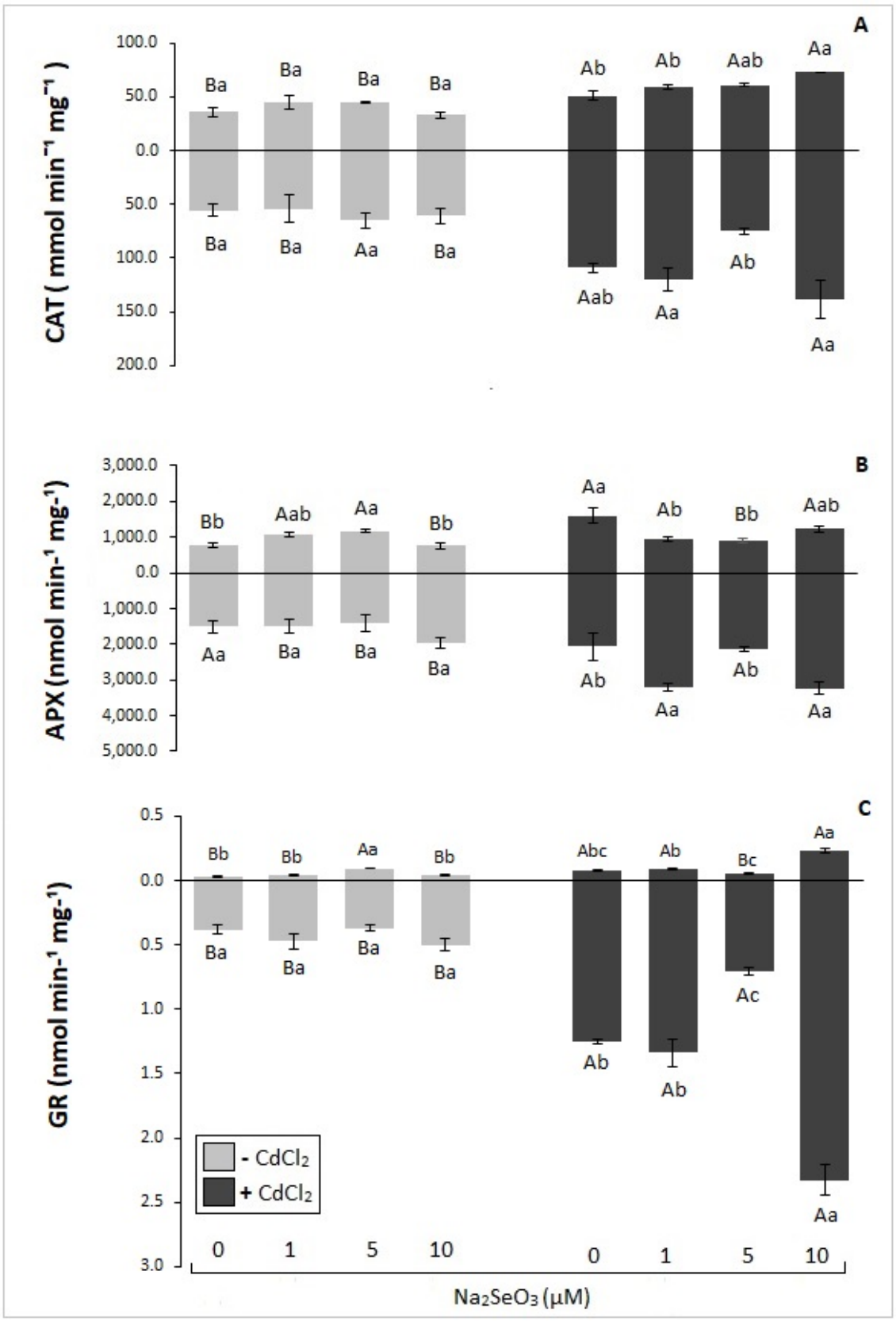


Figure 5


A
CAT (mmol min⁻¹ mg⁻¹)
100.0
50.0
0.0
50.0
100.0
150.0
200.0
Ba Ba Ba Ba
Ba Ba Aa Ba
Ab Ab Aab Aa
Aab Aa Ab Aa
B
APX (nmol min-1 mg-1)
3,000.0
2,000.0
1,000.0
0.0
1,000.0
2,000.0
3,000.0
4,000.0
5,000.0
Bb Aab Aa Bb
Aa Ba Ba Ba
Aa Ab Bb Aab
Ab Aa Ab Aa
C
GR (nmol min-1 mg-1)
0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Bb Bb Aa Bb
Ba Ba Ba Ba
Abc Ab Bc Aa
Ab Ab Ac Aa
- CdCl2
+ CdCl2
0 1 5 10 0 1 5 10
Na2SeO3 (µM)

9. Tables

Table 1. Cd and Se concentration (µg g-¹ dry weight) in MT plants grown over a 45-day period in the presence of 0,5 mM CdCl2. Same letters indicates no significantly different among Se concentrations and * indicates difference in the presence or absence of Cd at P < 0.05 by Tukey test.

Se uml^{-1}	Root		Leaves	
	+ Cd	- Cd	+ Cd	- Cd
	----------		----------	
	----------Se----------			
0	0.03 ± 0.21d	0.08 ± 0.20 d	0.05 ± 0.07 c	0.05 ± 0.08 d
1	7.71 ± 0.65 c	15.64 ± 0.88 c*	1.11 ± 0.22 b	1.45 ± 0.27 c
5	17.03 ± 0.04 b	63.48 ± 1.88 b*	2.44 ± 0.10 a	4.48± 0.36 b*
10	57.84 ± 0.37 a	96.86 ± 2.75 a*	1.96 ± 0.13 ab	10.45 ± 0.13 a*
	----------Cd----------			
0	2378.20 ± 48.26 a*	0.10 ± 58.67 a	286.47 ± 4.98 c*	0.04 ± 1.77 a
1	2238.20 ± 360.77 a*	0.10 ± 58.70 a	633.82 ± 11.81 a*	0.06 ± 1.78 a
5	1330.77 ± 140.86 b*	0.10 ± 58.69 a	394.15 ± 3.97 b*	0.04 ± 1.78 a
10	2533.44 ± 34.37 a*	0.13 ± 58.61 a	306.35 ± 22.54 c*	0.06 ± 1.77 a

Table 2. Nutritional analysis of macronutrients (mg g^{-1}). Same letters indicates no significantly different among Se concentrations and * indicates difference in the presence or absence of Cd at $P < 0.05$ by Tukey test.

	Root		Leaves	
Se μml^{-1}	+ Cd	- Cd	+ Cd	- Cd
	----------	----------	----------	----------
	----------P----------			
0	1457.13 ± 30.18 a	1576.40 ± 20.85 b	1268.39 ± 369.39 b	3060.27 ± 97.77 b*
1	1462.00 ± 46.48 a	2013.82 ± 193.77 a*	3648.42 ± 61.60 a*	2143.98 ± 81.93 a
5	1302.74 ± 67.71 a	2053.45 ± 34.40 a*	3420.34 ± 20.56 a	3789.23 ± 187.45 a
10	1057.33 ± 6.00 a	1676.08 ± 263.26 a*	3695.39 ± 154.22 a	4393.23 ± 235.84 a*
	----------K----------			
0	26934.81 ± 510.2 ab	30181.74 ± 272.40 a	15290.28 ± 1593.46 c	15720.8 ± 1945.86 b
1	25549.26 ± 440.2 ab	23901.33 ± 279.94 b	30655.45 ± 422.74 a	13655.2 ± 297.49 b
5	24318.79 ± 2089.5 b	26564.83 ± 952.34 ab	24697.07 ± 1128.72 b	21210.8 ± 671.91 a
10	30251.97± 1422.8a*	24169.9 ± 1546.2 b	25698.88 ± 686.31 b	22445.9 ± 370.55 a
	----------Ca----------			
0	8066.72 ± 553.88 ab	9708.44 ± 315.98 a*	22319.1 ± 1195.55 b*	14289.3 ± 4294.6 b
1	6282.83 ± 191.25 b	8972.23 ± 700.1 ab*	17103.46 ± 1724.4 a	33758.6 ± 1345.4 b*
5	6890.46 ± 220.67 ab	7911.84 ± 47.41 ab	28592.26 ± 332.6 a	32715.9 ± 650.1 a
10	8608.79 ± 196.13 a	7347.02 ± 763.56 b	29825.51 ± 2798.0 a	33407.2 ± 1476.0 a
	----------Mg----------			
0	6497.09 ± 489.19 a	7661.48 ± 73.62 a*	3741.99 ± 182.44 b	4058.14 ± 247.91 b
1	6274.77 ± 70.17 a	6916.35 ± 23.80 ab	6508.25 ± 158.62 a*	3560.57 ± 80.13 b
5	6395.82 ± 50.29 a	6653.74 ± 23.89 b	6138.99 ± 335.30 a	5904.65 ± 177.74 a
10	6235.06 ± 83.10 a*	5506.69 ± 325.74 c	6274.06 ± 401.47 a	57.16.88 ± 391.65 a
	----------S----------			
0	4574.15 ± 345.55 a	5220.67 ± 100.00 a	3134.37 ± 961.82 c	5227.31 ± 212.93 a*
1	4477.36 ± 137.08 a	4883.82 ± 37.23 a	7870.38 ± 282.82 a*	4469.90 ± 568.58 a
5	4135.58 ± 333.69 a	4464.4 ± 241.04 ab	7154.57 ± 197.01 a	6495.32 ± 265.7 ab
10	4285.32 ± 321.01 a	3647.46 ± 202. 11 b	5590.15 ± 248.33 a	5300.05 ± 231.64 a

Table 3. Nutritional analysis of micronutrients (µg g-¹). Same letters indicates no significantly different among Se concentrations and * indicates difference in the presence or absence of Cd at P < 0.05 by Tukey test.

Se µml^{-1}	Root		Leaves	
	+ Cd	- Cd	+ Cd	- Cd
	----------		----------	
	----------Bo----------			
0	36.21 ± 5.44 a	29.41 ± 0.48 a	53.50 ± 3.97 b	60.45 ± 4.30 a
1	24.10 ± 2.68 a	27.53 ± 0.33 a	55.67 ± 5.27 b*	44.61 ± 0.84 b
5	29.80 ± 2.37 a	27.51 ± 0.63 a	67.30 ± 1.12 ab	63.89 ± 1.77 a
10	29.07 ± 2.61 a	31.62 ± 1.71 a	80.97 ± 3.12 a*	63.49 ± 3.07 a
	----------Fe----------			
0	690.31 ± 2.97 ab	562.10 ± 2.11 ab	168.87 ± 18.46 b	155.17 ± 31.06 a
1	552.95 ± 24.18 b	843.53 ± 133.34 a*	244.86 ± 2.79 ab*	137.89 ± 28.93 b
5	993.44 ± 163.75 a*	569.85 ± 66.90 ab	340.63 ± 20.94 a*	241.71 ± 8.40 a
10	687.70 ± 34.76 ab*	384.93 ± 26.44 b	270.47 ± 11.14 ab	242.94 ± 43.89 a
	----------Zn----------			
0	17.79 ± 3.68 a	17.91 ± 0.28 a	3.63 ± 0.96 b	4.20 ± 0.16 b
1	11.40 ± 0.61 a	16.10 ± 0.76 a	8.85 ± 0.07 a*	5.51 ± 0.57 b
5	13.15 ± 1.32 a	18.77 ± 0.73 a	8.17 ± 0.50 a	9.86 ± 0.30 a*
10	9.73 ± 0.54 a	17.91 ± 4.21 a*	6.69 ± 0.31 a	8.79 ± 0.51 a*
	----------Cu----------			
0	10.07 ± 0.53 ab	7.71 ± 0.05 a	2.62 ± 0.16 b	2.15 ± 0.12 b
1	11.31 ± 0.04 a	6.62 ± 0.33 a	6.16 ± 0.26 b*	3.03 ± 0.22 a
5	8.06 ± 0.05 c	6.98 ± 0.01 a	5.46 ± 0.21 a	7.08 ± 0.12 a*
10	9.72 ± 0.61 b	6.71 ± 0.26 a	5.43 ± 0.08 a	6.24 ± 0.25 a*
	----------Mn----------			
0	54.68 ± 8.66 a	32.86 ± 0.80 c	30.05 ± 1.74 b	32.26 ± 3.26 a
1	31.87 ± 7.19 a	52.89 ± 6.46 bc	52.04 ± 1.59 a*	12.08 ± 4.85 b
5	35.90 ± 0.46 a	99.68 ± 6.83 a*	45.29 ± 1.98 a*	24.21 ± 1.35 ab
10	28.43 ± 0.98 a	85.85 ± 17.67ab*	55.61 ± 2.04 a*	20.05 ± 5.15 ab
	----------Ni----------			
0	4.73 ± 0.02 a	10.69 ± 0.24 a*	1.59 ± 0.07 b	1.86 ± 0.12 b
1	3.89 ± 0.26 a	8.93 ± 2.54 ab*	2.88 ± 0.04 a*	1.99 ± 0.23 b
5	8.83 ± 3.04 a	5.96 ± 1.10 ab	3.18 ± 0.09 a	3.16 ± 0.02 a
10	4.64 ± 0.01 a	3.16 ± 0.06 b	3.07 ± 0.034 a	3.31 ± 0.034 a

CHAPTER III - The new insights into cadmium stressful-conditions: role of ethylene in selenium-mediated antioxidant enzymes

Abstract

Cadmium (Cd) contamination has generated an environmental problem worldwide, leading to harmful effects on human health and damages to plant metabolism. Selenium (Se) is non essential for plants, however it can improve plant growth and reduce the adverse effects of abiotic stress. In addition, ethylene may interplay the positive effects of Se in plants. In order to investigate the role of ethylene in Se-modulation of antioxidant defence system in response to Cd-stress, we used hormonal mutant *Epinastic (epi)* with a subset of constitutive activation of the ethylene response and Micro-Tom (MT) plants. For this purpose, Se mineral uptake, Cd and Se concentrations, pigments, MDA and H_2O_2 contents, ethylene production, GSH compound, and SOD, APX, CAT, GR and GSH-Px activities were analysed in MT and *epi* plants submitted to 0.5 mM $CdCl_2$ and 1 μM of selenate or selenite. MT plants treated with both Se forms increased growth in the presence or not of 0.5 mM $CdCl_2$, whilst both Se forms reduced Cd uptake, mainly in *epi* plants. P, Mg, S, K and Zn uptake increased in *epi* plants with Se aplication, irrespective to Cd exposure. Chlorophylls and carotenoids contents decreased in both genotypes under Cd exposure, in contrast to observed in *epi* leaves in the presence of Se. When antioxidant enzymes activities were concerned, Se application increased Mn-SOD, Fe-SOD, and APX activities. In the presence of Cd, MT and *epi* plants exhibited decreased SOD activity and increased CAT, APX and GR activities. MT and *epi* plants with Se supply exhibited increased APX and GR activities in the presence of Cd. Overall, these results suggest that ethylene may be involved in Se induced-defence responses, that triggers a positive response of antioxidant system and improve growth under Cd stress. This results add further information that should help unraveling integrative roles of ethylene and Se in regulating the cell responses to stressful-conditions.

Key words: antioxidant system, ethylene, mutants, reactive oxygen species, tomato.

1. Introduction

In the last decades, there was a considerable increase in heavy metals concentration in the atmosphere due to anthropic factors (Alves et al., 2016). Among the toxic metals, cadmium (Cd) is one of the main environmental contaminants, and this concentration in environment increased due to metallurgical, forest fires and as a contaminant from phosphate fertilizers, fertilization with sewage sludge and contaminated industrial effluents (Gratão et al., 2015).

Among the toxic metals, Cd is one of the most dangerous pollutants to agriculture because of its high mobility in the soil-plant system. Cd may affect biochemical and structural mechanisms in plants, such as reduction of cell redox state control, inducing oxidative stress and rupture of membrane composition and function (Gratão et al., 2009). Moreover, Cd can induce severe disturbances in physiological processes of plants, such as photosynthesis, water relations and nutrient absorption (Lavres et al., 2019).

Studies have shown that Cd induces an overproduction of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). These ROS can accelerate lipid peroxidation, thus affecting membrane fluidity and permeability due to changes in lipid composition (Manquián-Cerda et al., 2016; Foyer, 2018). Consequently, these responses trigger stress response mechanisms. In fact, during stress caused by Cd, occurs an induction of systems capable of preventing uncontrolled oxidation, which includes key enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), glutathione reductase (GR, EC 1.6.4.2), catalase (CAT, 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9) and ascorbate peroxidase (APX, EC 1.11.1.11) (Gratão et al., 2015; Bashri et al., 2016). Furthermore, a number of non-enzymatic mechanisms are used in this defense, including antioxidants such as proline and glutathione (GSH) (Hasan et al., 2016), as well as vitamins, flavonoids, alkaloids, sugars, and carotenoids (Ahmad et al.2010; Guo et al., 2016a; Rabêlo et al., 2018a).

Many agricultural practices are used to avoid damages to plant metabolism caused by Cd toxicity. Plant supplementation with mineral elements is one of the strategies that can be adopted, and beneficial minerals can minimize the toxicity

generated by Cd (Feng et al., 2013a). Studies have shown that essential or beneficial elements such as nitrogen (N), iron (Fe), sulfur (S) and selenium (Se) can restore photosynthetic capacity, improve the antioxidant response and yield of cultivated plants (Khan et al., 2015b; Rabêlo et al., 2018a, b).

Selenium can reduce the adverse effects of the abiotic stress by modulation of different mechanisms (Feng et al., 2013b; Andrade et al., 2018). For instance, low concentrations of this element stimulate plant growth and alleviate negative effects of Cd stress (Saidi et al., 2014). In addition, Se regulates ROS metabolism and induce the activity of antioxidant enzymes (Castillo-Godina et al., 2016; Silva et al., 2018), reduces absorption (Lin et al., 2012; Abd et al., 2016) and translocation of Cd; (Wan et al., 2016; Sun et al., 2016) and can delay fruit senescence (Pezzarossa et al., 2014). A study conducted by Khan et al. (2015b) demonstrated that positive effects induced by Se-mediated attenuation during Cd stress was suppress by application of ethylene biosynthesis inhibitor. Thus, Se could reverse oxidative stress induced by Cd due to regulation of ethylene biosynthesis, resulting in an increased proline and glutathione contents (Khan et al., 2015b).

Cadmium can increases the biosynthesis of ethylene by regulating the enzymes involved in the biosynthesis of this hormone (Schelligen et al., 2014). Ethylene is closely related to stress physiology responses and the plant defense mechanism (Keunen et al., 2016). Application of exogenous ethylene stimulates the stress induced by Cd, and treatment with Cd induces the production of endogenous ethylene (Iakimova et al., 2005). These factors lead to the induction of senescence and the increase of ROS production, causing damage to the metabolism and consequent plant death. Moreover, Gratão et al. (2012) demonstrated that ethylene-insensitive *Never ripe* tomato mutant withstand Cd stress due to retains a partial sensitivity to ethylene, which reinforce the relative importance of ethylene in regulating the cell responses under stressful conditions.

Tomato is one of the most cultivated vegetables worldwide and has a great agricultural importance (Costa and Heuvelink, 2018), although a large part of its production is affected by abiotic stresses. The cultivar Micro-Tom tomato (MT) is considered a model plant for agronomic studies (Meissner et al., 1997), and has well-characterized hormone mutants (Monteiro et al., 2012). For instance, the mutant *epi*

exhibits a subset of constitutive activation of the ethylene response required for normal vegetative growth and development (Barry et al., 2001). The use of hormonal mutants has been a powerful tool to understand the mechanisms involved in Cd stress sensitivity and tolerance (Gratão et al., 2012, 2015; Alves et al., 2017).

The use of these mutants may add information of stress attenuation mechanisms, as well as the interplay between Se and ethylene by providing novel and relevant information on the role of ethylene in the mechanismis modulated by Se. Moreover, Khan et al. (2013b) reinforced the necessity of researches using ethylene mutants to reveal more comprehensive information about the role of ethylene in regulation of nutrients optimization in plants under normal and stressful environment. Since Se is a powerful antioxidant, we hypothesized that ethylene may be envolved in the role of Se-attenuation of Cd stress conditions by increasing ROS-scavenging enzymes, which will lead to the maintenance of biomass growth. This, our study aimed to investigate the role of ethylene in Se-modulation of antioxidant defence system in response to Cd-stress in MT and *epi* plants, which may lead to enhance Cd stress tolerance in plants.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of the tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom (MT) and *epinastic (epi)* were treated using a sodium hypochlorite solution (5%) to sterilization, rinsed three times and sown in boxes filled with a 1:1 (by volume) mixture of commercial pot mix (Plantmax HT Eucatex, Brazil) and vermiculite supplemented with 1 g L^{-1} 10:10:10 nitrogen-phosphorus-potassium and 4 g L^{-1} lime ($MgCO_3$ + $CaCO_3$). The boxes were maintained in a greenhouse until two true leaves were completely formed. After that, one seedling was transplanted per 0.350 L Leonard vassel (Vincent, 1975) containing sterilized sand, polystyrene (4:3) and modified Hoagland´s nutrient solution, pH 5.6 (250 mL). When plants completed thirty six-day-old, the treatment was applied following (1) control; (2) 0.5 mM of $CdCl_2$; (3) 1μM of selenate; (4) 1 μM of selenate + 0.5 mM of $CdCl_2$; (5) 1 μM of selenite and (6) 1 μM

of selenite + 0.5 mM of $CdCl_2$ in both MT and *epi* plants, which was changed every five days.

After a period of 75 days post germination, corresponding 40 days under Cd and Se exposure, samples of roots, leaves and fruits were collected, rinsed and immersed in liquid N_2 and stored at -80ºC for analysis of lipid peroxidation, H_2O_2, enzyme extraction and protein determination. Tomato plants were separated in root, leaf, and fruit and kept in paper bags for drying (60ºC) until achieve constant weight for dry mass measurement and nutritional analysis.

2.2. Nutritional analysis, Cd and Se content

Dry root, leaf and fruit samples (200.0 mg DW) were submitted to a controlled pressure of 2 MPa, milled and microwave digested in 2 mL 70% HNO_3, 2 mL H_2O_2 and 2 mL Milli-Q water (18.2 MX cm a 25ºC) (Chilimba et al., 2011). The digested samples were diluted to 10 mL with Milli-Q water. The presence and concentration of Cd, Se, P, K, Ca, Mg, S, Cu, Fe, Mn and Zn were determined by radial view inductively coupled plasma optical emission spectrometry (ICP-OES JobinYvon, JY50P Longjumeau, France) equipped with a nebulization chamber. The following emission lines were used: Cd II 228.80; Se II 196.09; P I 213.618 nm; K I 769.897 nm; Ca I 422.673 nm; Mg I 280.270 nm; S I 181.972 nm; Cu I 324.754 nm; Fe II 259.940 nm; Mn II 259.373 nm and Zn II 231.865 nm. The accuracy and precision of the analytical method were assured by the use of standard reference materials – SRM (NIST 1515 – apple leaves, and NIST 1573a – tomato leaves). Elemental recovery from SRM ranged from 93% to 98%.

2.3. Lipid peroxidation

Lipid peroxidation was analysed from the content of thiobarbituric acid (TBA) reactive substances (TBARS) as defined by Heath and Packer (1968). Three independent replicates from each samples of root, leaf and fruit masses were ground with 20% (w/v) polyvinylpyrrolidone (PVPP) and 0.1% trichloroacetic acid (TCA). The mixture was centrifuged at 11,000 xg for 10 min and the supernatant was reacted to

a solution of 20% TCA and 5% TBA and kept in a water bath at 95ºC for 30 min. Samples were then incubated in an ice bath for 10 min to stop the reaction and then centrifuged at 11,000 xg for 5 min. The concentration of malondialdehyde (MDA) equivalents was measured spectrophotometrically between 535 and 600 nm; data were calculated using an extinction coefficient of 1.55 x 10^{-5} mol^{-1} cm^{-1} (Gratão et al. 2012).

2.4. H_2O_2 content

Three independent replicates from each samples of root, leaf and fruit masses were homogenized in trichloroacetic acid (0.1%) and centrifuged at 10,000 x g for 10 min. The supernatant was reacted to 100 mM potassium phosphate buffer (pH 7.5) and 1 M potassium iodide solution. This solution was kept on ice for one hour, the absorbance was measured at 390 nm, and the H_2O_2 content was assessed using a known H_2O_2 concentration curve as a standard following the method of Gratão et al. (2012).

2.5. Ethylene production

The ethylene production analyses were performed on the following day post-harvest. For the analysis, five tomato fruits were placed into 200 mL glass flasks and hermetically sealed for 2 h at 20°C. A 0.5 mL sample of internal atmosphere was collected through a silicone septum fitted in each flask lid and measured by flame ionization gas chromatography using a gas chromatograph (model Trace GC Ultra, Thermo Electron Corporation, MA, USA), which was equipped with two flame ionization detectors (FID), two injectors, two Porapack N columns and one methanator set to 350 °C. The results were determined considering the chromatographic values, fruit mass, flask volume and the time it remained closed. The respiratory rate was expressed in ethylene production in μL kg^{-1} h^{-1} of C_2H_4 (Tezotto-Uliana et al., 2014).

2.6 GSH content

To determine the GSH content, the reaction was analyzed with of 5,5′ -dithiobis (2-nitrobenzoic acid) (DTNB) and Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) incubated in phosphate buffer at 143 mM and pH 7.5 for 10 min at 30°C in a water bath. After the incubation period, the plant extract was added. 2-nitro-5-thiobenzoic acid (TNB) production was determined using UV–vis spectrophotometer at the wavelength of 412 nm (Griffith, 1980).

2.7 Enzyme extraction and protein determination

Enzyme extraction from roots, leaves and fruit was performed by Boaretto et al. 2014. The tissues were homogenized in a cold chilled mortar with a pestle using an extraction buffer containing 100 mM potassium phosphate buffer (pH 7.5), 1 mM ethylenediamine tetraacetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v) insoluble PVPP in 3:1 volume/fresh weight ratio to leaves and fruits and 2:1 to roots. The homogenate was centrifuged at 10,000 xg for 30 min, and the supernatant was stored at -80ºC for further determination of SOD, CAT, GR, GSH-Px and APX activities. The protein concentration was assayed following the method of Bradford (1976) using bovine serum albumin as a standard.

2.8 Superoxide dismutase assay

Superoxide dismutase (SOD) activity was analysed by activity staining using non-denaturing PAGE (Azevedo et al. 1998). Root, leaf and fruit extracts were inserted to non-denaturing-PAGE separation. After that, the gels were rinsed in distilled-deionized water and incubated in the dark for 30 min in 50 mM potassium phosphate buffer (pH 7.8) containing 0.05 mM riboflavin, 0.1 mM nitro blue tetrazolium, 1 mM EDTA, and 0.3% N,N,N´,N´-tetramethylethylenediamine. The gels were rinsed with deionized water and were exposed to artificial light under the water

until the achromatic bands of SOD activity were visible on a purple-stained gel. SOD isoenzymes were differentiated by their sensitivity or inhibition to 5 mM hydrogen peroxide (H_2O_2) or 2 mM potassium cyanide (Azevedo et al., 1998).

2.9 Catalase assay

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

2.10 Ascorbate peroxidase assay

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

2.11 Glutathione reductase assay

The glutathione reductase (GR) activity was determinated by a mixture containing plant extract, 100 mM potassium phosphate buffer (pH 7.5), 1 mM 5, 5'-dithiobis (2-nitrobenzoic acid), 1 mM glutathione disulphide (GSSG) and 0.1 mM NADPH. The rate of decrease of GSSG was monitored by the intensification in absorbance at 412 nm over 1 min. GR activity was measured spectrophotometrically at 30ºC and expressed as μmol min^{-1} mg^{-1} protein as described in Gratão et al. (2012).

2.12 Glutathione peroxidase assay

The glutathione peroxidase (GSH-Px) was determined by Flohé and Gunzler (1984). A mixture containing plant extract, 100 mM potassium phosphate buffer (pH 7.0), EDTA 3 mM, glutathione 0.24U, reduced glutathione 10 mM and sodium azide 1 mM was kept in 37°C for 10 min. The reaction started with the addition of NADPH 1.5 mM and H_2O_2 1.5 mM. GSH-Px activity was assayed by monitoring the NADPH oxidation at 340 nm over 5 min and expressed as nmol min^{-1} mg^{-1} protein.

2.13 Chlorophyll and caroteinoids determination

The chlorophyll and carotenoids content was assayed spectrophotometrically. Fresh leaves (0.50 mg) were added in tube with 2 ml acetone (100%). After shaking for 72 h at 60 g at 4ºC the sample was read in 470 nm for carotenoids and 645 nm and 662 nm for chlorophyll a and b, respectively**.** (Lichtenthaler, 1987).

2.14 Statistical analysis

The experimental design was randomized using six plants per treatment from three replicate pots. The result of three independent replicates of each extract for plant growth, Cd concentration and nutritional analyses, MDA, H_2O_2, mineral elements, ethylene, GSH and GSSG contents, CAT, APX, GSH-Px and GR activities were expressed as the mean and standard error of the mean (±SEM). A multiple comparison between means by the Duncan test followed the application of an individual ANOVA for each characteristic at a 0.05 level of significance. The statistical analyses were performed using Agro Estat® software (Barbosa and Maldonado Júnior, 2015).

3. Results

3.1 Plant growth

After a period of 75 days post germination, corresponding to 40 days of exposure to Cd and Se forms, MT plants exhibited an increase in root growth, being more pronounced with selenate application (Fig. 1A). On the other hand, no difference was found in leaves. Selenite application was more efficient to improve fruits dry mass (Fig. 1C). When MT plants were exposed to Cd stress, all tissues exhibited a pronounced reduction in growth. However, when selenate or selenite were applied we observed that both Se forms were effective to reduce damages caused by Cd. For instance, selenate + Cd enhance 52% in roots (Fig. 1A), 57% in leaves (Fig. 1B) and 33% in fruits (Fig. 1C), when compared to only Cd application. Whereas selenite + Cd enhanced 50% in roots, 54% in leaves and 49% in fruits. In *epi* plants, Se application was not effective to promote plant growth (Fig. 1). Cadmium provoked a reduction of 60% in *epi* roots, leaves and fruits dry mass. No difference was found when Se forms was applied in *epi* plants exposed to Cd.

3.2 Selenium and Cd content

In MT plants, both selenium forms were uptake by plants (Suplementar material 1). However, selenate and selenite exhibited a distinct trend in accumulation and root-to-shoot Se-translocation. For instance, Se concentration in roots of MT plants with selenite application was four times higher than selenite supply. On the other hand, Se exhibited highly translocated to shoots in selenate form, whilst selenite form induced high Se concentration only in roots and restrained Se translocation to leaves and fruits. MT plants treated with selenate + Cd exhibited the same pattern of Se uptake and translocation than plants grown with only selenate application. However, selenite + Cd plants, Cd caused a reduction of 45% in Se accumulation in roots, and also decreased Se translocation to above parts. Mutants *epi* plants exhibited similar results to MT plants in accumulation and translocation of Se forms. However, Se content in roots treated with selenite was not affected by Cd exposure, like found in MT plants.

Cadmium was uptake by plants, translocated, and accumulated in tomato fruits (Supplementary material 1). When Cd content was analysed in MT plants, Se forms application have a pronounced effect in Cd uptake. Selenate + Cd and selenite + Cd treatment induced a decrease of 45% and 41% in root concentration, respectively. However, plants treated with Se forms was not exhibited a decreased of Cd content in fruits. When Cd concentration was analysed in *epi* plants, the root concentration decreased 35% when compared with MT roots exposed to Cd. In selenate + Cd and selenite + Cd treatment, we observed an increase in Cd concentration of 17% and 26% in roots treated with selenate and selenite forms, respectively. Moreover, the Se application induce higher translocation of Cd to fruits of *epi* plants.

3.3 Nutritional analysis

Macronutrients and micronutrients were uptaked and its partitioning varied among plant genotypes depending on the Cd exposure and Se forms treatment (Supplementary material 2). Both Se forms of supply decreased P, K and augmented Mg and Fe content (only to selenate) in MT roots. In *epi* roots, selenate reduced Zn and P and increased Ca contents, when compared with roots of *epi* grown under control condition. Se application in *epi* plants induced an increase in P, Mg, S, K and Zn concentration in fruits, but any changes was found in MT fruits. Cadmium exposure caused a decrease of Mn in MT roots and increase in Zn content, when compared to control plants. Whereas, increased Mn concentration was observed in *epi* roots. In leaves, Cd exposure decreased P, Mg, K and Cu content in MT plants, whilst Cu, Mn and Zn contents decreased in *epi* leaves. In MT and *epi* fruits, Cd treatment caused a reduction in Cu, Mn and Zn contents. The application of Se forms under Cd exposure induced decrease in P and S contents in MT roots, when compared with plants under Cd exposure. When leaves were concerned, selenate + Cd treatment exhibited a decrease in K content, whilst *epi* leaves with selenate and selenite exhibited a decreased in Cu content in the presence of Cd. Se forms + Cd treatment increased in P, S and Zn concentration of *epi* fruits (Supplementary material 2).

3.4 Lipid peroxidation and H_2O_2 content

Lipid peroxidation, expressed in MDA content, was higher in leaves when compared with roots and fruits (Fig. 2). In general, the application of Se forms did not induced an increase in MDA content in roots, leaves or fruits. When MT and *epi* plants grown under Cd exposure, the MDA content increased. Selenate or selenite + Cd treatment induced a reduction of MDA content in MT roots (Fig. 2A) and fruits (Fig. 2C), when compared to control. In *epi* plants, selenite + Cd decreased MDA content in fruits.

The application of Se sources not caused expressive alteration in H_2O_2 content in both MT and *epi* plants. Roots (Fig. 2D), leaves (Fig. 2E) and fruits (Fig. 2F) of MT and *epi* plants exhibited a pronounced increase in H_2O_2 content in the presence of Cd, in contrast to observed for *epi* roots. Roots and fruits of MT plants grown under selenate or selenite + Cd exhibited a reduction in H_2O_2 content (Fig. 2D-E).

3.5 Chlorophylls and carotenoids content

Both Se sources did not change chlorophylls and carotenoids contents in MT and *epi* genotypes (Supplementary material 3). On the other hand, MT and *epi* plants exhibited pronounced reduction in chlorophylls and carotenoids contents under Cd exposure, irrespective to Se forms. The only exception was in *epi* plants following Cd and selenate applications, which exhibited an increase of 11% and 14% in chlorophylls and carotenoids, respectively, when compared with *epi* plants under Cd exposure.

3.6 Ethylene production

There was no significant differences in ethylene production between fruits of tomato genotypes (Supplementary material 4). The application of Se did not induce alterations in ethylene production in fruits of MT and *epi* plants, when compared to control plants. With exception, fruits of MT plants with selenite exhibited an increased in ethylene production. Fruits of plants submitted to Cd, exhibited a pronounced

increase in ethylene production, being more pronounced in *epi* plants (50% higher than MT). MT fruits exhibited an increase of 97% in ethylene production when compared with control plants, whereas fruits with Se exhibited an increase of 40% and 50% in ethylene production, with selenite and selenate, respectively. By the other way, fruits of *epi* plants exhibited an increase of 95% in ethylene production in the presence of Cd, whereas fruits with Se exhibited an increase of 45% and 62% in ethylene production, with selenate and selenite, respectively (Supplementary material 3).

3.7 GSH content

GSH content was not affected by Se forms application in roots and fruits of MT plants, but increased 32% with selenite application in leaves, when compared with control plants (Supplementary material 5). However, GSH content increased 84% in roots in the presence of Cd. Se forms + Cd treatment decreased GSH content in roots and fruits.

The *epi* plants exhibited lower GSH content in leaves and fruits than MT plants (Supplementary material 5), whereas the application of Cd improved GSH content in all tissues, when compared with control *epi* plants. Fruits of *epi* plants exhibited an increase of GSH content in the presence of Cd, irrespective to Se sources.

3.8 Antioxidant enzymes activities

Three common SOD isoenzymes were detected in roots (Fig. 3A), leaves (Fig. 3B) and fruits (Fig. 3C), which were characterized as Mn/ SOD (SOD I), Fe/SOD (SOD II) and Cu/Zn-SOD (SOD III). Changes were observed between tissue type and Se and Cd treatment. Following Cd application, the activity of SOD decreased in all tissues and genotypes, although the SOD activity was differently affected, independent of the Se sources, leading to an increase in SOD activity. For instance, SOD I and SOD II exhibited an increase in MT roots with selenite and Cd, (Fig 3A – lane 5), whereas *epi* roots exhibited increased SOD II acivity with selenate and Cd (Fig 3A – lane 9).

The activities of SOD II and SOD III increased in leaves of MT plants grown with selenite application (Fig 3B – lane 5), whilst leaves of *epi* plants exhibited high SOD II and III activities following Cd exposure, irrespective to Se forms (Fig 3B – lanes 9-12). In MT fruits, the changes in SOD activity was less intense, and the selenite application caused an increase of SOD II and SOD III activities (Fig 3C – lane 3), when compared with control plants (Fig 3C – lane 1).

CAT (Fig. 4), APX (Fig. 5), GR (Fig. 6) and GSH-Px (Fig. 7) activities are crucial for the detoxification of any excess H_2O_2 produced by SOD and/or by other metabolic processes. Leaves of MT plants exhibited an increased CAT activity with selenite application (Fig. 4B). On the other hand, all tissues and genotypes exhibited a pronounced increase of CAT activity under Cd exposure. With respect to Se application, roots of *epi* plants, exhibited an increase of 21% in CAT activity in the presence of selenite and Cd (Fig. 4A).

In response of Se application, roots and fruits of MT and *epi* plants exhibited augmented APX activity (Fig. 5). The Cd exposure caused a pronounced increase in APX activity of all tissues analysed, mainly in the presence of both Se sources. Selenate + Cd treatment induced an increased of 22% in APX activity in *epi* roots, while selenite + Cd treatment increased 26% in APX activity.

Both Se sources increased GR activity mainly in *epi* plants exposed to Cd (Fig. 6). In the presence of Cd, GR activity increased in all tissues analysed. Roots and leaves of *epi* plants exhibited low GR activity when compared to MT genotype. Roots of *epi* plants showed a decreased GR activity with selenite + Cd treatment, in contrast to observed for roots submitted to selenite + Cd. A distinct trend was observed in *epi* fruits, where Se forms + Cd treatment increased GR activity under Cd exposure (Fig. 6C).

Application of selenate or selenite did not change GSH-Px activity in MT roots, but caused a pronounced increase in GSH-Px activity in *epi* roots (Fig. 7A). On the other hand, both Se sources induced an increase in GSH-Px activity of MT fruits (Fig. 7C). Cadmium exposure caused an increase in GSH-Px activity in leaves, being more intense to MT plants, irrespective to Se application.

4. Discussion

Environmental stresses, including Cd, may cause serious imbalance to plant metabolism, and bring losses to crop production (Asgher et al, 2014). Selenium application is an interesting strategy that can reduces the adverse effects of the abiotic stress by modulation of different mechanisms, including activity of antioxidante enzymes and antioxidant compunds (Khan et al. 2015b; Alyemeni et al., 2018; Silva et al., 2018). Based on this hypothesis, we used tomato MT and hormonal mutant *epi* to verified the role of ethylene in modulate Se-attenuation of Cd stress conditions in tomato genotypes with distinct ethylene perception during Cd stress.

Although Se is not considered an essential nutrients to plants according to direct criteria proposed by Stout and Arnon (1939), this element may improve plant growth of several crops (Castillo-Godina, et al., 2016; Reis et al., 2018). In our study, MT plants treated with Se exhibited an increase in root growth, been more pronounced with selenate application (Fig. 1A), whilst selenite form was more efficient to improve fruits dry mass (Fig. 1C). However, Se application was not effective to promote plant growth *epi* plants (Fig. 1). It is well known that Cd provokes negative affects to plant growth (Rizwan et al., 2017). When MT plants were exposed to Cd stress, all tissue exhibited an intense reduction in growth (Fig. 1).

However, when selenate or selenite were applied, both Se sources were effective to protect the cell membranes damages caused by Cd. For instance, selenate + Cd increased 52% of dry mass in MT roots (Fig. 1A), 57% in leaves (Fig. 1B) and 33% in fruits (Fig. 1C), when compared to plants with only Cd application. Whereas selenite + Cd increased 50% of dry mass in roots, 54% in leaves and 49% in fruits. Selenate and selenite exhibit distinct distribution and accumulation pattern in plant tissue (Li et al., 2008). Se sources distribution may explain why Se may reverse damages caused by Cd in different ways. In *epi* plants, Cd caused a reduction of 60% in roots, leaves and fruits dry masses (Supplementary material 1). However, no difference was found in *epi* plants grown under Se sources + Cd treatment. Thus, the different ethylene perception in *epi* metabolism may alter the role of Se. For instance, Khan et al. (2015) demonstrated that plants exposed to ethylene biosynthesis inhibitor may reverse the effects of Se on Cd-induced photosynthesis, which could reflect an increase in plant growth. As well as, high ethylene production could reverse

the effects of Se in growth of plants under Se+Cd treatment.

In MT and *epi* plants, Se concentration increased in root, leaf and fruit in response to Se sources (Supplementary material 1). Se content was four times higher in MT roots supplied with selenite in constrast to selenate. Selenate is generally soluble, mobile and quickly available for plant uptake, while selenite is rapidly converted in selenocysteine and selenomethione in roots showing less translocation to shoots (Kaur et al., 2018). Similar results were found in wheat (Kaur et al., 2018), *Brassica* (Sharma et al., 2010; Yu et al., 2019), which report that selenate showed higher Se translocation from root to shoot when compared to selenite. Interestingly, selenate was more translocated from roots to shoots for *epi* than MT plants (Supplementary material 1). Ethylene induces an increase in levels of sulphate transporter and S assimilatory genes (Tamaoki et al., 2008; Wang et al., 2018). Selenate is uptake by plants via S transport system (Shinmachi et al., 2010), thus, our data in *epi* hormonal mutant reinforce that ethylene may be involved in Se uptake and transport.

In MT plants treated with selenite + Cd, Cd caused a reduction of 45% in Se content in roots, and also decreased Se translocation to shoot. Se ions are co-transported with Cd ions by the same protein carriers, resulting in the decrease of Cd in metabolically active membrane sites (Lin et al., 2012), but also may reduce selenite content. In *epi*, Se content in roots was not affected in selenite + Cd treatment, like found in MT plants. Probably it can be due *epi* plants exhibited higher expression of genes involved to sulphate transporter (Wang et al., 2018), that also may transport Se.

Cadmium can be uptaken by root cells through different channels and via several metal transporters, which are controlled by parameters such as Cd content, plant species, and nutrients levels (Ismael et al., 2019). When Cd content was analysed in MT plants treated with selenate or selenite, we note that Se application strong affected Cd uptake. Selenate + Cd and selenite + Cd treatments induced a decrease of 45% and 41% in root Cd content, respectively. It probably is related with the competition of Se an Cd for the same specific binds sites in proteins, causing a reduced Cd uptake and avoid its toxicity to plant metabolism (Lin et al., 2012). Moreover, Se may contribute to the sequestration of Cd in the root, trigger a

reduction in the Cd content in shoot of plants (Yu et al., 2019). When Cd content was analysed in *epi* plants, the root Cd-content decreased 35%, when compared with MT exposed to Cd. Thus, the *epi* plants, that exhibits high ethylene production induced a reduction in Cd content. Similar results were found by Chen et al. (2017), where exogenous ethylene application reduced Cd content in *Catharanthus roseous.* When Se was applied, we observed an increase of 17% and 26% in roots Cd content following selenate and selenite forms, respectively. Moreover, Se application can induce higher translocation of Cd to fruits of *epi* plants (Supplementary material 1). At low Cd concentrations, Se may decrease Cd uptake in paddy rice, however, at high Cd concentration, the addition of Se increased the Cd uptake, especially at high Se concentrations (Feng et al., 2013a). MT and *epi* plants were exposed to the same Se and Cd concentrations and exhibited distinct trends of Cd content in tissues. Probably, it indicates that ethylene signalling or perception may alter the competition of Se and Cd for the same specific binds sites in proteins.

The interactions between Se uptake and other mineral elements reported ambiguous conclusions and remains undefined. Some reports have confirmed our results that adequate Se concentrations could improve the uptake of some mineral elements, such Mg and Fe in MT roots, and Ca in *epi* roots (Li et al., 2018). Se application in *epi* plant induced an increase in P, Mg, S, K and Zn content in fruits, in contrast to observed in MT fruits (Supplementary material 2). On the other hand, application of Se sources decreased P, K in MT roots. In *epi* roots, Se forms reduced Zn and P. Se uptake also can decrease the content of other elements, induced by ionic effect of antagonism (Feng et al., 2009). Thus, the effects of Se on the uptake of other mineral elements depend on Se concentrations, Se sources, and concentrations of other elements. Cd and other cations compete for metal uptake channels in root surface and metal transporters within plants. This competition subsequently may lead to a deficiency in essential metals and cause negative impacts on the mineral content in most of the plants grown under Cd exposure, which induce nutrient deficiency (Khan et al., 2015a; Ismael et al., 2019).

In our data, Cd exposure caused a decrease of Mn in MT roots and an increase in Zn content, when compared to control plants. Whereas, increased Mn content was found in *epi* roots. In leaves, Cd exposure decreased P, Mg, K and Cu content in MT,

whereas decreased Cu, Mn and Zn in *epi* leaves, and increased P and Mg content. In MT and *epi* fruits, Cd treatment caused a reduction of Cu, Mn and Zn content. The decreased content of Cu, Zn and Mn in leaves and fruits of plants exposed to Cd could be part to reduced root growth (Fig. 1). Similar results were found by Jinadasa et al. (2016), probably due to competition for transport channel at the root surface, formation of non-absorbable complexes in the solution (Smeyers-Verbeke et al., 1978), and inhibited nutrient transport in the xylem (Lamoreaux and Chaney, 1977).

Selenium forms +Cd treatment caused P and S content reduction in MT roots, when compared with plants under Cd exposure. When leaves were concerned, selenate + Cd treatment occurred an decrease in K content in leaves of MT, and selenate and selenite + Cd treatment caused a decreased in Cu in *epi* leaves. Se forms + Cd treatment caused an improvement in P, S and Zn content of *epi* fruits (Supplementary material 2). Although Cd and Se effects on the nutrient content are known in different plant tissues, the mechanism of how these elements affect nutrients is still unclear (Ismael et al., 2019). Certainly, the alterations caused by Se loading and unloading of elements maybe related with ethylene modulation.

Studies shows that adequate Se concentrations may protect plants against oxidative stress trigged by Cd (Saidi et al., 2014; Castillo-Godina et al., 2016). MT and *epi* plants submitted to Cd exhibited an increase in lipid peroxidation rate, expressed as MDA content and an overproduction of H_2O_2 content in roots (Fig. 2A-D), leaves (Fig. 2B-E) and fruits (Fig. 2C-F), when compared to control plants. H_2O_2 is a dangerous ROS that interfering with the electron transport chain, leading to damaged cell membrane and causing an imbalance in plant metabolism (Alves et al., 2017). Selenium may act in the improvement of ROS scavenging, and change membrane physicochemical characteristics, which contribute to plant cell integrity (Lin et al., 2012). Selenium sources + Cd treatment demonstrated that Se may alleviate Cd induced oxidative stress due to alteration in H_2O_2 and MDA contents (Fig. 2), and to restore cell membrane. For instance, MT plants with selenate or selenite + Cd exposure trigged a reduction in MDA and H_2O_2 content in roots (Fig. 2A-D) and fruits (Fig. 2C-F), when compared to Cd exposure. In roots of *epi* plants, selenite + Cd induced an increase in MDA and H_2O_2 contents. Ethylene plays a crucial role in responses against abiotic stress (Massod et al., 2012). However, the

up regulation in ethylene biosynthesis of *epi* plants may induce an increased MDA and H_2O_2 contents.

Although Se may enhance photosynthesis rate (Khan et al., 2015b; Andrade et al., 2018), selenate and selenite supply did not change the chlorophylls and carotenoids content in MT and *epi* genotypes (Supplementary material 3). Cadmium toxicity can reduce the chlorophyll content of several plant species (Lysenko et al., 2015; Muradoglu et al., 2015). Our data exhibited that Cd exposure also reduced chlorophylls and carotenoids content in all treatments. It can be due Cd can accumulate in chloroplasts, which exhibit changes in organelle shape and internal organization (Gratão et al., 2009). These alterations in the chloroplasts of plants exposed to Cd may occur due to an increase in ROS production in the leaves (Fig. 2). Selenium has a protective effect against damages caused by Cd in *epi* plants following selenate + Cd exposure, that exhibited an increase of 11% in chlorophylls and 14% in carotenoids, when compared with *epi* plants under only Cd exposure (Supplementary material 3). It can be due Se restored membrane lipids, structure and properties of envelope membranes (Filek et al., 2010). Moreover, the ethylene perception may influence the role of Se under Cd stress, due to MT leaves not exhibit these improvements in chlorophylls and carotenoids content. Khan et al., (2014) reports that under metal stress, ethylene protected photosynthetic potential by through improvement of PS II activity and increased activity of ribulose-1,5-bisphosphate carboxylase and photosynthetic nitrogen use efficiency.

Although Se application may delay senescence (Pezzarossa et al., 2014), no significant alteration was observed between ethylene production of MT and *epi* fruits, when compared to control plants (Supplementary material 4). With exception to fruits of MT treated with selenite, that exhibited an increased ethylene production. Cd stress induces ethylene production, triggering senescence of fruits (Yu et al., 2018). In this study, we found a similar results, where Cd exposure caused an intense enhance in ethylene production of 97% in MT and 95% in *epi* fruits, compared with controls plants. On the other hand, plants with selenate + Cd exposure exhibited an increased of 40% in ethylene production, whereas selenite + Cd exposure exhibited an increased of 50% in ethylene production. The *epi* plants with selenate + Cd exposure exhibited an increase of 45% in ethylene production, whilst plants with

selenite + Cd exposure exhibited an increase of 62%, when compared with fruits of *epi* plants grown under Cd application (Supplementary material 4).

The ascorbate-glutathione cycle is an efficiently strategy of antioxidant system to eliminate the excessive H_2O_2 production. Se may modulate this cycle though induce the expression of genes coding for GSH-related enzymes (Wang et al., 2018). MT leaves exhibited an increase of 32% in GSH content with selenite application, when compared with control plants (Supplementary material 5). Moreover, Se may be involved in modulate GSH responses though modulation of ethylene, once there are evidences regarding a close interplay between ethylene and GSH metabolism during metal stress (Keunen et al., 2016). In this study, *epi* plants exhibit a distinct GSH content when compared with MT plants, mainly with Se application. For instance, *epi* plant exhibit lower GSH content than MT plants without Cd, whereas both Se forms caused an increase in GSH content only in *epi* fruits. (Supplementary material 5). Moreover, Se forms can stimulate the efficiency of ascorbate-glutathione cycle under Cd stress by increasing the GSH and ascorbate (AsA) concentrations in Chinese cabbage tissues (Wu et al., 2017). In the presence of Cd, the GSH content in MT roots enhance 84%. GSH and phytochelatins have high affinity for metal, and can sequestrate Cd (Clemens et al., 2013; Rabêlo et al., 2018a,b). MT fruits exhibited a decrease in GSH content when submitted to Se and Cd, in contrast to observed by *epi* fruits. Moreover, Se can contribute to the sequestration of the metal by stimulating the production of GSH (Kumar et al., 2014). Plants with both Se forms and cadmium exposure exhibited an increased GSH levels, which may contribute with reverse reduced plant growth caused by Cd (Fig. 1B).

Selenium application may improve antioxidant defence systems against stresses by enhancing the expression of genes and increased enzymes activities related to stress responses. It allows plants to avoid or tolerate oxidative stress and survive Cd contamination (Wang et al., 2018). Selenium may plays a role in ethylene that regulates antioxidant defence mechanisms and alleviates adverse effects of Cd stress on photosynthesis and growth (Khan et al., 2015b). In this study, we selected key antioxidant enzymes SOD, CAT, APX, GR and GSH-Pox based on several reports on their responses to Cd stress in plants.

SOD, which converts $O^{\cdot -}$ into H_2O , has been shown to be induced in a number

of plant species treated with Se under Cd stress (Wu et al., 2017). Different isoenzymes contribute to cell protection against different toxic substances, such as Cd (Aksmann et al., 2014). In this study, three SOD isoenzymes (SOD I, II and III) were identified following non denaturing PAGE analysis (Fig. 3), with a similar isoenzyme classification as previously reported by Gratão et al. (2012, 2015) and Alves et al. (2017) for MT plants. Three common SOD isoenzymes were detected in roots (Fig. 3A), leaves (Fig. 3B) and fruits (Fig. 3C), which were characterized as Mn/ SOD (SOD I), Fe/SOD (SOD II) and Cu/Zn-SOD (SOD III). It is clear that Cd exposure trigger reduction of SOD activity in all tissue and genotypes. Cd exposure may decline SOD activity due to the fact that heavy metals disturb plant metabolism such as the synthesis of enzyme protein (Wu et al., 2017).

However, the application of Se forms caused different responses in tissues and may enhance SOD activity in specifics treatments. For instance, SOD I and SOD II exhibited an improvement in MT roots with selenite and Cd application (Fig 3A – lane 5), and selenate treatment in *epi* roots (Fig 3A – lane 9). SOD II and SOD III increased in leaves of MT plants grown with selenite application (Fig 3B – lane 5) and *epi* leaves , irrespective to Se and Cd application (Fig 3B – lanes 9-12). In fruits, the changes in SOD activity was less pronounced, and the selenite application in MT caused an increase of SOD II (Fig 3C – lane 3), when compared with control plants (Fig 3C – lane 1). Cao et al. (2006) demonstrated higher transcription of Cu/Zn SOD in ethylene insensitive *ein2-1 A. thaliana* mutants, indicating that ethylene may affect plants antioxidant defence. Therefore, it is possible to indicates Se modulates ethylene and SOD activity under normal or Cd-stressful condition.

High H_2O_2 content in response to SOD activity or other metabolic activities may be reduced to H_2O by the action of CAT, APX and other peroxidases. Selenium application improved the activity of antioxidant enzymes under normal and Cd stress conditions, providing additional protection to tomato plants to counteract Cd stress (Alyemeni et al., 2018). However, among enzymes analysed in this study, Se application cause upregulation mainly in APX activity of roots (Fig. 5A) and fruits (Fig. 5C) of MT and *epi* plants (Fig. 5), while Se application did not alter CAT activity, with exception for MT leaves, where selenite application caused an increase in CAT activity (Fig. 4B). On the other hand, Cd exposure upregulated the activity of CAT

(Fig. 4), APX (Fig. 5) and GR (Fig. 6) activities in all tissues and genotypes. These finding were observed by other author, such as Khan et al. (2015b) in *Triticum aestivum* and Alyemeni et al. (2018) in tomato. Although Se application did not cause strong changes in enzymes activities, the application of Se under Cd expusure induced the enhancement of CAT, APX and GR activities. For instance, plants with selenate + Cd exhibited a increase of 21% in CAT activity in *epi* roots, when compared with *epi* following only Cd exposure. This difference was not evident in MT roots (Fig. 4A). Therefore, ethylene could mediate the upregulate CAT activity trigged by selenate. This hypothesis could be reinforced by Cao et al. (2006), that demonstrate that CAT3 genes exhibited higher transcription in ethylene sensitive *ein2-1* mutants plants.

Roots subjected to both Se sources and Cd exposure exhibited an increase in APX activity, mainly in *epi* genotype (22% higher to selenate and 26% to selenite), which reinforce that APX activity also may influenced by interplay between Se and ethylene. Selenate or selenite influenced GR activity mainly in *epi* plants exposed to Cd (Fig. 6). The *epi* plants exhibit low GR activity in roots and leaves, when compared with MT genotype. MT roots treated with selenite + Cd application exhibited a increased GR activity, when compared with plants submitted to selenate + Cd or Cd alone. A distinct trend was observed in *epi* fruits, where Se forms application increased GR activity under Cd exposure (Fig. 6C). The application of Se forms not altered GSH-Px activity in MT roots, but caused a pronounced increase in GSH-Px activity in *epi* roots (Fig. 7A). In fruits (Fig. 7C), Se sources induced an increase in GSH-Px activity of MT genotype. Cadmium exposure enhanced GSH-Px activity only in leaves, being more pronounced in MT leaves without Se application.

Thus, it is possible to note different regulation between MT and *epi* plants, which demonstrate that Se positive effects in modulate of antioxidant systems may be ethylene-dependent. The enhancement of these enzymes may alleviate the consequences of ROS to plant metabolism induced by Cd exposure. Khan et al. (2015b) indicates that Se increased APX and GR activities in *B. juncea*, thus protecting photosynthetic electron transport by Cd oxidative damages via preservation of NADP/ NADPH ratio. In rapeseed, adequate Se application improved growth by enhancing ascorbate-glutathione cycle (Kaklewski et al., 2008). Therefore,

Se may maintain higher activity of antioxidant enzymes, and it can be achieve thought ethylene-modulation of Se responses against Cd stress and help to protected tomato plants from oxidative damage to cellular macromolecules, including lipids, proteins, and nucleic acids (Khan et al., 2015b; Ahmad et al. 2016).

5. Conclusion

Se may promote positive alteration in antioxidant metabolism and nutrient uptake in plant tissue under non stress and stress conditions. Application of Se promotes regulation of GSH biosynthesis, activity of antioxidant enzymes and alleviates adverse effects of Cd stress on growth. The distinct physiological response between MT and tomato mutant *epi* plants indicates that ethylene may be involved in Se induced-defence responses, that triggers a positive response of antioxidant system and improve growth under Cd stress.

Further studies using ethylene-insensitive *Never ripe* tomato mutant, may help to explore more details about how ethylene signalling works in the Se-modulation of antioxidant system under normal or stressful conditions. Such responses based on redox signalling pathways involving Se and ethylene in the control of growth and cross-tolerance to stress can be used to manipulate ethylene regulated gene expression to induce heavy metal tolerance.

6. Acknowledgement

This work was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - Grant n° 2017/04787-6). PLG also thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the research fellowship (Grant nº 314380/2018-3) - Brazil. We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001- for scholarship granted (L.R.A). This work was partial financially supported by a grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant number 448783/2014-2). ARR also thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the research fellowship (Grant nº 309380/2017-0).

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8. Figures legends

Fig. 1. Roots (A), leaves (B) and fruits (C) dry mass (g dry wt) in MT and *epi* plants grown under Cd and Se forms. Different letters on the top of the columns indicates significantly different at $P < 0.05$ by Duncan test.

Fig. 2. Content of lipid peroxidation measured as malondialdehyde (MDA) (nmol g^{-1} fresh weight) in roots (A), leaves (B) and fruits (C) and hydrogen peroxide (H_2O_2) (µmol g^{-1} fresh weight) in roots (D), leaves (E) and fruits (F) of MT and *epi* plants grown under Cd and Se forms. Different letters on the top of the columns indicates significantly different at $P < 0.05$ by Duncan test.

Fig. 3. Superoxide dismutase (SOD) activity stained following non-denaturing polyacrylamide gel electrophoresis of roots (A), (B) leaves and fruits (C) isolated from MT and *epi* plants grown under Cd and Se forms. The lanes listed are: (S) bovine SOD standard; (1) MT (-Cd), (2) MT (+Cd), (3) MT with Selenate (-Cd), (4) MT with Selenate (+Cd), (5) MT with Selenite (-Cd), (6) MT with Selenite (+Cd), (7) *epi* (-Cd), (8) *epi* (+Cd), (9) *epi* with Selenate (-Cd), (10) *epi* with Selenate (+Cd), (11) *epi* with Selenite (-Cd), (12) *epi* with Selenite (+Cd). The SOD isoforms are (I) Mn-SOD, (II) Fe-SOD and (III) Cu/Zn-SOD.

Fig. 4. CAT specific activity (µmol min^{-1} mg^{-1} protein) in roots (A), leaves (B) and fruits (C) dry mass (g dry wt) in MT and *epi* plants grown under Cd and Se forms. Different letters on the top of the columns indicates significantly different at $P < 0.05$ by Duncan test.

Fig. 5. APX specific activity (nmol min^{-1} mg^{-1} protein) in roots (A), leaves (B) and fruits (C) dry mass (g dry wt) in MT and *epi* plants grown under Cd and Se forms. Different letters on the top of the columns indicates significantly different at $P < 0.05$ by Duncan test.

Fig. 6. GR specific activity (μmol min^{-1} mg^{-1} protein) in roots (A), leaves (B) and fruits (C) dry mass (g dry wt) in MT and *epi* plants grown under Cd and Se forms. Different letters on the top of the columns indicates significantly different at $P < 0.05$ by Duncan test.

Fig. 7. GSH-Px specific activity (nmol min^{-1} mg^{-1} protein) in roots (A), leaves (B) and fruits (C) dry mass (g dry wt) in MT and *epi* plants grown under Cd and Se forms. Different letters on the top of the columns indicates significantly different at $P < 0.05$ by Duncan test.

9. Supplementary material legends

Supplementary Material 1. Cd and Se content in roots, leaves and fruits (mg/kg dry wt) in MT and *epi* plants grown under Cd an Se forms. Different letters indicates significantly different at $P < 0.05$ by Duncan test.

Supplementary Material 2. Nutritional analysis of macronutrients (mg/kg dry wt) and micronutrients (mg/kg dry wt) in MT and *epi* roots, leaves and fruits grown under Cd and Se forms. Different letters indicates plants with significantly different at $P < 0.05$ by Duncan test.

Supplementary Material 3. Total chlorophyll and carotenoids content (μg cm^{-2}) measured in leaves of MT and *epi* plants grown under Cd and Se forms. Different letters on the top of the columns indicates significantly different at $P < 0.05$ by Duncan test.

Supplementary Material 4. Ethylen production (μL C_2H_4 kg/h) measured in fruits of MT and *epi* plants grown under Cd and Se forms. Different lowercase indicates plants with significantly different at $P < 0.05$ by Duncan test.

Supplementary Material 5. GSH content (nmol/g FW) measured in MT and *epi* roots, leaves and fruits grown under Cd and Se forms. Different lowercase indicates plants with significantly different at $P < 0.05$ by Duncan test.

10. Figures and supplementary material

Figure 1

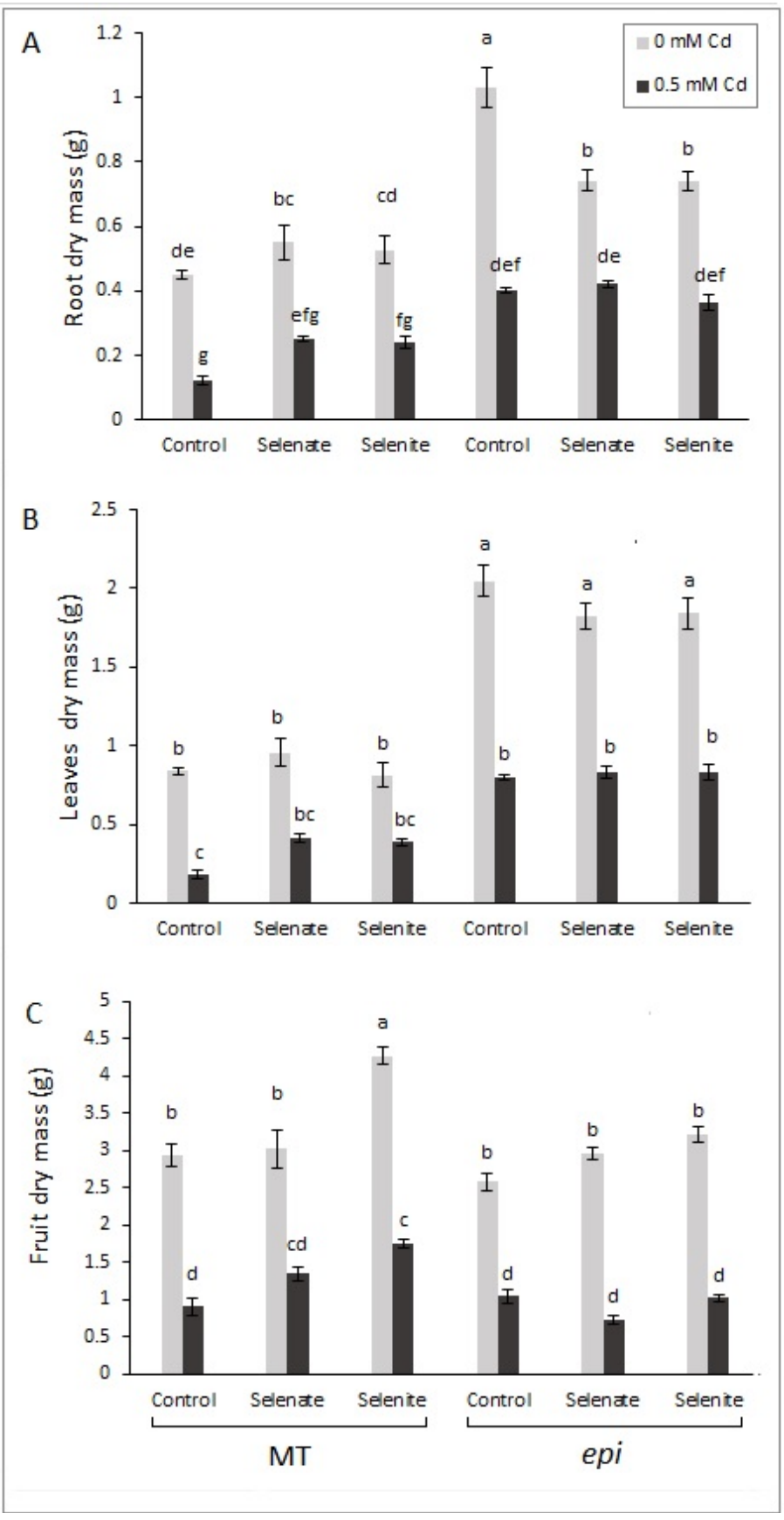


Figure 2

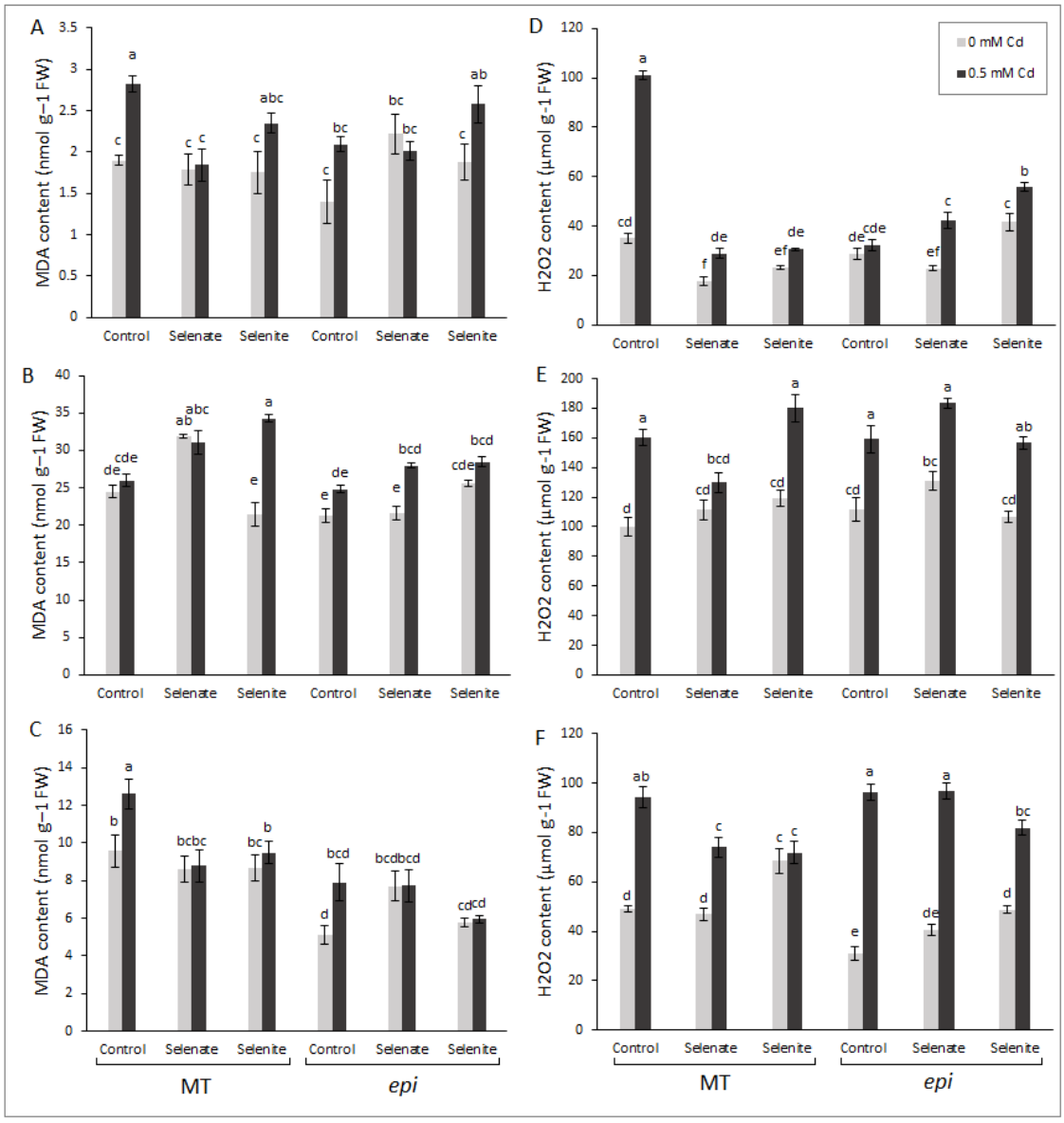

A
MDA content (nmol g−1 FW)
B
MDA content (nmol g−1 FW)
C
MDA content (nmol g−1 FW)
D
H2O2 content (µmol g-1 FW)
0 mM Cd
0.5 mM Cd
E
H2O2 content (µmol g-1 FW)
F
H2O2 content (µmol g-1 FW)
Control
Selenate
Selenite
MT
epi

Figure 3

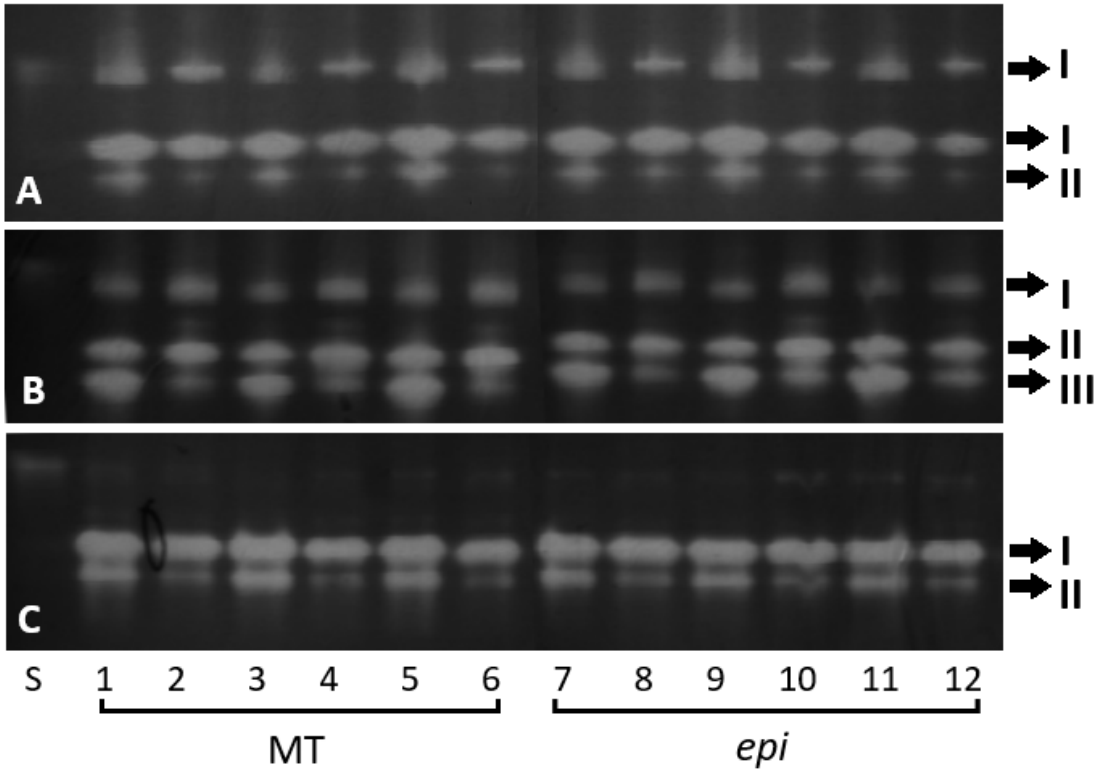


Figure 4

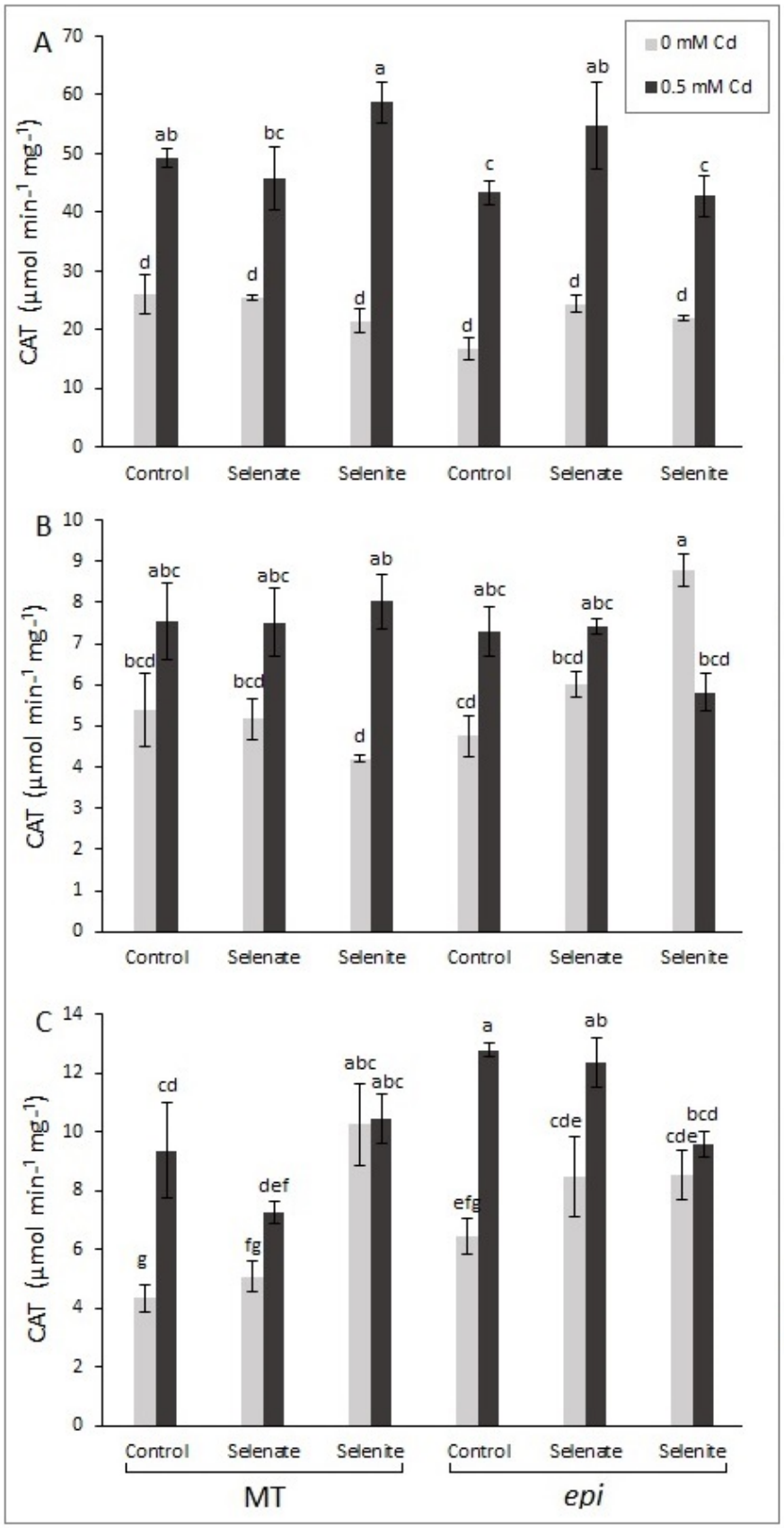

A
B
C
0 mM Cd
0.5 mM Cd
CAT (µmol min-1 mg-1)
Control
Selenate
Selenite
MT
epi

Figure 5

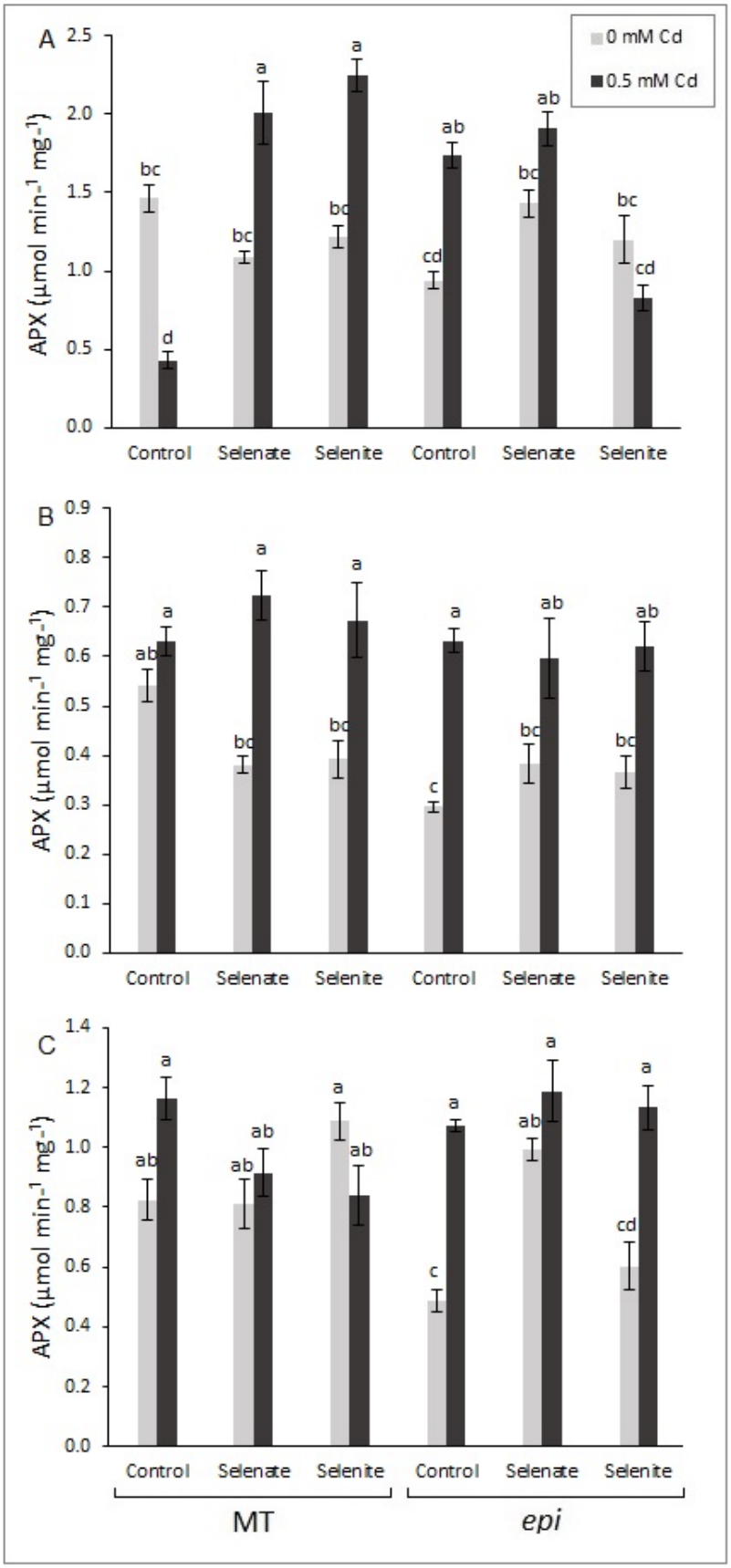

A
B
C
0 mM Cd
0.5 mM Cd
APX (µmol min-1 mg-1)
Control
Selenate
Selenite
MT
epi

Figure 6

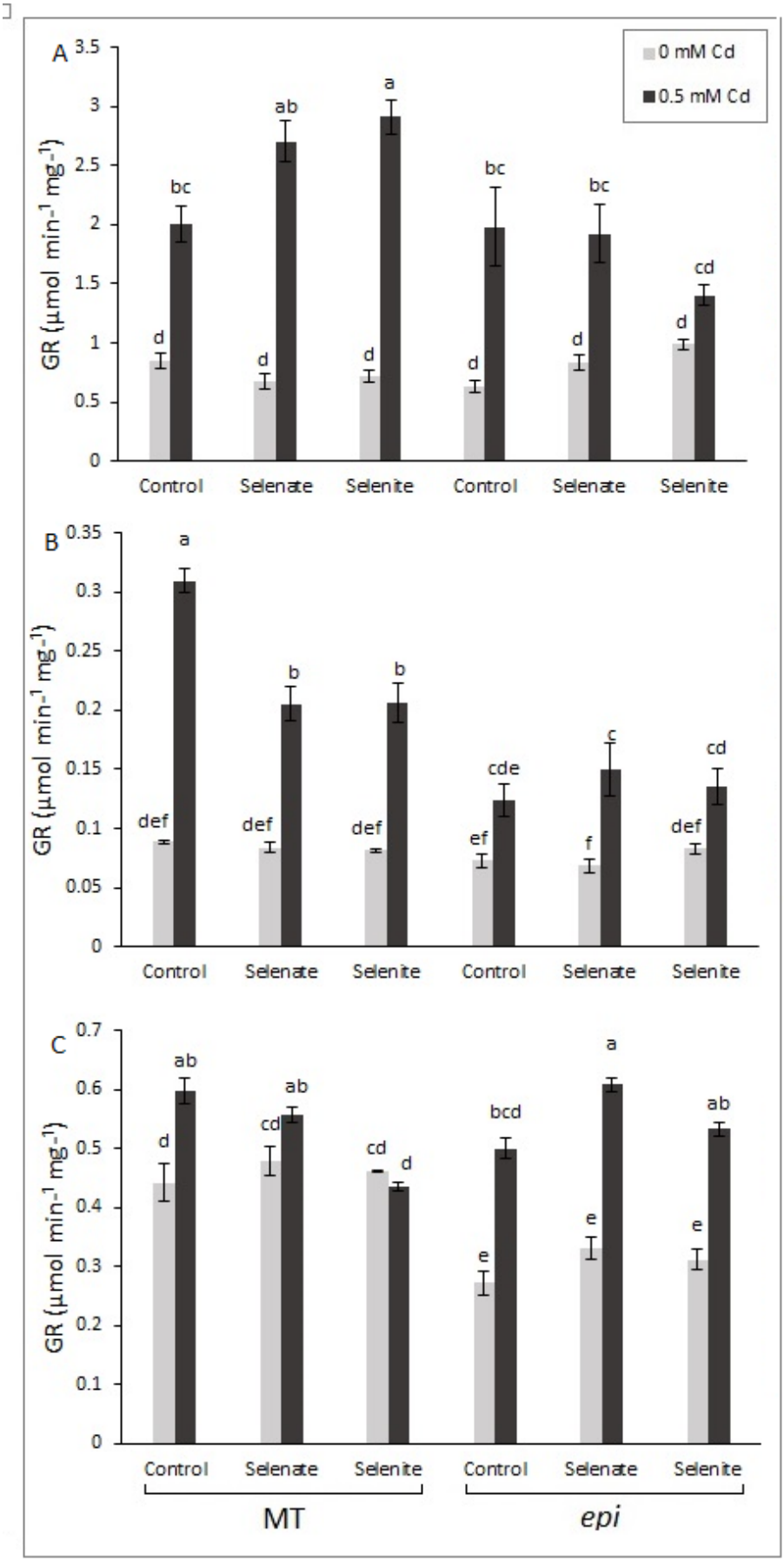
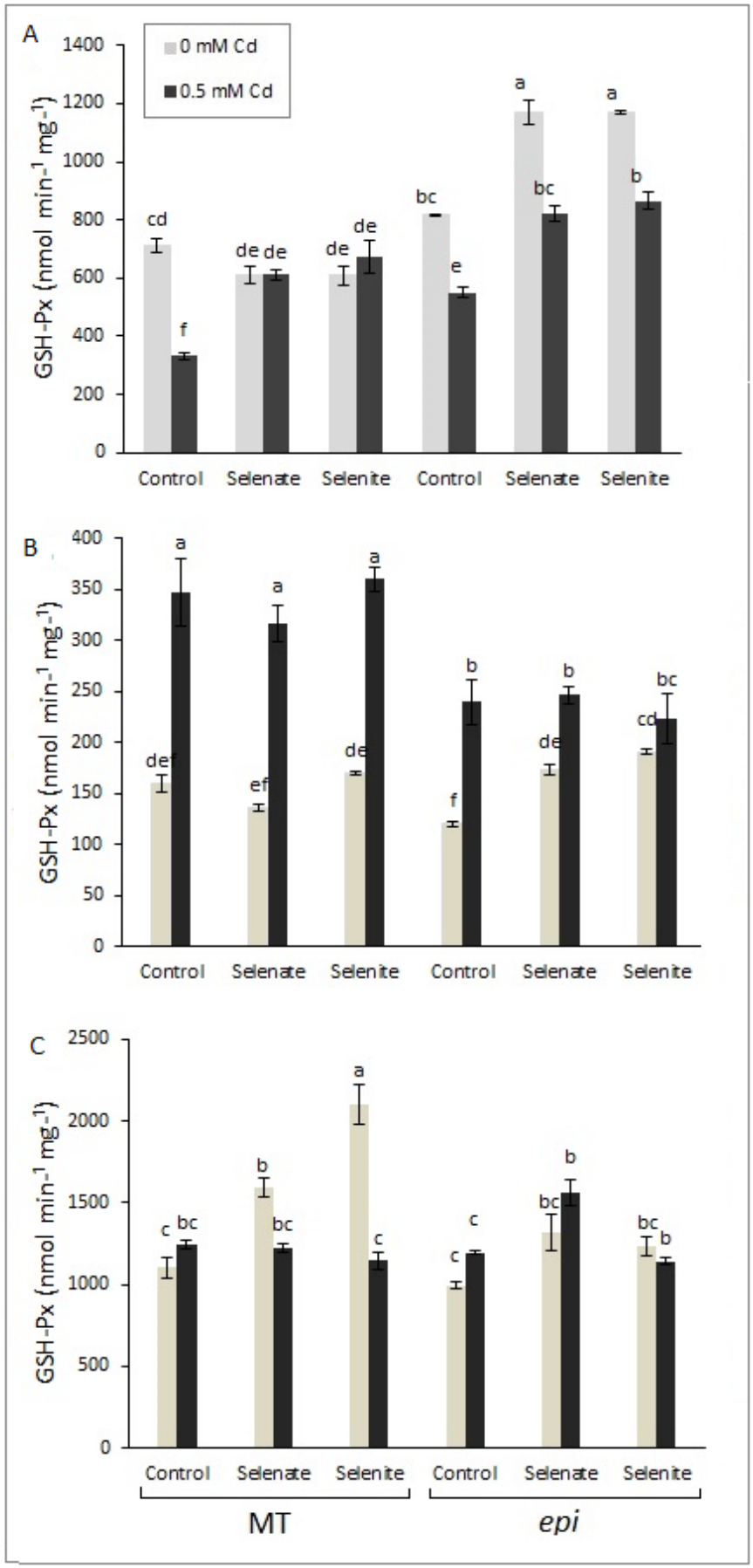

A
B
C
0 mM Cd
0.5 mM Cd
GR (µmol min-1 mg-1)
Control
Selenate
Selenite
MT
epi

Figure 7


A
0 mM Cd
0.5 mM Cd
GSH-Px (nmol min-1 mg-1)
1400
1200
1000
800
600
400
200
0
cd
f
de de
de
de
bc
e
a
bc
a
b
Control
Selenate
Selenite
Control
Selenate
Selenite
B
GSH-Px (nmol min-1 mg-1)
400
350
300
250
200
150
100
50
0
def
a
ef
a
de
a
f
b
de
b
cd
bc
Control
Selenate
Selenite
Control
Selenate
Selenite
C
GSH-Px (nmol min-1 mg-1)
2500
2000
1500
1000
500
0
c
bc
b
bc
a
c
c
c
bc
b
bc
b
Control
Selenate
Selenite
Control
Selenate
Selenite
MT
epi

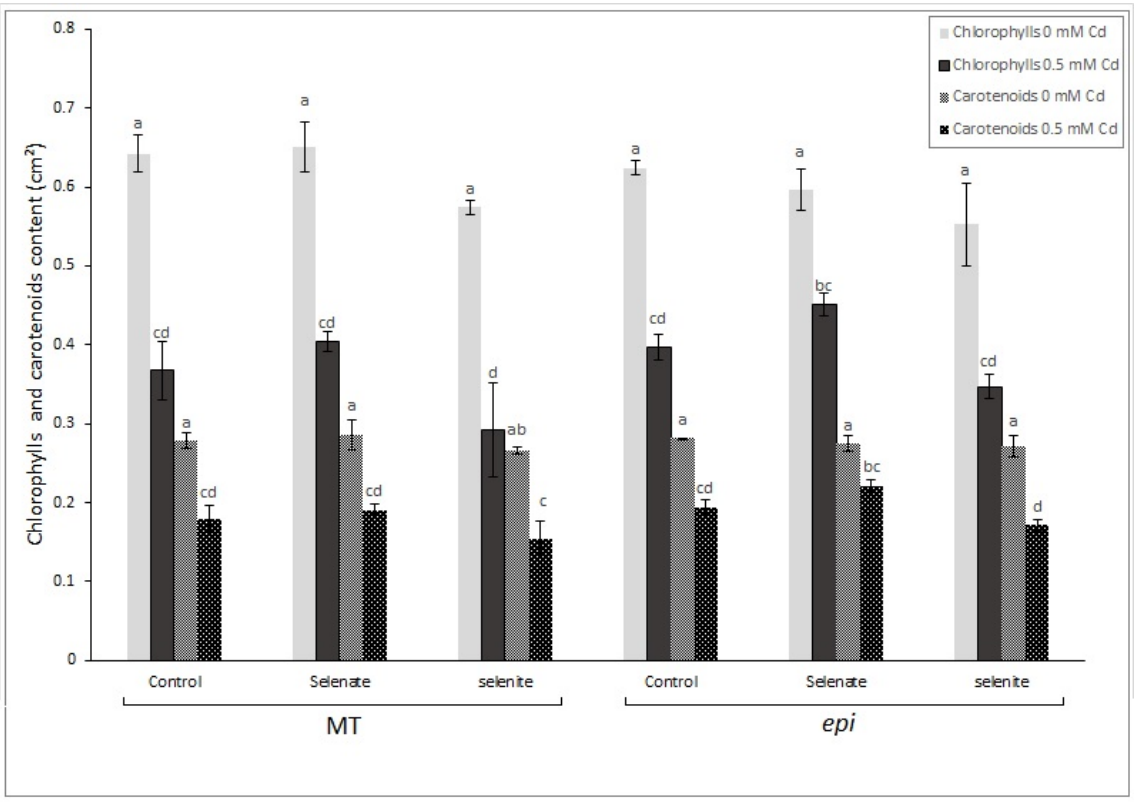
Supplementary material 1

	Treatments		Se	Cd
			mg/kg	
Micro Tom	Control	Root	0.00 ± 0.00 c	8.10 ± 1.32 e
		Leaf	0.00 ± 0.00 e	3.53 ± 0.21 c
		Fruit	0.00 ± 0.00 f	0.63 ± 0.10 c
	Cd	Root	0.00 ± 0.00 c	5614.00 ± 524.86 a
		Leaf	0.00 ± 0.00 e	1615.59 ± 112.26 a
		Fruit	0.00 ± 0.00 f	30.77 ± 1.10 b
	Selenate	Root	4.92 ± 0.44 b	13.32 ± 7.69 e
		Leaf	10.37 ± 0.42 b	3.55 ± 0.26 c
		Fruit	3.40 ± 0.06 b	0.55 ± 0.05 c
	Selenate + Cd	Root	5.41 ± 0.37 b	3102.25 ± 230.92 d
		Leaf	9.16 ± 0.47 c	1415.40 ± 31.70 ab
		Fruit	3.40 ± 0.21 b	29.12 ± 2.37 b
	Selenite	Root	16.07 ± 1.09 a	12.58 ± 7.26 e
		Leaf	0.70 ± 0.27 de	5.06 ± 0.19 c
		Fruit	1.25 ± 0.21 c	0.70 ± 0.11 c
	Selenite + Cd	Root	8.83 ± 0.34 b	3323.22 ± 90.78 d
		Leaf	0.00 ± 0.00 e	1331.01 ± 75.70 b
		Fruit	0.00 ± 0.00 f	20.14 ± 1.82 b
epinastic	Control	Root	0.00 ± 0.00 c	11.60 ± 3.70 e
		Leaf	0.00 ± 0.00 e	3.78 ± 0.83 c
		Fruit	0.00 ± 0.00 f	0.50 ± 0.00 c
	Cd	Root	0.00 ± 0.00 c	3598.18 ± 81.97 cd
		Leaf	0.00 ± 0.00 e	1381.62 ± 8.82 b
		Fruit	0.00 ± 0.00 f	55.56 ± 3.71 a
	Selenate	Root	7.80 ± 0.48 b	9.37 ± 0.66 e
		Leaf	14.68 ± 0.77 a	3.14 ± 0.20 c
		Fruit	3.90 ± 0.09 a	0.50 ± 0.00 c
	Selenate + Cd	Root	6.93 ± 0.40 a	4317.14 ± 329.62 bc
		Leaf	9.93 ± 0.82 bc	1354.33 ± 32.53 b
		Fruit	3.50 ± 0.07 b	60.37 ± 6.20 b
	Selenite	Root	19.76 ± 3.14 a	16.03 ± 2.90 e
		Leaf	1.79 ± 0.01 d	4.45 ± 1.13 c
		Fruit	1.30 ± 0.27 c	0.43 ± 0.06 c
	Selenite + Cd	Root	18.18 ± 2.58 a	4869.24 ± 646.96 ab
		Leaf	0.08 ± 0.01 e	1232.29 ± 202.27 b
		Fruit	0.00 ± 0.00 f	49.78 ± 11.69 a

	Treatments		P	Ca	Mg	S	K	Cu	Fe	Mn	Zn
							mg g^{-1}				
Micro Tom	Control	Root	4080.6 ± 327.2 abc	8097.5 ± 495.4 d	6930.7 ± 204.5 ab	3394.0 ± 233.1 d	47268.4 ± 2919.6 ab	21.1 ± 3.1 ab	457.4 ± 87.9 b	329.4 ± 57.2 abc	40.3 ± 4.5 bc
		Leaf	6791.5 ± 111.2 f	33477.7 ± 1338.6 de	8907.7 ± 691.4 a	6711.8 ± 255.9 f	42935.0 ± 1566.9 c	14.7 ± 0.1 ab	371.3 ± 38.8 abc	113.5 ± 6.6 def	26.9 ± 1.7 c
		Fruit	4663.1 ± 136.6 e	1611.3 ± 89.8 abc	1875.0 ± 61.4 d	1556.3 ± 61.3 c	32263.7 ± 1211.4 f	9.1 ± 0.2 ab	49.3 ± 2.9 de	329.4 ± 57.2 b	17.1 ± 0.1 c
	Cd	Root	4308.5 ± 268.1 abc	8657.1 ± 255.8 cd	6115.9 ± 87.2 abc	5282.5 ± 350.8 a	53527.6 ± 1039.3 a	31.1 ± 2.9 ab	452.0 ± 75.6 b	207.4 ± 6.9 cd	76.7 ± 14.6 a
		Leaf	11699.8 ± 379.6 bc	31467.8 ± 647.4 e	8525.1 ± 249.1 cd	6753.1 ± 110.7 f	52979.2 ± 3188.7 a	8.4 ± 0.6 de	265.4 ± 30.6 c	201.9 ± 13.0 ab	25.8 ± 1.6 cd
		Fruit	6040.4 ± 72.4 bcd	1534.8 ± 80.6 bc	1827.0 ± 47.9 d	1652.5 ± 105.8 c	34332.6 ± 312.7 def	5.9 ± 0.2 cde	63.1 ± 4.6 abcd	207.4 ± 6.9 c	10.4 ± 0.6 efg
	Selenate	Root	3462.2 ± 535.6 cde	9083.6 ± 31.9 bcd	7170.4 ± 763.4 a	3484.5 ± 227.7 cd	43259.9 ± 1900.2 bc	24.3 ± 7.9 ab	1026.7 ± 155.2 a	203.1 ± 37.3 cd	32.9 ± 3.9 c
		Leaf	7873.4 ± 240.6 def	41013.3 ± 3842.5 abc	9736.7 ± 380.2 cd	7970.1 ± 373.7 ef	44583.0 ± 2042.8 bc	17.7 ± 2.2 ab	440.4 ± 61.5 a	119.4 ± 10.5 cdef	26.1 ± 1.0 cd
		Fruit	4820.5 ± 190.5 e	1678.8 ± 223.6 abc	1868.0 ± 42.3 d	1781.2 ± 72.7 bc	33148.6 ± 1649.0 ef	8.2 ± 0.6 b	43.2 ± 2.5 e	203.1 ± 37.3 ab	16.1 ± 0.7 cd
	Selenate + Cd	Root	2869.7 ± 227.2 de	10798.6 ± 504.9 bc	7157.1 ± 234.5 a	3973.1 ± 213.7 bcd	43707.7 ± 1054.4 bc	21.2 ± 2.7 ab	530.8 ± 19.5 b	199.5 ± 14.2 cd	24.8 ± 3.6 c
		Leaf	11911.0 ± 335.8 bc	36011.6 ± 935.2 cde	8666.1 ± 193.1 cd	7561.7 ± 535.3 f	46408.8 ± 539.1 bc	9.1 ± 0.3 de	313.4 ± 19.9 bc	193.1 ± 25.5 ab	26.3 ± 0.9 c
		Fruit	5779.3 ± 367.0 bcd	1657.6 ± 31.2 abc	1984.8 ± 135.6 d	1670.0 ± 62.6 c	36026.5 ± 3037.4 def	5.4 ± 0.2 cd	58.9 ± 4.5 bcd	199.5 ± 14.2 c	8.6 ± 0.5 fg
	Selenite	Root	3561.2 ± 275.3 cde	10046.7 ± 455.1 bcd	5228.5 ± 444.5 c	3682.2 ± 279.5 bcd	44470.2 ± 986.5 bc	17.6 ± 1.6 b	507.7 ± 5.0 b	293.8 ± 41.6 abcd	35.7 ± 3.9 c
		Leaf	7349.8 ± 334.0 ef	35550.1 ± 1538.6 cde	9288.8 ± 632.8 cd	7412.7 ± 158.9 f	43210.4 ± 1706.4 c	14.3 ± 1.0 abc	314.5 ± 10.6 bc	141.6 ± 23.3 cde	25.1 ± 2.3 cd
		Fruit	5237.8 ± 116.2 de	1681.3 ± 74.1 abc	2136.9 ± 75.6 cd	1790.4 ± 39.8 bc	35713.1 ± 162.4 def	9.2 ± 0.2 ab	52.4 ± 2.7 cde	293.8 ± 41.6 a	15.9 ± 0.6 cd
	Selenite + Cd	Root	2638.5 ± 122.4 e	10691.0 ± 223.7 bcd	5772.9 ± 91.4 bc	3827.0 ± 192.3 bcd	38236.0 ± 1121.6 c	19.4 ± 1.5 b	772.6 ± 147.3 ab	184.1 ± 3.2 cd	21.8 ± 2.2 c
		Leaf	2638.5 ± 122.4 cd	36577.6 ± 925.3 bcde	9563.6 ± 365.9 cd	6586.9 ± 296.0 f	49027.3 ± 2925.0 ab	8.3 ± 0.7 e	261.8 ± 11.4 c	203.8 ± 12.3 a	23.5 ± 1.6 cd
		Fruit	5256.4 ± 149.0 cde	1450.6 ± 148.6 c	1842.7 ± 62.3 d	1646.4 ± 25.3 c	33338.3 ± 1244.4 ef	5.6 ± 0.1 cd	67.7 ± 5.2 ab	184.1 ± 3.2 c	7.3 ± 1.0 g
epinastic	Control	Root	4816.1 ± 771.6 ab	11250.7 ± 1779.4 bc	5608.7 ± 301.2 bc	4554.4 ± 605.9 ab	45669.1 ± 5192.7 abc	23.2 ± 5.4 ab	803.6 ± 170.6 ab	415.3 ± 105.6 a	56.9 ± 12.2 ab
		Leaf	9338.1 ± 414.3 cdef	39930.3 ± 1282.9 abc	10365.0 ± 532.2 bc	10758.1 ± 316.5 abc	46766.2 ± 484.2 bc	17.3 ± 1.3 a	381.6 ± 20.6 ab	103.4 ± 10.0 ef	38.4 ± 0.5 a
		Fruit	5408.2 ± 225.3 cde	1851.2 ± 21.9 abc	2189.5 ± 150.2 bcd	1731.2 ± 91.3 bc	37896.9 ± 1852.5 bcde	9.9 ± 0.7 a	59.3 ± 2.1 bcd	415.3 ± 105.6 ab	20.5 ± 2.2 b
	Cd	Root	3933.8 ± 270.7 abcd	11414.1 ± 347.4 b	5271.2 ± 135.0 c	4648.6 ± 85.4 ab	45566.6 ± 1387.9 abc	31.0 ± 1.0 ab	458.7 ± 5.9 b	159.3 ± 3.9 d	25.9 ± 1.4 c
		Leaf	14822.3 ± 848.0 a	45121.4 ± 2512.1 a	11073.1 ± 319.3 a	9618.1 ± 410.9 bcd	47191.3 ± 1084.0 bc	11.8 ± 0.3 bcd	335.6 ± 47.8 abc	159.1 ± 8.3 bc	21.5 ± 1.1 d
		Fruit	6135.2 ± 327.6 bcd	2548.7 ± 272.4 a	2478.8 ± 160.0 abc	1823.0 ± 48.7 bc	36775.9 ± 1785.8 cdef	6.5 ± 0.6 c	74.0 ± 2.3 a	159.3 ± 3.9 c	11.0 ± 0.6 ef
	Selenate	Root	4953.6 ± 147.7 cde	14783.0 ± 984.0 a	5559.1 ± 229.5 c	4468.0 ± 159.1 abc	46275.6 ± 1037.4 abc	23.1 ± 4.8 ab	635.8 ± 32.8 ab	370.7 ± 50.7 ab	59.5 ± 7.2 ab
		Leaf	9752.2 ± 1012.2 cde	41900.0 ± 1599.3 ab	10150.5 ± 288.6 abc	10861.1 ± 523.4 ab	41471.4 ± 606.7 c	17.1 ± 1.9 a	328.9 ± 18.4 bc	98.0 ± 2.6 ef	31.5 ± 0.9 b
		Fruit	5761.5 ± 501.0 bcd	2505.1 ± 563.2 ab	2552.2 ± 288.9 abc	2079.8 ± 101.5 b	39322.9 ± 2339.7 bcd	10.5 ± 0.8 a	67.0 ± 6.2 abc	370.7 ± 50.7 a	20.4 ± 1.8 b
	Selenate + Cd	Root	3739.9 ± 264.3 de	11486.1 ± 451.6 bc	4974.8 ± 137.3 c	4597.5 ± 234.8 ab	46170.4 ± 1406.5 abc	32.1 ± 0.2 ab	621.4 ± 133.6 ab	209.9 ± 15.8 cd	23.2 ± 2.0 c
		Leaf	14096.0 ± 735.7 ab	40660.4 ± 459.0 abc	10492.3 ± 754.7 ab	9428.7 ± 390.3 cd	46158.0 ± 797.0 bc	10.5 ± 0.6 de	300.0 ± 25.2 bc	149.5 ± 3.5 cd	21.7 ± 0.2 d
		Fruit	7104.0 ± 223.6 a	2328.6 ± 320.3 abc	2678.6 ± 129.3 abc	2419.1 ± 130.5 a	42589.2 ± 1268.7 ab	5.8 ± 0.6 cd	63.7 ± 2.7 abcd	209.9 ± 15.8 c	17.0 ± 0.4 c
	Selenite	Root	3800.3 ± 316.1 cde	11614.2 ± 1329.4 bcd	5599.2 ± 658.8 bc	4193.0 ± 438.8 bcd	45392.1 ± 2833.4 abc	18.6 ± 3.3 b	702.1 ± 108.0 ab	335.1 ± 68.2 abc	35.1 ± 8.5 c
		Leaf	9757.3 ± 635.7 cde	35941.3 ± 1279.6 cde	9636.1 ± 696.0 cd	11157.0 ± 876.2 a	42570.7 ± 971.7 c	17.2 ± 1.0 a	323.7 ± 31.4 bc	91.5 ± 4.7 f	26.3 ± 1.6 c
		Fruit	6161.1 ± 96.6 bc	1932.4 ± 244.3 abc	2630.9 ± 72.1 ab	2441.3 ± 95.8 a	45191.5 ± 965.2 a	9.5 ± 0.4 ab	52.6 ± 0.4 cde	335.1 ± 68.2 a	24.4 ± 1.0 a
	Selenite + Cd	Root	3966.1 ± 214.7 abcd	10671.1 ± 890.4 bcd	5458.7 ± 749.6 c	5270.2 ± 341.2 a	47283.0 ± 4095.9 ab	35.9 ± 9.5 a	767.2 ± 267.4 ab	226.3 ± 26.9 bcd	24.9 ± 1.9 c
		Leaf	13529.0 ± 2135.7 ab	38766.3 ± 523.0 bcd	11183.6 ± 526.1 ab	9012.7 ± 507.2 de	44140.7 ± 2020.2 bc	11.1 ± 0.9 cde	376.3 ± 38.3 ab	161.5 ± 18.2 abc	23.2 ± 1.8 cd
		Fruit	6650.7 ± 460.2 ab	2115.4 ± 637.0 abc	2727.0 ± 276.5 a	2463.1 ± 272.9 a	41691.3 ± 1995.9 abc	4.9 ± 0.4 d	63.0 ± 9.5 abcd	226.3 ± 26.9 c	12.8 ± 1.5 de

Supplementary material 2

Supplementary material 3



Supplementary material 4

Treatment		Ethylene µL C2H4 kg/h		
Micro Tom	SN	0.185 ±	0.01	e
	Cd	5.790 ±	2.23	de
	Selenate	0.126 ±	0.02	e
	Cd + Selenate	14.530 ±	3.56	bc
	Selenite	8.866 ±	1.09	cd
	Cd + Selenite	11.512 ±	1.19	cd
epinastic	SN	0.562 ±	0.20	e
	Cd	11.530 ±	1.59	cd
	Selenate	1.156 ±	0.21	e
	Cd + Selenate	21.160 ±	3.94	bc
	Selenite	0.146 ±	0.04	e
	Cd + Selenite	30.713 ±	3.06	a

Supplementary material 5

	Treatments		GSH nmol/g FW			
Micro Tom	Control	Root	550.01	±	183.34	d
		Leaf	1514.19	±	504.73	bc
		Fruit	1188.32	±	396.11	a
	Cd	Root	3411.85	±	1969.83	a
		Leaf	1772.60	±	1023.41	b
		Fruit	1162.22	±	671.01	a
	Selenate	Root	402.11	±	134.04	d
		Leaf	1800.62	±	600.21	b
		Fruit	1139.07	±	379.69	a
	Selenate + Cd	Root	1363.85	±	787.42	c
		Leaf	2256.47	±	1302.77	a
		Fruit	755.57	±	436.23	bc
	Selenite	Root	362.76	±	120.92	d
		Leaf	2212.41	±	737.47	a
		Fruit	1157.96	±	385.99	a
	Selenite + Cd	Root	1252.98	±	723.41	c
		Leaf	2360.03	±	1362.56	a
		Fruit	637.12	±	367.84	bc
epinastic	Control	Root	571.33	±	190.44	d
		Leaf	1006.27	±	335.42	ef
		Fruit	543.92	±	181.31	c
	Cd	Root	2035.76	±	1175.35	b
		Leaf	1747.21	±	1008.75	b
		Fruit	741.90	±	428.34	bc
	Selenate	Root	572.28	±	190.76	d
		Leaf	738.24	±	246.08	f
		Fruit	846.67	±	282.22	b
	Selenate + Cd	Root	1643.46	±	948.85	bc
		Leaf	1354.37	±	781.95	cd
		Fruit	1096.43	±	633.02	a
	Selenite	Root	672.45	±	224.15	d
		Leaf	853.11	±	284.37	f
		Fruit	796.50	±	265.50	b
	Selenite + Cd	Root	1738.49	±	1003.72	bc
		Leaf	1182.59	±	682.77	de
		Fruit	1155.01	±	666.84	a

CHAPTER IV - Exogenous selenium improves photosynthesis and preserves cell ultrastructure, but does not restore cadmium stress damages in tomato plants

[1]This chapter was submitted to Plant Growth Regulation and we are waiting for evaluation.

Abstract

Selenium (Se) is not considered an essential nutrient to plants, however it can affect some anatomical and physiological aspects since its application improves growth and enhances antioxidant defense mechanisms against Cd-stressful conditions. Cadmium (Cd) is a damaging metal that can be uptake by plants, leading to cell death and metabolic pathways disruption. The application of Se in plants growing under Cd contamination, may become an alternative to minimize Cd damages. Although several studies have indicated the role of Se to minimize Cd stress effects in plants, there is no specific information available if Se can act on the anatomical structure and on photosynthetic rates in plants under Cd stress. To address questions related to Se-protective responses under Cd stress, we evaluated structural and ultrastructural aspects, photosynthetic rates and growth of tomato cv. Micro-tom plants exposed to 0.5 mM $CdCl_2$ and further supplemented by 1.0 μM of selenite or selenate forms. The overall results revealed different trends according to Se sources and Cd application. Both Se sources improved growth, photosynthesis, leaf and middle lamella thickness between mesophyll cells. In contrast, decrease in photosynthesis, growth and damages to ultrastructure of the chloroplast, increased the presence of mitochondria, peroxisomes, starch grains, plastoglobules, thylakoids and middle lamella in plants in the presence of Cd or Cd+Se. Se plays an important role in plant cultivation under normal conditions, which was corroborated with the identification of specific structural changes in Se-treated plants, which could benefit plant development. However, a reversion of Cd stress effects was not observed in the presence of Se

Keywords: abiotic stress, selenate, selenite, microscopy, *Solanum lycopersicum.*

1. Introduction

Selenium is considered a non-essential nutrient to plants, because it is not fundamental for plant structure or metabolism. However, its foliar or soil solution application at low concentrations triggers numerous benefits to plants. Recent studies indicate that Se application in plants improves plant growth (Ashraf et al., 2018; Yin et al., 2019), photosynthetic attributes (Haghighi et al., 2016), decreases Cd uptake in tomato plants (Abd et al., 2016), and Cd translocation from root to shoot in rice (Wan et al. 2016). Moreover, Se seems to play an important role in plant tolerance during biotic and abiotic stresses (Hawrylak-Nowak et al., 2018). This is especially the case when plants are exposed to heavy metals, and Se has been shown to alleviate negative effects of Cd-induced stress by increasing the production of stress-responsive proteins (Sun et al., 2016), regulating ROS scavenger metabolism (Castillo-Godina et al., 2016), improving flowering (Shekari et al., 2019), and the antioxidant defense systems (Alyemeni et al., 2018).

Cadmium (Cd) is one of the most toxic heavy metal to living organisms. Due to its increasing and widespread use in industry, Cd is becoming a hazard in a worldwide scale (Alves et al., 2016; Edelstein et al., 2018). Plants can easily absorb this heavy metal, which lead to uncontrolled oxidation, disrupting cell balance and causing electrolyte leakage (Jarvis et al., 1976; Gratão et al., 2015). Even at low concentrations, Cd can increase the content of reactive oxygen species (ROS), which can trigger drastic alteration in metabolic pathways, also affecting cells and tissues structure (Alves et al., 2017; Shanying et al., 2017; Zhu et al., 2018). Moreover, Cd causes structural damages in roots cells, leading to smaller nucleus, and ruptured nuclear envelope (Ali et al., 2014). Cd induced damages in aerial plant parts, include decreased intercellular air spaces and modifications in chloroplast structure, with disrupted and dilatied thylakoid membranes in tomato plants (Djebali et al., 2005). Yue et al. (2018) have demonstrated disorders in lamellar structure of the chloroplast grana and the thylakoid membranes, with increased interlamellar spaces. In addition, Cd significantly reduced the quantity and size of starch grains in the chloroplasts of wheat, inhibiting starch accumulation (Yue et al., 2018). These results suggest that Cd can drastically affect the photosynthetic capacity of plants,

thus reflecting in plant survival and productivity. Additionally, Gratão et al. (2009) showed that tomato cv. Micro Tom, under Cd stress, exhibited stomata closure, reduced root diameter and leaf epidermis disintegration, relating the impact of Cd in gas exchange and nutrient uptake capacity.

The miniature tomato cultivar Micro-Tom (MT) exhibits short life cycle and limited growth, making this as a plant model for agronomical studies (Meissner et al., 1997), especially regarding experimental approaches to understand isolated and combined effects of abiotic stresses in plant survival and productivity. Although several studies have indicated the beneficial effects of Se in plants to minimize Cd stress effects, there is no specific information available as to how Se can act protecting the ultrastructural aspects of chloroplast membranes, conserving photosynthetic capacity, and plant growth. Thus, to better understand the role of Se in structural changes and photosynthesis attributes during Cd stress, we analyzed structural aspects of chloroplasts in MT tomato plants, as well plant attributes related to root and leaf biomass, as well as photosynthetic rates. We expected to find plants under Cd stress, and without Se application, to show damages in their chloroplast structure, leading to photosynthesis reduction, which would decrease biomass production of the whole plant.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of tomato (*S. lycopersicum* L.) cultivar Micro-Tom (MT) were sterilized with sodium hypochlorite solution (5%), rinsed three times and sown in boxes filled with a mixture of commercial pot mix (Plantmax HT Eucatex, Brazil) and vermiculite (1:1, by volume) supplemented with 1 g L^{-1} 10:10:10 nitrogen-phosphorus-potassium and 4 g L^{-1} lime ($MgCO_3$ + $CaCO_3$). When two true leaves were completely expanded, each seedling was transplanted to one Leonard pot (0.35 L) (Vincent 1975), a hydroponic cultivation system, with sterilized sand and polystyrene (4:3), and a Hoagland´s nutrient solution (250 ml). Thirty-day-old plants were grown in a greenhouse, with the same nutritive solution supplemented with (1) control; (2) 0.5 mM of $CdCl_2$; (3) 1µM of selenate; (4) 1 µM of selenate + 0.5 mM of $CdCl_2$; (5) 1 µM of selenite and (6) 1 µM of selenite + 0.5 mM of $CdCl_2$ in MT plants. The nutritive solution was which was

replaced every five days. All of these procedures were performed in the year 2018, using installations from the Plant Physiology Laboratory and a greenhouse of the Department of Applied Biology, from the Faculty of Agrarian and Veterinary Sciences, Jaboticabal, São Paulo, Brazil.

2.2 Photosynthetic rates

Sixty days after germination, gas exchange parameters under saturating light were carried out at the beginning of the rainy season (October 2018). The measurements were taken using a portable system to measure CO_2 and H_2O exchanges (LcPro-SD, ADC BioScientific Ltd., Hoddesdon, UK). The gas exchange measurements were made under a constant light intensity of 1200 $\mu.mol.m^{-2}.s^{-1}$ emitted by a blue-red LED light source. The measurements were made using the terminal leaflet of fully expanded leaves, from nodes 4 to 5. The leaf temperature was maintained at 29.00 ± 0.46 ° C. Measurements were performed under ambient concentrations of CO_2 (between 413-427 ppm). Maximum area-based rate of light-saturated CO_2 assimilation (A), leaf transpiration (E) and stomatal conductance (gs) were measured after a stabilization period (3-5 minutes). Data collection was performed during the morning, between 08:00 and 10:00.

2.3 Sample preparation for light microscopy (LM) and transmission electron microscopy (TEM)

For histological characterization, samples of leaves were processed for light (LM) and transmission electron microscopy (TEM). Small pieces of leaf blade were collected right after the photosynthetic measurements, and immediately fixed in a modified Karnovsky solution (2% glutaraldehyde, 2% paraformaldehyde, and 5 mM $CaCl_2$ in 0.05 M sodium cacodylate buffer, pH 7.2) (Karnovsky, 1965), for 48 h. The samples were then rinsed in cacodylate buffer (0.1 M) and post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, pH 7.2, at room temperature, for 1 h. The samples were dehydrated in a graded acetone series and embedded in Spurr epoxy resin (EMS, Electron Microscopy Sciences, Hatfield, PA, USA), for 48 h. Semithin

sections (120–200 nm) were collected in glass slides, stained with toluidine blue (2% in water), for 5 min, rinsed in distilled water, and air-dried.

The sections were permanently mounted in Entellan®, observed and documented using an upright light microscope (Axioskop 40 Carl Zeiss, Jena, Germany).

Ultrathin sections of leaves (60–90 nm) were obtained with ultramicrotome (Porter Blum MT2- Dupont-Sorvall) equipped with a diamond knife, collected on copper grids (300 mesh), and stained with uranyl acetate (2.5%), followed by lead citrate (0.1%) (Reynolds, 1963). Sections were observed at 80 kV under a transmission electron microscope (JEM1400 JEOL, Tokyo, Japan) and the images digitalized.

Root samples were fixed as described above, dehydrated in an ethanol series (35% to 100%), at 30-min intervals. The infiltration was done at 4 °C using ethanol:infiltration medium (glycol methacrylate, Historesin kit, Leica, Heidelberg, Germany) (3:1, 1:1, 1:2, minimum of 2 h each step), followed by infiltration in 100% infiltration medium for 48 h. Polymerization was done at room temperature for 48 h. Histological serial sections (5 mm thick) were obtained in a rotary microtome (Leica RM 2155, Nussloch, Germany), and stained with 1% acid fuchsin and 0.05% (w/v) toluidine blue in water, mounted in Entellan® synthetic resin (Merck, Darmstadt, Germany), covered with coverslips, and observed under an optical microscope (Axioskop 40 Carl Zeiss, Jena, Germany).

The effect of Se and Cd on the leaf blade thickness (µm) and root diameter was determined in three samples of three different roots and leaves. The tissues were measured using the Image J 1.46r program (RASBAND, 2012) ===(RASBAND, W. S. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012.)

2.4 Biomass

After a period of 60 days post germination, samples of roots and leaves were collected to determine de total biomass produced. Samples were rinsed with distilled water and dried at 60ºC until constant weight, and the biomass produced was determined in a analytical scale.

2.5 Statistical analysis

The experimental design was completely randomized, with three replicates, two Se forms and presence or absence of Cd. The result of three independent replicates of root and leaves dry masses, maximum area-based rate of light-saturated CO_2 assimilation (A), leaf transpiration (E) and stomatal conductance (gs), leaf mesophyll thickness and root diameter was expressed as the mean and standard error of the mean (±SEM). A multiple comparison between means by the Duncan test followed the application of an individual ANOVA for each characteristic at a 0.05 level of significance. The statistical analyses were performed using AgroEstat® software (Barbosa; Maldonato Júnior, 2015).

3. Results and discussion

Selenium application may improve plant growth and photosynthesis attributes under normal and stress condition, due to its capacity to re-establish photosynthesis and growth (Alyemeni et al., 2018; Yin et al., 2019). In our study, the application of either selenite or selenite forms, under normal conditions, at 1 μM, improved root and shoot dry mass, when compared with control plants (Table 1). However, Cd decreased plant growth. Although, Se application has been shown to restore plant growth under Cd stress (Alyemeni et al., 2018), in our study there were no differences in root and shoot dry mass of plants treated with Cd and Se (Table 1). Similar results were reported for *Brassica chinensis* (Yu et al., 2019). Therefore, Se not always is effective to avoid losses in plant growth under Cd stress. This can be due to photosynthesis attributes impaired by Cd stress, which are not restored by Se application.

When photosynthetic attributes were analyzed, leaf transpiration (E), stomatal conductance (gs) and maximum area-based rate of light-saturated CO_2 assimilation (A) increased with Se application, in comparison with control plants (Table 1). Our data is in accordance with Djanaguiraman et al. (2010), which reports that Se application increased photosynthesis attributes in sorghum, because Se tends to minimize damages to the chloroplasts, enhancing chlorophyll content. These positive

responses may be related to the fact that Se can reduce ROS production, leading to the capacity to restore the structure of damaged chloroplasts and stimulate other vital metabolites production (Feng et al., 2013a).

Treatments submitted to Cd exhibited a decrease in photosynthetic rates (Table 1). These responses can be explained by an imbalance caused by Cd in cell homeostasis that can destroy chloroplasts, leading to decreased electron transport rate (Gratão et al., 2015). The use either form of Se during Cd stress was not efficient in avoiding damages caused by Cd in the photosynthetic attributes. Thus, Se application may not be efficient in avoiding the reduction in E, gs and A of MT plants grown under Cd stress, as a result of damages caused by Cd in chloroplasts.

Histological analyses revealed that both Se forms leaded to an increase in intercellular spaces in the mesophyll (Fig. 1 C, E), producing thicker leaves (Fig. 2) and increased dry mass (Table 1), when compared to control plants (Fig. 1 A). The increase in thickness observed in plants treated with Se might be related to the increased photosynthetic and transpiration rates also observed here (Table 1). Otherwise, Cd stress caused a serious reduction on leaf mesophyll thickness (Fig. 1 B and Fig. 2). When Se was applied in plants under Cd stress, we can observe a decrease in leaf thickness, when compared with only Cd application (Fig. 1D, F and Fig. 2). A study conducted by Feng et al. (2013b) demonstrated that Se application induced Cd uptake and reduced growth in rice plants exposed to high Cd concentrations (89–178 μM). In accordance with this information, in our study neither Se form used were effective to reverse the negative effects caused by 0.5 mM Cd on plant growth and development.

In roots, the Se application did not cause significant changes in root diameter, when compared to the control plants (Fig. 3-4). On the other hand, Se increased intercellular spaces (Fig. 3C, E arrows). The presence of Cd caused a reduction in root diameter and Se application was ineffective to restore root diameter in plants under Cd stress (Fig. 3-4). Selenite is more efficient than selenate to induce the antioxidant system and decrease Cd uptake (Wan et al. 2016) and translocation to the aerial parts, which causes a higher accumulation of Se in roots (Lin et al., 2012). Although selenite and selenate exhibited distinct trends in plant metabolism, neither Se forms used were able to to restore root diameter in plants concomitantly exposed

to Cd.

The ultrastructural analysis (TEM) of palisade parenchyma cells, shows differences among treatments (Figs 5). Cells treated with Se exhibited an increased thickness of the middle lamella (Fig. 5H, I, N, O). Thus, an increased thickness of middle lamella in plants treated with Se is interesting, because priming with Se could be a strategy to improve the plants tolerance before heavy metal exposure. The exposure of plants to Cd caused alterations in the ultrastructure of the chloroplast, increased mitochondria, peroxisomes, starch grains, plastoglobules, disorganization of thylakoids and middle lamella, and presence of autophagic bodies (Fig. 5D-F).

The increased number of mitochondria may be a consequence of the cell need for higher amounts of energy, which is necessary for the Cd detoxification process (Zorov et al., 2014). Chloroplast ultrastructure changes maybe be caused by an increase in the production of reactive oxygen species (ROS), which in high concentration in the cellular environment, can cause oxidative damage to cellular ultrastructure and function (Alyemeni et al., 2018).

Stressful conditions impose direct effects on chloroplast ultrastructure and biosynthesis of sugars and starch (Verma and Dubey 2001). In this study, the effect of Cd on the ultrastructure of chloroplasts involved thylakoid disorganization with dilated thylakoid membranes, increased size and number of plastoglobules and starch grains (Fig. 5D-F). The changes in chloroplast ultrastructure could indicate metabolic dysfunction leading to a reduction on photosynthesis rate (Table 1). The decrease in photosynthesis attributes can, in turn, be related to reduced carbon fixation (Devi et al., 2007) and consequently, lower number of starch grains (Yue et al., 2018). However, our data reveals an increase in starch grains (Fig. 5D-F), in the presence of Cd. Starch is a major metabolite and product of photosynthesis, involved in several essential biological activities in the plant response against Cd stress, including osmoregulation and detoxification (He et al., 2011). Thus, the increase in starch grains may play a role in increasing responses against Cd toxicity in MT leaf cells.

Cd stress also increases the number and size of plastoglobuli and peripherical vesicles (Hakmaoui et al., 2007), as also observed in this study (Fig. 5D-F, J-L, P-R). The increased numbers and size of plastoglobuli in chloroplasts is a consequence of

stress exposure (Olmos et al., 2006). For instance, plastoglobuli may have a function in the synthesis and recycling of lipophilic products formed by oxidative metabolism during stress (Olmos et al., 2006). These chloroplast changes are visible effects of Cd stress, as previously reported in tomato (Gratão et al., 2009), rice (Wang et al. 2014), and wheat (Yue et al., 2018). Disintegration of chloroplasts caused by Cd stress compromises photosynthesis rate, thus, maintenance of the ultrastructural integrity is fundamental for plant growth and development (Yue et al., 2018). At low concentrations, selenium exerts positive activities of enzymes related to sucrose and starch metabolism (Kaur et al., 2018). However, plants treated with both forms of Se under Cd stress exhibited similar changes compared to those observed in plants submitted to Cd only. Thus, the disintegration of chloroplasts and quantity of starch grains under Cd stress was not altered by Se application and the potential benefits caused by Se possibly does not revert structural damages in palisade parenchyma cells caused by Cd.

4. Conclusions

Recently, several studies reported Se as an efficient strategy to avoid damages derived from abiotic stresses, including Cd. Although the biochemical responses have been widely reported, structural changes underlying this interplay were not enlightened until now. We found that plants treated with both Se forms used exhibited an improvement in photosynthesis attributes and growth, which are related to structural modifications in leaves. In contrast, Cd stress induced severe damages to chloroplast and others cell structures, which caused decreased photosynthetic rates, affecting growth. The application of Se in plants under stress did not restore cell damages caused by Cd. Although our results did not find that Se may induce ultrastructural and physiological changes (ie, alleviation) during Cd stress, Se plays an important role under non-stress conditions, due to the identification of specific Se-induced structural changes which could promote benefits to plant development. We conclude that the novelty information provided by this study had not yet been described in plant anatomical studies, being a fundamental step towards obtaining a

better understanding of structural changes, caused by Se and the elucidation of its role in plant metabolism.

5. Acknowledgements

This work was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - Grant n° 2017/04787-6) - Brazil. PLG also thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the research fellowship (Grant nº 314380/2018-3) - Brazil. We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Brasil - Código de Financiamento 001 - for the scholarship granted (L.R.A). We also thank to Centro de Microscopia e Imagem, (FOP/UNICAMP) for the access to the transmission electron microscope.

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7. Figures legends

Figure 1. Tomato leaves from plants of cultivar Micro-Tom grown under $CdCl_2$ stress with selenate and selenite application, observed in cross section by light microscopy. A) control; B-F) supplemented with 0.5 mM $CdCl_2$ (B); 1µM selenate (C); 0.5 mM $CdCl_2$ + 1µM of selenate (D); 1µM of selenite (E); 0.5 mM CdCl2 + 1µM selenite (F). ep = epidermis; is = intercellular space; pp = palisade parenchyma. Bars = 100 µm.

Figure 2. Micro-Tom leaf blade thickness of plants grown for 60 days in absence or presence of 0,5mM $CdCl_2$ and 1 µM of either Na_2SeO_3, or Na_2SeO_4. Different letters indicate significant difference at $P < 0.05$ by Duncan test.

Figure 3. Cross sections of Micro-Tom roots grown under $CdCl_2$ stress with selenate or selenite application, observed by light microscopy. A) control; B-F) supplemented with 0.5 mM $CdCl_2$ (B); 1µM selenate (C); 0.5 mM $CdCl_2$ + 1µM of selenate (D); 1µM of selenite (E); 0.5 mM CdCl2 + 1µM selenite (F). cc = central cylinder; co = cortical cell; arrows: intercellular spaces. Bars = 200 µm

Figure 4. Diameter of roots of Micro-Tom plants grown for 60 days in absence or presence of 0.5mM $CdCl_2$ and 1 µM of either Na_2SeO_3, or Na_2SeO_4. Different letters indicate significant difference at $P < 0.05$ by Duncan test.

Figure 5. Ultrastructure of palisade parenchyma cells from plants grown under $CdCl_2$ stress with selenate and selenite application, observed by transmission electron microscopy. A-C) control; Supplemented with 0.5 mM $CdCl_2$ (D-F); 1µM selenate (G-I); 0.5 mM $CdCl_2$ + 1µM selenite (J-L); 1µM selenite (M-O); 0.5 mM $CdCl_2$ + 1µM selenite (P-R); ch = chloroplast; mi = mitochondria; ml = middle lamella; pe = peroxisome; pl = plastoglobuli; sg = starch grain; th = thylakoids; small arrows = autophagosomes. Bars: A, D, G, J, M, P = 5 µm; B, E, H, K, N, Q = 1 µm; C, F, I, L, O, R = 0.5 µm.

8. Figures and table

Table 1

Treatment	Leaves DM (g)		Roots DM (g)		E (mol.m^{-2} s^{-1})		gs (mol.m^{-2} s^{-1})		A (µmol m^{-2} s^{-1})	
Control	1.57 ± 0.17	b	0.35 ± 0.04	bc	2.570 ± 0.386	b	0.360 ± 0.104	b	15.66 ± 1.73	b
Cd	0.89 ± 0.18	c	0.25 ± 0.07	c	1.920 ± 0.453	bc	0.193 ± 0.073	bc	4.94 ± 0.99	c
Selenate	2.24 ± 0.05	a	0.55 ± 0.03	a	3.784 ± 0.233	a	0.651 ± 0.073	a	19.90 ± 1.32	ab
Selenate +Cd	0.59 ± 0.10	c	0.19 ± 0.08	c	1.250 ± 0.155	c	0.087 ± 0.022	c	4.41 ± 0.65	c
Selenite	1.85 ± 0.10	a	0.45 ± 0.03	ab	4.216 ± 0.266	a	0.689 ± 0.061	a	22.79 ± 2.28	a
Selenite +Cd	0.96 ± 0.09	c	0.28 ± 0.03	bc	2.061 ± 0.165	bc	0.184 ± 0.037	bc	7.15 ± 1.04	c

Table 1 Dry mass (g) of leaves and roots and photosynthesis attributes - leaf transpiration (E), stomatal conductance (gs) and maximum area-based rate of light-saturated CO2 assimilation (A) of MT plants grown for 60 days in the presence of 0 mM or 0.5mM $CdCl_2$ and 1 µM of Na_2SeO_3 or Na_2SeO_4. Different letters in each column indicate significant difference at $P < 0.05$ by Duncan test.

Figure 1

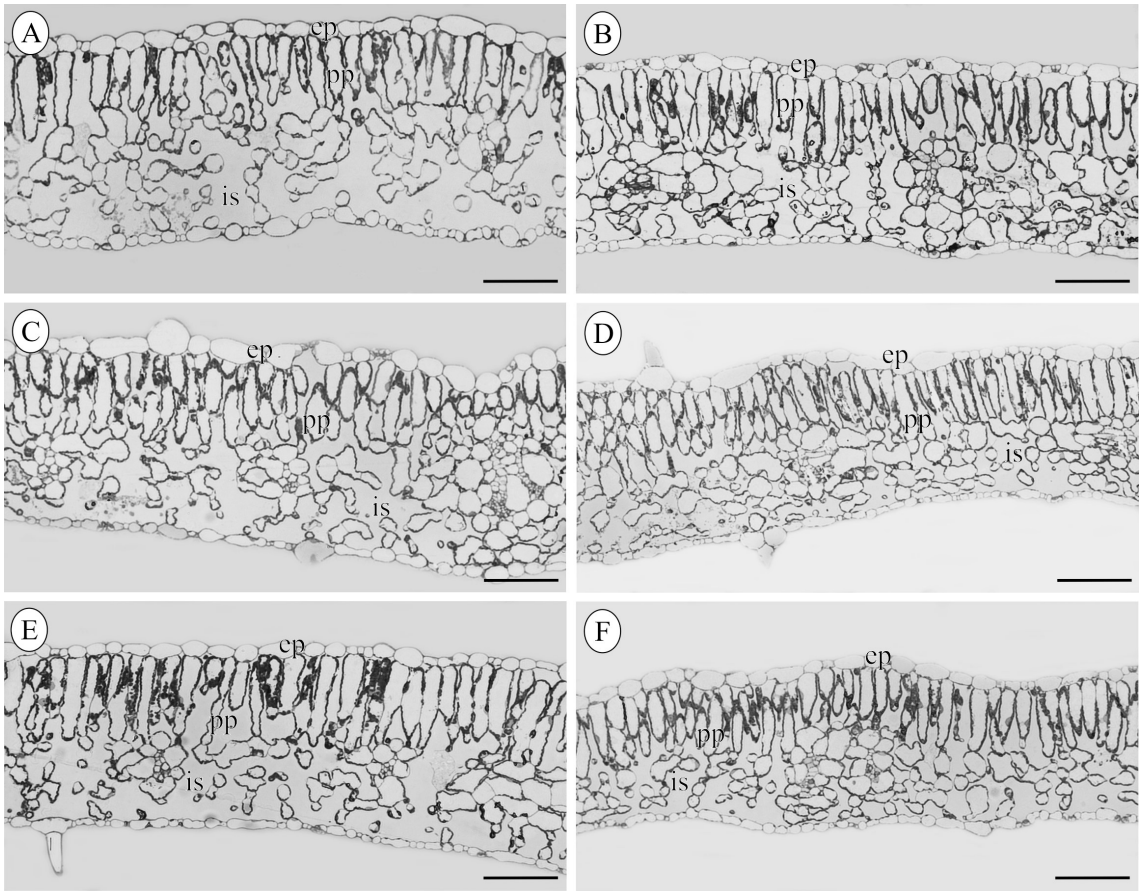


Figure 2

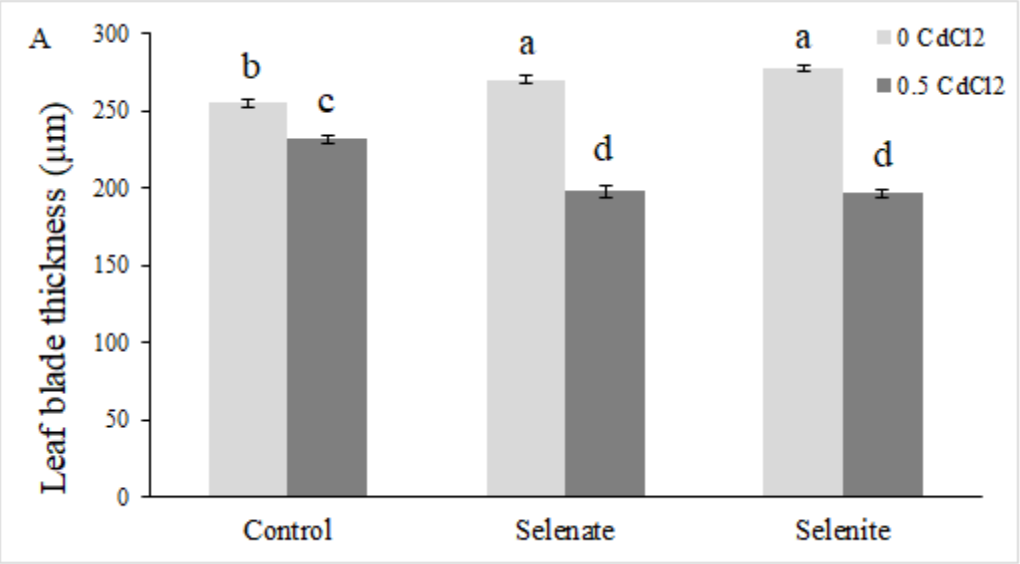

A
Leaf blade thickness (µm)
300
250
200
150
100
50
0
b
c
a
d
a
d
0 CdCl2
0.5 CdCl2
Control
Selenate
Selenite

Figure 3

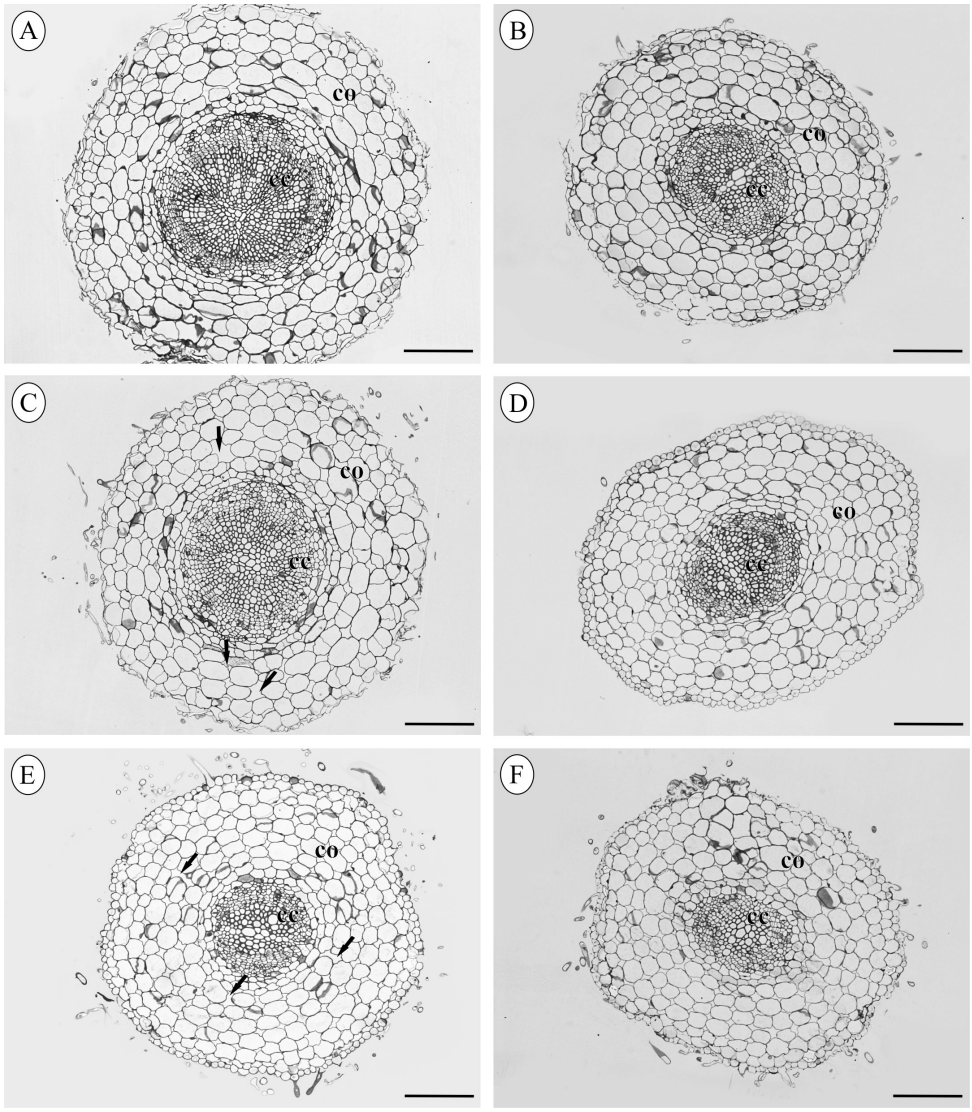


Figure 4

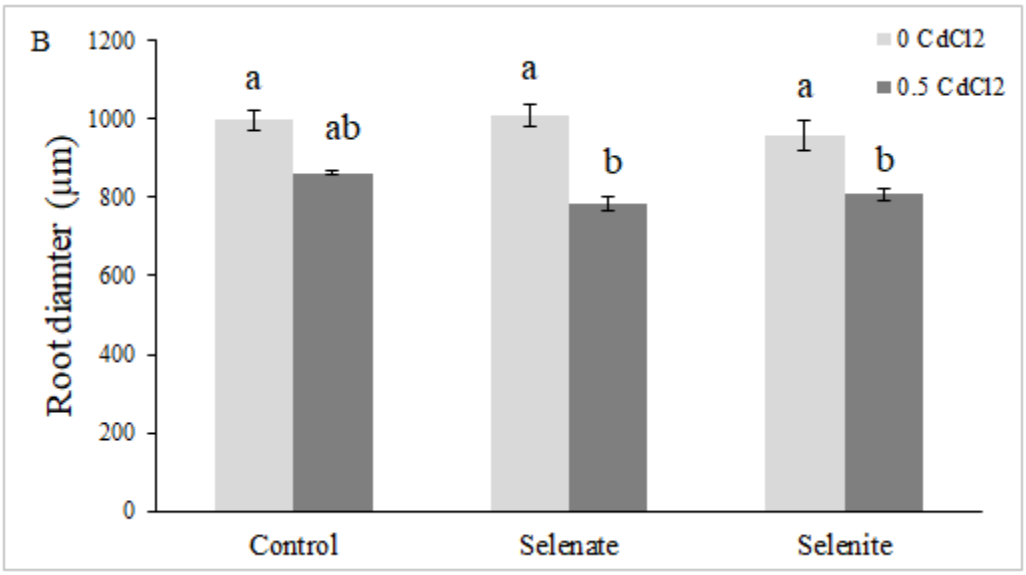
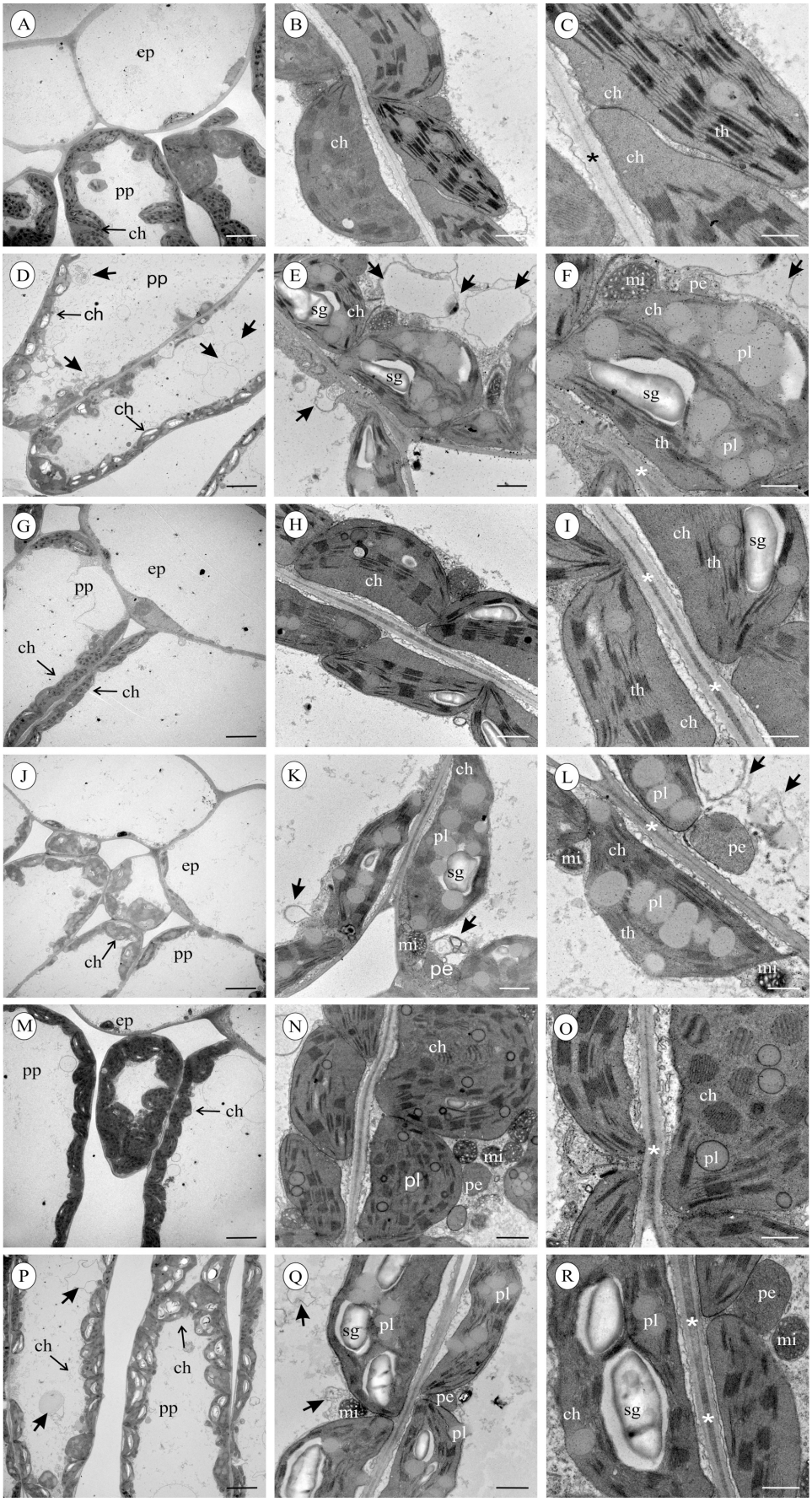

B
1200
1000
800
600
400
200
0
Root diamter (µm)
a
ab
a
b
a
b
0 CdCl2
0.5 CdCl2
Control
Selenate
Selenite

Figure 5


A
ep
pp
ch
B
ch
C
ch
th
ch
D
pp
ch
ch
E
sg
ch
sg
F
mi
pe
ch
pl
sg
th
pl
G
ep
pp
ch
ch
H
ch
I
ch
sg
th
th
ch
J
ep
ch
pp
K
ch
pl
sg
mi
pe
L
pl
mi
ch
pe
pl
th
mi
M
ep
pp
ch
N
ch
mi
pl
pe
O
ch
pl
P
ch
ch
pp
Q
pl
sg
pl
mi
pe
pl
R
pe
pl
mi
ch
sg