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Integrated approach of anatomical, physiological and biochemical parameters for the study of tolerance mechanisms to cadmium in tomato accessions

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

Piracicaba
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATORY

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I also offer it to Marcos, for his companionship and patience during the hard moments.

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SUMMARY

RESUMO

Abordagem integrada de parâmetros anatômicos, fisiológicos e bioquímicos para o estudo de mecanismos de tolerância ao cádmio em acessos de tomateiro

O consumo de tomate (*Solanum lycopersicum* L.) tem aumentado a cada ano devido à atratividade dos frutos, suas diversas utilizações e efeitos benéficos para a saúde humana. No entanto, os frutos de tomate podem acumular uma concentração de cádmio (Cd) que excede o limiar de segurança para o consumo humano, mesmo quando as plantas são cultivadas em solo com níveis aceitáveis de Cd. Cádmio é um elemento não-essencial, extremamente perigoso para os sistemas biológicos, desencadeando várias doenças em seres humanos. Nas plantas, o Cd perturba a maquinaria antioxidante, altera o estado nutricional e prejudica a produção e /ou o particionamento de fotoassimilados, frequentemente reduzindo a produtividade e qualidade de frutos. No entanto, diferentes acessos de tomateiros podem apresentar contrastantes graus de tolerância à toxicidade gerada pela exposição ao Cd, como detectado em estudos anteriores de nosso grupo. O uso desses acessos é uma abordagem poderosa para identificar as estratégias empregadas pelas plantas para lidar com os desafios induzidos pelo Cd; e o conhecimento de tais estratégias pode ser potencialmente utilizado em programas biotecnológicos e de melhoramento genético. Deste modo, o conjunto de estudos que compõem a presente tese objetivou (i) identificar os principais mecanismos que suportam o grau de tolerância contrastante à toxicidade induzida por Cd em acessos de tomate após exposição a curto e longo prazos a este metal pesado; (ii) avaliar a relação entre o grau de tolerância e os atributos físico-químico de frutos oriundos de tomateiros cultivados em solo contendo Cd, e (iii) determinar os efeitos transgeracionais do estresse induzido por Cd. No primeiro experimento, nove acessos de tomateiro com graus variados de tolerância à exposição ao Cd, baseado na acumulação de biomassa, foram cultivados em solução hidropônica contendo 35 μM de $CdCl_2$ durante 6 dias. O impedimento de elevada concentração de magnésio (Mg) em raízes foi identificado como possível estratégia da planta para mitigar a toxicidade de Cd, por meio da evitação da formação de pêlos radiculares. Em relação ao modo de ação da toxicidade induzida por Cd, o excesso de Mn, em adição à elevada concentração de Cd, parece estar acoplado aos danos foliares que são acentuados ainda mais pelas altas concentrações de zinco (Zn) e boro (B) nos tecidos fotossintéticos de plantas sob exposição ao Cd. No segundo experimento, os genótipos tolerantes (Yoshimatsu) e sensíveis (Tropic Two Orders) foram cultivados em solo contendo Cd, a fim de avaliar os parâmetros de produção. O genótipo tolerante apresentou frutos com maior diâmetro, altura e peso após o cultivo em solo contendo Cd, quando comparado às plantas controle. Em ambas as cultivares, a concentração de Cd variou de acordo com a seguinte ordem descendente: raízes = folíolos> (receptáculo floral, pedúnculo e sépalas) > caule = casca de fruta = polpa de fruta. Além disso, dados sugerem que o receptáculo floral e suas estruturas atuaram como uma barreira ao transporte de Cd para os frutos, entretanto, ela não foi suficiente para evitar que o Cd atingisse os frutos. Em adição, a exposição ao Cd provocou notáveis reduções na concentração de Mg nas raízes de genótipos sensíveis e tolerantes, revelando que a aclimatação das plantas depende do baixo status de Mg em tecidos radiculares. Desde que ambas as cultivares são capazes de empregar este mecanismo, os dados sugerem que, durante a exposição a curto prazo ao Cd, acessos tolerantes são capazes de ativá-lo ou mais cedo ou mais rápido do que acessos sensíveis. Ademais, efeitos transgeracionais positivos na germinação e vigor das sementes do genótipo tolerante foram desencadeados pelo cultivo planta-mãe em solo com Cd, apesar do aumento de anormalidades cromossômicas. Este trabalho reportou novos conhecimentos sobre os efeitos da exposição ao Cd sobre o desenvolvimento do tomateiro, mecanismos de tolerância, qualidade e rendimento de frutos, bem como a distribuição de Cd dentro da planta.

Palavras-chave: *Solanum lycopersicum*; Metais pesados; Segurança alimentar; Estresse oxidativo; Remobilização de nutrientes; Efeitos transgeracionais

ABSTRACT

Integrated approach of anatomical, physiological and biochemical parameters for the study of tolerance mechanisms to cadmium in tomato accessions

Tomato (*Solanum lycopersicum* L.) consumption has increased every year due to the fruit attractiveness, several utilizations, and beneficial effects for human health. However, tomato fruits can accumulate a Cd concentration that exceeds the safety threshold for human consumption of vegetables, even when plants are grown in soil with acceptable Cd level. Cd is a non-essential, hazardous element to biological systems, triggering several diseases in humans. In plants, Cd disturbs the antioxidant machinery, changes the nutritional status, and impairs the photoassimilate production and/or partitioning, hence reducing fruit yield and quality. However, distinct tomato accessions can present contrasting tolerance degree to Cd toxicity, as detected by our group in previous studies. The use of these accessions is a powerful approach to identify strategies employed by plants to cope with Cd-induced challenges, and the acknowledgement of such strategies can be potentially used in breeding and biotechnological programs to improve fruit yield and quality in crops that were cultivated in contaminated fields. The set of studies that compose the present thesis aimed (i) to identify the main mechanisms for the contrasting tolerance degree to Cd-induced toxicity in tomato accessions after short and long-term Cd exposure; (ii) to evaluate the relationship among tolerance degree and fruits attributes in plants that were grown in Cd-containing soil, and (iii) to determine the transgenerational effects of Cd-induced stress. In the first experiment, nine tomato accessions with a varied tolerance degree, which was based on biomass accumulation, to Cd exposure were grown in hydroponic solution containing $CdCl_2$ 35 μM for 6 days. Avoidance of high Mg concentration in roots was identified as a plant strategy to mitigate Cd toxicity by preventing formation of root hairs. Regarding the mode of action of Cd toxicity, Mn excess in leaves, in addition to the high Cd concentration *per se*, seems to be coupled to leaf damages that are enhanced by the increased Zn and B concentrations in the photosynthetic tissues. In the second experiment, tolerant (Yoshimatsu) and sensitive (Tropic Two Orders) genotypes were grown in Cd-containing soil, in order to evaluated production parameters. After plant exposure to Cd, the tolerant genotype presented an increased fruit diameter, height and weight, when compared to the control plants. In both cultivars, Cd concentration varied according to the following descending order: roots = leaf blade > (floral receptacle, peduncle and sepals) > stem = fruit peel = fruit pulp. Moreover, data suggested that floral receptacle and its related-structures acted as a barrier to the Cd transportation to the fruits, but it was not enough to avoid Cd reaching the fruits. Furthermore, Cd exposure provoked remarkable reductions in the Mg concentration in roots of sensitive and tolerant genotypes, revealing that both tomato cultivars are able to employ this mechanism for plant acclimation to long-term Cd exposure. Considering such information, it is possible that, under the short-term Cd exposure, tolerant accessions activate this mechanism either early or faster than sensitive genotypes. In addition, positive transgenerational effects on seed germination and vigor of the tolerant genotype were triggered by the plant-mother cultivation in Cd-containing media, despite of the increased chromosomal abnormality. This work reported new insights about the effects of Cd exposure on tomato development, tolerance mechanisms, fruit quality and yield of tomato, as well as Cd distribution in the plants.

Keywords: *Solanum lycopersicum*; Heavy metals; Food security; Oxidative stress; Nutrient remobilization; Transgenerational effects

SAMENVATTING

Een geïntegreerde studie op basis van anatomische, fysiologische en biochemische parameters van tolerantiemechanismen tegen cadmium in verschillende tomatenvariëteiten

Consumptie van tomaten (*Solanum lycopersicum* L.) neemt jaarlijks toe omwille van de aantrekkelijkheid van deze groente, verschillende toepassingen ervan in de voeding en zijn gunstige effecten op de humane gezondheid. Tomaten kunnen echter een concentratie van cadmium (Cd) accumuleren die de veiligheidsdrempel overschrijdt voor humane consumptie, zelfs wanneer planten in grond met een toegelaten Cd niveau worden geteeld. Cd is een niet-essentieel, toxisch element voor biologische systemen, en kan bij de mens verschillende ziekten veroorzaken. In planten verstoort Cd de antioxidatieve mechanismen en beïnvloedt het de nutriëntenbalans en de fotosynthese, waardoor de opbrengst en de kwaliteit van de tomaten worden verminderd. Er zijn echter heel wat tomatenvariëteiten die een verschillende graad van tolerantie voor Cd vertonen zoals reeds door onze onderzoeksgroep in eerdere studies is aangetoond. Het gebruik van deze variëteiten biedt een interessante manier om strategieën die planten gebruiken om om te gaan met deze Cd-geïnduceerde stress te bestuderen. Een betere kennis van deze mechanismen kan potentieel worden gebruikt in specifieke teelt en biotechnologische programma's om de opbrengst van gewassen en de kwaliteit ervan te verbeteren. De verschillende hoofdstukken waaruit het proefschrift is samengesteld, focussen op (i) de identificatie van mechanismen die de tolerantiegraad voor Cd-geïnduceerde toxiciteit bij verschillende tomatenvariëteiten na korte en langdurige blootstelling aan Cd; (ii) de evaluatie van de relatie tussen tolerantiegraad en fysico-chemische parameters van de tomaten die in Cd-houdende grond werden geteeld en (iii) de analyse van de transgenerationele effecten van Cd-geïnduceerde stress. In het eerste hydropoon experiment werden negen tomatenvariëteiten met een verschil in tolerantiegraad, gebaseerd op accumulatie in de biomassa, blootgesteld gedurende 6 dagen aan 35 μM $CdCl_2$. Het vermijden van hoge concentraties van Mg in wortels werd geïdentificeerd als een mogelijke plantstrategie om Cd toxiciteit te verminderen door de wortelintegriteit te ondersteunen en de vorming van wortelharen te voorkomen. Wat de werking van Cd-geïnduceerde toxiciteit betreft, lijkt een overmaat aan Mn naast een hoge Cd concentratie gekoppeld aan bladschade die nog verder wordt verhoogd door de verhoogde Zn- en B-concentraties in de fotosynthetische weefsels. In het tweede experiment werden tolerante (Yoshimatsu) en gevoelige (Tropic Two Orders) genotypes gegroeid voor de volledige biologische cyclus in Cd-houdende grond om de productieparameters te evalueren. Beide genotypes toonden geen verschillen in het aantal vruchten na blootstelling aan Cd. Er werd echter een verhoogde vruchtdiameter alsook de grootte en het gewicht van de tomaten waargenomen in tolerante tomaten na blootstelling aan Cd in vergelijking met de controle planten. In beide cultivars varieerden de Cd concentraties volgens de volgende aflopende reeks: wortels = blad> (bloemboden, bloemsteel en kelkbladeren) > stengel = schil van de tomaat = vlees van de tomaat. Bovendien konden we uit de gegevens afleiden dat de bloembodem als een barrière voor het Cd transport naar de vruchten optreedt, maar onvoldoende om te voorkomen dat Cd de vruchten bereikt. Bovendien veroorzaakte Cd blootstelling duidelijke reducties in de Mg concentratie in wortels van gevoelige en tolerante genotypen, waarbij duidelijk werd dat beide tomaten cultivars dit mechanisme kunnen gebruiken voor de aanpassing aan langdurige Cd blootstelling. Dit mechanisme wordt waarschijnlijk eerder of sneller geactiveerd in de tolerante variëteit in vergelijking met het gevoelige genotype. Tot slot werden positieve transgenerationele effecten op zaadkieming en kiemkracht van het tolerante genotype waargenomen wanneer de moederplant op Cd-houdende media werden opgegroeid ondanks de verhoogde chromosomale abnormaliteit. Dit werk beschrijft nieuwe inzichten over de effecten van Cd blootstelling op de ontwikkeling van tomaten, tolerantiemechanismen, vruchtkwaliteit en tomaatopbrengst, evenals Cd distributie in de planten.

Trefwoorden: *Solanum lycopersicum*; Zware metalen; Voedselveiligheid; Oxidatieve stress; Nutriëntenherverdeling; Transgenerationeel effecten

1. GENERAL INTRODUCTION

Human health can be affected by exposure to cadmium (Cd), which triggers infertility (Alaee et al., 2014), causes kidney and bone diseases, and increases cancer risk (Järup and Åkesson, 2009). The diet is the main source of Cd to non-smokers, who intake more than 80% of the food-Cd from cereals, vegetables and products made from them (Olsson et al., 2002). Therefore, many countries are regulating Cd concentration in edible portions of crops, as well as in agricultural soils from where plants uptake this heavy metal (Commission of the European Communities, 2014; CETESB, 2014). Usually, Earth's crust has a natural and low-Cd concentration that ranges from 0.01 to 0.3 mg kg^{-1} and 0.2 to 0.8 mg kg^{-1} in uncontaminated sandy and loamy soils, respectively (Kabata-Pendias, 2011). However, contamination of agricultural land by heavy metals has increased since the first industrial revolution, especially near urban and industrial centers, where several vegetables are commonly grown (Loganathan et al., 2012; Piotto, 2012).

The major source of soil cadmium is atmospheric deposition from metal smelters and phosphorus (P) fertilizers, although a substantial amount is released through mining, metal-based pesticides, industrial waste, and battery production (Alloway and Steinnes, 1999; Kabata-Pendias, 2011; Loganathan et al., 2012). In soils, Cd mobility is influenced by pH, texture and organic matter, but pH is the most important factor (Basta et al., 2001; Kabata-Pendias, 2011). The highest Cd mobility is found in acidic soils within the range of pH 4.55 – 5 (Kabata-Pendias, 2011). Most of the Cd (55 to 90%) is presented as free metal ion that is readily available to plants, which absorbs Cd through roots and spreads it to shoots after a short period of exposure (Taylor and Percival, 2001; Lux et al., 2011; Chou et al., 2011; Kudo et al., 2015; Nogueirol et al., 2016; Pompeu et al., 2017). Once inside the plants, Cd triggers oxidative stress, disturbs nutrient uptake and distribution, decreases biomass production due to the damages in the photosynthetic apparatus, and reduces fruit quality and yield (Gratão et al., 2005; 2008, 2009, 2012, 2015; Hédiji et al., 2010, 2015; Haouari et al., 2012; Hartke et al., 2013; Cuypers et al., 2016; Alves et al., 2017).

The current threshold for Cd concentration in vegetables is 0.05 mg kg^{-1} (Commission of the European Communities, 2014); however, tomato fruits have been shown to present a Cd concentration higher than the maximum level (Hussain et al., 2015). Depending on tomato cultivar, Cd concentration ranged from 0.0107 to 0.0970 mg kg^{-1} in fruits from plants grown in soil containing 3 ppm of Cd (Hussain et al., 2015), which is a suitable value for arable soils (CETESB, 2014). Therefore, tomato fruits are a pathway for Cd entry into the food chain, being a potential threat to human health. Considering this information, our group has studied Cd effects on development of several tomato accessions in order to understand the mode of action of Cd-induced toxicity, as well as the mechanisms that plants employ to cope with the challenges imposed by this metal. Tomato (*Solanum lycopersicum* L.) was selected for this study due to its agricultural and economical relevance, which is coupled to its advantages as a scientific tool when compared to several model plants.

Tomato is a worldwide-cultivated crop that presents the highest market value among vegetables, which are included in the group of the 50 most produced commodities nowadays (FAOSTAT, 2016). Its consumption has increased every year due to fruit attractiveness, several utilizations, and positive effects on human health (Bergougnoux, 2014), such as the prevention of cardiovascular diseases due to the presence of flavonoids (Willcox et al., 2003). Additionally, tomato intake presented a hepatoprotective potential against Cd toxicity by reducing its accumulation in the liver (Nwokocha et al., 2012). Tomato is also the best model organism for fleshy-fruited plants to be used in research programs because it presents i) a sequenced and small genome (approximately 900 Mb); ii) a large set of spontaneous and artificial mutants; iii) a short life cycle; iv) photoperiod insensitivity, and v) specific morphological

traits that are not shared with other model plants (Bergougnoux, 2014). Furthermore, tomato can be grown in different conditions (from soils to hydroponics), and also be propagated asexually by grafting, or regenerated from distinct parts of the plant (Ajenifujah-Solebo et al., 2013; Gratão et al., 2015; Nogueirol et al., 2016).

The cultivated tomato is a self-pollinating plant (2n = 2x = 12 chromosomes) with a high morphological diversity, but low genetic diversity due to the successive genetic bottlenecks and the modern breeding practices (Miller and Tanksley, 1990; Ranc et al., 2012; The Tomato Genome Consortium, 2012). Tomato was domesticated from its wild relative *S. pimpinellifolium*, and the first domesticated form was represented by the cherry tomato *S. lycopersicum* var. *cerasiforme* (Ranc et al., 2012). Breeders have used the wild germplasm through interspecific crosses since 1940's, in order to select positive features for fruits and tolerance to a wide range of stresses (Bai and Lindhout, 2007). Among the 13 wild relatives, *S. pimpinellifolium* is probably the most varied source of agronomic traits of interest. It can be used to improve several plant characteristics (height, growth habit and self-pruning); fruit quality and yield (weight, length, shape, diameter, firmness, viscosity, soluble solid content, ripening, maturity and locule number) and tolerance to abiotic (cold, drought and salt) and biotic stresses (virus, bacterium, fungi and insect) (Bergougnoux, 2014). Therefore, the choice of tomato accessions that were used in the present studies (*S. pimpinellifolium*, *S. lycopersicum* and *S. lycopersicum* var. *cerasiforme*) was based on their importance in tomato breeding, as well as to their differential tolerance degree to Cd-induced stress.

The magnitude of Cd effects on tomato development depends on cadmium concentration in the growth media, time length of exposure, growth media properties and plant features, such as genotype and phenological stage (Gratão et al., 2008, 2009, 2012; Monteiro et al., 2011; Piotto, 2012; Nogueirol et al., 2016; Pompeu et al., 2017; Alves et al., 2017). Furthermore, microorganism-plant interaction can influence Cd effects on tomatoes, since inoculation of *Burkholderia* ssp., *Pseudomonas* sp. and *Bacillus* sp. decreased Cd toxicity in *S. lycopersicum* (He et al., 2009; Dourado et al., 2013). Cd accumulates preferentially in tomato roots than shoots, and its translocation to aboveground organs is probably driven by transpiration (Gratão et al., 2015; Delpérée and Lutts, 2008; Hédiji et al., 2010, 2015; Lux et al., 2011; Hartke et al., 2013; Hussain et al., 2015; Kumar et al., 2015). Inside plants, Cd activates several biochemical, physiological, anatomical, and molecular mechanisms. Frequently, there is an overproduction of reactive oxygen species (ROS), which act as both signaling and toxic compounds to cell integrity (Gratão et al., 2005; Cuypers et al., 2016), hence harming proteins, lipids, carbohydrates and DNA (Gill and Tujeta, 2010).

In tomato, lipid peroxidation can be increased after Cd exposure through different modes that ranged from ROS overproduction (mainly due to the reductions in the activity of enzymatic and non-enzymatic antioxidants) to changes in the lipid biosynthesis and/or its degradation pathways (Ouariti et al., 1997; Ben Ammar et al., 2007; 2008; Monteiro et al., 2011; Gratão et al., 2015; Nogueirol et al., 2016; Pompeu et al., 2017). Protein carbonylation was also reported in roots and leaves of Cd-stressed tomato (Djebali et al., 2008). Furthermore, ROS is probably related to the increased chromosomal aberrations and decreased mitotic index in cells from tip roots of tomato seedlings that were exposed to Cd (Pizzaia, 2013). In order to scavenge ROS excess, Cd-stressed tomato changes the enzymatic pattern in all tissues, being noted differences in superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPOX, EC 1.11.1.7), glutathione reductase (GR, EC 1.6.4.2), and/or ascorbate peroxidase (APX, EC 1.11.1.11) (Dong et al., 2006; Gratão et al., 2005, 2008, 2012, 2015; Monteiro et al., 2011; Nogueirol et al., 2016; Pompeu et al., 2017). Early studies also reported modifications in the metabolisms of non-enzymatic compounds that are related to ROS scavenging, such as α-tocopherol (Hédiji et al., 2010).

Cd also activates mechanisms related to the maintenance of ion homeostasis through Cd complexation by amino acids, organic acids, glutathione (GSH) and glutathione-derived peptides (Hall, 2002), namely phytochelatin

(Steffens et al., 1986; Scheller et al., 1987; Gupta and Goldsbrough, 1991; Chen and Goldsbrough, 1994; Mediouni et al., 2006; Ben Ammar et al., 2008a). It seems that roots are less affected by Cd than tomato leaves, where this heavy metal triggers quickly chlorosis and necrosis and decreases the leaf area and biomass (Dong et al., 2005; Ben Ammar et al., 2008ab; Djebali et al., 2008; Gratão et al., 2008; Hédiji et al., 2010). Histological and ultrastructural investigations showed Cd changes the shape and internal organization of chloroplasts, formation of intercellular spaces, and size of mesophyll cells in leaves of 'MicroTom' wild-type and its mutants (Gratão et al., 2009; Pompeu et al., 2017). Interestingly, Cd presented different effects on these genotypes, since the intercellular spaces were decreased in *dgt* and *sit*, but increased in *Nr*.

Anatomical modifications were also found in tomato roots after Cd exposure, which triggered disintegration of epidermis and external cortical cell layers of roots (Gratão et al., 2009). The damages in root tissues changed the nutrient and water absorption that, in addition to the injuries in the photosynthetic apparatus, decreased fresh and dry weight of the whole plant (Dong et al., 2005; Delpérée and Lutts, 2008; Gratão et al., 2009; Gratão et al., 2015; Pompeu et al., 2017). In shoots, Cd caused the closure of stomatal pores, reductions in the transpiration and photosynthetic rates, decreases in the chlorophyll and carotenoid contents, as well as injuries in the chloroplast structures (Chaffei et al., 2004; Ben Ammar et al., 2007; Gratão et al, 2009, 2015; Hédiji et al., 2010; Pompeu et al., 2017). Therefore, the primary carbon metabolism is generally disturbed in tomato subjected to Cd stress, decreasing plant potential to produce photoassimilates and, consequently, reducing biomass accumulation (Hédiji et al., 2010, 2015; Rodríguez-Celma et al., 2010).

However, the magnitude of Cd side-effects on tomato can be potentially managed. Gratão et al. (2008) showed that plant exposure to gradual increasing concentrations of $CdCl_2$ (from 0.05 to 1 mM) enhances tomato tolerance to Cd stress. According to Hédiji et al. (2010, 2015), tomato is able to acclimate to the long-term exposure to Cd. Moreover, there are reports about the maintenance or even increases in the biomass of Cd-stressed tomato when compared to control plants (Nogueirol et al., 2016; Pompeu et al., 2017). For instance, tomato cv. Calabash Rouge presented similar biomass in shoots and roots of plants that were grown, during 34 days, in sandy and clay soils with 0, 3, and 6 mg kg-1 of Cd (Nogueirol et al., 2016). In addition, plant exposure to $CdCl_2$ 10 and 100 μM during 96 hours provided increments in the biomass of 'MicroTom' wild-type and *sit* mutant, when compared to the non-treated tomato (Pompeu et al., 2017). Therefore, it is clear that tomato tolerance to Cd exposure depends on genotype, and that plants can use some time-depended strategies to overcome challenges imposed by Cd stress. Thus, this work aimed (i) to identify the main mechanisms for the contrasting tolerance degree to Cd-induced toxicity in tomato accessions after short and long-term Cd exposure; (ii) to evaluate the relationship among tolerance degree and fruits attributes in plants that were grown in Cd-containing soil, and (iii) to determine the transgenerational effects of Cd-induced stress on seed germination, vigor and nutrition.

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2. TOMATO CULTIVARS WITH SIMILAR TOLERANCE DEGREE EMPLOY DIFFERENT STRATEGIES TO COPE WITH CADMIUM-INDUCED STRESS

ABSTRACT

Tolerant plant genotypes frequently exhibit a greater cadmium (Cd) concentration in shoots than sensitive genotypes. The disadvantage is the high Cd accumulation in edible portions of crops, hence increasing the potential intake of toxic amounts of Cd by humans. Therefore, tomato cultivars (Rey de Los Tempranos, Nagcarlang and Moneymaker) with intermediary tolerance to Cd toxicity were selected in order to investigate the mechanisms that could be coupled to reduced Cd translocation and decreased sensitivity to Cd exposure. Although differences in the root-to-stem Cd translocation were observed, they were not enough to provide decreases in the leaf Cd concentration of tomato cultivars. An increased lipid peroxidation, which was not coupled to the H_2O_2 overproduction, was detected in leaves of Rey de Los Tempranos and Nagcarlang under Cd exposure. In all cultivars, Cd induced a high manganese (Mn) and boron (B) accumulation in leaves, suggesting that both micronutrients enhance the damages in photosynthetic tissues. For Mn, increases in root-to-leaf translocation in Cd-challenged plants were observed. By contrast, a high B uptake, rather than changes in its translocation, provided the great B accumulation. Boron excess and potassium (K) deficiency, in addition to the high Cd concentration, may contribute for reductions of the chlorophyll content in leaves. Phosphorus (P), iron (Fe), calcium (Ca) and copper (Cu) concentrations in leaves were not affected by Cd exposure probably because of their remobilization from roots and, especially stems. This study shows that each genotype employed different strategies to manage the mineral cycling under Cd exposure, and such varied behavior may be related to the distinct effects of this metal on organ development and biomass partitioning.

Keywords: Boron toxicity; Heavy metals; Manganese translocation; Nutrient remobilization; Oxidative stress; *Solanum lycopersicum*

2.1. Introduction

Cadmium (Cd) is naturally found as a trace element, but industrial and agricultural activities have increased its concentration in several areas, from where plants quickly absorb this metal (Kabata-Pendias, 2011; Gallego et al., 2012; Teklić et al., 2013; Pompeu et al., 2017). The direct consequence is the reduced crop development and yield, but risks to human health through intake of contaminated plant-origin products must be considered (Gallego et al., 2012; Augustsson et al., 2015; Dziubaneka et al., 2017). In humans, Cd toxicity has been associated to kidney and bone diseases, as well as increased cancer incidence (Järup and Åkesson, 2009; Nair et al. 2013). Within the plant, Cd triggers misbalances in the antioxidant machinery and disturbances in the nutrient homeostasis (Fidalgo et al., 2011; Gallego et al., 2012; Štolfa et al., 2015; Cuypers et al., 2016; Alves et al., 2017; Bayçu et al., 2017a, b), which is also observed in response to a variety of abiotic and biotic stresses (Gratão et al., 2005; Fidalgo et al., 2013; Hippler et al., 2016; Soares

et al., 2016; Peters et al., 2017). Moreover, disruptions in the photosynthetic apparatus is usually coupled to Cd translocation to leaves, in which this metal can be found after a short period of plant exposure, hence preventing the overall plant development due to the lower biomass production (Gratão et al., 2012, 2015; Iannone et al., 2015; Nogueirol et al., 2016; Sebastian and Prasad, 2016a, b; Pompeu et al., 2017).

Cadmium reduces the leaf conductance (Delpérée and Lutts, 2008; Gratão et al., 2015) due to inductions in the stomata closure (Gratão et al., 2009), disturbing water balance and carbon assimilation in Cd-challegend plants (Perfus-Barbeoch et al., 2002). Thus, it is tempting to suppose that variations in Cd absorption, transportation and accumulation may explain the diverse tolerance degree as well as phenotypes of cultivars/varieties of plant species under Cd exposure, however, the results in the literature are contradictory. In black oat plants (*Avena strigose* Schreb.), genotypes with the highest Cd translocation and concentration in leaves presented slight decreases in plant dry weight, while the low-Cd accumulator, sensitive cultivar exhibited major biomass losses (Uraguchi et al., 2009a). By contrast, in rice genotypes with distinct root-to-shoot Cd translocation, which was the main factor determining shoot and grain Cd accumulation, no differences in the plant dry weight were observed (Uraguchi et al., 2009b). However, the use of alternative approaches, such as grafted and genetically modified plants, have shown that a high Cd concentration in leaves can be related to the reductions in plant growth and development (Gratão et al., 2015; Iannone et al., 2015).

In tomato (*Solanum lycopersicum* L.), a varied tolerance degree to Cd exposure has been detected, but this phenomenon is not always linked to distinct Cd uptake and/or translocation to shoots (preliminary data). Therefore, the differential Cd accumulation did not explain completely (i) the superior performance, which was based on biomass production, of some of the genotypes during exposure to this metal, and (ii) the distinct effects on biometric parameters of genotypes with similar tolerance degree to this metal (data obtained by our group but not yet published). These observations suggested that plants used distinct strategies to cope with Cd-induced challenges, a phenomenon that should be explored by researchers in order to discover mechanisms that can improve crop tolerance against heavy metal-induced stress. Therefore, this work aimed to investigate some aspects related to the side effects and protective mechanisms that are triggered by Cd exposure in tomato with an intermediary tolerance to this metal.

2.2. Materials and Methods

2.2.1. Plant material and growth conditions

Three tomato cultivars (*Solanum lycopersicum* cvs.) with intermediary tolerance to Cd exposure (i.e. plants that were not ranked in the group of the most tolerant or sensitive accessions after plant growth in Cd-containing media) were selected according to the studies carried out in our laboratory (data obtained by our group but not yet published). Tomato seeds were chemically scarified by stirring in 2% HCl (v:v) for 15 minutes, in order to standardize germination. Subsequently, seeds were sown in polystyrene trays filled with thin exfoliated vermiculite, which was irrigated four times a day. During germination and seedling establishment, trays were kept in a greenhouse with temperature and relative humidity of 24.9 ± 1.58 °C and 78.9 ± 5.22%, respectively. After seedling emergence, daily application of macro- and micronutrients (Peters Professional 20-20-20 at 1 g L^{-1}) was initiated in order to maintain suitable seedling development. After one week, this concentration was increased to 1.5 g L^{-1}, which was used until the 18-days-old seedlings were transplanted to the hydroponics.

Seedlings were removed from the trays and their roots were washed and then transferred to hydroponic system (tanks) containing nutrient solution at 10% ionic strength that is used for adult tomato plants (Hoagland and

Arnon 1950). Seedlings were fixed in 200 mm thick styrofoam plates using foam pieces, where plants were spaced from each other by 8 cm. Plants were maintained in hydroponics for 6 days as an adaptation period in order to mitigate stress generated by seedling transplantation, and also to increase nutrient concentration from 10 to 50% ionic strength. This procedure (gradual increase of salt concentration) was carried out to diminish plant stress due to an increased content of salts in solution.

Twenty-four-day-old plants (three/four-leaf stage) were then subjected to Cd exposure by adding 35 μM $CdCl_2$ to the nutrient solution, which was monitored through electrical conductivity and pH checking that exhibited 1.2 mS cm^{-2} and 6.54 ± 0.07 average values, respectively. Seedlings were grown under control (Cd free) and Cd-containing hydroponic solution for 6 days, which was chosen because it is sufficient to detect the onset of most frequent toxicity symptoms (i.e. chlorosis, necrosis and decreased height) in tolerant accessions, but avoiding severe damages in the sensitive genotype group. During the experiment, distilled-deionized water was added to the tanks daily, in order to replace water lost through evapotranspiration. The homogeneous distribution of nutrient solution and suitable oxygenation level were maintained by air pump systems in each tank.

2.2.2. Biometric variables

The stem and root lengths were evaluated with a millimeter ruler. The stem diameter was measured in the region immediately above cotyledonar leaves (or their scars) using digital caliper. For leaf area evaluation, fully expanded leaves were detached from plants and measured through a leaf area meter (LI-COR®, LI-3100). Samples of roots, stems and leaves were kept in paper bags and dried in an oven (60 ºC) until constant weight for dry mass determination. The specific leaf area – SLA (leaf area / leaf dry weight), leaf area ratio – LAR (leaf area/ total plant dry weight) and leaf weight ratio LWR – (leaf dry weight/ total plant dry weight) were also calculated. All growth analysis-related variables were obtained from evaluations carried out in nine plants.

2.2.3. Chlorophyll content

Chlorophyll content was indirectly evaluated using a Soil Plant Analysis Development (SPAD) chlorophyll meter (Konica Minolta, SPAD-502 model) through two measurements in the biggest terminal leaflets of two youngest and fully expanded leaves in each of the nine plants per cultivar.

2.2.4. Biochemical analyses

In order to evaluate the oxidative stress triggered by Cd exposure, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents as well as the enzymatic activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and glutathione reductase (GR, EC 1.6.4.2) were analyzed in completely expanded leaves from the shoot middle third of plants that composed each of the three replications.

In the greenhouse, leaves were collected and rapidly stored in liquid nitrogen. Next, all samples were stored in -80 ºC freezer until the analyses. Before their onset, leaf tissues were grinded to a fine powder in liquid nitrogen. Lipid peroxidation was measured as MDA content according to Heath and Packer (1968), and hydrogen peroxide

content was determined using procedures of Alexieva et al. (2001). The extraction of antioxidant enzymes was carried out according to Azevedo et al. (1998). Protein content was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard. CAT and GR total activities were determined as described by Azevedo et al. (1998). SOD total activity was determined as described by Cembrawska-Lech et al. (2015).

2.2.5. Cadmium, macro and micronutrient concentrations

The dried samples were milled to determine Cd effects on nutrient uptake and translocation by quantifying the concentrations of Cd, calcium (Ca), potassium (K), magnesium (Mg), phosphorous (P), sulphur (S), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn) and boron (B) through ICP-OES (inductively coupled plasma optical emission spectrometry) analysis, which was preceded by nitro-perchloric digestion of root, stem and leaf tissues of Cd-exposed (stressed) and control (non-stressed) plants. Three replicates composed of three plants were used. All procedures were carried out in the Instituto Agronômico de Campinas (IAC, Campinas, Brazil).

2.2.6. Tolerance and translocation indexes

Tolerance index (TI) calculation was based on Piotto (2012), according to the following formula (1):

$TI = (DWfCdi - DWoi) / (DWfCti - DWoi)$ - (1)

Where $DWfCdi$ = dry weight of plants of i accession exposed to Cd; $DWoi$ = dry weight of i accession in the moment of Cd application, and $DWfCti$ = dry weight of control plants of i accession. Therefore, TI values can range from 0 to 1 (100%), where 0 indicates the maximum sensitivity, and 1 designates the maximum tolerance. In addition, the translocation index (Cd concentration in leaves or stems divided by Cd concentration in roots) was also estimated.

2.2.7. Statistical procedures

The experiment was carried out in a completely randomized design and factorial scheme 3 x 2 (tomato cultivars - Rey de Los Tempranos, Nagcarlang and Moneymaker *vs* Cd concentration - 0 and 35 μM). Before analysis of variance (ANOVA), data were subjected to tests through the "Guided Data Analysis" tool of statistical software SAS (SAS Institute, 2011) in order to check the assumptions for the ANOVA performance (i.e. normal distribution, variance homogeneity and error independence). Moreover, data transformations were performed when indicated by this tool. Next, ANOVA ($p \leq 0.05$) were performed, and the means of the treatments were compared by the Tukey's test ($a \leq 0.05$). In addition, the Pearson's correlation analysis was employed to evaluate the cause-effect relations among some of the variables using SAS software (SAS Institute, 2011).

2.3. Results

2.3.1. Cadmium concentration in roots, stems and leaves

Increases in Cd accumulation in all vegetative tissues of the three tomato cultivars under Cd exposure were observed, when compared to the control plants (Table 1). Roots exhibited a higher Cd concentration than stems and leaves, regardless cultivar (Table 1). However, Cd concentration in stems depended on tomato cultivar (Table 1).

Table 1. Cadmium (Cd) concentration in roots, stems and leaves of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd, that were grown in hydroponic solution with 0 and 35 μM $CdCl_2$ for 6 days

Treatments	Cd (mg kg^{-1} DW)		
	Roots	Stem	Leaves
$CdCl_2$ concentrations			
0	0.45 b	0.12 b	0.22 b
35	1904.76 a	105.97 a	278.63 a
Tomato cultivars (TC)			
RLT	913.29 a	53.95 ab	143.59 a
NGL	939.82 a	45.49 b	132.87 a
MNM	1004.71 a	59.68 a	141.82 a
Significance			
$CdCl_2$	***	***	***
TC	NS	0.052	NS
$CdCl_2$*TC	NS	NS	NS

The distinct letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations, or for comparisons among tomato cultivars. NS and ***: Nonsignificant and significant at $p \leq 0.001$, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker

2.3.2. Macro and micronutrient concentrations

2.3.2.1. Roots

The exposure to Cd caused changes in the mineral profile in all organs of the 30-day-old tomato cultivars. Decreases in the P and Mn concentrations in roots of plants that were grown in Cd-containing media were observed, when compared to the control plants (Table 2).

Table 2. Macro and micronutrient concentration in roots of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd, that were grown in hydroponic solution with 0 and 35 μM $CdCl_2$ for 6 days

Treatments	Root mineral profile						
	Macronutrient (g kg^{-1} DW)			Micronutrient (mg kg^{-1} DW)			
	P	Ca	Mg	B	Mn	Fe	Cu
$CdCl_2$ concentrations							
0	13.89 a	16.11 a	10.66 a	64.36 b	2134.98 a	8341.86 a	10.53 b
35	11.99 b	17.42 a	10.96 a	168.97 a	614.47 b	7788.39 a	12.18 a
Tomato cultivars (TC)							
RLT	11.62 b	17.25 a	10.52 ab	110.12 a	1294.28 b	8236.68 a	10.92 a
NGL	12.83 b	16.87 a	11.88 a	118.77 a	1243.12 b	7389.97 a	10.92 a
MNM	14.37 a	16.18 a	10.02 b	121.10 a	1586.77 a	8568.72 a	12.23 a
Significance							
$CdCl_2$	**	NS	NS	***	***	NS	*
TC	**	NS	*	NS	**	NS	NS
$CdCl_2$*TC	NS	NS	NS	NS	NS	NS	NS

The distinct letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations, or for comparisons among tomato cultivars. NS, ***, ** and *: Nonsignificant and significant at $p \leq 0.001$, 0.01 or 0.05, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker

By contrast, Cd-challenged tomato exhibited an increased B and Cu concentrations (Table 2). Moreover, P, Mg, and Mn concentrations in root tissues depended on cultivar (Table 2). Potassium, S and Zn concentrations were significantly affected by both factors (Fig. 1). Potassium concentration was maintained in 'Rey de Los Tempranos', whereas decreased in 'Nagcarlang' and 'Moneymaker' tomato when submitted to the Cd exposure, in comparison to the control plants (Fig. 1a). However, only Moneymaker exhibited reductions in S concentration when plants were grown in Cd-containing media (Fig. 1b). In addition, Cd treatment led to a reduction in Zn concentration in the roots of tomato cvs. Moneymaker and Rey de Los Tempranos (Fig. 1c).

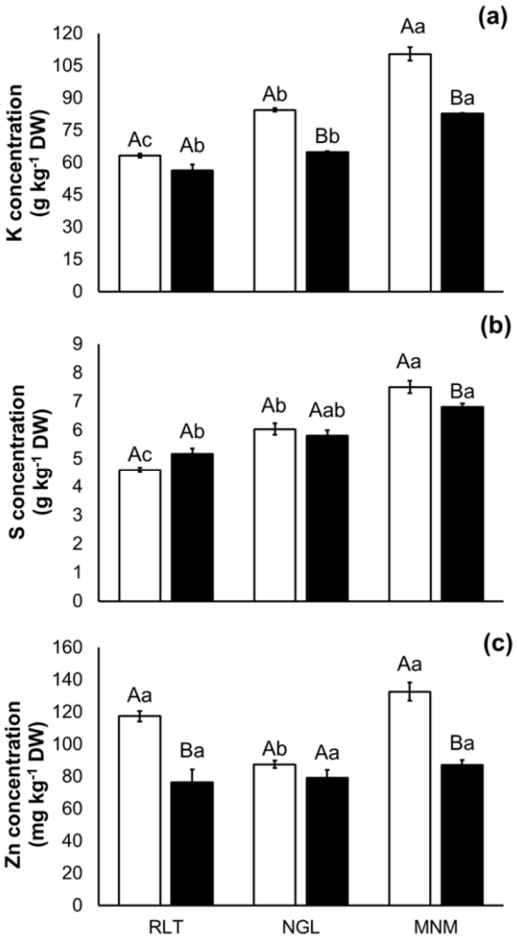

Figure 1. Potassium – K (**a**), sulfur – S (**b**), and zinc – Zn (**c**) concentrations in roots of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd exposure, that were grown in hydroponic solution with 0 (white columns) and 35 μM $CdCl_2$ (black columns) for 6 days. Distinct uppercase and lowercase letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations in the growth media, and for comparisons among tomato cultivars in a same growth media condition, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. Bars represent the standard errors of the means

2.3.2.2. Stems

In stems, reductions in the K, Ca, Mg, Fe and Zn concentrations in Cd-challenged plants were observed when compared to the control plants (Table 3). On the other hand, Cd induced increments in the B concentration in all plants that were grown in Cd-containing media, regardless tomato cultivars (Table 3).

Table 3. Macro and micronutrient concentration in stems of tomato (*Solanum lycopersicum*) cultivars, with intermediary tolerance to Cd, that were grown in hydroponic solution with 0 and 35 μM $CdCl_2$ during 6 days

Treatments	Stem mineral profile								
	Macronutrient (mg g^{-1} DW)					Micronutrient (mg g^{-1} DW)			
	P	K	Ca	Mg	S	B	Fe	Cu	Zn
$CdCl_2$ concentrations									
0	7.78 a	125.76 a	11.93 a	2.86 a	3.36 a	30.11 b	84.62 a	1.93 a	47.78 a
35	7.54 a	102.00 b	10.81 b	2.62 b	3.09 a	79.66 a	53.73 b	2.03 a	30.36 b
Tomato cultivars (TC)									
RLT	6.88 c	104.75 b	10.93 b	2.45 b	2.83 b	47.03 b	64.65 b	1.85 b	38.78 b
NGL	7.63 b	107.50 b	11.28 ab	2.90 a	3.08 b	56.97 ab	62.92 b	1.68 b	34.57 c
MNM	8.47 a	129.38 a	11.90 a	2.87 a	3.75 a	60.65 a	79.97 a	2.42 a	43.85 a
Significance									
$CdCl_2$	NS	***	***	*	NS	***	***	NS	***
TC	***	**	*	**	***	*	*	***	***
$CdCl_2$*TC	NS	NS	NS	NS	NS	NS	NS	NS	NS

The distinct letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations, or for comparisons among tomato cultivars. NS, ***, ** and *: Nonsignificant and significant at $p \leq 0.001$, 0.01 or 0.05, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker

Mn concentration was increased in Nagcarlang and Moneymaker, but not in Rey de Los Tempranos under Cd exposure when compared to the control plants (Fig. 2). Moreover, the concentrations of all nutrients were depended upon cultivars, being often higher in Moneymaker than Rey de Los Tempranos and Nagcarlang (Table 3).

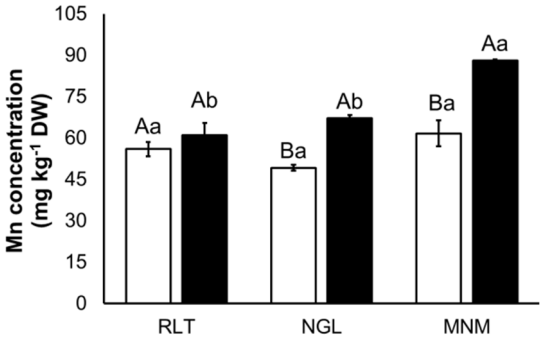

Figure 2. Manganese (Mn) concentration (mg g^{-1} DW) in stems of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd exposure, that were grown in hydroponic solution with 0 (white columns) and 35 μM $CdCl_2$ (black columns) for 6 days. Distinct uppercase and lowercase letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations in the growth media, and for comparisons among tomato cultivars in a same growth media condition, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. Bars represent the standard errors of the means

2.3.2.3. Leaves

Increases in the S, Mn, B and Zn concentrations and decreases in K concentration in leaves were observed in Cd-challenged plants in comparison to the control plants (Table 4). The S and B concentrations were also depended upon tomato cultivar, which was the only factor that influenced P and Ca concentrations. In general, Moneymaker presented the highest nutrient concentration among the genotypes tested (Table 4).

Table 4. Macro and micronutrient concentrations in leaves of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd, that were grown in hydroponic solution with 0 and 35 μM $CdCl_2$ for 6 days

Treatments	Leaf mineral profile									
	Macronutrient (g kg^{-1} DW)					Micronutrient (mg kg^{-1} DW)				
	P	K	Ca	Mg	S	B	Mn	Fe	Cu	Zn
$CdCl_2$ concentrations										
0	7.42 a	82.56 a	32.49 a	4.90 a	8.29 b	48.57 b	245.3 b	317.9 a	3.33 a	34.47 b
35	7.50 a	59.31 b	32.02 a	5.07 a	9.73 a	111.17 a	363.4 a	271.2 a	3.69 a	38.00 a
Tomato cultivars (TC)										
RLT	7.08 b	68.58 a	30.60 b	5.07 a	8.45 b	70.73 b	290.3 a	277.6 a	3.60 a	36.28 a
NGL	7.08 b	65.92 a	33.45 a	5.03 a	8.72 b	82.70 ab	294.2 a	290.3 a	3.28 a	35.65 a
MNM	8.22 a	78.30 a	32.72 ab	4.85 a	9.87 a	86.17 a	328.6 a	315.9 a	3.65 a	36.77 a
Significance										
$CdCl_2$	NS	***	NS	NS	***	***	***	NS	NS	*
TC	***	NS	*	NS	**	*	NS	NS	NS	NS
$CdCl_2$*TC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

The distinct letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations, or for comparisons among tomato cultivars. NS, ***, ** and *: Nonsignificant and significant at $p \leq 0.001$, 0.01 or 0.05, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker

The root-to-leaf translocation of nutrients exhibited Cd-dependent changes in leaves (Table 4) and roots (Table 2). Potassium translocation was reduced in plants under Cd exposure (Table 5). By contrast, P, S, Mn and Zn translocations were increased after Cd exposure, whilst B and Cu translocations were not modified in comparison to the control plants (Table 5).

Table 5. Root-to-leaf translocation factor of phosphorus (P), potassium (K), sulfur (S), boron (B), manganese (Mn), copper (Cu) and zinc (Zn) in tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd exposure, that were grown in hydroponic solution with 0 and 35 μM $CdCl_2$ for 6 days

Treatments	Translocation factor (leaf: root)						
	P	K	S	B	Mn	Cu	Zn
$CdCl_2$ concentrations							
0	0.54 b	1.07 a	1.41 b	0.76 a	0.12 b	0.32 a	0.31 b
35	0.63 a	0.90 b	1.65 a	0.72 a	0.61 a	0.32 a	0.48 a
Tomato cultivars (TC)							
RLT	0.62 a	1.15 a	1.73 a	0.75 a	0.33 a	0.37 a	0.40 ab
NGL	0.56 a	0.99 ab	1.48 b	0.77 a	0.42 a	0.30 b	0.43 a
MNM	0.58 a	0.80 b	1.39 b	0.72 a	0.34 a	0.30 b	0.36 b
Significance							
$CdCl_2$	**	*	***	NS	***	NS	***
TC	NS	**	***	NS	NS	**	*
$CdCl_2$*TC	NS	NS	NS	NS	*	NS	NS

The distinct letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations, or for comparisons among tomato cultivars. NS, ***, ** and *: Nonsignificant and significant at $p \leq 0.001$, 0.01 or 0.05, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker

2.3.3. Tolerance index, biometry and physiological indexes

All cultivars exhibited biomass losses after Cd exposure, as summarized by the tolerance index that which ranged from 0.78 to 0.66 (Fig. 3).

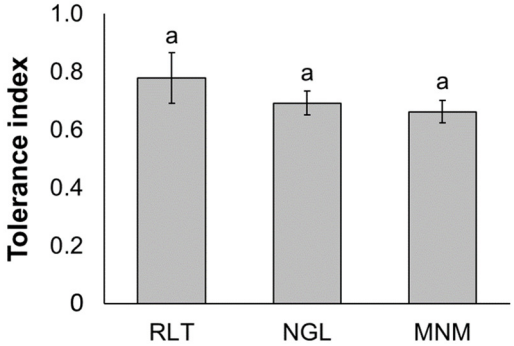

Figure 3. Tolerance index of tomato (*Solanum lycopersicum*) cultivars that were grown in hydroponic solution with 35 μM $CdCl_2$ for 6 days. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. There were no differences among tomato cultivars by F test ($p > 0.05$). Bars represent the standard errors of the means

In general, root dry weight was decreased by Cd exposure, affecting mainly 'Nagcarlang' tomato in comparison to the other cultivars (Fig. 4a). Nagcarlang also exhibited remarkable reductions in the root length and volume (Fig. 4b, c), as well as stem length, diameter and dry weight (Fig. 4d, Fig. 5a, b) in response to Cd stress.

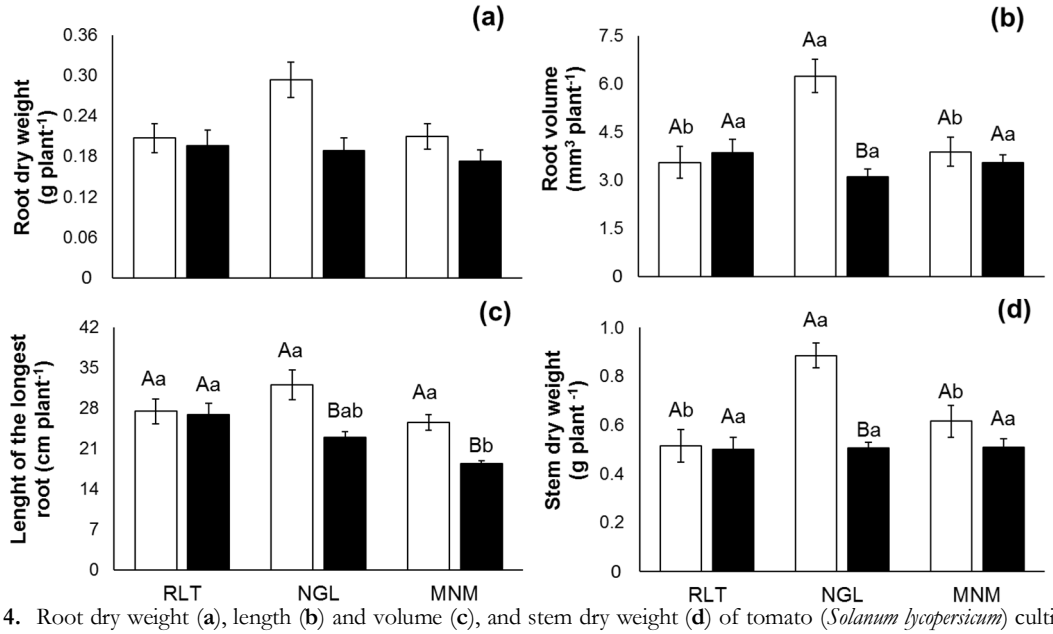

Figure 4. Root dry weight (**a**), length (**b**) and volume (**c**), and stem dry weight (**d**) of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd exposure, that were grown in hydroponic solution with 0 (white columns) and 35 μM $CdCl_2$ (black columns) for 6 days. Distinct uppercase and lowercase letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations in the growth media, and for comparisons among tomato cultivars in a same growth media condition, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. There was no interaction effect ($CdCl_2$ concentration vs tomato cultivars) on root dry weight, that was influenced only by $CdCl_2$ concentration, presenting mean values that ranged from 0.24 to 0.19 in Cd-free and Cd-containing growth media, respectively. Bars represent the standard errors of the means

Tomato cv. Moneymaker presented decreases in the root length after Cd exposure, when compared to the control plants (Fig. 4c). Moneymaker also exhibited significant reductions in the leaf dry weight duet to plant exposure to Cd, as noted in tomato cv. Rey de Los Tempranos (Fig. 5c). However, only 'Nagcarlang' tomato exhibited alterations in the root:shoot ratio, which was reduced in Cd-challenged plants in comparison to the control (Fig. 5d).

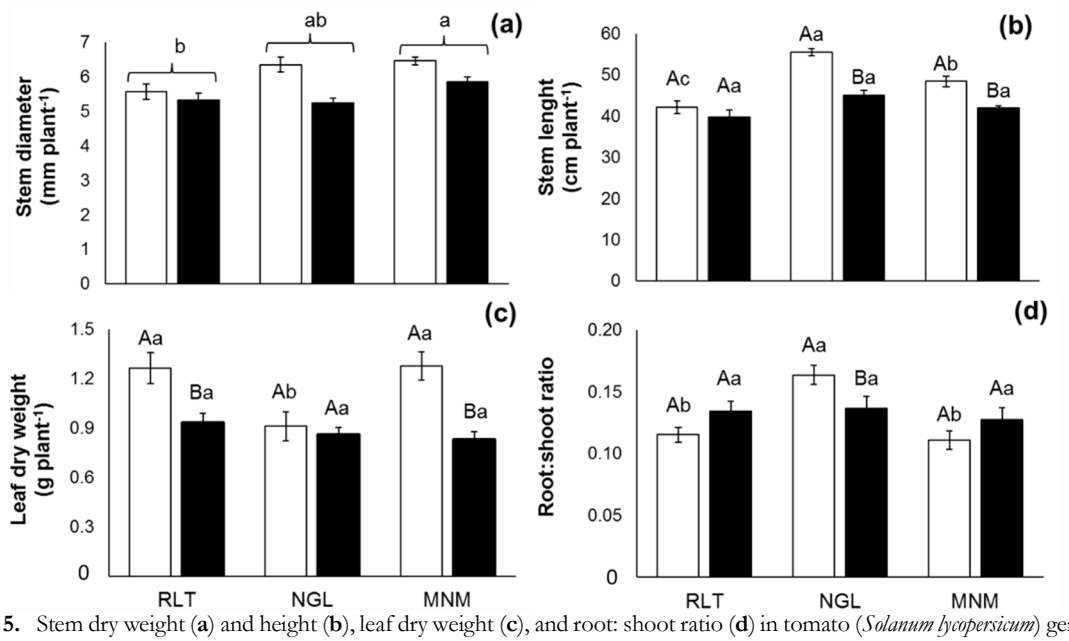

Figure 5. Stem dry weight (**a**) and height (**b**), leaf dry weight (**c**), and root: shoot ratio (**d**) in tomato (*Solanum lycopersicum*) genotypes with intermediary tolerance to Cd exposure, that were grown in hydroponic solution with 0 (white columns) and 35 μM $CdCl_2$ (black columns) for 6 days. Distinct uppercase and lowercase letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations in the growth media, and for comparisons among tomato cultivars in a same growth media condition, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. There was no interaction effect ($CdCl_2$ concentration vs tomato genotypes) on the stem diameter, which was separately affected by these two factors and presented mean values that ranged from 6.12 and 5.56 mm in Cd-free and Cd-containing growth media, respectively. Bars represent the standard errors of the means

When the physiological indexes are concerned, specific leaf area, leaf area ratio and leaf weight ration presented genotype-dependent modifications in plants under Cd exposure (Fig. 6b-d).

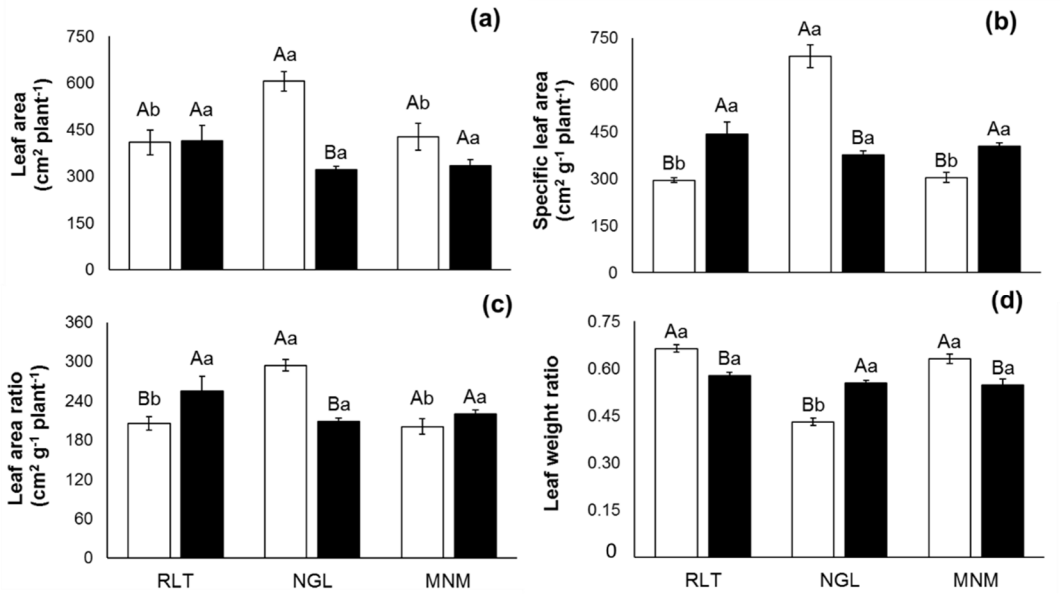

Figure 6. Leaf area (**a**), specific leaf area (**b**), leaf area ratio (**c**), and leaf weight ratio (**d**) of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd exposure, that were grown in hydroponic solution with 0 (white columns) and 35 μM $CdCl_2$ (black columns) for 6 days. Distinct uppercase and lowercase letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations in the growth media, and for comparisons among tomato cultivars in a same growth media condition, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. Bars represent the standard errors of the means

Specific leaf area and leaf area ratio were increased in tomato cv. Rey de Los Tempranos, but decreased in Nagcarlang after Cd exposure, when compared to the control plants (Fig. 6b, c). In addition, Cd caused decreases in the LWR of 'Rey de Los Tempranos' and 'Moneymaker' tomato, whereas increases in Nagcarlang were observed (Fig. 6d).

2.3.4. Indicators of Cd-induced stress and activity of antioxidant enzymes

Increased lipid peroxidation, which was expressed as MDA content, was observed in tomato cvs. Rey de Los Tempranos and Nagcarlang after plant exposure to Cd (Fig. 7a).

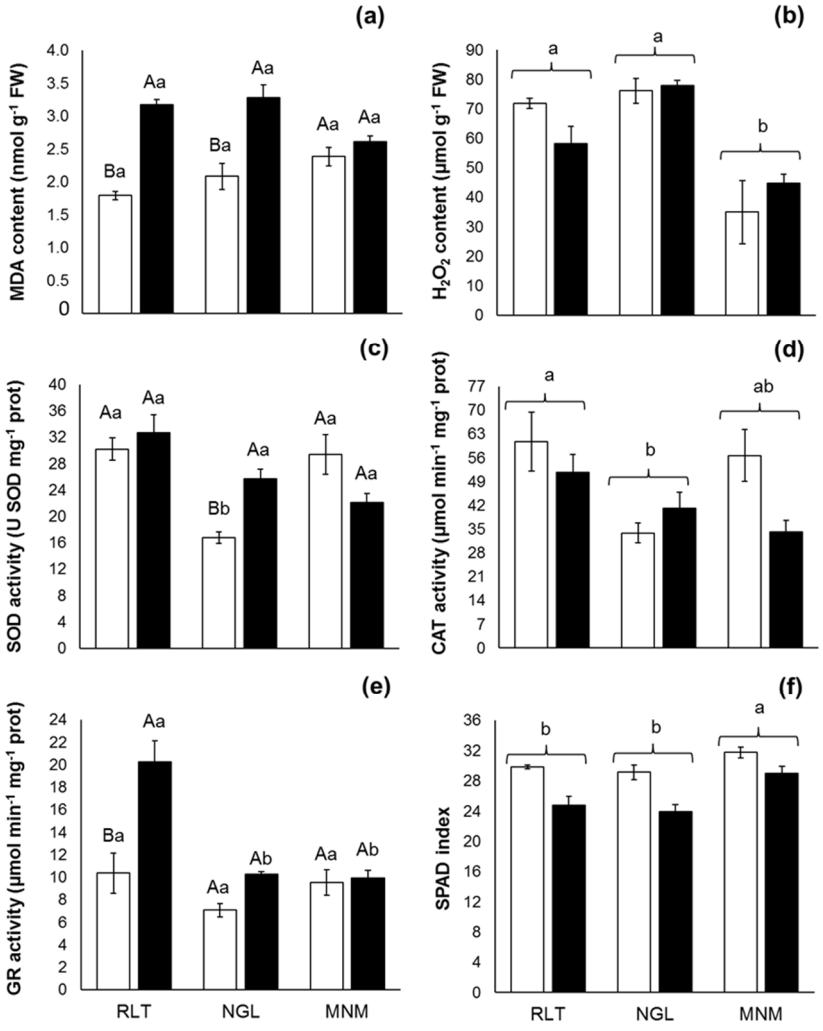

Figure 7. Malondialdehyde – MDA (**a**) content, hydrogen peroxide - H_2O_2 (**b**) content, superoxide dismutase – SOD (**c**) activity, catalase – CAT (**d**) activity, glutathione reductase – GR (**e**) activity, and chlorophyll content - SPAD index (f) in leaves of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd exposure, that were grown in hydroponic solution with 0 (white columns) and 35 µM $CdCl_2$ (black columns) for 6 days. Distinct uppercase and lowercase letters denote different means by Tukey's test ($\alpha \leq 0.05$) for comparisons between different $CdCl_2$ concentrations in the growth media, and for comparisons among tomato cultivars in a same growth media condition, respectively. RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. There was no interaction effect ($CdCl_2$ concentration vs tomato cultivars) on the H_2O_2 content, CAT activity and chlorophyll content. Both H_2O_2 content and CAT activity were affected only by tomato cultivars. Chlorophyll content was separately influenced by these two factors and presented mean values that ranged from 30.25 and 25.92 in Cd-free and Cd-containing growth media, respectively. Bars represent the standard errors of the means

The H_2O_2 content was not affected by Cd and was shown to be dependent only upon tomato cultivars, being high in Rey de Los Tempranos and Nagcarlang when compared to the Moneymaker tomato (Fig. 7b). When enzymes from the antioxidant machinery are concerned, SOD activity was increased in tomato cv. Nagcarlang under Cd exposure, but was not affected in the other cultivars (Fig. 7c). CAT activity exhibited genotype-dependent modifications, and varied in the following decreasing order Rey de Los Tempranos ≥ Moneymaker ≥ Nagcarlang (Fig. 7d). GR activity was only increased in Cd-challenged tomato cv. Rey de Los Tempranos, and presented no changes in the other cultivars after Cd exposure (Fig. 7e). In Cd-free and Cd-containing growth media, chlorophyll content dependent on tomato cultivar, being observed the highest value in Moneymaker tomato when compared to the other cultivars (Fig. 7f).

2.4. Discussion

2.4.1. Root-to-stem Cd translocation depends on tomato genotype, which presents an organ-specific sensitivity to Cd exposure

Cd accumulation in tomato is governed by the external $CdCl_2$ concentration, but different genotypes may exhibit distinct rates of Cd uptake, translocation and accumulation (Gratão et al., 2008, 2012; Monteiro et al., 2011; Nogueirol et al., 2016; Alves et al., 2017; Pompeu et al., 2017). In this study, tomato cultivars presented a varied Cd concentration in stems, but similar Cd concentration in roots (Table 1), providing evidences about a genotype-dependent Cd translocation within the plant. This mechanism may protect the photosynthetic apparatus by reducing Cd translocation to the leaves (Carrió-Seguí et al., 2015; Nogueirol et al., 2016), where this metal may impair the photosynthetic activity (Gratão et al., 2015) by disorganizing thylakoid system and stroma (Gratão et al., 2009; Pompeu et al., 2017), and decreasing the number of reaction centers and inhibiting electron transfer to acceptors of PSII (Delpérée and Lutts, 2008). In 'Nagcarlang' tomato, differences in the stem Cd accumulation were probably coupled to the reduced leaf area (Fig. 6a) that can diminish the transpiration rate, which drives Cd translocation to the shoots (Lux et al., 2011; Gratão et al., 2015). A lower transpiration surface also can be a strategy to avoid water losses by the Cd-challenged plants that often present a diminished water absorption capacity and, consequently, decreased plant growth (Perfus-Barbeoch et al., 2002; Delpérée and Lutts, 2008) due to the limitations in cell expansion. Nevertheless, the reduced leaf area (Fig. 6a) also means a decreased potential for sunlight harvesting to photosynthesis performance, possibly impairing biomass production and accumulation (Fig. 3, Fig. 4a, d, Fig. 5c).

However, since tomato cultivars exhibited a similar Cd concentration in leaf tissues (Table 1), the data indicated that (i) changes in the root-to-stem Cd translocation were not enough to decrease Cd accumulation in leaves after a short-term exposure, and (ii) the accumulation of this metal in leaf tissues is coupled to the biomass losses in Cd-challenged tomato (Fig. 3, Fig. 4a, d, Fig. 5c). Moreover, there are evidences about an organ-specific sensitivity to Cd exposure, a phenomenon that varies among cultivars and does not depend strictly on the amount of Cd accumulated (Table 1, Fig. 5c, Fig. 6a-d). The last assumption can be supported by the behavior of stems and roots, in addition to the leaves, when plants were subjected to Cd exposure. The genotype that presented the lowest Cd concentration in stems, Nagcarlang, (Table 1) was the only one that exhibited remarkable reductions in the stem diameter, length (Fig. 5a, b) and, consequently, dry weight (Fig. 4d). In leaves of Cd-treated plants, biomass losses were

found in Rey de Los Tempranos and Moneymaker, but Nagcarlang exhibited no changes in leaf dry weight (Fig. 5c). Furthermore, only Nagcarlang suffered great decreases in the root dry weight after Cd exposure (Fig. 4a), but all cultivars exhibited a similar Cd concentration in roots (Table 1). Therefore, different tomato genotypes employ distinct strategies to cope with Cd-induced challenges to the plant development, a feature that can, and should be explored by breeders and researchers. One of these strategies is related to the root length maintenance in plants under Cd exposure, a feature that probably supported the suitable P uptake in Rey de Los Tempranos, which presented slight reductions in the root P concentration (-3.7%) when compared to Nagcarlang and Moneymaker (from 14.9 and to 20.0%, respectively - Table 2, Fig. 4c, Table S1).

Cd-induced anatomical modifications, such as formation of intercellular air spaces and disintegration of epidermis and external cortical cell layers, may provoke reductions in the root diameter (Gratão et al. 2009; Pompeu et al. 2017), length and volume (Fig. 4b, c), hence impairing P absorption (Table 2, Table S1). Mn, Zn and K absorption was also prevented in plants under Cd exposure, regardless genotype (Table 2, Fig. 1a, c). The antagonist effect of Cd on Mn and Zn absorption, a well-documented phenomenon in Cd-treated plants, may be related to the Cd-mediated modulation in the activity of plasma membrane transporters (Migocka and Klobus, 2007), and also to the Cd ability to use the same transporters of these micronutrients because they present the same valence (Hernández et al., 1998; Korshunova et al., 1999; Dong et al., 2006; Sasaki et al., 2012; Tkalec et al., 2014; Wu et al., 2016). As another consequence of the similarities between cadmium and some nutrients, Cd ions may bind to membrane and enzyme active sites and inactivate their functions (Cherif et al., 2012).

In addition, Cd-induced reductions in the K concentration, which is the most important nutrient for tomato development (EMBRAPA, 2003; Kanai et al., 2007), were observed in both roots and shoots of all cultivars (Fig. 2a, Tables 3-4), probably due to reductions in its root-to-leaf translocation (Table 5). According to Rigas et al. (2001), suitable K nutrition is needed for root hair elongation and maintenance of cell turgor (by consequence, cell expansion and growth), so explaining the decreases in the root length, volume and weight in 'Nagcarlang' tomato under Cd exposure (Fig. 4a-c). This genotype also exhibited other features that are strongly related to the effects of K deficiency in tomato, especially reductions in the development and biomass accumulation of stems without great effect on leaf dry weight (Fig. 4d, Fig. 5a-c). Such symptoms are due to changes in biomass partitioning that K deficiency can trigger by reducing sink activity, without a rapid stop in the photosynthesis activity (Kanai et al., 2007), so photosynthates are initially accumulated and used in leaves (Fig. 4d, Fig. 5c, Fig. 6d). Furthermore, the reduced leaf K concentration (Table 4) can be considered one of the factors that decreased chlorophyll content in tomato leaves (Fig. 7f), as reinforced by the proportional and significant relation between K concentration and SPAD index ($r = 0.575$, $p = 0.0158$). However, other tomato genotypes did not exhibit the typical symptoms of the reduced K concentration on the plant growth, probably due to the interactive effects among Cd and others misbalanced nutrients (Table 1-4).

2.4.2. Increased Mn, B and Zn concentrations in leaves enhance Cd-induced damages in photosynthetic tissues

In order to cope with the Cd-induced disturbances in the mineral profile, plants may activate mechanisms related to the uptake and remobilization of nutrients (Tables 2-5). A high absorption capacity could be coupled to the increases in the root Cu concentration (Table 2) that, below the threshold for Cu toxicity, may benefit the plant development under Cd-induced stress (Carrió-Seguí et al., 2015) by enhancing both primary and secondary

metabolisms because Cu plays a key role in photosynthesis, respiration, C and N metabolism, and antioxidant mechanisms (Marschner, 2012). In addition, the use of nutrients that was stocked in stems seems to play an important role for the restoration of nutritional homeostasis in tomato leaves (Table 3, 4), similarly to the node in graminaceous plants (Yamaji and Ma, 2014). The data suggested that Mg, Ca and Fe from stems were used to maintain their status in leaf tissues (Table 3, 4), as a probable strategy to avoid the deficiency of these elements in the photosynthetic organs, where all play key functions. For instance, Mg is the central element in the chlorophyll molecule, Fe is a cofactor in several enzymes, being necessary for chlorophyll synthesis, and Ca is needed for cell wall building, signaling and stomatal aperture (Taiz et al., 2015).

However, an increased ability to absorb and cycle certain nutrients seems to be a 'double-edged sword' strategy in plants under Cd exposure, which often trigger black spots and chlorosis in leaves of the Cd-challenged plants. Leaf chlorosis is frequently coupled to the reductions in the Fe status (Hermans et al., 2011), but there were no significant changes in the Fe concentration (Table 4) as reported by Nogueirol et al. (2016). By contrast, Cd exposure increased Mn and B accumulation in leaves (Table 4), indicating a link between Cd-induced damages and high concentrations of these nutrients. For Mn, an increased root-to-shoot translocation could drive the accumulation of this element in leaves (Table 5, Fig. 2). On the other hand, an increased B uptake, rather than changes in its translocation, can be coupled to the increments of B concentration in all organs of tomato Cd exposure (Table 2-4). This hypothesis is reinforced by the similarity among the toxicity symptoms from Cd, Mn and B excess (Eysinga and Smilde, 1981; González et al., 1998; Kaya et al., 2009; Reid and Fitzpatrick, 2009).

According to Kaya et al. (2009), the visual damages of B toxicity in tomato started as a yellow–green interveinal chlorosis, which developed first in the oldest leaves and progressed to the youngest. Afterwards, small patches of necrotic tissue appeared between the minor veins and extended to the midribs resulting into a reduced photosynthetic leaf area, as reported by Kaya et al. (2009), who also suggested that a significant B accumulation (≥ 74 mg kg^{-1}) might have occurred before the effects became apparent. In the present study, leaves of plants under Cd exposure presented a B concentration that varied from to 96.5 to 120 mg kg^{-1} (Table 4, Table S2). Moreover, B status was significantly, inversely related to the SPAD index ($r = -0.608$, $p = 0.0074$), suggesting that a high B concentration is one of the factor that caused decreases in the chlorophyll content in leaves of tomato under Cd exposure, in addition to the high Cd accumulation ($r = -0.691$, $p = 0.015$).

The negative role of the Mn excess in the biochemical and visual changes of tomato leaves under Cd exposure was also considered, since the threshold for Mn toxicity is 250 mg kg^{-1} DW (Alvarenga, 2013), and Cd-challenged plants exhibited a Mn concentration that ranged from 325.7 to 398.9 mg kg^{-1} DW (Table 4, Table S2). According to Ramos et al. (2002), Mn excess is stored mainly in chloroplasts, where it disorganizes the chloroplast lamellae and increases ROS production (González et al., 1998; Lidon and Teixeira, 2000; Lavres Junior et al., 2010), causing effects that resemble those reported in tomato grown in Cd-containing media (Baszyński et al., 1980; Djebali et al., 2005; Gratão et al., 2009; Pompeu et al., 2017). Curiously, the initial effect of the high Mn supply to leaves of Cd-treated tomato can be beneficial by reorganizing the grana stack and restoring the photosystem II activity (Baszyński et al., 1980). However, a high concentration may trigger Mn-induced phytotoxicity, as already supposed by Baszyński et al. (1980).

In addition, tomato leaves also presented a high Zn concentration in plants under Cd exposure, probably due to its quickly remobilization from stems (Nagcarlang and Moneymaker – Fig. 2) and roots (all cultivars – Table 2-5). According to Marschner (2012), Zn is the second most abundant transition metal in living organisms, but excess of Zn triggers lipid peroxidation (Pramanick et al., 2017), inhibits photosynthesis and quickly causes chlorosis due to

reduction in both chlorophyll *a* and *b* (Cherif et al., 2012), and provokes toxicity symptoms that are indistinguishable from that caused by Cd (Smith and Brennan, 1983) or similar to Fe deficiency (Eysinga and Smilde, 1981; Yara, 2017). Furthermore, it was shown that the higher Zn and Cd concentrations are synergistic in their effect on plant growth parameters and oxidative stress (Cherif et al., 2011). Then, biomass production can be directly affected by Cd exposure, as well as by the nutritional misbalance, through decreases in the chlorophyll content (Fig. 7f), reductions in the leaf area (i.e. plant potential to intercept light - Fig. 6a), and prevention of root development (Fig. 4a-c).

2.4.3. Cd triggers genotype-dependent changes in the antioxidant machinery

Cd induced oxidative stress (Fig. 7a) and modifications in leaf anatomy, as estimated by the specific leaf area – SLA (Fig. 6b). Higher SLA values may indicate the occurrence of thinner leaves in 'Rey de Los Termpranos' and 'Moneymaker' tomato under Cd exposure, a phenomenon which is probably due to the smaller palisade parenchyma cells and decreased number and size of the spongy parenchyma cells (Gratão et al., 2009). By contrast, changes in the SLA of tomato cv. Nagcarlang can be coupled to the great reductions in the leaf area with increases in cell size in the palisade parenchyma and lowered intercellular spaces (Pompeu et al., 2017), hence augmenting the mesophyll resistance to carbon dioxide uptake (Lamoreaux and Chaney, 1978), an event that can be enhanced by the stomatal closure (Gratão et al., 2009). When the Cd-induced oxidative stress is concerned, data indicated that unbalances in the antioxidant machinery were not coupled to the H_2O_2 overproduction (Fig. 7a, b), suggesting that lipid peroxidation in leaves was triggered by other compounds, such as $O_2^{\bullet-}$ (Iannone et al., 2015), nitric oxide and S-nitrosothiol (Hasan et al., 2016).

Plants under Cd exposure have been shown to be able to increase the activity of antioxidant enzymes such as SOD, for the dismutation of $O_2^{\bullet-}$ into oxygen and H_2O_2, as well as CAT and other peroxidases for H_2O_2 scavenging (Gratão et al., 2005, 2008; Iannone et al., 2015; Cuypers et al., 2016; Hasan et al., 2016). SOD activity was only increased in the Nagcarlang tomato (Fig. 7c), hence indicating increases in the generation of superoxide ion, which is the SOD subtract. However, an increased SOD activity was not sufficient to avoid oxidative stress (Fig. 7a). CAT activity exhibited a slight, but non-significant variations in all tomato genotypes under Cd exposure, corroborating the report by Ianonne et al. (2015) who indicated that CAT does not play a crucial role in protection against Cd toxicity in tobacco. According to these authors, plants may activate alternative defense mechanisms to avoid H_2O_2 overproduction, explaining why its content was not affected in CAT-deficient tomato that were grown in Cd containing media.

This hypothesis is reinforced by the fact that an improved CAT activity is not always coupled to better plant performance, as observed in Never ripe (*Nr*) mutant (blockage of ethylene perception that result in incomplete fruit ripening) that exhibited increased CAT activity concurrently to the biomass losses after 12 days of plant exposure to 0.2 mM $CdCl_2$ (Gratão et al., 2012). The inverse was also observed in *diageotropica* (*dgt*) tomato mutant (loss of sensitivity to auxin) that presented decreases in the CAT activity but no changes in root and shoot biomass (Gratão et al., 2012). Another possible explanation is the Cd-induced overproduction of compounds that can reduce CAT activity, such as $O_2^{\bullet-}$ (Kono and Fridovich, 1982).

By contrast, it seems that an increased GR activity provided a certain advantage for the biomass production in Rey de Los Tempranos in comparison to the other tomato cultivars (Fig. 3, Fig. 7e). This enzyme is mainly stored in chloroplasts (70–80%), where it may protect the PSII function by maintaining the electron transport in PSII acceptor side and stabilizing PSII complexes (Ding et al., 2012). In both Micro-Tom and *Nr* tomato mutant, a high GR activity

was detected in leaf tissues of Cd-challenged plants, from the 7th to the 36th day after the onset of Cd-exposure, indicating that this enzyme is important for the plant development (Monteiro et al., 2011). The direct function of GR is to convert oxidized to reduced glutathione (GSH), a disulphide reductant that participates directly and also indirectly in H_2O_2 detoxification through GPX and APX activation (GSH is used for ascorbate regeneration) as well as reacts with other ROS like $^1O_2$ and OH (see review of Gill et al., 2013). Anyways, the increased GR activity was not enough to avoid oxidative stress (Fig. 7a).

Possibly, increases in the GR activity may relate to the counteraction of Cd side-effects through a mechanism that involves the balance of reduced/oxidized GSH for chelation of Cd ions through production of GSH-derived peptide phytochelatin (Gallego et al., 2012; Gill et al., 2013; Yamaguchi et al., 2016). Since GSH is made from cysteine, in addition to the amino acids histidine and glutamine, an adequate S supply is needed for GSH production (Gallego et al., 2012; Gill et al., 2013; Yamaguchi et al., 2016). In effect, the root-to-leaf S translocation was enhanced in all tomato cultivars under Cd exposure, especially in 'Rey de Los Tempranos' that exhibited the highest values (Table 5). Probably the fastest S supply to tomato shoots (Table 5), which are more sensitive to Cd exposure than roots (Hédiji et al., 2010), in addition to the sustenance of S assimilation in Cd-stressed plants (Table 2) were able to provide some advantages to Rey de Los Tempranos' tomato when compared to the other genotypes (Fig. 3). For instance, $CdCl_2$ treatment induced the overexpression of genes related to the S uptake (mainly SULTR1;2), stimulated the distribution of sulfate to shoots and shifted the sulfur metabolism to phytochelatin synthesis in *Arabidopsis thaliana* (Yamaguchi et al., 2016).

2.5. Conclusions

Although tomato genotypes were shown to have intermediate tolerance to Cd exposure, they were able to employ different strategy to cope with Cd-challenges. Changes in the root-to-stem Cd translocation, choice of maintenance of either root or shoot development, as well as distinct management of the nutrient cycling are part of the plant mechanisms to mitigate Cd side-effects. As result of the different strategies, tomato genotypes exhibit varied sensitivity to Cd exposure in an organ-level, a feature that can be used in biotechnological and breeding programs. On the other hand, there are similarities regarding the Cd-induced effects on tomato, including the high translocation of Zn, Mn and B to leaves, as well as the low K concentration in plants under Cd exposure. It is possible that the increased Mn and B accumulation be coupled to the biochemical and visual damages in the photosynthetic apparatus, such as necrosis and chlorosis. The latter is often related to the reduced Fe concentration, but there were no significant changes in the Fe concentration in leaves. Moreover, the Zn excess in addition to the K deficiency may be related to the low chlorophyll content in Cd-challenged plants.

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Supplementary materials

Table S1. Phosphorus (P) concentration (g kg^{-1} DW) in roots of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd, that were grown in hydroponic solution with 0 and 35 μM $CdCl_2$ for 6 days

Tomato cultivar	P concentration					
	0 μM			35 μM		
RLT	11.83	±	0.36	11.40	±	0.68
NGL	13.87	±	0.63	11.80	±	0.34
MNM	15.97	±	0.36	12.77	±	0.22

RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. Means ± standard errors.

Table S2. Boron (B) and manganese (Mn) concentrations (mg kg^{-1} DW) in leaves of tomato (*Solanum lycopersicum*) cultivars with intermediary tolerance to Cd exposure, that were grown in hydroponic solution with 0 and 35 μM $CdCl_2$ for 6 days

Tomato cultivar	B concentration						Mn concentration					
	0 μM			35 μM			0 μM			35 μM		
RLT	45.0	±	3.59	96.5	±	6.08	254.9	±	14.06	325.7	±	21.57
NGL	48.4	±	0.79	117.0	±	1.38	222.8	±	6.00	365.5	±	6.88
MNM	52.3	±	2.14	120.0	±	8.33	258.3	±	21.21	398.9	±	14.92

RLT: Rey de Los Tempranos, NGL: Nagcarlang and MNM: Moneymaker. Means ± standard errors.

3. TOMATO TOLERANCE TO CADMIUM TOXICITY IS COUPLED TO MAGNESIUM-DEPENDENT MECHANISMS THAT PREVENT THE ROOT HAIR FORMATION

ABSTRACT

The misbalanced mineral profile is emerging as a protective mechanism rather than a side-effect from the plant exposure to cadmium (Cd). Little is known about this plant strategy against Cd toxicity, and such knowledge is essential for crop management and breeding programs. Physiological, biochemical, anatomical, and nutritional analyses were performed in six tomato accessions with contrasting tolerance degree to short-term Cd exposure. Data indicated that avoidance of high magnesium (Mg) concentration in roots appears to be a plant strategy to mitigate Cd toxicity by decreasing formation of root hairs and, consequently, reducing Cd entrance and accumulation. Moreover, plant capacity to maintain the leaf blade expansion is associated to the sustaining of biomass production under the short-term Cd exposure. Regarding the mode of action of Cd toxicity, this metal causes drastic misbalances in the plant mineral profile by changing nutrient uptake and distribution. In leaves, Mn excess enhances Cd-induced damages that are further amplified by increases in the Zn and B concentration. In conclusion, changes in the nutritional status are a 'double-edge sword' mechanism for overcoming Cd-toxicity. Therefore, plant ability to quickly manage the uptake and distribution of certain nutrients is necessary to mitigate the Cd-induced damages.

Keywords: Boron phytotoxicity; Environmental contamination; Heavy metal; Manganese excess; *Solanum lycopersicum*; Zinc toxicity

3.1. Introduction

Cadmium (Cd) is a non-essential, hazardous element to biological systems, triggering disturbances in the antioxidant machinery and nutritional status of plants even at low concentration in the growth media (Gratão et al., 2005, 2015; Gallego et al., 2012; Fidalgo et al., 2011; Štolfa et al., 2015; Cuypers et al., 2016; Alves et al., 2017; Bayçu et al., 2017a, b). In plants under Cd exposure, mechanisms related to the prevention of oxidative stress are the most studied aspect, but not always they are the first protective strategy to be used (Weber et al. 2006). It has also been shown that plants may activate "tools" to balance ion homeostasis prior to the maintenance of the antioxidant status, and even before an actual nutrient deficiency (Weber et al., 2006). For instance, changes in the concentration of nitrogen (N) and sulfur (S) are part of a protective mechanism against Cd toxicity through the production of cysteine-rich compounds, namely glutathione, phytochelatin and metallothionein (Ruttkay-Nedecky et al., 2013; Khan et al., 2016; Yamaguchi et al., 2016; Gielen et al., 2017). A high manganese (Mn) and zinc (Zn) accumulations in leaves were also shown to be related to the decreased sensitivity to Cd exposure by supporting chloroplast integrity and photosynthetic activity (Cherif et al., 2012; Rahman et al., 2016). Moreover, the maintenance of suitable iron (Fe) concentration in leaves of Cd-challenged plants was coupled to the mitigation of Cd-induced damages in photosynthetic tissues (Sebastian and Prasad, 2016a, b), such as chlorosis and necrosis. Curiously, even the

accumulation of non-essential elements like selenium (Se) and silicon (Si), was able to enhance plant tolerance to Cd stress through increases in the activity of antioxidant enzymes and/or reductions of root-to-shoot Cd translocation (Abd_Allah et al., 2016; Wu et al., 2016).

Despite the relevance of mineral interactions against Cd-induced toxicity, the significance of the nutritional status alterations is frequently reported as a Cd side-effect rather than a protective mechanism that can be actively modulated by plants. Currently, the positive role of the suitable management of magnesium (Mg) status in plants under Cd exposure is emerging, but the mechanisms behind this event are superficially known. Magnesium, a macronutrient that is the central element in the chlorophyll molecule, also regulates the cellular pH, cation–anion equilibrium, photoassimilate partitioning and carbon/nitrogen balance (Marschner, 2012; Bloom and Kameritsch, 2017). However, information about the Mg-dependent protective mechanism against Cd-induced stress is contradictory. The low Mg status was coupled to an enhanced antioxidant potential in rice (Chou et al., 2011), and beneficial outcomes in *Arabidopsis* leaves (Hermans et al., 2011). By contrast, a high Mg accumulation alleviated Cd-induced damages in Japanese mustard (Kashem and Kaway, 2007) and reduced the root-to-shoot Cd translocation in barley (Kudo et al., 2015). Furthermore, increases in the Mg concentration mitigated lead (Pb) phytotoxicity in *Torreya grandis* seedlings (Shen et al., 2016). In this context, the present research aimed to identify how changes in the mineral profile can improve the plant tolerance to Cd exposure.

Differently from previous studies, the nutrient concentration in hydroponic solution was not varied because extra effects on plant development can occur due to nutrient excess and/or starvation. Moreover, tomato accessions with contrasting tolerance/sensitivity degrees were used as a tool for the identification of divergent mechanisms that can provide advantageous (or disadvantageous) plant features to Cd toxicity. Tomato was chosen because it is one of the most cultivated and consumed vegetable nowadays, and also the best model organism for fleshy-fruited plants to be used in research programs (The Tomato Genome Consortium, 2012; Bergougnoux, 2014). In this work, we show that Mg management is part of an important strategy for mitigation of Cd toxicity by controlling root hair development. Moreover, plant capacity to maintain the leaf blade expansion is associated to the sustaining of biomass production upon the short-term Cd exposure. In addition, new insights about the mode of action of Cd within plants were obtained, showing that the Cd-induced remobilization of Mn, B and Zn to leaves may enhance the Cd-coupled damages in leaf tissues. Data also indicate that reduction in the leaf Fe concentration, which is normally considered a Cd side-effect, is not associated to the highest sensitivity to Cd stress. The current study revealed plant mechanisms to cope with Cd-induced stress, and such knowledge can be used in breeding programs, especially for tomato and its-related species (potato, tobacco, pepper, eggplant etc) that are cultivated in areas containing naturally or accidentally high heavy metal concentrations.

3.2. Materials and Methods

3.2.1. Plant material and growth conditions

Six tomato accessions with contrasting tolerance to Cd exposure were selected according to the studies carried out in our laboratory (preliminary data). The group of tolerant plants comprised *S. lycopersicum* var. *cerasiforme* (CNPH0263), and tomato cultivars Indigo Rose and Yoshimatsu. The group of sensitive plants encompassed *Solanum lycopersicum* var. *cerasiforme* (CNPH0920), the tomato cultivar Tropic Two Orders, and the tomato relative *S. pimpinellifolium* (LA0122). These genotypes presented varied biomass when they were grown in Cd-free media for 15

days, exhibiting from 163.33 to 243.33 g plant^{-1} DW. After Cd exposure for seven days, a distinct impact on the biomass production were observed, and tolerance index ranged from 0.97 to 0.91 and from 0.23 to 0.07 in tolerant and sensitive accessions, respectively (see later 'Tolerance index and translocation factor' section).

Tomato seeds were chemically scarified by stirring in 2% HCl (v:v) for 15 min, in order to standardize germination. Subsequently, seeds were sown in polystyrene trays filled with thin exfoliated vermiculite, which was irrigated four times a day. During germination and seedling establishment, trays were kept in a greenhouse with temperature and relative humidity of 24.9 ± 1.58 °C and 78.9 ± 5.22%, respectively. After seedling emergence, daily application of macro- and micronutrients (Peters Professional 20-20-20 at 1 g L^{-1}) was initiated in order to maintain suitable seedling development. After one week, this concentration was increased to 1.5 g L^{-1}, which was used until the 18-days-old seedlings were transplanted to the hydroponics system.

Seedlings were removed from the trays and their roots were washed and then transferred to a hydroponic system (tanks) containing nutrient solution at 10% ionic strength that is used for adult tomato plants (Hoagland and Arnon, 1950). Seedlings were fixed in 200 mm thick styrofoam plates using foam pieces, where plants were spaced from each other by 8 cm. Plants were maintained in hydroponics for 6 days as an adaptation period in order to mitigate stress generated by seedling transplantation, and also to increase nutrient concentration from 10 to 50% ionic strength. This procedure (gradual increase of salt concentration) was carried out to diminish plant stress due to an increased content of salts in solution.

Twenty-four-day-old plants (three/four-leaf stage) were then subjected to Cd exposure by adding 35 μM $CdCl_2$ to the nutrient solution, which was monitored through electrical conductivity and pH checking that exhibited 1.2 mS cm^{-2} and 6.54 ± 0.07 average values, respectively. Seedlings were grown under control (Cd free) and Cd-containing hydroponic solution for 6 days, which was chosen because it is sufficient to detect the onset of most frequent toxicity symptoms (i.e. chlorosis, necrosis and decreased height) in tolerant accessions, but avoiding severe damages in the sensitive genotype group. During the experiment, distilled-deionized water was added to the tanks daily, in order to replace water lost through evapotranspiration. The homogeneous distribution of nutrient solution and suitable oxygenation level were maintained by air pump systems in each tank.

3.2.2. Plant biometry

The length (cm) of stems and the longest roots were evaluated with a millimeter ruler. The stem diameter (mm) was measured in the region immediately above cotyledonar leaves (or their scars) using digital caliper. For leaf area (cm^2) evaluation, fully expanded leaves were detached from the plants and measured with a leaf area meter (LI-COR®, LI-3100). Samples of roots, stems and leaves were kept in paper bags and dried in a drying oven (60 ºC) until a constant weight was achieved for dry mass determination. The specific leaf area [leaf area divided per leaf dry weight (cm^2 g^{-1})] was also calculated. All growth analyses and related variables were obtained from evaluations carried out in 9 plants, which were used to calculate the average value for each of the three replicates.

3.2.3. Chlorophyll content

Chlorophyll content was indirectly evaluated by using a chlorophyll meter SPAD equipment (Konica Minolta, SPAD-502 model). Two measurements were carried out in the middle third of the biggest terminal leaflets of two youngest and fully expanded leaves of three plants that composed each replicate (i.e. 12 evaluations per experimental unit).

3.2.4. Lipid peroxidation, H_2O_2 production and antioxidant enzyme activities

In order to evaluate the oxidative stress triggered by Cd exposure, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents as well as the activities of the antioxidant enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and glutathione reductase (GR, EC 1.6.4.2) were analyzed in completely expanded leaves from the shoot middle third of plants that composed each of the three replications.

In the greenhouse, leaves were collected and rapidly stored in liquid nitrogen. All samples were immediately stored in a -80 ºC freezer until the analyses. Before analysis onset, leaf tissues were grinded to a fine powder in liquid nitrogen. Lipid peroxidation was measured as MDA content according to Heath and Packer (1968), and hydrogen peroxide content was determined using procedures of Alexieva et al. (2001). The extraction of antioxidant enzymes was carried out according to Azevedo et al. (1998). Protein content was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard. Catalase and GR total activities were determined as described by Azevedo et al. (1998). Superoxide dismutase total activity was determined as described by Cembrawska-Lech et al., (2015).

3.2.5. Cadmium, macro- and micronutrient concentrations

The dried samples were milled to determine Cd and nutrient concentrations [calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P), sulphur (S), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn) and boron (B)] through inductively coupled plasma optical emission spectrometry (ICP-OES) analysis, which was preceded by nitro-perchloric digestion of root, stem and leaf tissues of Cd-exposed (stressed) and control (non-stressed) plants. Three replicates composed of three plants were used. All procedures were carried out in the Instituto Agronômico de Campinas (IAC, Campinas, Brazil).

3.2.6. Tolerance index and translocation factor

Tolerance index (TI) calculation was based on Piotto (2012), according to the following formula (1):

$TI = (DWfCdi - DWoi) / (DWfCti - DWoi)$ - (1)

Where $DWfCdi$ = dry weight of plants of i accession exposed to Cd; $DWoi$ = dry weight of i accession in the moment of Cd application, and $DWfCti$ = dry weight of control plants of i accession. Therefore, TI values can

range from 0 to 1 (100%), where 0 indicates the maximum sensitivity, and 1 designates the maximum tolerance. The translocation factors (TF= Cd concentration in leaves or stems / Cd concentration in roots) was also estimated.

3.2.7. Root anatomy

Anatomical analyses were performed in two of the three tomato accessions from the tolerant (*S. lycopersicum* cvs. Indigo Rose and Yoshimatsu) and sensitive groups (*S. pimpinelifolium* and *S. lycopersicum* cv. Tropic Two Orders). Root samples were obtained from the cap region (3 mm) and were fixed in modified Karnovsky's solution (2% glutaraldehyde, 2% paraformaldehyde and 5 mM calcium chloride in 0.05 M sodium cacodylate buffer pH 7.2) for 48 hours. Subsequently, the samples were dehydrated by an increasing ethanol series (from 35 to 100%) followed by 100% propanol.

The infiltration was performed slowly in butanol: infiltration medium (Leica) (3:1, 1:1, 1:2) at 4 ºC, and finally through infiltration media for 10 days. The polymerization was performed in infiltration medium and hardener at room temperature for 48 hours, according to the manufacturer's recommendation. Histological sections (5 μm) were obtained in a rotary microtome (Leica, RM2155), contrasted with acid fuchsin 1% in water and toluidine blue 1% (Feder and O'Brien, 1968), and covered with slip and Entellan®. The images were recorded in a light microscope (Zeiss, Axioskop 40).

3.2.8. Statistical analysis

The experiment was carried out under a completely randomized design with factorial scheme (accessions *vs* Cd concentrations) with 3 replications, totalizing 36 experimental units that were composed by 12 plants that were used for growth (three), anatomical (three) and biochemical analysis (six). Each experimental unit was limited by rows of plants, which were not used for analyses. Data were subjected to analysis of variance (ANOVA, $p \leq 0.05$) through SAS® statistical software (SAS Institute, 2011). Tukey tests were used to compare means of treatments (accessions). Some variables were transformed when indicated by the "Guided data analysis" tool, in order to be according to the ANOVA's assumptions (SAS Institute, 2011). This tool also indicated some outliers or influential data, which were removed before ANOVA (SAS Institute, 2011). In addition, the Pearson's correlation analysis was employed to evaluate the cause-effect relations among some of the variables using SAS software (SAS Institute, 2011).

3.3. Results

3.3.1. Cadmium uptake and translocation

Twenty-four-day-old tomato accessions with contrasting tolerance degrees to Cd toxicity were grown in hydroponic solution containing 0 or 35 μM $CdCl_2$ for six days. After this short-term exposure, increased Cd concentration was observed in all tissues of sensitive (LA0122, CNPH0920 and Tropic Two Orders) and tolerant accessions (CNPH0263, Indigo Rose and Yoshimatsu), when compared to the control plants (Fig. 1a-c).

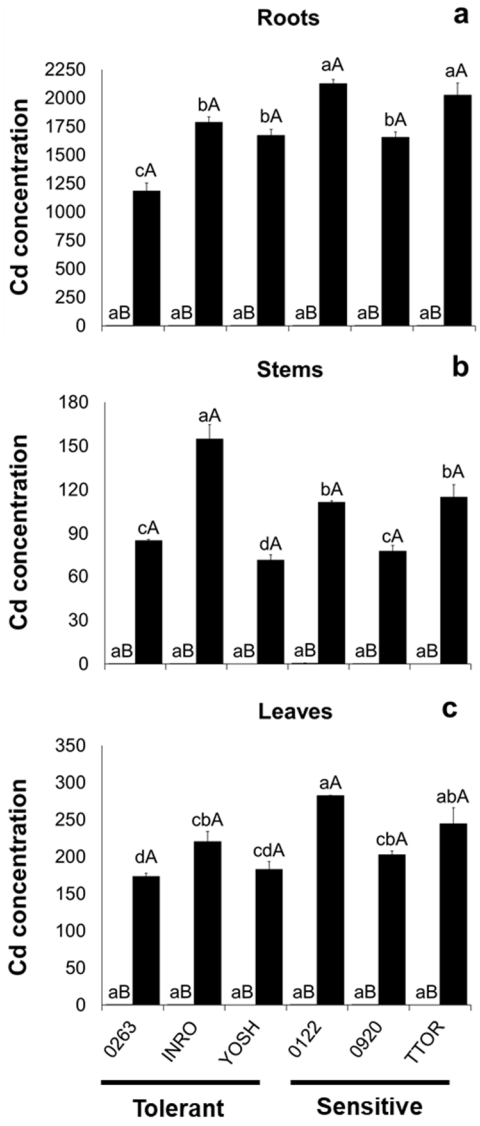

Figure 1. Cadmium concentrations (Cd, mg kg^{-1} DW) in roots (**a**), stems (**b**), and leaves (**c**) of tolerant and sensitive tomato accessions that were grown in hydroponic solution with 0 (white columns) or 35 μM $CdCl_2$ (black columns) for six days. Distinct uppercase and lowercase letters denote different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solution, and for comparisons among accessions in the same growing condition, respectively. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. Bars represent the standard errors of the means.

Among the evaluated tissues, roots presented the highest Cd concentration, regardless accession (Fig. 1a-c). A decreasing trend in the root-to-stem Cd translocation was detected in sensitive accessions when compared to the tolerant plants (Fig. 2a). By contrast, sensitive accessions exhibited an increasing trend in the stem-to-leaf Cd translocations (Fig. 2b). However, the root-to-leaf Cd translocation presented no differences among contrasting plants (Fig. 2c).

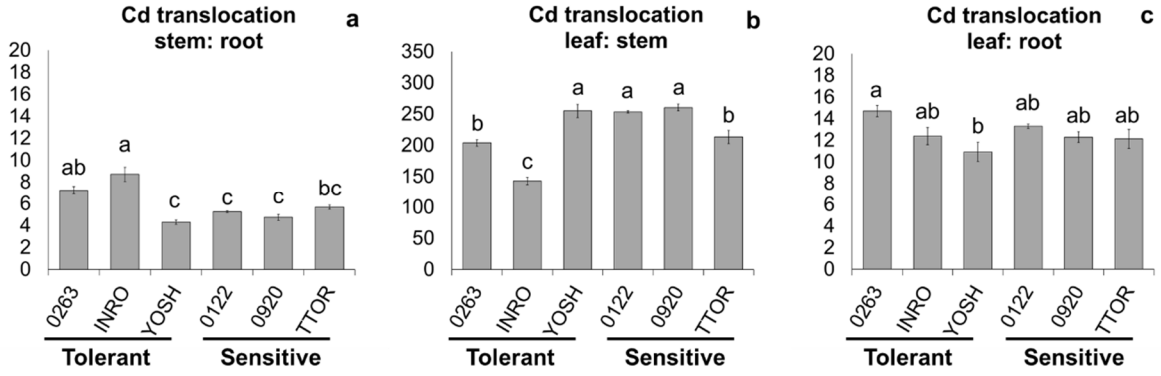

Figure 2. Translocation factors of cadmium (Cd) from roots to stem (a), from stem to leaves (b), and from roots to leaves (c) in tolerant and sensitive tomato accessions after plant exposure to 35 μM $CdCl_2$ for six days. Distinct letters denote different means by Tukey test ($p \leq 0.05$). $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. Bars represent the standard errors of the means.

3.3.2. Mineral profile modifications

Mineral profile analysis revealed that only sensitive accessions presented an increased Mg concentration in roots after Cd exposure (Fig. 3a). In leaves, two of the tolerant accessions, Indigo Rose and CNPH0263, exhibited reductions in Mg concentration, whereas other accessions generally showed an increased concentration (Fig. 3c). By contrast, Mg concentration in tomato stems was not changed in Cd-stressed when compared to the control plants, regardless accession (Fig. 3b). Sulfur concentration was increased in roots of all plants grown in Cd-containing media, and five of them also exhibited an elevated S concentration in leaves (Fig. 3d, f). In stems, variations in the S concentration depended on tomato accessions (Fig. 3e). For others macronutrients, a pattern in the plant response to Cd exposure could not be detected, when compared to the control plants (Tables S1-S3).

When micronutrients are concerned, an increased B concentration was observed in roots and leaves of almost all plants after Cd exposure (Fig. 3g, i). The highest increments were observed in two of the tree tolerant accessions, CNPH0263 (roots and leaves) and Yoshimatsu (roots and stems) (Fig. 3g-i). Interestingly, the tolerant accession Yoshimatsu, was the only tomato line that did not exhibited changes in B concentration in any of the organs (Fig. 3g-i). Cd-stressed plants exhibited remarkable decreases in Mn concentration in roots, but increases in their leaves (Fig. 4a, c).

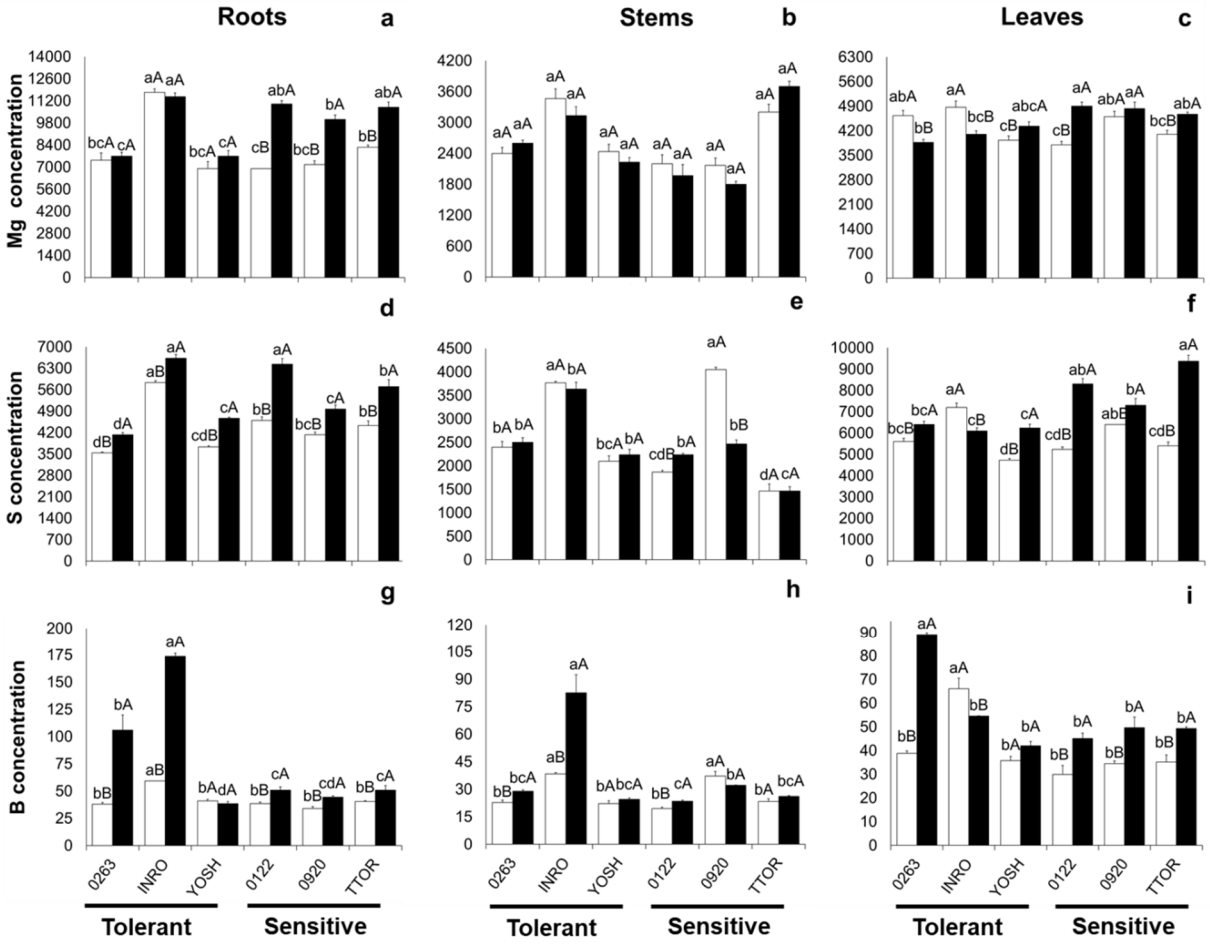

Figure 3. Magnesium – Mg (**a**, **b**, **c**), sulfur – S (**d**, **e**, **f**), and boron – B (**g**, **h**, **i**) concentrations (mg kg^{-1} DW) in roots, stems and leaves of tolerant and sensitive tomato accessions that were grown in hydroponic solution with 0 (white columns) or 35 μM $CdCl_2$ (black columns) for six days. Distinct uppercase and lowercase letters denote different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solution, and for comparisons among accessions in the same growing condition, respectively. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. Bars represent the standard errors of the means.

Zn concentration exhibited a pattern similar to that shown by Mn in leaves and roots (Fig. 4d, f). However, these micronutrients presented contrasting behaviors in stems, with decreases in Zn concentration after Cd exposure, while Mn concentration was generally increased (Fig. 4b, d). Fe concentration in roots was not changed in 4 accessions cultivated in Cd-containing media (Fig. 4g). The exceptions were LA0122 and Indigo Rose accessions, which exhibited an increased and decreased Fe concentration, respectively, in plants under Cd exposure in comparison to the control plants (Fig. 4g). In stems, Fe concentration was decreased in stems of all accessions after Cd exposure (Fig. 4h). In leaves, Fe concentration exhibited the most remarkable reductions in tolerant accessions when compared to the sensitive accessions (Fig. 4i). For others micronutrients, a pattern in the plant response to Cd exposure could not be detected, when compared to the control plants (Tables S1-S3).

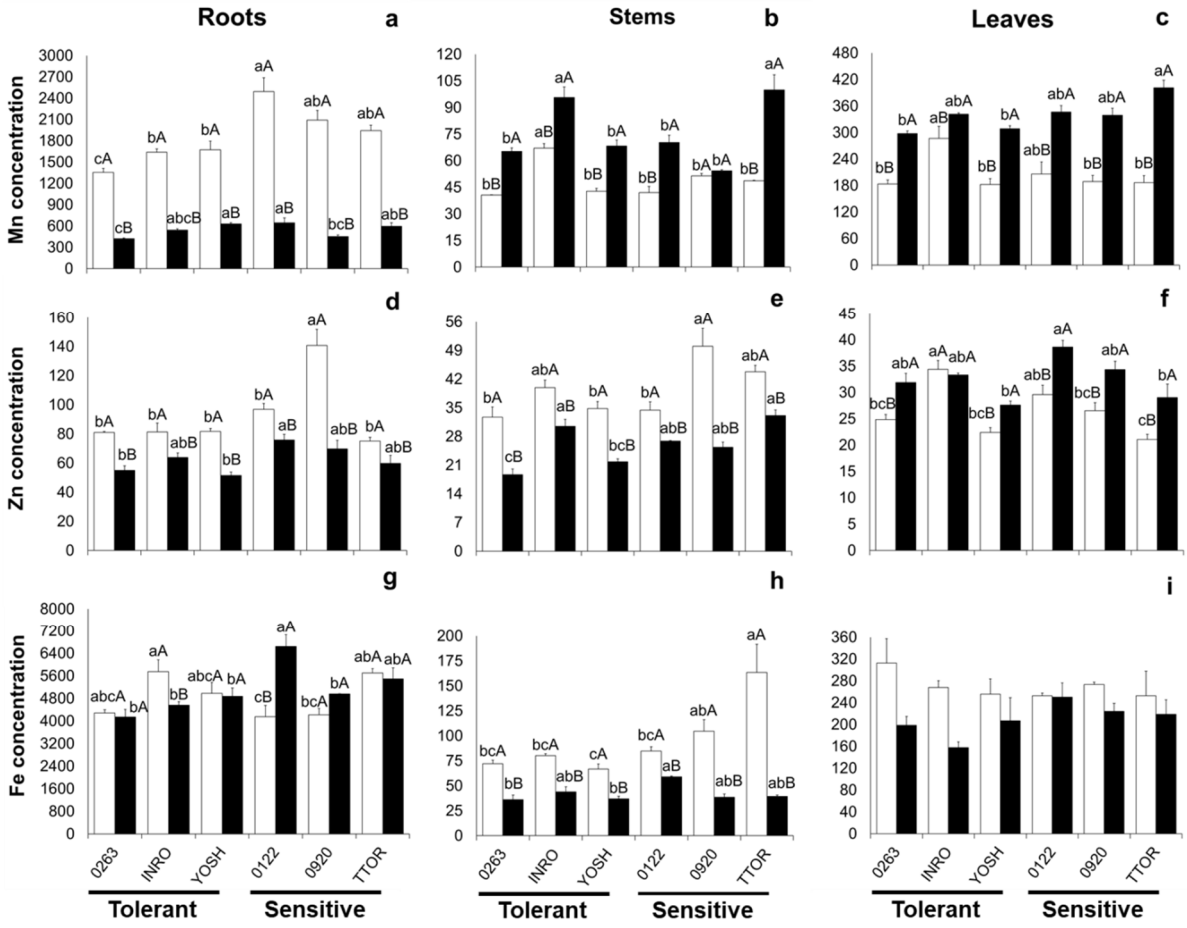

Figure 4. Manganese – Mn (**a**, **b**, **c**), zinc – Zn (**d**, **e**, **f**), and iron – Fe (**g**, **h**, **i**) concentrations (mg kg^{-1} DW) in roots, stems and leaves of tolerant and sensitive tomato accessions that were grown in hydroponic solution with 0 (white columns) or 35 μM $CdCl_2$ (black columns) for six days. Distinct uppercase and lowercase letters denote different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solution, and for comparisons among accessions in the same growing condition, respectively. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. There was no interaction effect (tomato accessions vs $CdCl_2$ concentrations) on the leaf Fe concentration, which was affected only by '$CdCl_2$ concentration' factor (0 μM – 269.09 and 35μM – 209.39 mg kg^{-1} DW). Bars represent the standard errors of the means.

3.3.3. Cd-induced impacts on plant development

Tomato cv. Tropic Two Orders and *S. pimpinellifolium* exhibited reductions in root and stem dry weights, while no changes were observed in the tolerant accessions under Cd exposure, when compared to the control plants (Fig. 5a, b). Leaf dry weight in sensitive accessions was more decreased than in tolerant accessions after cultivation in Cd-containing media (Fig. 5c). The length of the longest root was generally reduced in Cd-stressed plants when compared to the control ones (from 19.4 to 38.4%), the only exception was Yoshimatsu, which exhibited no changes in the root length (Fig. 5d). The highest reductions in the leaf area were observed in sensitive accessions under Cd exposure, but the leaves of the tolerant accession CNPH0263 were also significantly affected (Fig. 5e). Specific leaf area exhibited a varied response in plants grown in Cd-containing media (Fig. 5f), suffering decreases in CNPH0263 and *S. pimpinellifolium* (from 14 to 44%), while increases up to 18% in CNPH0920 in comparison to the control plants (Fig. 5f).

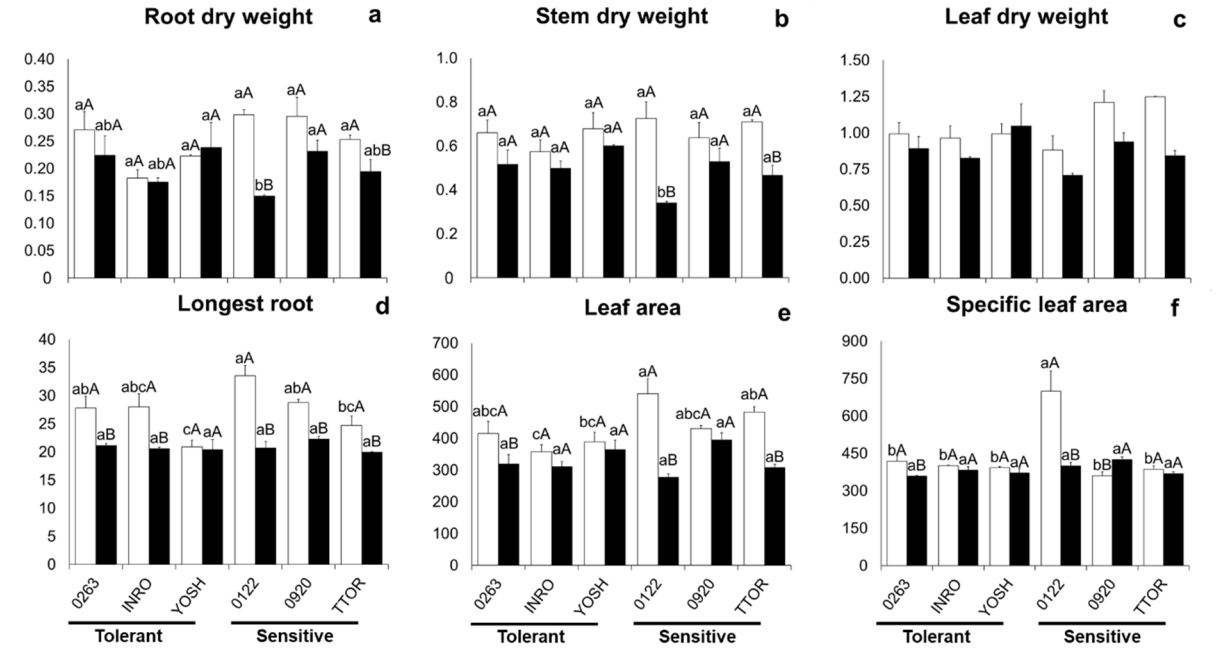

Figure 5. Dry weight of roots (**a**), stems (**b**) and leaves (**c**), length of the longest root (**d**), leaf area (**e**) and specific leaf area (**f**) in roots, stems and leaves of tolerant and sensitive tomato accessions that were grown in hydroponic solution with 0 (white columns) or 35 μM $CdCl_2$ (black columns) for six days. Distinct uppercase and lowercase letters denote different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solution, and for comparisons among accessions in the same growing condition, respectively. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. There was no interaction effect (tomato accessions vs $CdCl_2$ concentrations) on the leaf dry weight, which was affected separately by 'accessions' and '$CdCl_2$ concentration' factors (0 μM – 1.05 and 35μM – 0.87 g). Bars represent the standard errors of the means.

3.3.4. Disturbances in root structure and development

Thirty-day-old seedlings of tomato cv. Tropical Two Orders exhibited an increased formation of root hairs bulges and large holes in Cd-stressed plants (Fig. 6) when compared to the control ones (Fig. 6). The number of epidermal layers was also increased in roots of stressed plants, whereas the size of parenchymal cells was decreased (Fig. 6). Similar effects were observed in roots of *S. pimpinellifolium* under Cd-induced stress (Fig. 6). Tomato cv. Indigo Rose presented a higher number of epidermal and exodermal cells, which exhibited an increased number of vacuoles with smaller size in Cd-treated plants when compared to the control plants (Fig. 6). On the other hand, roots of the tomato cv. Yoshimatsu exhibited changes in shape and size of parenchymal cells, which showed a decreased longitudinal length and presented increased invaginations along the whole walls (Fig. 6).

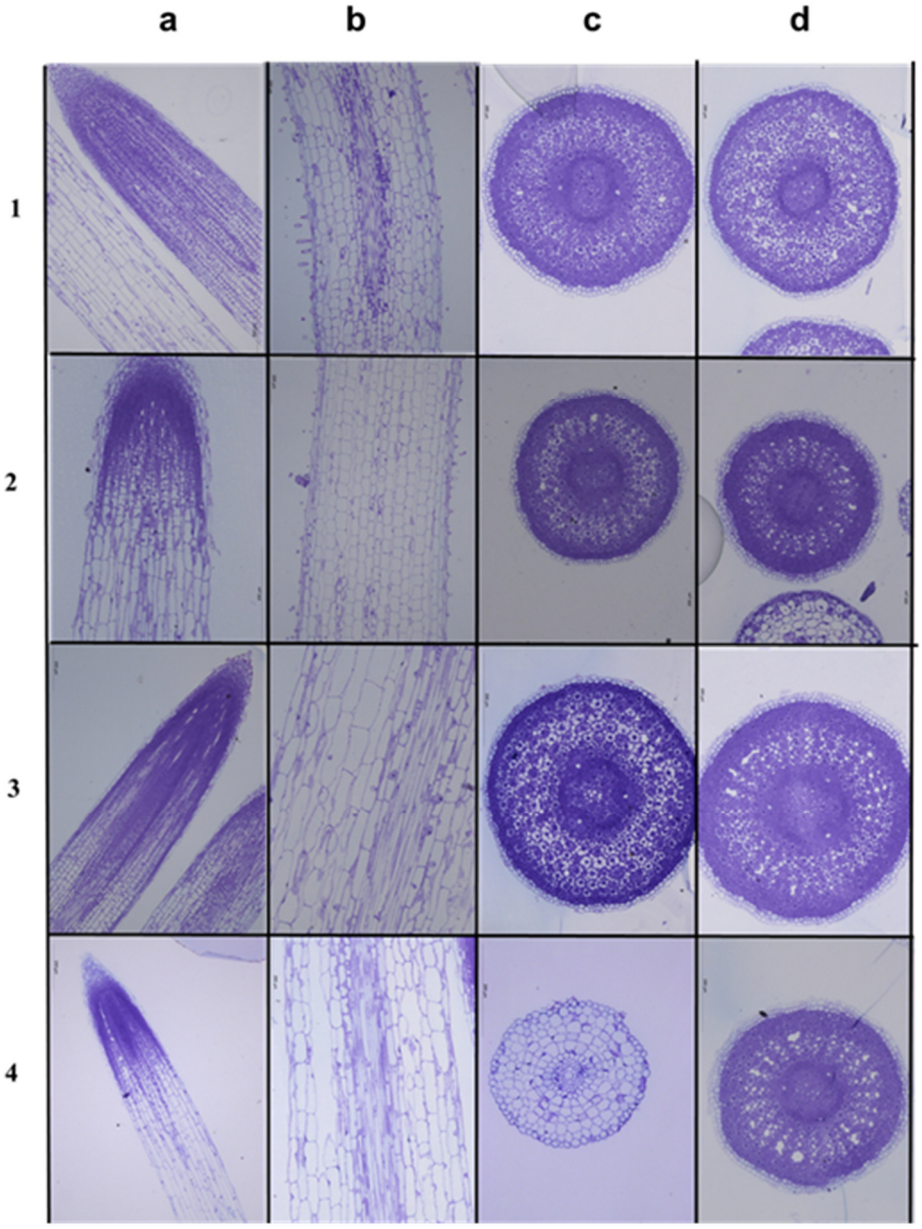

Figure 6. Longitudinal and transversal cross sections from roots of thirty-day-old tomato accessions *Solanum pimpinellifolium* (1 - sensitive) and *S. lycopersicum* cvs. Tropic Two Orders (2 - sensitive), Indigo Rose (3 - tolerant) or Yoshimatsu (4 - tolerant) that were grown in hydroponic solution containing 0 (**a**, **c**) or 35 μM $CdCl_2$ (**b**, **d**) for six days.

3.3.5. Tolerance degree to Cd toxicity and oxidative stress indicators

Sensitive accessions exhibited the lowest tolerance indexes, however, CNPH0920 showed a tolerance degree similar to that from tolerant plants (Fig. 7a). After Cd exposure, *S. pimpinellifolium*, Indigo Rose and also Yoshimatsu presented an increased H_2O_2 content (Fig. 7b). Concurrently, a high lipid peroxidation level was observed in *S. pimpinellifolium*, and tomato cvs. Tropic Two Orders and Indigo Rose, when compared to the control plants (Fig. 7c).

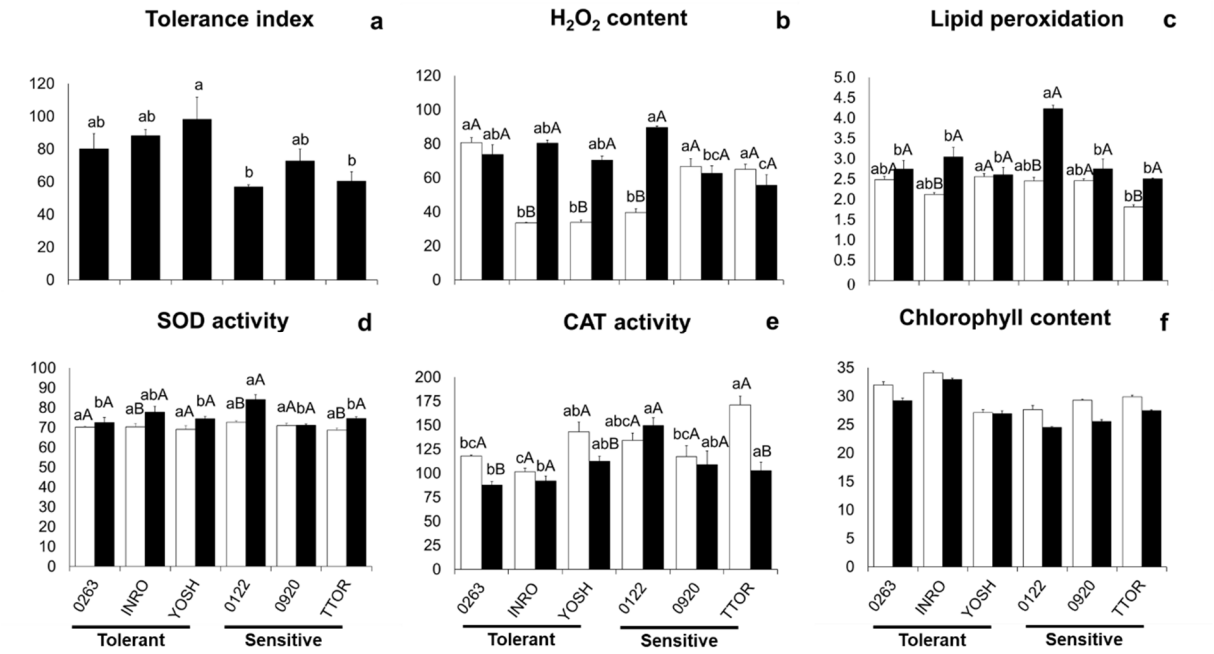

Figure 7. Tolerance index (**a**), H_2O_2 – hydrogen peroxide content (μmol g^{-1} FW, **b**), lipid peroxidation - malondialdehyde (MDA) content (nmol g^{-1} FW, **c**), SOD – superoxide activity (U SOD g^{-1} FW, **d**), catalase - CAT activity (μmol min^{-1} g^{-1} FW, **e**) and chlorophyll content (SPAD index, **f**) in leaves of tolerant and sensitive tomato accessions that were grown in hydroponic solution with 0 (white columns) or 35 μM $CdCl_2$ (black columns) for six days. Distinct uppercase and lowercase letters denote different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solution, and for comparisons among accessions in the same growing condition, respectively. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. There was no interaction effect (tomato accessions vs $CdCl_2$ concentrations) on the chlorophyll content, which was influenced separately by 'tomato accessions' and '$CdCl_2$ concentration' (0 μM -29.98 and 35μM -27.74 SPAD values). Bars represent the standard errors of the means.

Upon Cd exposure, *S. pimpinellifolium*, and tomato cvs. Tropic Two Orders and Indigo Rose showed a high SOD activity in leaf tissues, however CAT activity presented no changes or even decreases (Fig. 7d, e). In tomato leaves, GR activity was unaffected by Cd exposure, regardless accession (data not shown). In Cd-stressed plants, a decreasing trend in the leaf chlorophyll was observed, but sensitive accessions were more affected than tolerant ones (Fig. 7f).

3.4. Discussion

Although several aspects of Cd-induced effects on plant development have been studied, the tolerance mechanisms against Cd toxicity are still poorly understood. One of the main reasons is related to the numerous and complex factors that make experimental settings difficult. For instance, plant responses to Cd toxicity depend on the Cd concentration in the growth media, time-lenght of plant exposure, plant features (genotype, age, target organ and tissues), growth media properties, among others (Gratão et al., 2008, 2009, 2012, 2015; Nogueirol et al., 2016; Pompeu et al., 2017). Since previous studies indicated that modifications in the plant mineral profile are coupled to the tolerance mechanisms against Cd toxicity (Hermans et al., 2011; Kudo et al., 2015; Sebastian and Prasad, 2016a), hydroponic solution with the same nutrient composition (except by the $CdCl_2$ addition) was used to avoid extra outcomes from nutrient excess or starvation, as well as potential variations in the Cd speciation. In this context, the present study was performed to address two main questions: (i) Are changes in the nutrient status associated to tomato tolerance to the short-term Cd exposure? and (ii) How are these changes coupled to the mechanisms for mitigation of Cd toxicity?

3.4.1. Avoidance of high Mg uptake mitigates Cd toxicity by preventing formation of root hairs

The use of tomato accessions with different tolerance degrees to Cd exposure was a powerful approach to identify plant strategies for mitigation of Cd toxicity. Since Mg concentration was increased only in roots of sensitive accessions (Fig. 3a), data indicate that the plant's ability to quickly manage the Mg status is associated to mechanisms that regulate tomato tolerance to the short-term Cd exposure. Such capacity is specifically linked to the avoidance of Mg uptake by roots (Fig. 3a), validating previous studies in which the low Mg concentration was coupled to improvements in the plant tolerance to Cd toxicity. In such studies, however, the low Mg status was related to enhancements in the antioxidant status in rice (Chou et al., 2011) and reductions in leaf damages in *Arabidopsis* (Hermans et al., 2011). Nevertheless, a common feature is the maintenance of root development in Cd-treated plants when compared to the control ones - see results of Chou et al. (2011), Hermans et al. (2011) and Kudo et al. (2015). Consistently, the root dry weight of tolerant tomato accessions was sustained after Cd exposure, while sensitive accessions exhibited major reductions (Fig. 5a). These data corroborated findings from Niu et al. (2014), who showed that a high Mg supply decreases root growth, but enhances trichoblast initiation. Accordingly, sensitive accessions presented an increased formation of root hairs bulges (Fig. 6) concurrently with the high Mg concentration (Fig. 5a). At the same time, sensitive accessions exhibited an increasing trend of Cd concentration in roots in comparison to the tolerant plants (Fig. 1a), suggesting that stimulation of root hair formation enhances the Cd uptake and accumulation, which in turn impairs plant development (Fig. 5a-e).

3.4.2. Plant's ability to maintain the leaf blade expansion is associated to the biomass production in plants under Cd exposure

Consistently, as higher Cd concentration in leaves, lower tolerance index ($r = -61.96\%$ and $p = 0.0061$, Fig. 1c, Fig. 7a), so strategies to decrease the amount of Cd that reaches tomato leaves are relevant for reductions in tomato sensitivity to Cd exposure. Although accession-dependent mechanisms in the root-to-stem and stem-to-leaf translocations of Cd were detected, they were not enough to provoke changes in the proportion of Cd that was sent to leaves, in comparison to the amount of Cd absorbed by roots (Fig. 2a-c). Thus, since Cd concentration in leaves depended on Cd concentration in roots ($r = 80.91$ and $p < 0.0001$, Fig. 1a, c), preventions in the Cd uptake are a key mechanism to reduce Cd toxicity. Anyways, once Cd reaches the leaves, the plant's capacity to maintain the leaf blade expansion seems to be a strategy to sustain the development of Cd-stressed plants. This hypothesis is supported by the fact that the only sensitive accession with no changes in the leaf area, CNPH0920, exhibited tolerance index similar to that from tolerant accessions (Fig. 5e, Fig. 7a). Otherwise, the only tolerant accession that showed remarkable reductions in the leaf area, CNPH0263, exhibited decreasing trend in the tolerance index in comparison to the other tolerant plants (Fig. 5e, Fig. 7a). Moreover, there was a significant and proportional relation ($r = 73.81$ and $p = 0.0007$) between tolerance index and accessions' capacity to sustain leaf area. In this context, data suggested that the continuous leaf blade expansion in plants under the short-term Cd exposure is associated to the increased plant potential to produce biomass, which in turn is negatively affected by Cd accumulation in leaves.

3.4.3. Leaf damages are coupled to increases in leaf Cd concentration, which triggers ROS overproduction

Moreover, Cd may impair the photosynthetic tissues by causing damages in biological membranes (Fig. 7c), since as higher Cd concentration in leaves, higher the lipid peroxidation level ($r = 81.49$ and $p < 0.0001$). Such damages seem to be linked to H_2O_2 overproduction (*S. lycopersicum* cv. Indigo Rose and *S. pimpinellifolium* LA0122), but the generation of others ROS and NOS may be involved in this Cd induced-effect too, because tomato cv. Tropic Two Orders presented an elevated lipid peroxidation and no changes in the H_2O_2 content (Fig. 7b-c). Under certain conditions, tomato is able to modulate the activity of some antioxidant enzymes to counteract effects of ROS (Gratão et al. 2005). Accordingly, some accessions under Cd exposure presented a high SOD activity (Fig. 7d) that is an enzyme with three isoform-dependent localization in plants: Fe-SOD in chloroplasts, Mn-SOD in mitochondria, and Cu/Zn-SOD in cytoplasm (Gratão et al., 2005; Azevedo et al., 1998). Although increases in the Zn, Cu and Mn concentrations (Fig. 4c, f) could be related to enhancements of SOD activity, further data indicated that Zn and Mn excess may enhance Cd-induced damages in photosynthetic tissues (see the next Discussion' sections).

Moreover, not always an increased SOD activity depends on Cu accumulation, so a cause-effect relation between these variables is weak (Table S3). In addition, since a high SOD activity is associated to superoxide ($O_2^{\bullet-}$) overproduction, this ROS may increase the lipid peroxidation in Tropic Two Orders, and enhance the H_2O_2-dependent lipid peroxidation in Indigo Rose and LA0122 (Fig. 7b-d). In Yoshimatsu, however, SOD activity was not increased (Fig. 7d), CAT activity was decreased (Fig. 7e) and GR activity was unaffected after Cd exposure (data not shown), so other enzymes and/or non-enzymatic compounds may counteract the H_2O_2 excess. According to Iannone et al. (2015), CAT does not play a crucial role in protection against Cd toxicity, hence alternative defense mechanisms to avoid H_2O_2 overproduction should be activated. As another possibility, H_2O_2 excess in this accession is enough to act as signals without damaging leaf tissues (Cuypers et al., 2016).

3.4.4. Manganese remobilization to leaves amplify the Cd side-effects on photosynthetic tissues

Concurrently to the Cd accumulation in shoots (Fig. 1b-c), all tomato accessions exhibited a high Mn concentration in leaves (Fig. 4c) and low Mn concentration in roots (Fig. 4a), indicating that Cd induced Mn remobilization from roots to photosynthetic tissues (Fig S1c). Since the root Mn concentration was drastically reduced after the short-term exposure to Cd (Fig. 4a), data also suggested that Mn uptake was prevented in Cd-stressed plants, corroborating previous reports that showed strong inhibitions in the Mn absorption in other tomato genotypes under Cd exposure (Dong et al., 2006; López-Millán et al., 2009). Such event may be related to the Cd-mediated modulation in the activity of plasma membrane transporters (Migocka and Klobus 2007), and/or by the Cd ability to use Mn transporters (Korshunova et al., 1999; Sasaki et al., 2012; Wu et al., 2016). Mn excess in shoots causes stunted growth, chlorosis, crinkled leaves and brown speckles (González et al., 1998), symptoms that are similar to the ones observed in all tomato accessions under Cd exposure. According to Alvarenga (2013), the threshold for Mn toxicity in tomato starts from 250 mg kg^{-1} DW, supporting evidences about the negative effects of the high Mn concentration on the

development of plants under Cd exposure. In Cd-stressed plants, Mn excess is stored mainly in the chloroplasts (Ramos et al., 2002), where it disorganizes the chloroplast lamellae and increases ROS production (González et al., 1998; Lidon and Teixeira, 2000; Lavres Junior et al., 2010), causing effects that resemble those reported in tomato plants grown in Cd-containing media (Baszyński et al., 1980; Djebali et al., 2005; Gratão et al., 2009; Pompeu et al., 2017). Considering this information, the results indicate that the high Mn concentration in leaves is associated to damages in photosynthetic tissues of tomato under short-term Cd exposure.

3.4.5. Zinc and boron excess in leaves aggravates the Cd-induced damages in photosynthetic tissues

The classical symptoms of Cd phytotoxicity in leaves, chlorosis and necrosis, were observed in all tomato accessions under Cd exposure, but Cd-sensitive tomato exhibited them earlier than the Cd-tolerant accessions. Since all sensitive accessions presented Zn and B excess in leaves (Fig. 3i, 4f), such micronutrients may be associated to damages in photosynthetic tissues. Although Zn is the second most abundant transition metal in living organisms, its excess inhibits photosynthesis and quickly causes chlorosis (Marschner, 2012), triggering toxicity symptoms that are indistinguishable from that caused by Cd (Smith and Brennan 1983). In tomato cv. Rutgers, Cd stimulated Zn translocation from roots to leaves, where Zn enhanced leaf injuries caused by Cd (Smith and Brennan, 1983). In line with these findings, Zn seems to be remobilized from roots and stems to the leaves of tomato accessions (Fig. 4d-e), and sensitive genotypes exhibited concurrently high Zn and Cd concentrations (Fig. 1c). By contrast, B translocation to leaves was generally unaffected in tomato genotypes growing in Cd-containing media (Table S4), indicating that increases in the B uptake was the determining factor for B excess in photosynthetic tissues (Fig. 3i). According to Kaya et al. (2009), B toxicity in tomato started as a yellow–green interveinal chlorosis, which developed first in the oldest leaves and progressed to the youngest leaves. Afterwards, small patches of necrotic tissue appeared between the minor veins and extended to the midribs resulting into a reduced photosynthetic leaf area. Taking together the results indicate that Zn and B excess in leaves enhances the Cd-induced damages in photosynthetic tissues.

3.4.6. Low Fe concentrations in leaves are uncoupled to the highest sensitivity to the short-term Cd exposure

Although Cd side-effects has been coupled to reductions in Fe accumulation in leaves (Rodríguez-Celma et al., 2010; Hermans et al., 2011), data indicated that highest sensitivity to Cd toxicity is not associated to this phenomenon, because only mild decreases in leaf Fe concentration were observed in the sensitive accessions when compared to the tolerant ones (Fig. 4i). Similarly, reductions in the chlorophyll content was not related to the Fe concentration in leaves (Fig. 7f). For the sensitive plants, data indicated that the Fe stored in stems was remobilized to the leaves in order to maintain the Fe status in photosynthetic tissues, as observed in CNPH0920 and Tropic Two Orders (Fig. 4g-i), despite no alterations in Fe uptake. In *S. pimpinellifolium* LA0122, however, increases in the Fe uptake in Cd-treated plants were necessary to implement the relatively low Fe concentration in stems (Fig. 4g-i). In line with these findings, reductions in the Fe status in leaves of tolerant accessions can be coupled to the low Fe concentration in stems, and also to their apparent incapacity to increase Fe absorption under Cd exposure (Fig. 4g-i). However, with

the current data, it was not possible to establish the mechanism by which changes in Fe concentration may act in tomato under Cd exposure.

One hand, tolerant accessions might activate the low Fe status-dependent protective mechanisms in Cd-stressed plants. For instance, proline accumulation was enhanced by iron depletion in leaves of plants under Cd exposure (Sharmila et al., 2017). On the other hand, decreases in the biomass production can be linked to reductions in the Fe concentration in photosynthetic tissues (Hermans et al., 2011). However, there are evidences that interaction of Fe with other nutrients is more important than Fe deficiency *per se*, since the Fe concentration was not changed or even increased in some plant species after Cd exposure (Root et al., 1975; Larbi et al., 2002; Thomine et al., 2000; Lopéz-Millán et al., 2009; Chou et al., 2011). When Fe concentration is the same in Cd-treated and non-treated plants, at least two events must be taken into consideration: (1) "dilution and concentration effects" on Fe concentration by changing the concentration of other nutrients and/or by altering the cell growth, and (2) modifications in the Fe distribution in sub-cellular compartments, which can play a key role in the protective effect against Cd exposure (Sárvári et al., 2011).

3.4.7. Differential tolerance to Cd toxicity is uncoupled to increases in the S uptake

Cd is one of the most dangerous metals to biological systems because it is a non-essential element with high water solubility, maintaining this feature under physiological conditions (Schützendübel and Polle, 2002). The direct consequence is its great potential to react with biological molecules and to impair their functions, a phenomenon that can be enhanced due to the chemical similarities shared by Cd and some nutrients (Korshunova et al., 1999; Dong et al., 2006; Cherif et al., 2012). Thus, it is possible that Cd binds to the functional membrane and enzyme active sites and impair or even inactivate their functions (Cherif et al., 2012). In this context, compounds able to chelate/ compartmentalize Cd ions may play an important role for mitigation of Cd toxicity in plants, and one of the most relevant are cysteine-rich compounds such as metallothionein, glutathione (GSH) and phytochelatin (Ruttkay-Nedecky et al., 2013; Yamaguchi et al., 2016; Hasan et al., 2016). According to Yamaguchi et al. (2016), an adequate S supply is needed for phytochelatin production concurrently to the maintenance of suitable GSH content, thus suggesting that plants able to enhance S absorption are also capable to be more tolerant to Cd toxicity (Hasan et al., 2016). However, increases in S absorption, as estimated by the high S concentration in roots, were not coupled to the differential tolerance degree to Cd toxicity because tolerant and sensitive accessions exhibited similar behaviors (Fig. 3d) that also resulted into, in general, higher S concentrations in leaves of Cd-stressed plants in comparison to the control plants (Fig. 3f).

By contrast, reductions in the leaf Mg status may be related to the advantageous outcomes in plants under Cd exposure, because tolerant accessions exhibited a decreasing trend of Mg concentration in leaves when compared to the sensitive accessions (Fig. 3c). Taking into account that Mg^{2+} stored in roots may readily be used to meet the demand in the Mg deficient-shoots (Hermans et al., 2005), the Mg transport to leaves is another tool to avoid the Cd accumulation in roots and, consequently, to prevent the root hair formation in tolerant accessions. In this context, the proportion of Mg concentration between root and leaf tissues can be associated to an important aspect for the regulation of Mg-dependent strategies to overcome the Cd toxicity during the short-term exposure of the plants to this heavy metal. Accordingly, root-to-leaf Mg translocation presented no differences in tolerant accessions after Cd-exposure, but sensitive accessions exhibited a decreasing trend in this variable in Cd-stressed plants when compared to the control plants (Table S4). Therefore, data indicated that tolerant plants should possess a fine regulation of the

internal Mg redistribution, in addition to the mechanism to decrease Mg entrance in plants. It is interesting to notice that high Mg concentration in leaves did not improve the chlorophyll content in sensitive accessions (Fig. 7f).

3.5. Conclusions

In conclusion, the use of tomato accessions with different tolerance degrees to Cd toxicity was a valuable approach to suggest the more likely Cd-tolerance mechanisms involved in tomato after a short-term exposure to this metal. Among the findings, avoidance of high Mg uptake inhibits root hair formation, hence decreasing Cd uptake and its translocation to leaves, where this heavy metal triggers ROS overproduction. Data also indicated that the plants' capacity to maintain the leaf blade expansion is an important strategy to allow biomass production in tomato under Cd exposure. Regarding the mode of action of Cd toxicity, this metal causes drastic misbalances in the plant mineral profile by changing the nutrient uptake and remobilization. In leaves, Mn excess enhances Cd-induced damages that are further amplified by increases in the Zn and B concentration in photosynthetic tissues. In addition, modifications in the Fe concentration of tomato leaves are uncoupled to the highest sensitivity to the short-term Cd exposure.

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Supplementary materials

Table S1 Copper – Cu, potassium – K, phosphorus – P and calcium – Ca concentrations (mg kg^{-1} DW) in roots of tolerant and sensitive tomato accessions that were grown in hydroponic solution with 0 or 35 μM $CdCl_2$ for six days.

Accessions	$CdCl_2$ 0 μM				$CdCl_2$ 35μM				Average			
					Cu							
0263	6.13	±	0.39		9.07	±	0.67		7.60	±	0.53	b
INRO	8.70	±	0.15		12.77	±	0.49		10.73	±	0.32	a
YOSH	6.97	±	0.03		8.40	±	0.49		7.68	±	0.26	b
0122	5.55	±	0.55		9.55	±	0.55		7.55	±	0.55	b
0920	7.00	±	0.65		8.87	±	0.27		7.93	±	0.46	b
TTOR	7.73	±	0.44		10.10	±	0.72		8.92	±	0.58	b
Average	7.01	±	0.37	b	9.79	±	0.53	a				
					K							
0263	61600.00	±	1493.32	bcA	60750.00	±	50.00	bA	61175.00	±	771.66	
INRO	94266.67	±	3030.03	aA	90000.00	±	2621.07	aA	92133.33	±	2825.55	
YOSH	55500.00	±	2055.08	cB	70700.00	±	600.00	bA	63100.00	±	1327.54	
0122	69050.00	±	50.00	bA	58500.00	±	866.03	bB	63775.00	±	458.01	
0920	61033.33	±	2418.22	bcA	42400.00	±	3203.64	cB	51716.67	±	2810.93	
TTOR	53666.67	±	1431.01	cB	68600.00	±	4000.00	bA	61133.33	±	2715.50	
Average	65852.78	±	1746.28		65158.33	±	1890.12					
					P							
0263	9133.33	±	88.19	bA	9366.67	±	338.30	bA	9250.00	±	213.24	
INRO	12500.00	±	458.26	aA	9800.00	±	152.75	bB	11150.00	±	305.51	
YOSH	8533.33	±	218.58	cB	9333.33	±	233.33	bA	8933.33	±	225.96	
0122	10700.00	±	0.00	abB	12100.00	±	503.32	aA	11400.00	±	251.66	
0920	9533.33	±	266.67	bA	9866.67	±	417.67	bA	9700.00	±	342.17	
TTOR	9900.00	±	115.47	bA	10266.67	±	317.98	bA	10083.33	±	216.72	
Average	10050.00	±	191.19		10122.22	±	327.22					
					Ca							
0263	11666.67	±	584.05		8600.00	±	100.00		10133.33	±	342.02	b
INRO	14200.00	±	100.00		13000.00	±	624.50		13600.00	±	362.25	a
YOSH	12133.33	±	560.75		9100.00	±	550.76		10616.67	±	555.76	ab
0122	12850.00	±	2450.00		15433.33	±	1794.75		14141.67	±	2122.37	a
0920	12833.33	±	2324.03		12500.00	±	2272.30		12666.67	±	2298.16	ab
TTOR	10700.00	±	152.75		9466.67	±	545.69		10083.33	±	349.22	b
Average	12397.22	±	1028.60	a	11350.00	±	981.33	b				

For K and P concentrations, variables in which the studied factors effect (tomato accessions vs $CdCl_2$ concentrations) presented a significant interaction ($p \leq 0.05$), distinct uppercase and lowercase letters denote different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solution, and for comparisons among accessions in the same growing condition, respectively. For Cu and Ca concentrations, distinct letters denote different means by Tukey test ($P \leq 0.05$) for comparisons among accessions or growing conditions. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. Mean ± standard error.

Table S2 Copper – Cu, potassium – K, phosphorus – P and calcium – Ca concentrations (mg kg^{-1} DW) in stems of tolerant and sensitive tomato accessions that were grown in hydroponic solution with 0 or 35 μM $CdCl_2$ for six days.

Accessions	$CdCl_2$ 0 μM				$CdCl_2$ 35μM				Average			
					Cu							
0263	1.65	±	0.05	abcA	1.73	±	0.09	abA	1.69	±	0.07	
INRO	1.77	±	0.09	abA	2.00	±	0.15	aA	1.88	±	0.12	
YOSH	1.40	±	0.06	bcA	1.17	±	0.09	cB	1.28	±	0.07	
0122	1.33	±	0.03	cB	1.60	±	0.00	abA	1.47	±	0.02	
0920	2.17	±	0.07	aA	1.37	±	0.03	bcB	1.77	±	0.05	
TTOR	1.85	±	0.05	abA	2.07	±	0.20	aA	1.96	±	0.13	
Average	1.69	±	0.06		1.66	±	0.09					
					K							
0263	70866.67	±	1971.74	bB	106566.67	±	5304.82	aA	88716.67	±	3638.28	
INRO	133900.00	±	4161.73	aA	114300.00	±	2672.70	aB	124100.00	±	3417.22	
YOSH	64566.67	±	3702.40	bB	96633.33	±	1017.08	aA	80600.00	±	2359.74	
0122	64366.67	±	6140.12	bB	104433.33	±	4223.08	aA	84400.00	±	5181.60	
0920	121900.00	±	19400.00	aA	61000.00	±	2818.39	aB	91450.00	±	11109.20	
TTOR	81833.33	±	2776.29	bB	108000.00	±	680.69	aA	94916.67	±	1728.49	
Average	89572.22	±	6358.71		98488.89	±	2786.13					
					P							
0263	5200.00	±	300.00	cB	6433.33	±	88.19	aA	5816.67	±	194.10	
INRO	8500.00	±	200.00	aA	7833.33	±	176.38	aA	8166.67	±	188.19	
YOSH	5533.33	±	375.65	bcA	6066.67	±	333.33	bA	5800.00	±	354.49	
0122	5166.67	±	392.99	cB	6766.67	±	66.67	aA	5966.67	±	229.83	
0920	8850.00	±	450.00	aA	6533.33	±	371.18	aB	7691.67	±	410.59	
TTOR	6666.67	±	296.27	bB	7633.33	±	218.58	aA	7150.00	±	257.43	
Average	6652.78	±	335.82		6877.78	±	209.06					
					Ca							
0263	7900.00	±	230.94		9866.67	±	352.77		8883.33	±	291.85	cd
INRO	14133.33	±	920.75		13400.00	±	818.54		13766.67	±	869.64	a
YOSH	8666.67	±	409.61		8733.33	±	296.27		8700.00	±	352.94	cd
0122	10800.00	±	1050.40		11566.67	±	328.30		11183.33	±	689.35	b
0920	9633.33	±	819.21		9233.33	±	392.99		9433.33	±	606.10	bcd
TTOR	10000.00	±	346.41		10833.33	±	520.68		10416.67	±	433.55	bc
Average	10188.89	±	629.55	a	10605.56	±	451.59	a				

For Cu, K and P concentrations, variables in which the studied factors effect (tomato accessions vs $CdCl_2$ concentrations) presented a significant interaction ($p \leq 0.05$), distinct uppercase and lowercase letters denote different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solution, and for comparisons among accessions in the same growing condition, respectively. For Ca concentration, distinct letters denote different means by Tukey test ($P \leq 0.05$) for comparisons among accessions or growing conditions. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. Mean ± standard error.

Table S3 Copper – Cu, potassium – K, phosphorus – P and calcium – Ca concentrations (mg kg^{-1} DW) in leaves of tolerant and sensitive tomato accessions that were grown in hydroponic solution with 0 or 35 μM $CdCl_2$ for six days.

Accessions	$CdCl_2$ 0 μM				$CdCl_2$ 35μM				Average			
					Cu							
0263	3.55	±	0.25		4.60	±	0.35		4.08	±	0.30	a
INRO	3.20	±	0.10		3.13	±	0.20		3.17	±	0.15	b
YOSH	2.23	±	0.15		2.53	±	0.19		2.38	±	0.17	c
0122	2.23	±	0.34		3.47	±	0.23		2.85	±	0.29	bc
0920	2.77	±	0.12		2.97	±	0.20		2.87	±	0.16	bc
TTOR	2.67	±	0.30		3.27	±	0.13		2.97	±	0.21	bc
Average	2.78	±	0.21	b	3.33	±	0.22	a				
					K							
0263	25600.00	±	1700.00	dB	41500.00	±	888.82	cdA	33550.00	±	1294.41	
INRO	93933.33	±	3418.74	aA	44566.67	±	2248.21	bcdB	69250.00	±	2833.47	
YOSH	52366.67	±	868.59	cB	58166.67	±	3643.41	aA	55266.67	±	2256.00	
0122	23150.00	±	5050.00	dB	52633.33	±	3227.14	abcA	37891.67	±	4138.57	
0920	67966.67	±	1047.75	bA	35033.33	±	592.55	dB	51500.00	±	820.15	
TTOR	47400.00	±	1997.50	cB	58033.33	±	4330.26	abA	52716.67	±	3163.88	
Average	51736.11	±	2347.10		48322.22	±	2488.40					
					P							
0263	5166.67	±	145.30	bA	5833.33	±	120.19	bcA	5500.00	±	132.74	
INRO	7500.00	±	251.66	aA	5666.67	±	145.30	cB	6583.33	±	198.48	
YOSH	5400.00	±	173.21	bA	6566.67	±	448.45	abcA	5983.33	±	310.83	
0122	5533.33	±	448.45	bB	7033.33	±	317.98	abA	6283.33	±	383.22	
0920	6133.33	±	33.33	bA	6700.00	±	173.21	abcA	6416.67	±	103.27	
TTOR	5266.67	±	185.59	bB	7500.00	±	173.21	aA	6383.33	±	179.40	
Average	5833.33	±	206.26		6550.00	±	229.72					
					Ca							
0263	22800.00	±	503.32	bA	23233.33	±	696.02	bA	23016.67	±	599.67	
INRO	31933.33	±	133.33	aA	23966.67	±	656.59	bB	27950.00	±	394.96	
YOSH	23066.67	±	656.59	bA	23500.00	±	692.82	bA	23283.33	±	674.71	
0122	28700.00	±	300.00	aA	29133.33	±	437.16	aA	28916.67	±	368.58	
0920	23900.00	±	971.25	bA	24800.00	±	404.15	bA	24350.00	±	687.70	
TTOR	24133.33	±	762.31	bB	27866.67	±	185.59	aA	26000.00	±	473.95	
Average	25755.56	±	554.47		25416.67	±	512.06					

For K, P and Ca concentrations, variables in which the studied factors effect (tomato accessions vs $CdCl_2$ concentrations) presented a significant interaction ($p \leq 0.05$), distinct uppercase and lowercase letters denote different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solution, and for comparisons among accessions in the same growing condition, respectively. For Cu concentration, distinct letters denote different means by Tukey test ($P \leq 0.05$) for comparisons among accessions or growing conditions. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. Mean ± standard error.

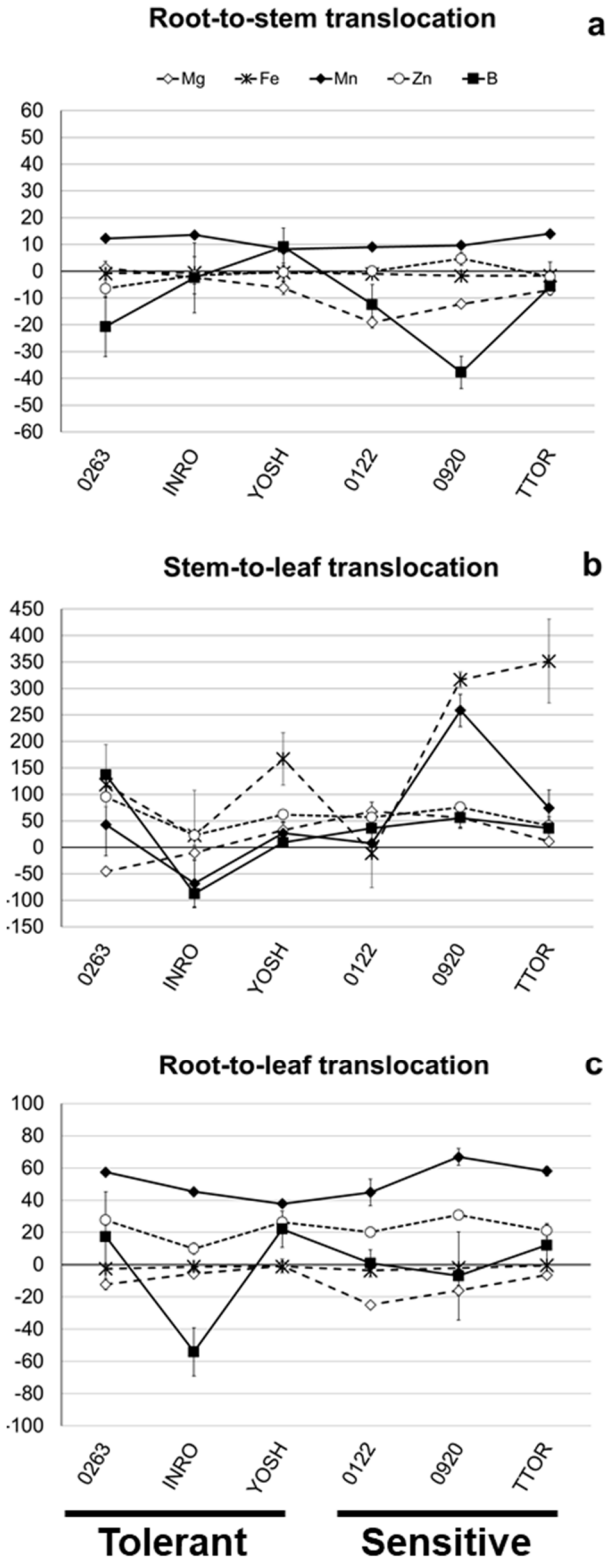

Fig. S1 Cadmium-induced changes (%) on the root-to-stem (**a**), stem-to-leaf (**b**) and root-to-leaf (**c**) translocation of magnesium (Mg), manganese (Mn), zinc (Zn), iron (Fe) and boron (B) in tolerant and sensitive tomato accessions that were grown in hydroponic solution with 35 μM $CdCl_2$ for six days. $n = 3$. *Solanum lycopersicum* accessions: 0263 – CNPH0263, INRO – Indigo Rose, YOSH – Yoshimatsu, 0920 – CNPH0920, TTOR – Tropic Two Orders, and *S. pimpinellifolium* accession: 0122 – LA0122. Bars represent the standard errors of the means.

4. NEW INSIGHTS ABOUT CADMIUM IMPACTS ON TOMATO: PLANT ACCLIMATION, NUTRITIONAL CHANGES, FRUIT QUALITY AND YIELD

ABSTRACT

Tomato is the second most consumed vegetable worldwide, but also a pathway for cadmium (Cd) entrance into the food chain. Since Cd concentrations in fruits depend on tomato genotype, this work aimed to study the relation between Cd accumulation, tolerance mechanisms, and fruit features in two tomato cultivars with contrasting tolerance to Cd stress. Tolerant (Yoshimatsu) and sensitive (Tropic Two Orders) plants were grown in control and contaminated soils from the seedling stage to the fruit production. Both cultivars were able to acclimatize to Cd exposure, probably through mechanisms associated to reductions in the magnesium (Mg) status. Cadmium concentrations varied according to the following descending order: roots = leaf blades > (peduncle + sepals) > stem = fruits. Although Cd reached the fruits from the first to the fourth bunches, peduncle and sepals may act as a barrier to Cd entrance in tomato pulp and peel. The Cd-induced changes in the fruit mineral profile varied according to plant cultivar, organ, tomato tissue and bunch position. Moreover, plant yield was not affected by the Cd stress, which was able to improve fruit size and weight in the tolerant cultivar. This work provided new insights about the impacts from long-term Cd exposure on tomato development, fruit quality and yield.

Keywords: Cadmium; Food security; Environmental contamination; Heavy metals; *Solanum lycopersicum*; Plant nutrition

4.1. Introduction

Tomato (*Solanum lycopersicum* L.) consumption increases every year due to the fruit attractiveness (many colors, shapes, sizes and flavors), multiple utilizations (from *in natura* consumption to processed sauces), and production of therapeutic compounds (Bergougnoux, 2014; FAOSTAT, 2016). However, tomato fruits are a potential pathway for cadmium (Cd) entrance into the food chain (Gratão et al., 2012; Hussain et al., 2015, 2017; Kumar et al., 2015), hence affecting human health by triggering infertility (Alaee et al., 2014), causing kidney and bone diseases, and increasing cancer risk (Järup and Åkesson, 2009; Nair et al., 2013). The threshold for Cd concentration in vegetables is set at 0.05 mg kg^{-1} (Commission of the European Communities, 2014), but tomato fruits can contain almost twice this limit (Hussain et al., 2015), even when plants are grown in soil with Cd concentrations accepted by the CETESB (CETESB, 2014).

In general, the amount of Cd translocated to the fruits is proportional to its concentration in the growth media (Gratão et al., 2012; Kumar et al., 2015; Hussain et al., 2017). The problem arose due to anthropogenic activities that strongly increased metal content in arable lands, augmenting Cd concentrations that range from 0.01 to 0.8 mg kg^{-1} in natural areas and up to 1500 mg kg^{-1} in contaminated areas (Kabata-Pendias, 2011). The environmental pollution occurs mainly near urban and industrial centers where a range of vegetables is commonly grown. The major source of

soil Cd is atmospheric deposition from metal smelters and phosphorous (P) fertilizers, and also a substantial amount is released through mining, metal-based pesticides, industrial waste, and battery production (Kabata-Pendias, 2011). Therefore, many countries implemented environmental legislations concerning Cd concentrations in edible portions of crops (Commission of the European Communities 2014), as well as in agricultural soils (CETESB, 2014) where plants uptake this metal.

In soil, most of the Cd (55 to 90%) is present as a free metal ion that is readily available to plants, being absorbed through roots and translocated to shoots after a short period of exposure (Kabata-Pendias, 2011; Gratão et al., 2015; Pompeu et al., 2017). Physicochemical characteristics of the soil such as pH, texture and organic matter contents affect Cd availability for plant absorption, which is particularly enhanced under acidic conditions (Castaldi and Melis, 2004; Kibria et al., 2006; Manciulea and Ramsey, 2006; Kabata-Pendias, 2011; Melo et al., 2011). Furthermore, soil microorganisms may influence Cd uptake as well as its effects in tomato plants (Madhaiyan et al., 2007; Dourado et al., 2013). In addition, similar to nutrients (Alvarenga, 2013), the uptake of non-essential elements may be enhanced in plants that were grown in hydroponic systems in comparison to soil. Therefore, studies about Cd translocation and accumulation in crops must be carried out in the growth media in which each species is usually cultivated.

Once within the plant, Cd triggers oxidative stress, disturbs nutrient uptake and distribution, decreases biomass production, avoids photoassimilate export and impairs yield (Gratão et al., 2005, 2012; Delpérée and Lutts, 2008; Gallego et al. 2012; Hédiji et al. 2015; Sebastian and Prasad 2016a, b; Bayçu et al. 2017a, b). Multiple studies have shown great damages in cell systems due to the overproduction of reactive oxygen and nitrogen species (the so-called ROS and NOS, respectively) as a consequence of plant exposure to heavy metals (Fidalgo et al., 2011, 2013; Gallego et al., 2012; Iannone et al., 2015; Cuypers et al., 2016; Alves et al., 2017). To a certain extent, plants can cope with the heavy metal-induced oxidative stress by employing enzymatic and non-enzymatic antioxidant machineries, which encompass the modulation of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) activities, among other enzymes, as well as the synthesis of amino acids, soluble sugars, glutathione and their derivates (Gallego et al., 2012; Jozefczak et al., 2012; Štolfa et al., 2015; Cuypers et al., 2016; Méndes et al., 2016).

Long-term exposure to Cd, however, generally impacts crop production by decreasing the weight and number of fruits, which is frequently coupled to reductions in the number of flowers and fruit setting rate (Hedĳi et al., 2010, 2015; Hussain et al., 2017). Moreover, Cd accumulation in fruits triggers stem-end yellowing in tomatoes (Kumar et al. 2015), causing visual damages that may reduce their commercial value. Interestingly, Cd accumulation and its effects on fruit quality and yield depend on tomato cultivars (Gratão et al., 2012; Hussain et al., 2015; Kumar et al., 2015), indicating a degree of tolerance/sensitivity to this metal. In this context, the use of tomato cultivars with contrasting sensitivity to Cd exposure can be a valuable tool to identify the relation between tolerance mechanisms, Cd accumulation and fruit quality and yield. For this purpose, the tolerant and sensitive tomato cultivars Yoshimatsu and Tropic Two Orders, respectively, were grown in soil rather than hydroponics, which is the most frequent system employed by researchers, in order to approach the reality of tomato cultivation and, consequently, to obtain information about the actual Cd concentration and its effects on plant development and fruit parameters after a long-term exposure to this toxic metal.

4.2. Materials and Methods

4.2.1. Plant material and growth conditions

Seeds of tomato *Solanum lycopersicum* cvs. Yoshimatsu (Cd-tolerant) and Tropic Two Orders (Cd-sensitive) were chemically scarified by stirring in 2% HCl (v:v) for 15 minutes in order to standardize germination. Subsequently, seeds were sown in polystyrene trays filled with thin exfoliated vermiculite, which were irrigated four times a day. After seedling emergence, daily application of macro- and micronutrients (Peters Professional 20-20-20 at 1 g L^{-1}) was initiated in order to maintain suitable seedling development. After one week, this concentration was increased to 1.5 g L^{-1}, which was used until the 29-days-old seedlings were transplanted to 20 dm^3 pots filled with control or Cd-containing soil (0.04 and 3.77 mg kg^{-1} Cd, respectively). Other chemical and physical properties of the soil are listed in Table S1.

In total, four treatments were tested, i.e. (i) tolerant cultivar in control soil, (ii) tolerant cultivar in Cd-contaminated soil, (iii) sensitive cultivar in control soil, and (iv) sensitive cultivar in Cd-contaminated soil. Fungicides, pesticides and fertilizers were applied to all plants, as recommended for tomato crop management. During the entire trial (since seed sowing), plants were cultivated in a greenhouse. From June to December 2015, plants were grown in control and contaminated soils (i.e., totalizing 131 days under Cd exposure) until the fruits of the four first bunches became mature, completely red. The monthly temperature and humidity were recorded, as provided by the meteorological station of Esalq/USP (Table S2).

4.2.2. Plant biometry and chlorophyll content

The plant height, from the root-stem transition region to the onset of the apical meristem, was evaluated with millimeter measuring tape in all replications, before the apex removal (i.e. apical pruning). In the end of the biological cycle, three replications of each treatment were used to determine the leaflet area from the seven youngest and fully expanded leaves, which were detached from the plants and measured in an area meter (LI-COR®, LI-3100). Samples of leaflets and stems were kept in paper bags and dried in an oven (65 ± 2 ºC) until constant weight for dry mass determination. The specific leaf area (leaflet area / leaflet dry weight) was also calculated. Chlorophyll content was indirectly evaluated using a Soil Plant Analysis Development (SPAD) chlorophyll meter (Konica Minolta, SPAD-502 model), through two measurements in the biggest terminal leaflets of the two youngest and fully expanded leaves in each experimental unit.

4.2.3. Production and quality parameters

The number of flowers and mature fruits, from the first to the third bunch, were recorded. Fruit diameter and height were evaluated by using a digital pachymeter, and the weight was determined through a digital scale. Subsequently, fruits were washed with water and gently dried with paper sheets. Only fruits from the first bunch were used for the determination of fruit firmness, pH, color, total soluble solids (SS), titratable acidity (TA), and SS/TA ratio, which indicates ripening and palatability (Araújo et al., 2016). The fruit firmness (N) was evaluated by using a

penetrometer with a 5 mm tip (Sammar 85261.0472 TR model) by two measurements in fruit's opposite sides, in which the peel was removed. For the determination of fruit external color [L* (luminosity), C* (saturation), a*, b* and h (tonality angles)], two assessments in fruit's opposite sides were performed by using the colorimeter Minolta CR-300 (Minolta 2017).

The pulps of two fruits (without peel and after removal of the placenta with seeds) were squeezed with gauze to obtain tomato juice that was used to estimate the SS through a digital refractometer (Atago PR-101, Palette). Two measurements per replication were performed in order to obtain the mean value, which was expressed as ºBrix. The pH of the fruit juice was measured with a digital pH meter (Mettler Toledo, Seven Easy model) upon dilution of 5 g tomato juice into 45 mL distilled-deionized water. Next, the potentiometric titration was evaluated by adding 0.1 N NaOH to reach pH 8.1. The percentage of citric acid was calculated based on the NaOH volume by using the following formula (Carvalho et al., 1990):

$$\%\ citric\ acid = \frac{64 \times NaOH \times N}{ws \times 10}$$

Where:
NaOH = volume of NaOH (mL);
N = normality of NaOH;
ws = weight of juice sample.

4.2.4. Quantification of Cd and nutrient concentration

Samples were dried in an oven at 60 ºC and subsequently grounded using mortar and pestle. Calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P), sulfur (S), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), boron (B) and Cd concentrations were evaluated through ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) analysis, which was preceded by nitro-perchloric digestion of the grounded samples. Three replications for each treatment were subjected to the analytical procedures carried out by the Soil Fertility Laboratory at Instituto Agronômico de Campinas (IAC, Brazil).

4.2.5. Statistical procedures

The experiment was carried out in a completely randomized design with a factorial scheme 4 x 4 (treatments x organs) to analyze the Cd-induced effects on the mineral profile of roots, stems, leaf blades and floral receptacle. The repeated measurement analysis was employed to assess the effect of treatments on plant height, stem diameter and chlorophyll content throughout the time. The split-plot analysis was used to evaluate the effect of treatments (plots) on size and weight of fruits from different bunches (sub-plots). For production parameters and fruit physicochemical attributes, a one-way analysis of variance (ANOVA) was performed ($p \leq 0.05$). The Tukey test was used to estimate the least significant range among means of treatments ($\alpha \leq 0.05$) for all variables, and a regression analysis ($p \leq 0.05$) was performed to evaluate the effect of treatments during the time. Before ANOVA, data were subjected to tests through the "Guided Data Analysis" tool of the statistical software SAS (SAS Institute, 2011), in

order to check whether they were in accordance to the assumptions for the ANOVA performance (i.e. normal distribution, variance homogeneity and error independence). Moreover, data transformations were performed when indicated by this tool.

4.3. Results

4.3.1. Plant development

Two tomato cultivars with a contrasting tolerance degree to Cd toxicity, Yoshimatsu (tolerant) and Tropic Two Orders (sensitive), were grown in soil containing 0.04 (control) and 3.77 mg kg^{-1} Cd (contaminated). After 39 days of exposure to this metal, the tolerant cultivar exhibited a lower height than control plants, but this effect disappeared in advanced stages of development (Fig. 1). The sensitive cultivar did not show significant differences in plant height (Fig. 1).

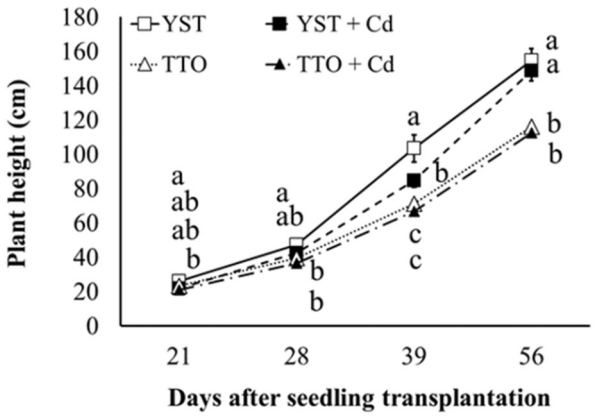

Figure 1. Plant height of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 10$. Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons among treatments within the same time of plant transplantation. Bars represent the standard errors of the means

The leaf area and dry weight were generally decreased in Cd-challenged plants, when compared to the control plants (Fig. 2a-b). Only the sensitive cultivar presented significant reductions in the stem dry weight after exposure to Cd (Fig. 2d). The specific leaf area (Fig. 2c) and stem diameter (Fig. S1) were not influenced by Cd, regardless of the tomato cultivar.

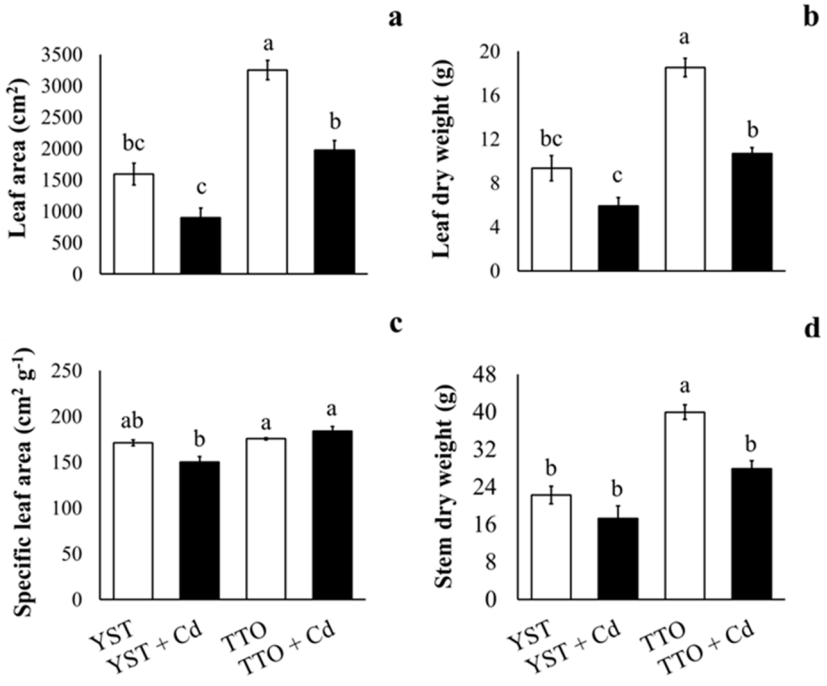

Figure 2. Leaf area (**a**) and dry weight (**b**), specific leaf area (**c**) and stem dry weight (**d**) of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control (white columns) and contaminated (black columns, + Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). n = 3. Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$). Bars represent the standard errors of the means

The chlorophyll content increased through plant development in both Cd-treated and control plants (Table S3). The long-term Cd exposure did not affect the chlorophyll content in tolerant and sensitive cultivars, when compared to the plants grown in control soil (Table S3).

4.3.2. Cd accumulation

Tomato cultivars exhibited Cd concentrations in the following descending order: leaf blades = roots > (peduncle + floral receptacle + sepals) > stem = peel and pulp of fruits from the first bunch (Fig. 3a-c). When the influence of fruit bunch position is concerned, there is a general decreasing trend in Cd accumulation in tomato pulp (Fig. 3b) and peel (Fig. 3c) concurrently to the advanced bunch position. Furthermore, the tolerant cultivar generally showed an increasing trend of Cd accumulation with respect to the sensitive cultivar, regardless of plant organ or tissue (Fig. 3a-c). This difference was significant in roots (Fig. 3a), as well as in fruits from the second (pulp and peel) and fourth bunches (pulp) (Fig. 3b, c).

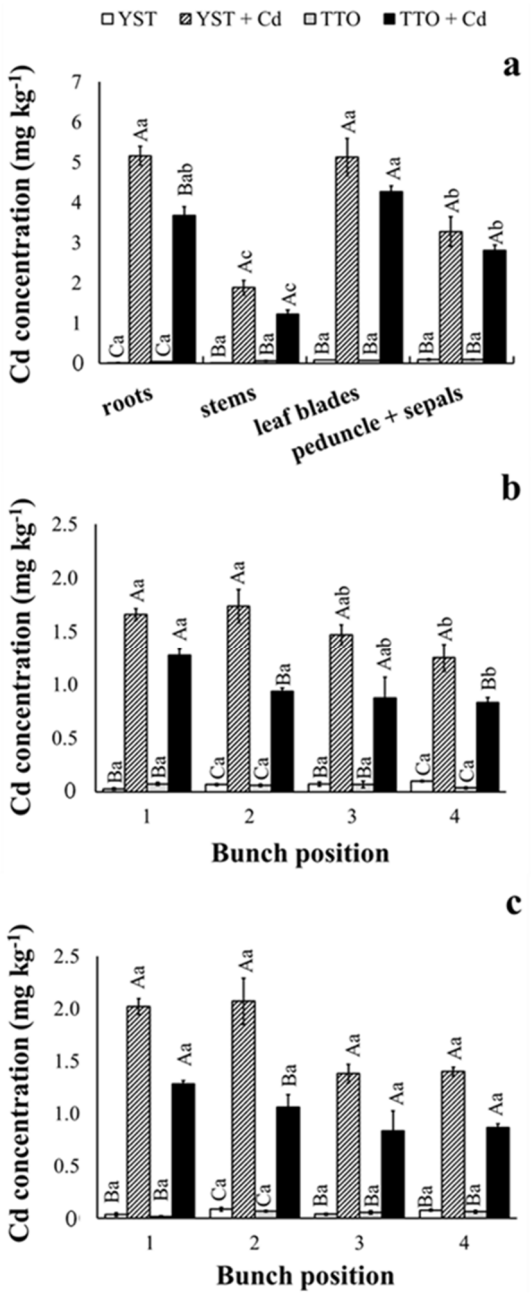

Figure 3. Cadmium (Cd) concentration in roots, stem, leaf blades, peduncle and sepals (**a**), as well as in fruit pulp (**b**) and peel (**c**) of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 3$. Distinct lowercase and uppercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of the same treatment in different organs/tissues, and for comparisons of all treatments inside each organ/tissues, respectively. Bars represent the standard errors of the means

4.3.3. Mineral profile

After exposure to Cd, both tolerant and sensitive tomato cultivars presented reductions in their root Mg concentration in comparison to plants that were grown in control soil (Fig. 4a). Also in roots, S, Cu, Zn, Mn and Fe concentrations showed a decreasing trend in Cd-challenged plants when compared to the control ones, regardless of the cultivar (Fig. 4c, Fig. 5a-c).

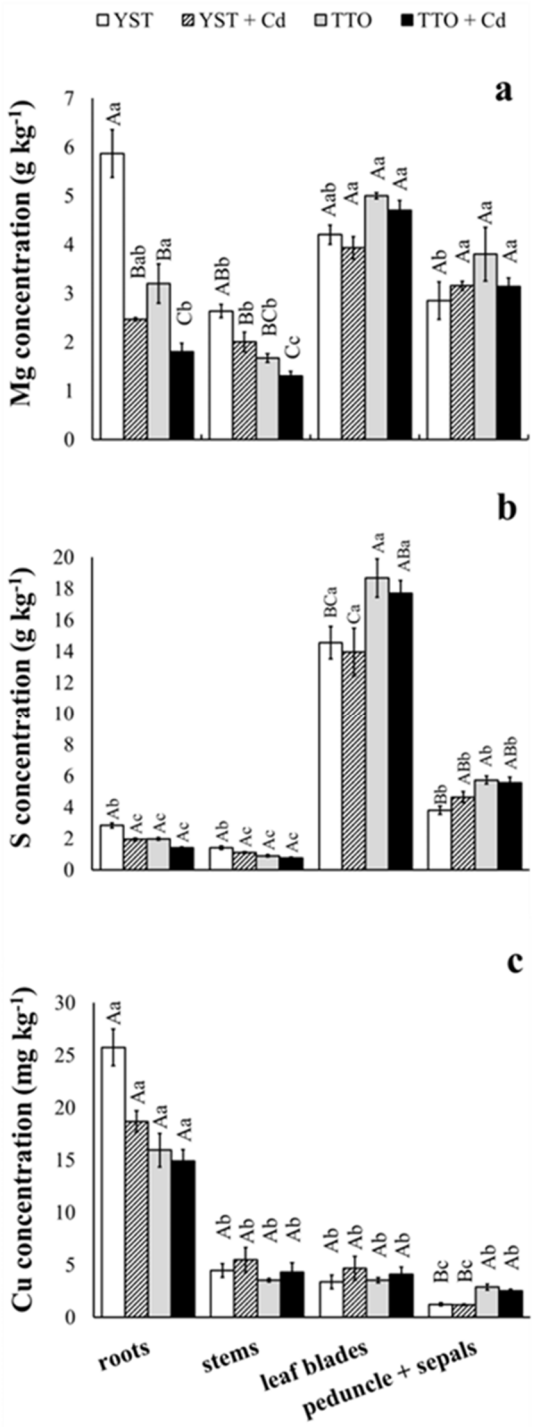

Figure 4. Magnesium – Mg (**a**), sulfur – S (**b**), and copper – Cu (**c**) concentration in different organs/tissues of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 3$. Distinct lowercase and uppercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of the same treatment in different organs/tissues, and for comparisons of all treatments inside each organ/treatment, respectively. Bars represent the standard errors of the means

However, the root B concentration was increased in the sensitive tomato cultivar after Cd exposure (Fig. 5d). For the others nutrients (N, P, K and Ca), Cd caused no significant differences between Cd-challenged and control plants (Tables S4- S5).

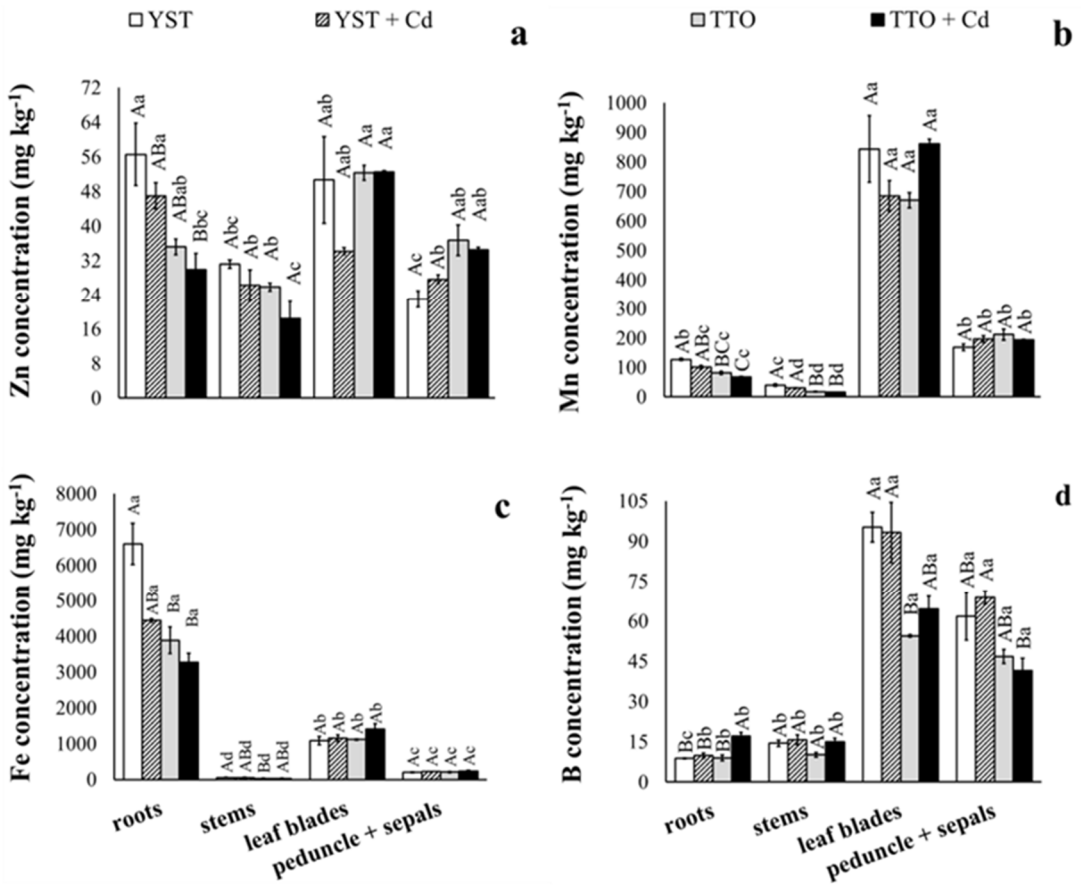

Figure 5. Zinc – Zn (**a**), manganese – Mn (**b**), iron – Fe (**c**) and boron – B (**d**) concentrations in different organs/tissues of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 3$. Distinct lowercase and uppercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of the same treatment in different organs/tissues, and for comparisons of all treatments inside each organ/tissue, respectively. Bars represent the standard errors of the means

In general, nutrient concentrations in fruits decreased in the youngest bunches when compared to the old ones (Fig. 6-10). However, depending on nutrient, fruit part, and genotype, the mineral profile of the fruits was also affected by Cd (Fig. 6-10). In fruits from the second bunch, the pulp P concentration was reduced in both Cd-treated cultivars (Fig. 6a). In contrast, Cd exposure increased the K concentration in the pulp of fruits from the second and third bunches in the tolerant cultivar (Fig. 6c). However, the peel K and P concentrations were not affected by Cd exposure (Fig. 6b, d).

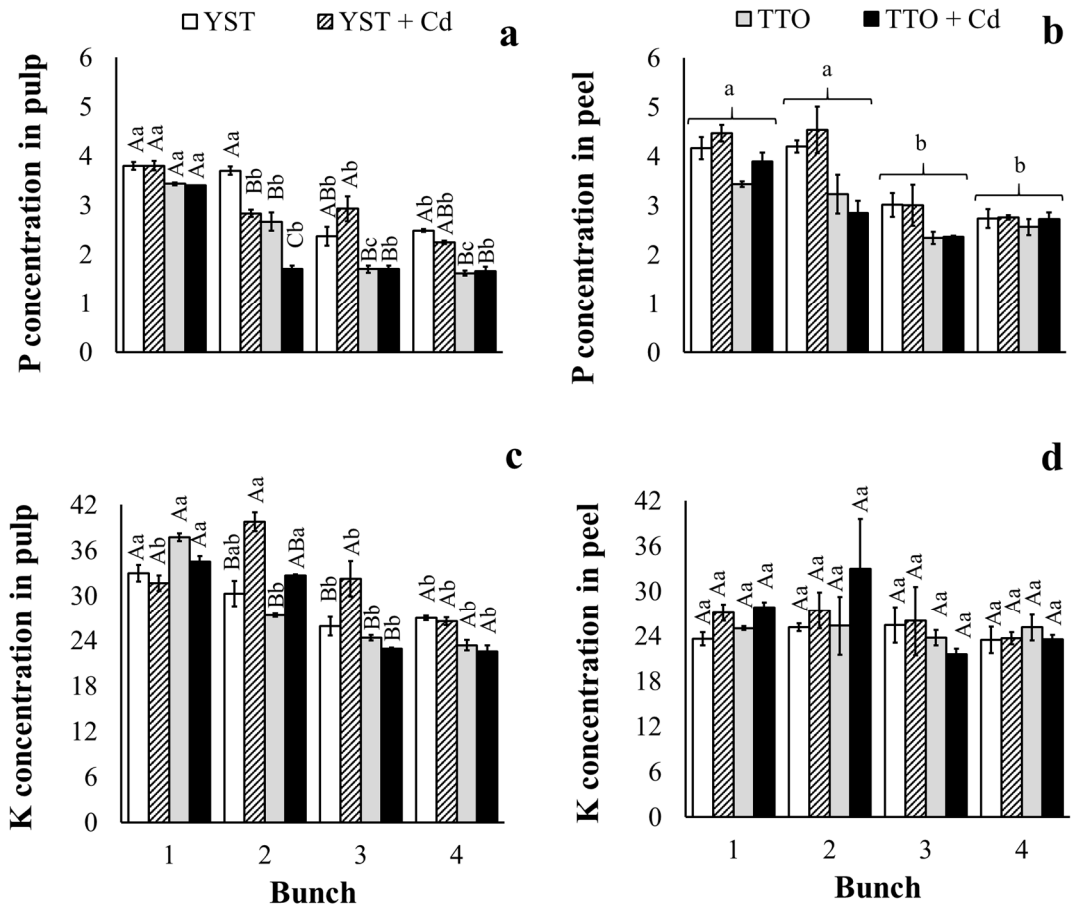

Figure 6. Phosphorus – P (**a**, **b**) and potassium – K (**c**, **d**) concentrations in pulp and peel of fruits from different bunches in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 3$. Distinct lowercase and uppercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of the same treatment in different organs/tissues, and for comparisons of all treatments inside each organ/tissue, respectively. Plant exposure to Cd and bunch position exerted no significant changes on K concentration in tomato peel ($p > 0.05$). Bars represent the standard errors of the means

Only the peel Ca concentration was strongly decreased in fruits from the first bunch in the tolerant cultivar after exposure to Cd (Fig. 7b). When the S concentration in tomato pulp and peel was examined, a general decreasing trend occured concurrently with the advanced bunch position, regardless of Cd exposure (Fig. 7c, d).

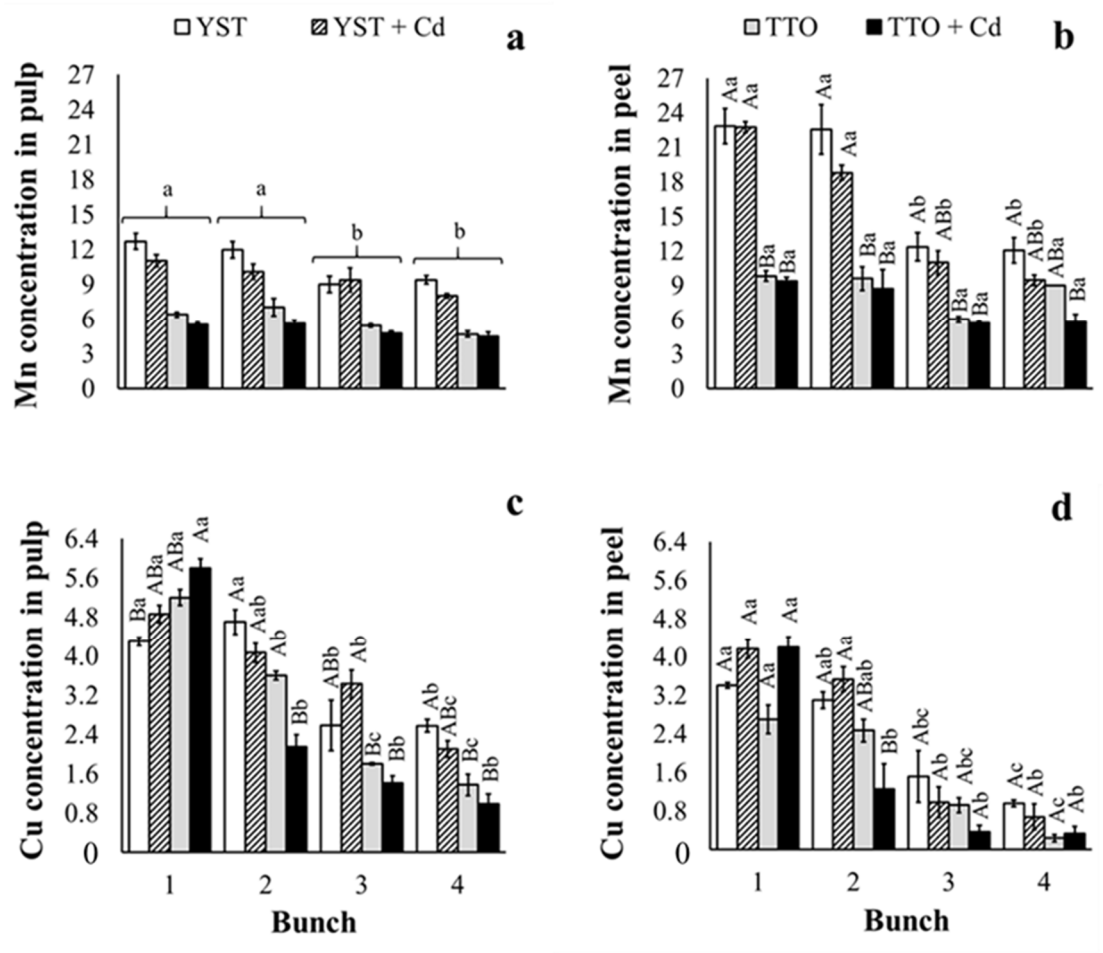

Figure 7. Calcium – Ca (**a**, **b**) and sulfur – S (**c**, **d**) concentrations in pulp and peel of fruits from different bunches in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 3$. Distinct lowercase and uppercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of the same treatment in different organs/tissues, and for comparisons of all treatments inside each organ/tissue, respectively. Bars represent the standard errors of the means

The Mg concentration in fruit pulp and peel was generally higher in the tolerant than the sensitive cultivar (Fig. 8a, b). Moreover, fruits from the first and second bunches contained higher Mg concentrations than those from the third and fourth bunches (Fig. 8a, b). The Fe concentration in tomato pulp and peel was lower in young than in old bunches (Fig. 8c, d).

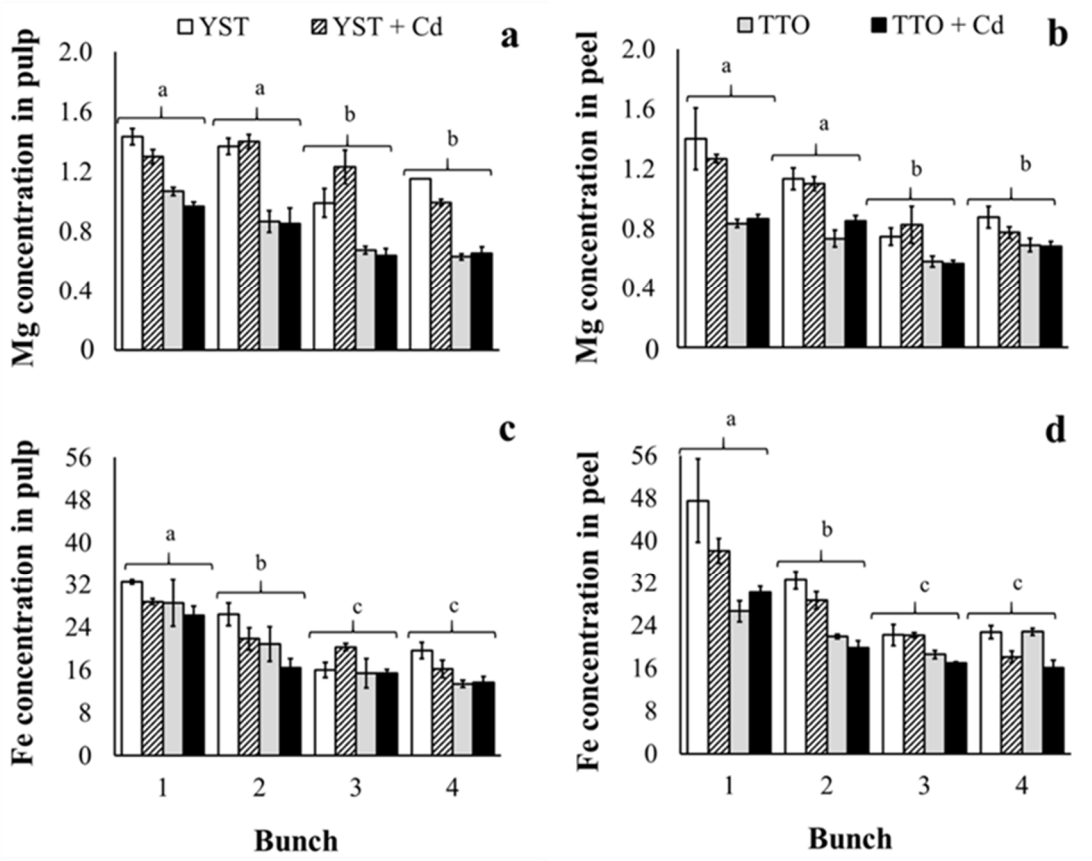

Figure 8. Magnesium – Mg (**a**, **b**) and iron – Fe (**c**, **d**) concentrations in pulp and peel of fruits from different bunches in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 3$. Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of the same treatment in different organs/tissues, and for comparisons of all treatments inside each organ/tissue, respectively. Bars represent the standard errors of the means

The Mn concentration in tomato pulp was higher in fruits from the tolerant than the sensitive cultivar, moreover, fruits from the oldest bunches (i.e. 1st and 2nd) accumulated more Mn than the youngest bunches (3rd and 4th, Fig. 9a). In fruit peel of the sensitive cultivar, Mn concentrations were maintained in distinct bunches, whereas the tolerant cultivar produced fruits with decreased Mn concentrations in advanced bunch position (Fig. 9b). Plant exposure to Cd provoked an increasing trend in the Cu concentrations in pulp and peel of fruits from the first bunch (Fig. 9c, d). In contrast, Cd caused significant reductions in the Cu concentration in the pulp of fruits from the second bunch in the sensitive cultivar (Fig. 9c).

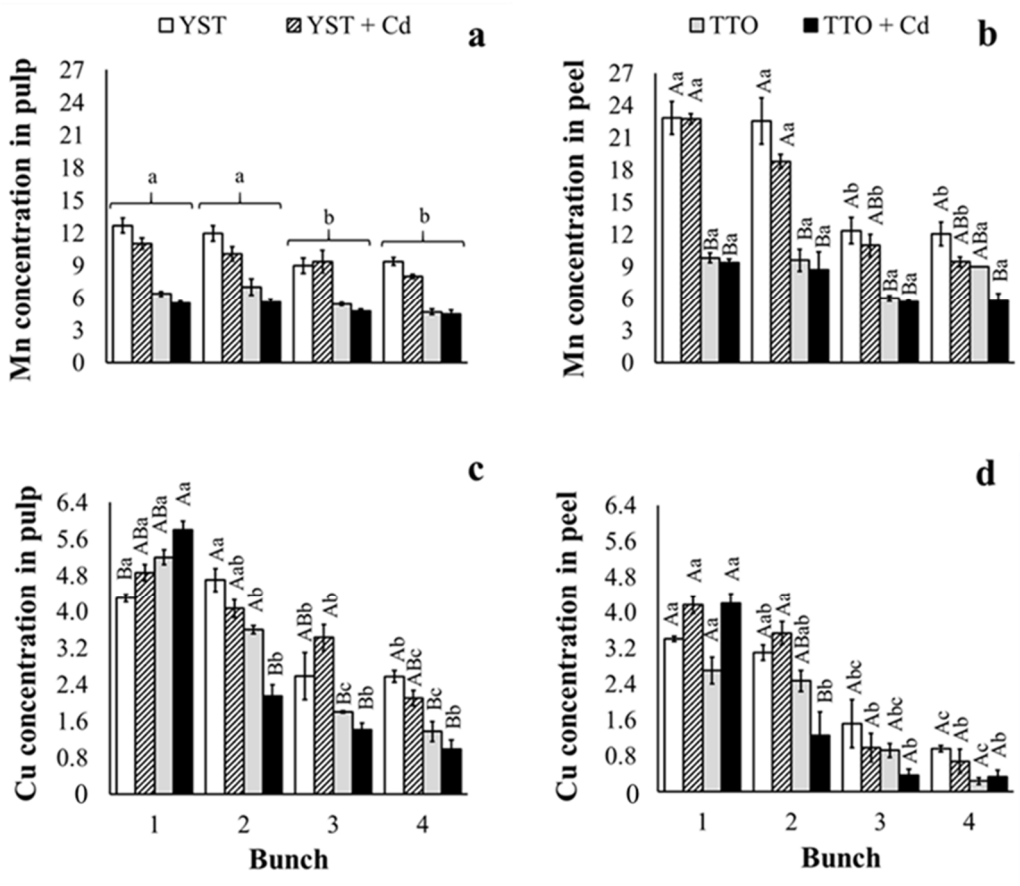

Figure 9. Manganese - Mn (**a**, **b**) and copper – Cu (**c**, **d**) concentrations in pulp and peel of fruits from different bunches in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 3$. Distinct lowercase and uppercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of the same treatment in different organs/tissues, and for comparisons of all treatments inside each organ/tissue, respectively. Bars represent the standard errors of the means

In plants under Cd exposure, the Zn concentration was reduced in the tomato pulp of tolerant and sensitive accessions, especially in fruits from the first and second bunches, but these variations were not enough to cause significant differences between Cd-treated and control plants (Fig. 10a, b). When B concentration in fruit pulp and peel is concerned, reductions were observed with advanced bunch position (Fig. 10c, d). However, in certain bunches, Cd enhanced this reduction as observed in the pulp of fruits from the third bunch in the sensitive cultivar (Fig. 10c), as well as in the peel of tomato fruits that were produced in the first bunch of the tolerant cultivar (Fig. 10d).

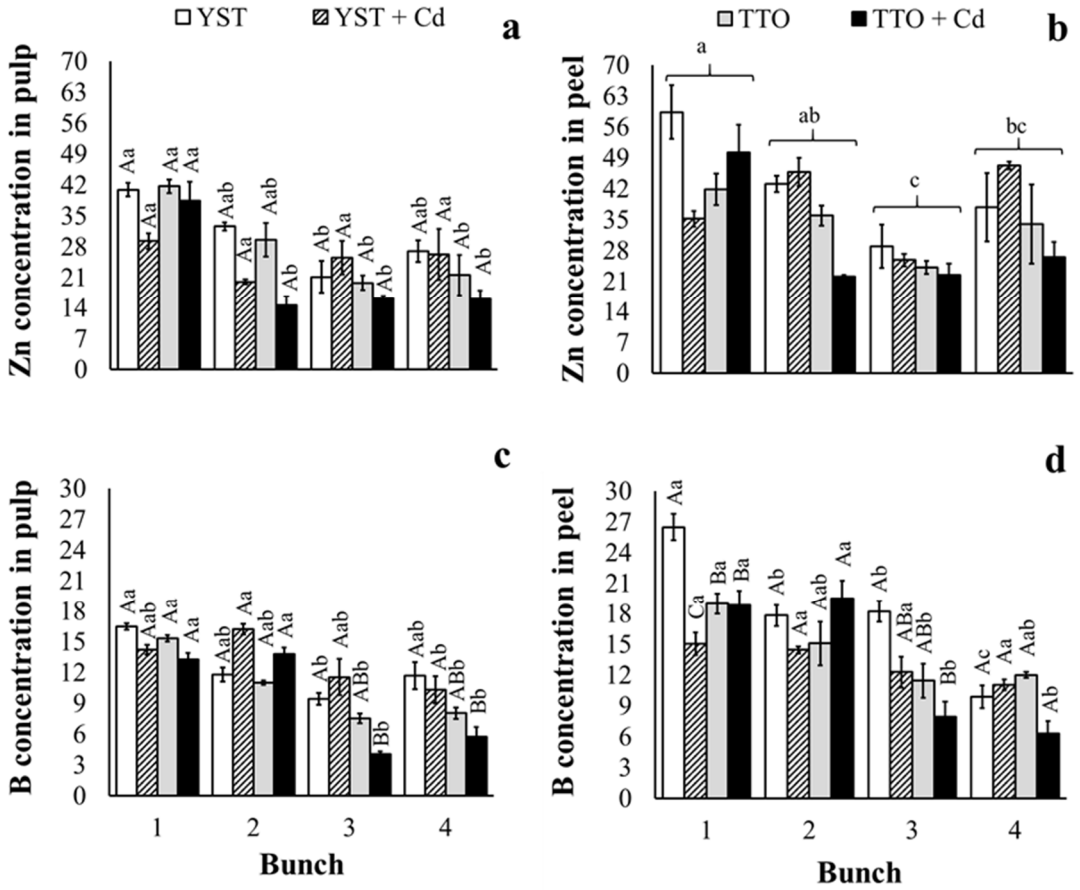

Figure 10. Zinc – Zn (**a**, **b**) and boron – B (**c**, **d**) concentrations in pulp and peel of fruits from different bunches in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 3$. Distinct lowercase and uppercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of the same treatment in different organs/tissues, and for comparisons of all treatments inside each organ/tissue, respectively. Bars represent the standard errors of the means

4.3.4. Production parameters

The number of flowers was not affected by either plant exposure to Cd nor bunch position, but the tolerant cultivar possessed generally more flowers than the sensitive one (Table S6). Although the fruit setting was decreased with the advanced bunch position, there were no significant changes between Cd-treated and control plants, regardless of the cultivar (Table S6). The number of fruits did show reductions in the youngest bunches when compared to the old ones, independent of genotype and Cd exposure (Fig. 11a). The fruit weight of the sensitive cultivar was naturally decreased in the youngest bunches, when compared to the old ones, and plant exposure to Cd was not enough to provide differences between control and Cd-treated plants (Fig. 11b). However, tolerant cultivars exhibited a trend of increasing fruit weight in plants under Cd exposure in comparison to control plants, being significantly higher in the youngest bunch (Fig. 11b).

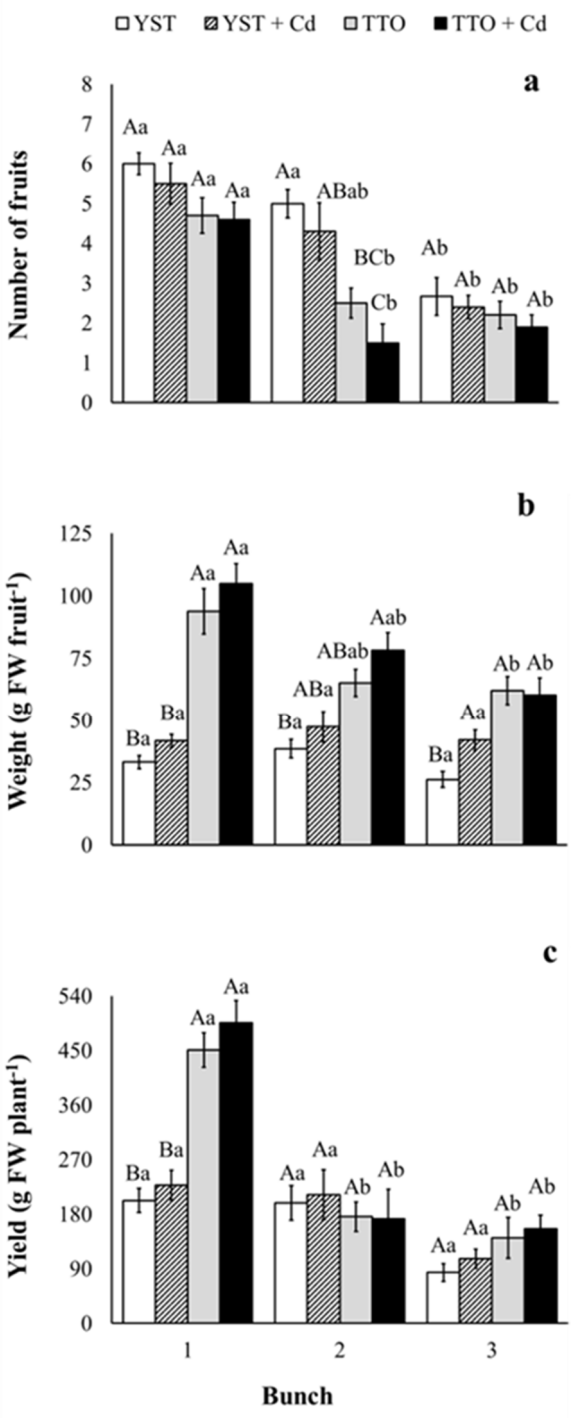

Figure 11. Number of fruits (**a**), fruit weight (**b**) and yield (**c**) from the first to the third bunch in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated (+Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 10$. Distinct lowercase and uppercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons the same treatment in different bunches and all treatments inside each bunch, respectively. Bars represent the standard errors of the means

Moreover, increases in fruit diameter and height were observed in the tolerant plants after cultivation in Cd-containing soil (Table 1). The sensitive tomato did not show differences in fruit dimensions due to Cd exposure (Table 1). Finally, plant yield of both sensitive and tolerant cultivars was not significantly affected by exposure to Cd (Fig. 11c).

Table 1. Diameter and height of fruits, from the 1st to the 3rd bunches of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated (+Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively)

Bunch	Treatments								Average	
	YST		YST + Cd			TTO		TTO + Cd		
					Diameter (mm)					
1	40.46 (1.27)		42.07 (1.19)			58.05 (2.04)		60.80 (1.63)	50.35 (1.53)	a
2	40.67 (1.41)		45.70 (1.91)			52.17 (1.59)		57.97 (2.36)	49.13 (1.82)	a b
3	37.24 (1.29)		44.36 (1.45)			51.81 (1.80)		54.35 (2.17)	46.94 (1.68)	b
Average	39.46 (1.33)	c	44.04 (1.52)	b		54.01 (1.81)	a	57.71 (2.06)	a	
					Height (mm)					
1	34.36 (0.96)		36.85 (0.97)			48.40 (1.83)		50.23 (1.15)	42.46 (1.23)	a
2	35.31 (1.24)		38.39 (1.65)			42.49 (1.35)		46.15 (1.88)	40.59 (1.53)	a b
3	30.76 (1.32)		37.72 (1.58)			42.74 (1.11)		43.88 (1.85)	38.78 (1.47)	b
Average	33.48 (1.18)	c	37.65 (1.40)	b		44.54 (1.43)	a	46.75 (1.63)	a	

Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons among treatments or bunch position. Values inside parentheses are the standard errors of the means. $n = 3$

4.3.5. Fruit physicochemical attributes

After plant exposure to Cd, tomato firmness presented slight increases in fruits from the tolerant cultivar, Yoshimatsu, but it was not enough to provoke significant differences between Cd-treated and control plants (Fig. S2a). Furthermore, plant cultivation in Cd-containing soil did not affect the total soluble solid content (TSS), which was similar in Yoshimatsu and Tropic Two Orders (Fig. S2b). Juice titratable acidity (TA), pH (Fig. S2c, d) and TSS/TA ratio (Table S7) depended on tomato cultivar, and none of these variables were influenced by the long-term exposure to Cd. The juice pH (Fig. S2d) and TSS/TA ratio (Table S7) were lower in the tolerant than in the sensitive cultivar. By contrast, TA was higher in tomato cv. Yoshimatsu than tomato cv. Tropic Two Orders (Fig. S2c), regardless of the presence of Cd in soil. When color parameters of the fruits are examined, no significant differences between Cd-treated and control plants occurred (Fig. S3a, b, Table S7). However, the color tonality, h, was higher in the sensitive tomato cultivar when compared to the tolerant cultivar (Fig. S3c).

4.4. Discussion

Yoshimatsu (tolerant) and Tropic Two Orders (sensitive) are two tomato cultivars with contrasting tolerance degrees to Cd exposure that were identified in previous studies, in which plants were subjected to a short period of Cd-induced stress using hydroponic systems with solutions containing 35 μM $CdCl_2$ (preliminary data). In the current work, both cultivars were grown from seedling stage (29-days-old) to fruit production in soil containing 0.04 (control) and 3.77 mg kg^{-1} Cd (contaminated). The latter is a concentration similar to that allowed for arable lands, i.e. 3.6 mg kg^{-1} Cd (CETESB, 2014). The study was performed in order to answer the following questions: (i) What are the effects of long-term Cd exposure on plant development and fruit features? (ii) How much Cd is translocated to the tomato fruits in plants that were grown in Cd-containing soil? (iii) Can tolerance mechanisms be associated to advantageous fruit attributes in commercial tomato cultivars under Cd exposure?

4.4.1. Low Mg concentration associated to plant acclimatization to long-term Cd exposure

The continuous plant development in Cd-containing soil validates previous reports that tomato is able to acclimate to long-term Cd exposure, reaching sexual maturity (Gratão et al., 2012; Hédiji et al., 2015; Hussain et al., 2017) and producing fruits (Fig. 11a) despite some impacts on plant growth (Figs. 1, 2, Fig. S1, Tables S3, S6). Although the mechanism behind this plant ability is poorly understood, data from the current study suggest a relation with reduced Mg concentration in roots, the only macronutrient that was altered between Cd-stressed and control plants' vegetative organs (Fig. 4a). This hypothesis is supported by previous studies in which the low Mg status was coupled to enhanced antioxidant potential in rice (Chou et al., 2011), and protective effects in *Arabidopsis* leaves (Hermans et al., 2011). In addition, the protective role of this Mg management was also important for reductions in the root-to-shoot Cd translocation in barley (Kudo et al., 2015), as well as against Pb toxicity in *Torreya grandis* seedlings (Shen et al., 2016), and Al injuries in wheat (Moustaka et al., 2016). Preliminary data associated Mg-driven tolerance mechanisms to the maintenance of root integrity in tomatoes under short-term Cd exposure (6 days), but the plant capacity to reduce Mg status was only observed in tolerant tomato accessions. However, the present study reveals that both tolerant and sensitive cultivars possess this ability (Fig. 4a), indicating that mechanisms coupled to a suitable management of the Mg status in Cd-challenged tomato plants may be activated earlier or faster in tolerant than in sensitive cultivars.

Another interesting point is that the tolerant cultivar showed stronger symptoms of Cd-induced phytotoxicity when compared to the sensitive cultivar, including decreased plant height in certain developmental stages (Fig. 1), large reductions in the fruit set –18.3% on average (Table S6) and clear visual changes in the leaf shape (data not shown), which support trends of modifications in the specific leaf area (Fig. 2c). However, at the same time, the tolerant cultivar produced bigger and heavier fruits in Cd-treated than in control plants (Fig. 1, Table 1), indicating that this cultivar is able to change photoassimilate distribution to favor fruit development during Cd-induced stress. It is not known whether such changes are a direct effect from the increased Cd accumulation (Fig. 3a-c) or a cultivar-specific ability to change plant features to cope with Cd-induced stress. Anyways, the immediate plasticity or capacity for a rapid adaptive response of the tolerant cultivar could be traits important for its survival and even its offspring under non-optimal environmental conditions. From the ecological point of view, improvement of fruit features might help tomato progeny fitness by supporting additional storage compounds (heavier fruits) or even to enhance tomato dispersion through increases in the fruit attractiveness (bigger fruits). According to Mueller et al. (2017), such fast responses were previously used by farmers to select the best accessions of other plant species (for example, *Polygonum erectum* L. – a seed crop used during the pre-maize agricultural systems) and, in a similar way, these features can be further explored by breeders to choose tomato accessions with superior adaptability in soils contaminated with Cd.

4.4.2. Cd changes the mineral homeostasis through modifications in the nutrient uptake and distribution

In contrast to the accumulation of Cd in plants (Fig. 1a), the concentration of several nutrients was decreased in tomato roots (Fig. 4, 5), indicating that Cd prevents their uptake. This antagonist effect, which was also reported in other tomato cultivars (Dong et al., 2006; López-Milan et al., 2009; Hédiji et al., 2015), is probably triggered

by the Cd-induced alterations in the activity of plasma membrane transporters (Migocka and Klobus, 2007), as well as by the fact that Cd uses the same transporters of certain essential elements (Korshunova et al., 1999; Thomine et al., 2000). The last assumption is especially consistent for Mn transporters that are enrolled in Cd absorption and translocation in several species (Thomine et al., 2000; Sasaki et al., 2012; Wu et al., 2016), indicating that Cd uptake occurs at the expense of Mn absorption. Modifications in the mineral profile (Figs. 4, 5, Table S4) can be coupled to changes in the plant development (Fig. 1, 2), and fruit setting (Table S6). In this context, reductions in Mn and Fe uptake (Fig. 5b, c) might enhance the decreases in Mn and Fe concentrations in tomato fruits, especially in the peel of those from the fourth bunch (Fig. 8d, Fig. 9b).

In contrast with previous findings that showed an increased S uptake in plants under short-term Cd exposure – from 10 to 14 days (Hasan et al., 2016; Yamaguchi et al., 2016), the S concentration was maintained in roots of both tomato cultivars (Fig. 4b). Such studies related the high S status to an increased production of glutathione and/or phytochelatins as a protective mechanism to cope with Cd-induced stress (Hasan et al., 2016; Yamaguchi et al., 2016). However, in the end of the biological cycle, S is probably remobilized from the vegetative organs to fruits to support their formation and/or development (Fig. 4b, Fig. 7c, d). Moreover, Cd exposure enhances B uptake in the sensitive cultivar, and such an event may be coupled to the increased B concentration in leaves (Fig. 5d). Preliminary data on short-term Cd exposure showed a strong correlation between Cd-induced excess of the leaf B concentration and tomato sensitivity to Cd toxicity, based on increased symptoms of chlorosis, necrosis and biomass losses. Despite reductions in the leaf and stem dry weight (Fig. 2b, d), the sensitive cultivar did not show changes in the chlorophyll content (Table S3), plant height (Fig. 1), stem diameter (Fig. S1), fruit size, weight (Table 1) or yield (Fig. 11a-c). This might be due to the lower magnitude of B accumulation in soil-cultivated plants when compared to hydroponic-cultivated plants, and/or the effects from the changes in the nutrient homeostasis might be more counter-balanced after long-term exposure when compared to short-term exposure.

4.4.3. Floral receptacle and its related-structures act as a barrier to Cd translocation to fruits

The current data concerning Cd accumulation in vegetative organs (Fig. 3a) are not in line with previous work, which showed that roots always possess a higher Cd concentration than leaves (López-Milan et al., 2009; Monteiro et al., 2011; Hédiji et al., 2015; Kumar et al., 2015; Alves et al., 2017). Four main hypotheses that do not exclude each other support this result: (i) The high transpiration rate of leaflets from the selected leaves (youngest and fully expanded leaves) provided the increased Cd accumulation, probably because they were one of the main organs for gas exchange at the end of the tomato biological cycle; (ii) Changes in Cd distribution and remobilization during the reproductive stage provoked Cd accumulation in the leaflets; (iii) The use of leaflets, rather than the complete tomato leaves, may overestimate Cd concentrations due to the exclusion of rachis that, as an extension of stems, may have a low Cd accumulation; (iv) A "dilution effect" on the root Cd concentrations might have occurred due to both an increased root development in adult plants and a reduced Cd uptake in soil-cultivated plants, when compared to the hydroponic-cultivated ones.

Cadmium also reached the peel and pulp of fruits from different bunch positions (Fig. 3b, c). Interestingly, data suggest that both cultivars presented a mechanism to limit Cd translocation to the fruits by depositing this metal in the floral receptacle and its related structures, i.e. peduncle and sepals (Fig. 3a-c). This mechanism may explain why

fruits, during distinct development stages, contained a lower Cd concentration than flowers, as observed previously by Hédiji et al. (2010, 2015). From the ecological point of view, this mechanism may protect tomato progenies from the potential side-effects of increased Cd accumulation in fruits and even seeds. However, this mechanism may cause fruit set reductions, which were accordingly higher in the tolerant than sensitive cultivar (Table S6), and even trigger fruit abortion (Hédiji et al., 2010, 2015). Yet, Cd concentration in fruits exceeded at least 300% the amount allowed in vegetables (Fig. 3b, c) (Commission of the European Communities, 2014). Therefore, agricultural and health organizations should run field experiments in order to check the actual Cd concentration in crops that are grown in contaminated soils with certain Cd concentrations that are allowed for arable lands. In such experiments, the influence of soil heterogeneity, texture, organic matter content, chemical and biological composition as well as plant species with contrasting root morphology must be considered since all these factors may affect Cd availability, mobility, absorption and/or accumulation in plants (Castaldi and Melis, 2004; Kibria et al., 2006; Manciulea and Ramsey, 2006; Kabata-Pendias, 2011; Nogueirol et al., 2016; Hirzel et al., 2017).

4.4.4. Consequences from the cultivar-dependent Cd accumulation in fruits on tomato features

The present study proved that Cd can enter the food chain by accumulating in tomato fruits from plants that were grown in Cd-containing soil (Fig. 1a-c). However, Cd accumulation can be potentially decreased by using cultivars with a reduced ability to translocate Cd to the fruits (Hussain et al. 2015), as well as by selecting fruits from the youngest bunches (Fig. 1b, c). There are evidences that reductions in Cd concentration may occur due to a "dilution effect", since fruits from the youngest bunches also have the highest size (Table 1) and weight (Fig. 11b). Moreover, fruits from different bunch positions were not suitable for human consumption. Another general problem induced by Cd is yield losses (Hédiji et al., 2015; Hussain et al., 2015; Kumar et al., 2015), but Cd exposure did not affect tomato yield in the current study (Fig. 11c). In the tolerant cultivar, Cd-stressed plants even showed up to 12.7% increased yield due to increments in fruit weight (Fig. 11) as a result of increased fruit size, especially from the youngest bunches (Table 1). According to Kumar et al. (2015), the presence of Cd in the growth media can increase the percentage of dry mass in fruits and, at the same time, reduce their water status, hence providing variations in fruit firmness, which was slightly increased in the tolerant cultivar (Fig. S2a). However, there were no significant changes between Cd-stressed and control plants, probably because fruits from the first bunch, in which Cd exerted diminished effects on their dimensions (Table 1), were used in the analyses. Other commercial attributes of tomato fruits, such as color, SS content, TA and pH, were not influenced by Cd exposure independently of plant cultivar (Fig. S2b-d, Fig. S3a-d, and Table S7).

When compared to other studies, the low impact of long-term Cd exposure on fruit quality and yield can be associated with the use of different cultivation systems (hydroponics or its variations vs soil). The direct implication is the overestimation of Cd accumulation because the uptake of essential and non-essential elements is generally enhanced in hydroponics when compared to soil (Alvarenga 2013). Accordingly, Cd concentration was 520 to 1969% higher in vegetative organs of plants that were grown in hydroponics with 20 μM $CdCl_2$ for 90 days (Hédiji et al., 2010), when compared to the present study in which plants were grown in soil with 3.77 mg kg^{-1} Cd (33.5 μM $CdCl_2$) for 131 days (Fig. 3a). Moreover, grafted plants that were cultivated in vessels filled with sand that received drip irrigation with 25 μM CdCl2, exhibited Cd concentrations that ranged from 525.1 to 119.5 mg kg^{-1} in roots and leaves, respectively

(Kumar et al. 2015). Similarly, fruits from hydroponic-cultivated plants presented an increased Cd accumulation that ranged from 5 to 14 mg kg^{-1} (Hédiji et al., 2010, 2015) after plant cultivation in hydroponics with 20 μM $CdCl_2$, whereas tomato fruits from the current study using contaminated soil contained only 0.83 to 2.07 mg kg^{-1} Cd (Fig. 3b, c). Therefore, Cd uptake and accumulation in tomato's vegetative and reproductive organs is decreased in plants that are grown in soil, when compared to hydroponics. Such phenomena are probably related to the soil properties (physical, chemical and/or biological features) that can retain Cd ions by reducing their mobility and/or availability to the plants (Kabata-Pendias, 2011), hence decreasing Cd-induced effects on plant development, nutrient homeostasis, and fruit quality and yield.

4.4.5. Modifications in the fruit nutritional status depend on genotype, fruit part and bunch position

In addition to the problems regarding Cd accumulation, modifications in the fruit mineral composition should be evaluated in order to avoid potential nutritional deficiencies in humans due to the low intake of the plant-origin nutrients as a consequence of Cd exposure (Teklić et al., 2013). It has been demonstrated that Cd disturbs the suitable translocation of nutrients to tomato fruits (Hédiji et al., 2015; Kumar et al., 2015). However, differences between tomato pulp and peel have not been considered before. Moreover, the present work demonstrated that the magnitude of such disturbances not only depends on tomato cultivar and fruit part, but also on bunch position (Fig. 6-10). For instance, only fruits from the second bunch exhibited reductions in their pulp P concentration, whereas fruits from the first, third and fourth bunches showed no modifications (Fig. 6a). In the tolerant cultivar, fruits from the first bunch showed a great loss in their peel Ca concentration after exposure to Cd, whereas it was not affected in tomato pulp (Fig. 7a, b). Curiously, B concentration was decreased in peel of fruits from the first bunch in the tolerant cultivar, as well as in the pulp of fruits from the third bunch in the sensitive cultivar (Fig. 10c, d). This result was surprising because Cd stimulated B uptake in the sensitive plants (Fig. 5d), hence a high B concentration was expected in fruits, as previously reported by Kumar et al. (2015). For this micronutrient, data indicate that Cd might induce B remobilization from fruits to others organs (Fig. 10c, d), explaining why tomatoes showed a lower B concentration even with increased B uptake (Fig. 5d).

Exposure to Cd did not cause significant misbalances in Mg and Fe concentrations in tomato pulp and peel (Fig. 8a-d, Fig. 9a, b). These results are in line with those from Kumar et al. (2015), who grew grafted tomato plants in pots containing quartziferous sand irrigated with a nutrient solution containing 25 μM $CdCl_2$. In contrast, Hédiji et al. (2015) observed decreases in the Mg concentration and increases in the Fe concentration in mature fruits of plants grown in Cd-containing hydroponic solutions (20 μM $CdCl_2$). When the K concentration in tomato fruits is concerned, there is contradicting information in literature. Reports from Hédiji et al. (2015) and Hussain et al. (2017) showed that K concentration in fruits was not changed by Cd exposure, whereas Kumar et al. (2015) observed increases in K concentration in Cd-treated plants. Since K homeostasis in distinct fruit parts were differently affected by Cd (Fig. 6c, d), the assessment of the pericarp (peel and pulp together) could explain the differences among these studies. In addition, the use of hydroponics (or a mixture of hydroponic solution and sand) to grow tomato plants under Cd exposure could also influence the mineral profile of fruits since the uptake of both essential and non-essential elements is enhanced in such cultivation systems (Alvarenga, 2013).

4.5. Conclusions

The impacts of long-term Cd exposure on plant development and fruit features depend on the tomato cultivar, which may present modifications in the plant height, leaf area, stem dry weight, and nutritional status. Even so, sensitive and tolerant cultivars are able to acclimatize to long-term Cd exposure, probably through mechanisms associated to reductions in the Mg status. Cadmium is accumulated in vegetative and reproductive organs of both cultivars, but the tolerant plant showed usually a higher Cd concentration than the sensitive cultivar. Tomato pulp and peel presented Cd concentrations that ranged from 0.83 to 2.07 mg kg^{-1}, also revealing that plants grown in soil accumulate less Cd in fruits than those cultivated in hydroponic systems, when compared to the previous studies. Although Cd reaches the fruits from the first to the fourth bunches, the floral receptacle and its related structures may act as a barrier to Cd entrance in fruits. The magnitude of the Cd-induced changes in the mineral profile varies according to plant cultivar, organ, tomato tissue and bunch position of fruit. Moreover, Cd exposure is able to improve fruit size and weight in the tolerant tomato cultivar.

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Supplementary materials

Table S1. Physicochemical properties of the uncontaminated and contaminated soils that were used for the growing of tomato plants

Properties	Unity	Soils					
		Contaminated			Control		
Organic matter	g dm^{-3}	43.00	±	0.35	42.25	±	0.96
pH		6.48	±	0.04	5.65	±	0.23
Phosphorus (P)	mg dm^{-3}	666.00	±	41.80	620.00	±	23.41
Potassium (K)	mmolc dm^{-3}	2.80	±	0.33	4.03	±	0.09
Calcium (Ca)	mmolc dm^{-3}	168.75	±	20.28	180.25	±	10.53
Magnesium (Mg)	mmolc dm^{-3}	23.00	±	2.52	30.25	±	2.19
Aluminium saturation	mmolc dm^{-3}	15.50	±	0.25	26.75	±	3.38
Base saturation	mmolc dm^{-3}	194.55	±	17.95	214.53	±	8.97
Cation exchange capacity	mmolc dm^{-3}	210.05	±	18.15	241.28	±	11.48
Volume	%	92.50	±	0.43	89.00	±	1.06
Boron (B)	mg dm^{-3}	0.66	±	0.02	0.82	±	0.03
Copper (Cu)	mg dm^{-3}	3.43	±	0.17	2.73	±	0.06
Iron (Fe)	mg dm^{-3}	123.75	±	14.72	89.25	±	1.95
Manganese (Mn)	mg dm^{-3}	11.68	±	1.46	14.45	±	0.11
Zinc (Zn)	mg dm^{-3}	17.03	±	0.36	14.55	±	0.28
Cadmium (Cd)	mg dm^{-3}	3.77	±	0.04	0.04	±	0.02

Means ± standard errors. $n = 4$

Table S2. Maximum, minimum and average temperature, and relative humidity from June to December 2015

Month	Temperature (°C)			Humidity (%)
	Maximum	Minimum	Average	
June	26.46	13.69	20.08	77.63
July	25.63	13.87	19.75	81.19
August	29.10	12.61	20.85	64.13
September	30.44	16.71	23.57	79.97
October	32.10	18.65	25.38	72.74
November	30.54	20.68	25.61	85.30
December	32.50	21.68	27.09	84.35

SOURCE: Meteorological station of the Biosystems Engineering at Escola Superior de Agricultura "Luiz de Queiroz "/ Universidade de São Paulo

Table S3. Chlorophyll content in leaves of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cv. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd)

DAT[1]	Treatments								Average	
	YST		YST + Cd		TTO		TTO + Cd			
21	34.91 (1.00)		35.28 (1.31)		35.49 (1.09)		32.27 (0.78)		34.49 (1.04)	b
54	44.96 (0.68)		45.18 (1.03)		43.08 (0.69)		42.15 (0.78)		43.84 (0.79)	a
Average	39.94 (0.84)	a	40.23 (1.17)	a	39.29 (0.89)	ab	37.21 (0.78)	b		

[1] DAT: days after transplanting of 29-days-old seedlings from the tray to the soil. Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons among treatments or day of evaluation. Values inside parentheses are the standard errors of the means. $n = 10$

Table S4. Phosphorus (P) and potassium (K) concentrations in different organs/tissues of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cv. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd)

Organs	Treatments								Average	
	YST		YST + Cd		TTO		TTO + Cd			
				P						
Roots	1.30 (0.20)		0.83 (0.09)		1.00 (0.06)		0.63 (0.03)		0.94 (0.09)	c
Stems	2.23 (0.34)		1.90 (0.17)		1.63 (0.07)		1.45 (0.15)		1.80 (0.18)	a
Leaf blades	1.77 (0.19)		2.10 (0.50)		1.03 (0.03)		1.10 (0.20)		1.50 (0.23)	b
Peduncle + sepals	1.57 (0.18)		1.78 (0.03)		1.13 (0.03)		1.10 (0.06)		1.40 (0.23)	b
Average	1.72 (0.23)	a	1.65 (0.20)	a	1.20 (0.05)	b	1.07 (0.11)	b		
				K						
Roots	16.43 (1.08)		12.73 (1.70)		12.83 (1.48)		8.77 (0.43)		12.69 (1.17)	b
Stems	23.07 (1.13)		21.03 (2.33)		16.43 (1.62)		14.25 (1.45)		18.70 (1.63)	a
Leaf blades	18.70 (1.23)		19.70 (1.70)		15.27 (1.85)		16.25 (1.95)		17.48 (1.68)	a
Peduncle + sepals	18.23 (3.43)		17.88 (0.98)		14.40 (0.74)		13.93 (0.92)		16.11 (1.52)	a
Average	19.11 (1.72)	a	17.84 (1.68)	a b	14.73 (1.42)	b c	13.30 (1.19)	c		

Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons among treatments or organs/ tissues. Values inside parentheses are the standard errors of the means. $n = 3$

Table S5. Nitrogen (N) and calcium (Ca) concentrations in different organs/tissues of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cv. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd)

Organs	**Treatments**							
	YST		YST + Cd		TTO		TTO + Cd	
				N				
Roots	14.73 (0.61)	Aa	14.17 (0.35)	Aa	14.67 (0.19)	Aa	13.43 (0.48)	Aa
Stems	5.03 (0.34)	Ac	7.37 (0.35)	Ab	5.70 (0.67)	Ac	4.95 (0.45)	Ab
Leaf blades	12.07 (0.84)	Aab	11.05 (0.85)	Aa	11.00 (0.15)	Ab	11.35 (0.15)	Aa
Peduncle + sepals	10.43 (0.39)	Ac	12.53 (0.58)	Aa	12.17 (0.79)	Aab	12.13 (0.77)	Aa
				Ca				
Roots	8.80 (0.61)	Ab	9.27 (0.69)	Ac	7.83 (0.64)	Ac	8.17 (0.27)	Ac
Stems	10.03 (0.55)	Ab	9.90 (1.43)	Ac	5.80 (0.12)	Ac	6.60 (1.00)	Ac
Leaf blades	34.20 (2.20)	Aa	36.83 (4.17)	Aa	32.70 (1.50)	Aa	36.10 (2.40)	Aa
Peduncle + sepals	11.65 (1.09)	Ab	13.75 (0.61)	Ab	15.77 (2.19)	Ab	14.37 (1.03)	Ab

Distinct uppercase and lowercase letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons of different treatments inside a same organ, and for each treatment in distinct organs, respectively. Values inside parentheses are the standard errors of the means. $n = 3$

Table S6. Number of flowers and fruit setting in distinct bunches of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cv. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in uncontaminated and contaminated soils (0.08 and 3.72 mg kg^{-1} of available Cd)

Bunch	Treatments								Average	
	YST		YST + Cd		TTO		TTO + Cd			
				Number of flowers						
1	7.76 (0.61)		8.00 (0.51)		7.30 (0.20)		6.70 (0.32)		7.42 (0.41)	a
2	8.13 (0.83)		9.80 (1.04)		6.70 (0.51)		6.70 (0.45)		7.83 (0.71)	a
3	9.67 (0.92)		8.20 (1.22)		6.10 (0.39)		7.10 (0.57)		7.77 (0.77)	a
Average	8.49 (0.79)	a	8.67 (0.92)	a	6.70 (0.37)	b	6.83 (0.45)	ab		
				Fruit setting (%)						
1	0.81 (0.05)		0.69 (0.06)		0.65 (0.06)		0.69 (0.05)		0.71 (0.05)	a
2	0.67 (0.09)		0.47 (0.09)		0.37 (0.04)		0.24 (0.06)		0.44 (0.07)	b
3	0.33 (0.06)		0.31 (0.03)		0.37 (0.06)		0.29 (0.05)		0.32 (0.05)	c
Average	0.60 (0.07)	a	0.49 (0.06)	ab	0.46 (0.05)	b	0.41 (0.05)	b		

Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$) for comparisons among treatments or bunches. Values inside parentheses are the standard errors of the means. $n = 3$

Table S7. Total soluble sugar/ titratable acid ratio (TSS/TA) and tonality angle (a*) in fruits from the first bunch of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cv. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in uncontaminated and contaminated soils (0.08 and 3.72 mg kg^{-1} of available Cd)

Fruit parameter	Treatments											
	YST			YST + Cd			TTO			TTO + Cd		
TSS/TA	7.97	(0.40)	b	7.74	(0.52)	b	13.54	(0.56)	a	13.73	(0.62)	a
a*	17.14	(0.29)	a	17.02	(0.64)	a	16.32	(0.73)	a	16.58	(0.59)	a

Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$). Values inside parentheses are the standard errors of the means. $n = 10$

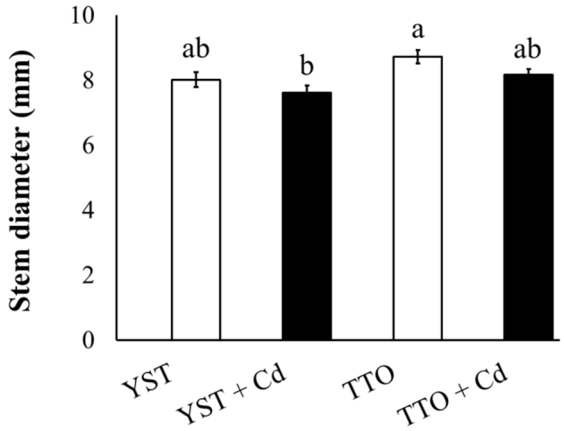

Figure S1. Stem diameter of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control (white columns) and contaminated (black columns, + Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$). Bars represent the standard errors of the means. $n = 10$

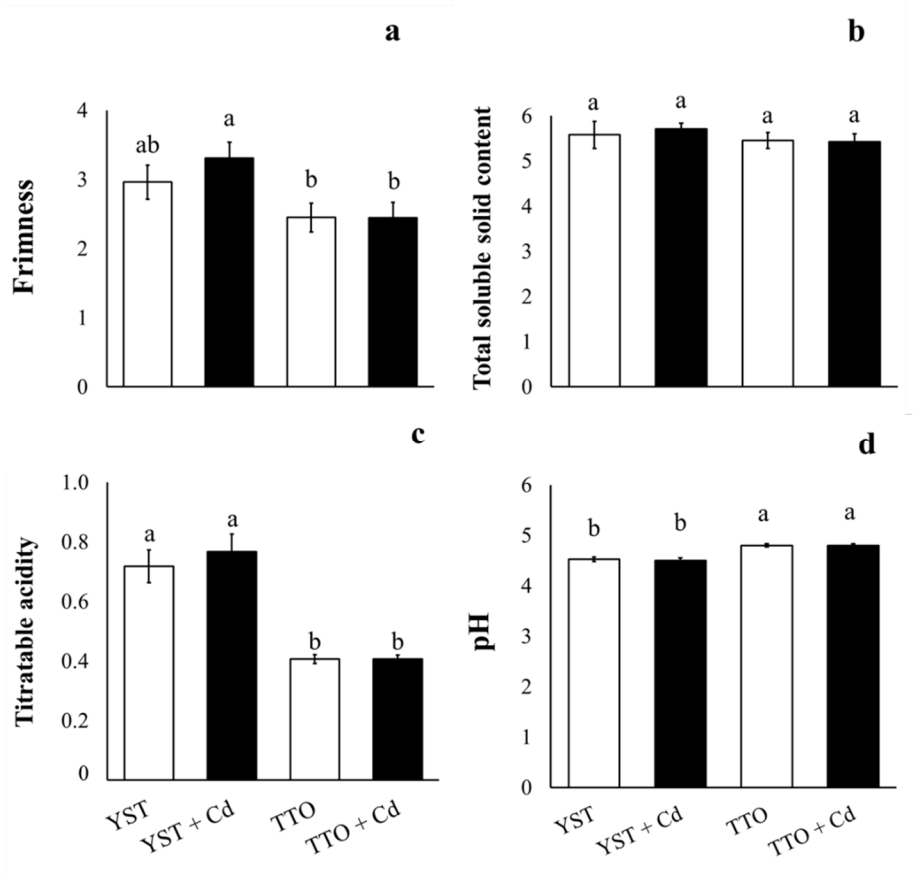

Figure S2. Firmness (N, **a**), total soluble solids content (ºBrix, **b**), titratable acidity (% citric acid, **c**) and pH (**d**) of fruits from tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control (white columns) and contaminated (black columns, +Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 9 – 10$. Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$). Bars represent the standard errors of the means

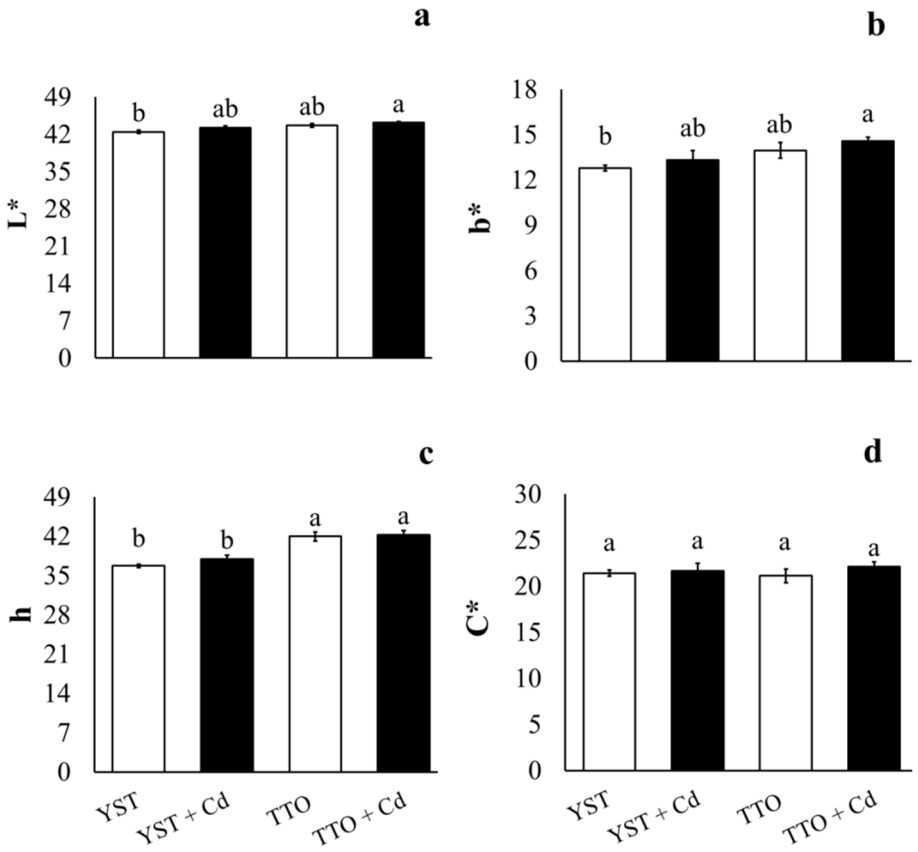

Figure S3. Fruit color as measured by luminosity - L*(**a**), tonality angles - b* (**b**) and h (**c**), and saturation - C* (**d**) in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). n = 9- 10. Distinct letters denote different means by Tukey test ($\alpha \leq 0.05$). Bars represent the standard errors of the means

5. CADMIUM EXPOSURE TRIGGERS GENOTYPE-DEPENDENT CHANGES IN THE SEED VIGOR AND GERMINATION OF TOMATO OFFSPRING

ABSTRACT

Although negative effects on the offspring fitness can be triggered by the mother-plant exposure to environmental stresses, some plants are able to 'remember' past incidents and enhance the progeny tolerance. Here, the mineral profile, cytogenetic modifications and physiological potential of seeds from two tomato cultivars, with contrasting tolerance degree to cadmium (Cd) toxicity, were evaluated after plant exposure to this metal. Both cultivars exhibited high Cd translocation to the seeds, however, the tolerant tomato accumulated more Cd than the sensitive one. As a consequence of the Cd accumulation, reductions in the Mn concentration in Cd-challenged plants were detected. Surprisingly, seed germination and vigor were increased in the tolerant tomato cultivar after Cd exposure, despite increases in the chromosomal abnormalities. By contrast, seeds from the sensitive cultivar exhibited no changes in their physiological potential after Cd exposure, despite of Cd-induced reductions in the mitotic index. Moreover, bunch position exerted effects on the vigor and type of chromosomal abnormality. The results show that maternal plant exposure to Cd can affect tomato offspring by changing the seed physiological potential, and such effect can be partially explained by alterations in the seed-derived elements (essential and non-essential) and genotype-dependent tolerance mechanisms.

Keywords: Cytotoxicity; Genotoxicity; Heavy metal; Seed-derived nutrient; Transgenerational effect; *Solanum lycopersicum*

5.1. Introduction

Embryo malformation and reductions in the seed provisioning can be triggered by environmental stresses by which mother-plant are submitted, potentially decreasing seed germination and seedling establishment (Marcos-Filho, 2016). However, some plants are able to remember past incidents and to use this stored knowledge - the so-called "memory" - to enhance the progeny tolerance to continuous or even upcoming more severe stresses (Herman and Sultan, 2011). Despite the importance of this issue, there are scarce information about the Cd-induced transgenerational effects on plants. Currently, we do not know whether germination, seedling establishment and yield can be affected by the Cd translocation to the seeds, which were able to accumulate high Cd concentrations after mother-plant exposure to this metal (Yan et al., 2016; Lin et al., 2017). Cadmium is one of the most dangerous metals to biological systems, being a matter of concern to farmers and health organizations because it impairs yield and quality of crops, which can potentially transfer hazardous levels of heavy metals to humans through food chain (Gallego et al., 2012; Dziubaneka et al., 2017).

In plants, Cd triggers several toxicity symptoms after a short-period of exposure, and even at low concentrations in the growing media (Benavides et al., 2005; Gratão et al., 2005, 2008; Gallego et al., 2012; Pompeu et

al., 2017). The visual damages consist of leaf necrosis and chlorosis, root brownish and reductions in the whole plant development, which is prevented due to antioxidant machinery disturbances, nutritional status imbalances and photosynthetic apparatus injuries (Iannone et al., 2015; Cuypers et al., 2016; Sebastian and Prasad, 2016a, b; Bayçu et al., 2017a, b; Pompeu et al., 2017). Moreover, Cd exposure impairs cell division, triggers chromosomal aberrations, damage DNA and also provokes unequal chromosome distribution (Seth et al., 2008; Shi et al., 2014, 2016), increasing the probability of mutations, ploidy abnormalities and even plant death. To a certain extent, plants can employ complex mechanisms to cope with the negative effects from Cd exposure (Gratão et al., 2005, 2008, 2012; Nogueirol et al., 2016; Cuypers et al., 2016; Alves et al., 2017).

The modulation of the activity of several antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11) are well-known protective strategies, which also include the overproduction of non-enzymatic antioxidant compounds such as phytochelatins and compatible osmolytes (Gratão et al., 2005, 2015; Hédiji et al., 2010; Fidalgo et al., 2011, 2013; Gallego et al., 2012; Štolfa et al., 2015; Cuypers et al., 2016; Hasan et al., 2016; Méndes et al., 2016; Nogueirol et al., 2016; Alves et al., 2017). Furthermore, variations in the mineral profile are associated to plant strategies against Cd toxicity, rather than a simply side-effect of Cd exposure (Kudo et al. 2015; Sharmila et al. 2017). In tomato (*Solanum lycopersicum* L.), a well-established model plant species that is also one of the most produced vegetable nowadays (Bergougnoux et al., 2014), the toxicity degree to Cd exposure varies according to Cd concentration in the growing media, time of exposure, growing media properties, plant features (such as genotype, phenological stage and target-organ or tissue), and even with plant associated-microorganisms (Gratão et al., 2005, 2015; Dourado et al., 2013; Nogueirol et al., 2016; Alves et al., 2017; Pompeu et al., 2017).

The differential responses of tomato cultivars to Cd exposure is one of the most interesting aspect among these factors (Gratão et al., 2012; Hussain et al., 2015; Kumar et al., 2015; Alves et al., 2017), since tolerant plants can be potentially used in breeding and biotechnological programs to improve fruit yield and quality in plants cultivated in naturally and/or artificially contaminated fields. Yoshimatsu (tolerant) and Tropic Two Orders (sensitive) are two tomato cultivars with contrasting tolerance degrees to Cd exposure that were identified in previous studies, in which plants were subjected to a short period of Cd-induced stress using hydroponic systems with solutions containing 35 μM $CdCl_2$ (preliminary data). In the current work, both cultivars were grown from seedling stage (29-days-old) to fruit production in soil containing 0.04 (control) and 3.77 mg kg^{-1} Cd (contaminated). The study was performed in order to evaluate (i) the influence of the plant-mother exposure to Cd on the translocation of this metal to tomato seeds, (ii) the consequences of Cd accumulation in the seed viability and vigor, and (iii) the effects of tomato cultivars with contrasting tolerance degree to Cd toxicity on the previous parameters.

5.2. Materials and methods

5.2.1. Plant materials and growth conditions

Seeds of the tolerant and sensitive tomato cultivars, Yoshimatsu and Tropic Two Orders, respectively, were chemically scarified using 2-2.5% HCl (v:v) during 15 minutes under stirring, in order to standardize cultivar's germination. Subsequently, seeds were sowed (June 24th 2015) in polystyrene trays filled with thin exfoliated vermiculite, which was irrigated four times a day. After seedling emergence, daily application of macro and micronutrients (Peters Professional 20-20-20 at 1 g L^{-1}) was initiated in order to maintain suitable seedling

development. After one week, this concentration was increased from 1 to 1.5 g L^{-1}, which was used until the 29-day-old seedlings were reached and then were transplanted to 20-dm3 pots that were filled with control or contaminated soil (0.04 and 3.77 mg kg^{-1} Cd, respectively). Other chemical and physical properties of the soils are in the Table S1 of the 4th chapter.

During all experiment, plants were cultivated inside a greenhouse. Fungicides, pesticides and fertilizers were applied to all plants, as following the recommendations for tomato crop management. Plants were grown in control and contaminated soil from July 23th to December 1st 2015 (131 days after transplanting), until the fruits of the 4 first bunches became mature (completely red). The monthly temperature and humidity were recorded in the Table S2 of the 4th chapter, as provided by the meteorological station of the Esalq/ USP (Esalq 2016). In order to obtain tomato seeds, the seed-containing mucilage from the fruits were extracted, and the seeds were immersed into a solution containing biological ferment (Saccharomyces cerevisiae) for 36 hours. Afterwards, the seeds were placed to dry at 21 °C, for 3 days. Seeds from the 1st to the 4th bunches were used for Cd and nutrient concentration analysis, whilst seeds from the 1st and 2nd bunches were selected for the germination and vigor tests, and seeds from the 3rd and 4th bunches were used to evaluate the cell cycle and chromosomal structures in each of the treatments, i.e. (i) tolerant cultivar in control soil, (ii) tolerant cultivar in Cd-contaminated soil, (iii) sensitive cultivar in control soil, and (iv) sensitive cultivar in Cd-contaminated soil.

5.2.2. Quantification of cd and nutrient concentration in seeds

The seeds were dried in an oven at 60 ºC and subsequently ground by using crucible and pistils. Cadmium, calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P), sulfur (S), iron (Fe), manganes (Mn), copper (Cu), zinc (Zn) and boron (B) status were evaluated through ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry) analysis, which was preceded by nitro-perchloric digestion of the ground samples. Each treatment had four replications, which were subjected to the analytical procedures that were carried out by the Soil Fertility Laboratory at Instituto Agronômico de Campinas (IAC, Brazil).

5.2.3. Germination and vigor tests

Four replications of 25 seeds for each treatment were distributed over two blotting paper sheets that were humidified with water (1: 2.5, g: mL) and placed inside plastic boxes (11.0 x 11.0 x 3.5 cm), which were subjected to 20-30 ºC, and 8-hour light and 16-hour darkness condition. Germination and first counting of germination were both measured as percentage of normal seedlings at 14th and 5th days after sowing, respectively (Brasil 2009). The number of death seeds and diseases seedlings were also evaluated (Brasil 2009). For the calculation of the speed of germination rate (SGR), the formula that was proposed by Maguire (1962) was used:

$$SGR = \frac{G1}{D1} + \frac{G2}{D2} \dots \frac{Gn}{Dn}$$

Where G_1, G_2 and G_n are the number of seedlings at the first, second and last count, respectively, and D_1, D_2 and D_n refer to the days after sowing at the first, second and last count, respectively.

5.2.4. Mitotic index and chromosomal abnormalities

Approximately 0.1 cm-length root tips of tomato seedlings were cut and fixed in Carnoy solution. Afterwards, for the slide preparation, the material was hydrolyzed with 1N HCl at 60 °C for 10 minutes and then stained with Schiff`s reagent, according to the Feulgen`s method (Feulgen and Rossenbeck, 1924). Approximately 3000 cells per treatment were evaluated, i.e. 1,000 cells per biological replicate. The chromosomal abnormalities encountered were calculated by measuring the percentage of cell with bridges, c-metaphase, sticky or lost chromosomes.

$$MI = (CI/CE)\, x\, 100$$

Where CI is the number of cells in division and CE is the total number of cells evaluated.

5.2.5. Statistical procedures

The experiment was arranged in a completely randomized design, with a one-way analysis of variance for the Cd and nutrient concentrations. The split-plot design was applied for variables from germination tests, mitotic index and chromosomal abnormalities, using treatments as primary plots and bunches as sub-plots. Before analysis of variance (ANOVA), we evaluated whether the data were according to assumptions for the ANOVA performance (viz. normal distribution, variance homogeneity and error independence) through the "Guided Data Analysis" tool of statistical software SAS (SAS Institute, 2011). Data transformations were performed when indicated by this tool. Moreover, an outlier, which belonged to the Mg concentration, was removed. Finally, the data were submitted to ANOVA ($P \leq 0.05$) and means were compared by LSD test ($P \leq 0.05$) using SAS software (SAS Institute, 2011). In addition, the Pearson's correlation analysis was employed to evaluate the cause-effect relations among some of the studied variables.

5.3. Results

5.3.1. Cd and nutrient concentration in seeds

Seeds of tomato cultivars with contrasting tolerance degree to Cd-induced stress were collected from plants that were grown from seedling stage (29-days-old) to fruit production in soil containing 0.04 (control) and 3.77 mg kg^{-1} Cd (contaminated). A high Cd concentration in the seeds of both cultivars was observed after plant-mother cultivation in contaminated soil, when compared to those from control soil (Fig. 1). However, the tolerant tomato cultivar, Yoshimatsu, exhibited a higher Cd accumulation than the sensitive cultivar, Tropical Two Orders, regardless soil contamination (Fig. 1).

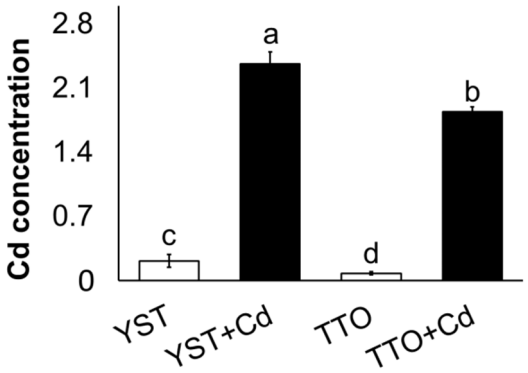

Figure 1. Cadmium concentration (μg g^{-1} DW) in seeds from tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control (white columns) and contaminated (black columns, +Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 4$. Distinct letters denote different means by LSD test ($P \leq 0.05$) for comparisons among treatments. Bars represent the standard errors of the means.

Neither tomato cultivar nor soil contamination influenced P, K, Ca, S and Fe status in seeds (Table 1). However, decreases in the Mn concentration in seeds of both tomato cultivars were observed after Cd exposure that were not enough to affect either Mg or Zn concentrations, which varied according to the genotype (Fig. 2a-c).

Table 1. Phosphorus (P), potassium (K), calcium (Ca), sulfur (S) and iron (Fe) concentration (μg g^{-1} DW) in seeds from tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated (+Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively).

Nutrients[ns]	Treatments			
	YST	YST + Cd	TTO	TTO + Cd
P	6.90 (0.118)	6.64 (0.080)	6.26 (0.240)	6.41 (0.127)
K	4.57 (0.240)	4.35 (0.200)	4.45 (0.230)	4.71 (0.030)
Ca	0.58 (0.071)	0.46 (0.039)	0.56 (0.073)	0.51 (0.049)
S	2.26 (0.081)	2.11 (0.076)	2.09 (0.075)	2.22 (0.034)
Fe	0.09 (0.015)	0.09 (0.008)	0.08 (0.007)	0.08 (0.003)

[ns]: non-significant differences among treatments by the F test ($P \leq 0.05$). Values inside parentheses are the standard errors of the means. $n = 4$.

The K/Mg ratio exhibited slight changes, but distinct behavior in tolerant and sensitive cultivars, with a decreased in Yoshimatsu, but an increased in Tropic Two Order tomato after Cd exposure (Fig. 2d).

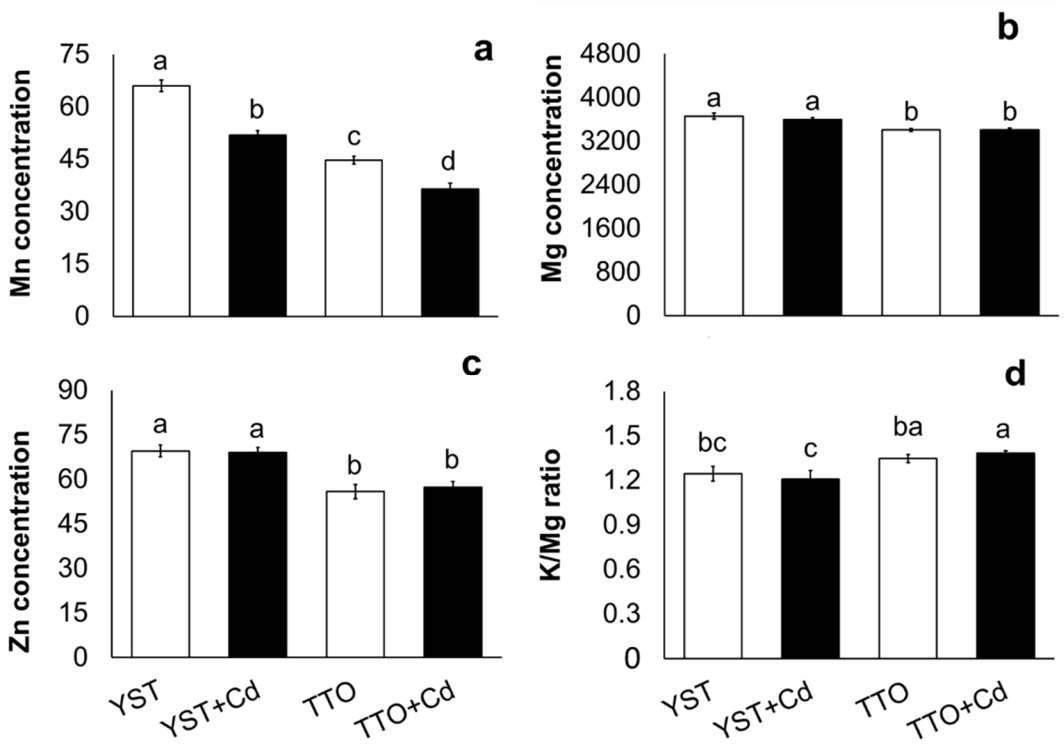

Figure 2. (**a**) Manganese - Mn, (**b**) zinc – Zn, and (**c**) magnesium -Mg concentrations (μg g^{-1} DW), as well as (**d**) K/Mg ratio in seeds from tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control (white columns) and contaminated (black columns, +Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively). $n = 4$. Distinct letters denote different means by LSD test ($P \leq 0.05$) for comparisons among treatments. Bars represent the standard errors of the means.

5.3.2. Seed physiological potential

No effects on the seed germination in Cd-challenged plants were observed in the sensitive cultivar Tropic Two Orders, when compared to its respective control (Table 2). On the other hand, the number of germinated seeds was increased by 27 to 32% in Yoshimatsu, which also exhibited a high vigor in comparison to the seeds of plants from control soil (Table 2). Moreover, seeds of fruits from the 2nd bunch exhibited a higher vigor than those from the 1st bunch, regardless tomato genotype or plant mother exposure to Cd (Table 2).

Table 2. Speed of germination index (SGI), percentage of normal seedlings (PNS) in the first and last count of the germination test, as well as percentage of diseased seedlings (DS) in seeds from the 1st and 2nd bunches of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated (+Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively).

Bunch	Treatments				Average
	YST	YST+ Cd	TTO	TTO + Cd	
		SGI			
1	2.4 (0.31)	3.1 (0.16)	3.3 (0.05)	3.4 (0.10)	3.1 (0.07) b
2	2.9 (0.34)	3.5 (0.24)	3.8 (0.15)	3.7 (0.13)	3.7 (0.13) a
Average	2.6 (0.24) b	3.3 (0.16) ab	3.6 (0.12) a	3.6 (0.10) a	
		PNS 1st count			
1	46.0 (4.36)	62.0 (4.58)	74.0 (3.00)	76.0 (3.46)	64.5 (2.39) b
2	59.0 (6.54)	77.0 (6.22)	82.0 (4.36)	77.0 (1.66)	73.8 (2.97) a
Average	52.5 (4.55) b	69.5 (4.69) a	78.0 (3.0) a	76.5 (1.93) a	
		PNS last count			
1	65.0 (9.53)	85.0 (3.57)	84.0 (1.41)	89.0 (1.66)	80.8 (3.39)
2	72.0 (8.49)	89.0 (5.36)	95.0 (3.28)	93.0 (2.18)	87.3 (1.50)
Average	68.5 (6.50) b	87.0 (3.30) a	89.5 (2.64) a	91.0 (1.54) a	
		DS			
1	30.0 (7.55)	3.0 (1.66)	9.0 (2.96)	1.0 (0.90)	10.8 (3.55)
2	23.0 (8.53)	3.0 (1.70)	0.0 (0.00)	4.0 (1.41)	7.5 (3.16)
Average	26.5 (5.83) a	3.0 (1.17) b	4.5 (2.17) b	2.5 (0.98) b	

Distinct letters denote different means by LSD test ($P \leq 0.05$) for comparisons among treatments or bunches. Values inside parentheses are the standard errors of the means. n = 4 with 25 seeds.

5.3.3. Cellular cycle and chromosome abnormalities

Maternal exposure to Cd caused decreases in the mitotic index of sensitive tomato offspring (Table 3) due to diminished percentage of cells in prophase, when compared to the control plants (Table 4).

Table 3. Percentage of mitotic cells (MI) and chromosomal abnormalities (CA) in the root tips of seedlings from seeds of the 3rd and 4th bunches in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated (+Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively).

Bunch	Treatments				Average
	YST	YST + Cd	TTO	TTO + Cd	
		MI			
3	31.27 (1.26)	30.40 (0.87)	28.43 (0.27)	24.17 (0.33)	28.57 (0.89)
4	31.45 (1.24)	29.37 (0.21)	34.00 (0.09)	26.73 (0.95)	30.39 (0.87)
Average	31.36 (0.89) a	29.88 (0.50) a	31.22 (1.15) a	25.45 (0.73) b	
		CA			
3	2.17 (0.14)	3.33 (0.21)	2.03 (0.12)	1.83 (0.10)	2.34 (0.18)
4	1.77 (0.14)	2.23 (0.40)	1.97 (0.15)	2.20 (0.08)	2.04 (0.13)
Average	1.97 (0.13) b	2.78 (0.32) a	2.00 (0.10) b	2.02 (0.10) b	

Distinct letters denote different means by LSD test ($P \leq 0.05$) for comparisons among treatments or bunches. Values inside parentheses are the standard errors of the means. n = 3 with 1000 cells.

On the other hand, the percentage of cells in metaphase, telophase and anaphase presented no changes after soil contamination (Table 4). The tolerant cultivar did not exhibit any differences in the cell cycle due to the addition of Cd to the growth media (Table 3).

Table 4. Percentage of cells under metaphase, telophase and anaphase in the root apex of seedlings from seeds of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated (+Cd) soils (0.04 and 3.77 mg kg^{-1} Cd, respectively).

Cellular phases	Treatments			
	YST	YST + Cd	TTO	TTO + Cd
Prophase*	17.65 (0.38) a	16.43 (0.56) a	16.90 (0.66) a	14.08 (0.54) b
Anaphase^{ns}	1.65 (0.16) a	1.60 (0.05) a	1.73 (0.04) a	1.27 (0.07) a
Metaphase^{ns}	8.51 (0.73) a	8.65 (0.13) a	8.52 (0.67) a	7.15 (0.23) a
Telophase^{ns}	3.46 (0.24) a	3.08 (0.42) a	3.88 (0.34) a	2.80 (0.06) a

* and ^{ns}: significant and non-significant differences among treatments by the F test ($P \leq 0.05$). Same letter letters denote no differences among means of treatments by LSD test ($P \leq 0.05$). Means are followed by the standard error (n= 3 with 1000 cells). n = 3 with 1000 cells.

However, Cd increased the occurrence of chromosomal aberrations in Yoshimatsu (Table 3), mainly due to the increments in the proportion of C-metaphase when compared to the control plants (Tables 5) since the percentage of sticky and lost chromosomes did not change (Table 6).

Table 5. Percentage of cells with chromosome abnormalities bridges and C-metaphase in the root apex of seedlings from seeds of the 3rd and 4th bunches in tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated (+Cd) soils (0.04 and 3.77 mg kg^{-1} of available Cd, respectively).

Bunch	Treatments			
	YST	YST + Cd	TTO	TTO + Cd
		Bridges		
3	0.30 (0.08) aA	0.33 (0.05) aA	0.13 (0.03) aA	0.03 (0.03) bA
4	0.11 (0.05) aA	0.26 (0.05) aA	0.17 (0.07) aA	0.17 (0.03) aA
		C-metaphase		
3	0.67 (0.07) bA	1.33 (0.12) aA	0.57 (0.12) bA	1.00 (0.05) abA
4	0.64 (0.16) aA	0.67 (0.31) aB	0.86 (0.06) aA	1.00 (0.05) aA

Distinct lowercase and uppercase letters denote different means by LSD test ($P \leq 0.05$) for comparisons among all treatments inside each bunch, a same treatment in distinct bunches. Bars represent the standard errors of the means. Values inside parentheses are the standard errors of the means. n = 3 with 1000 cells.

Table 6. Percentage of sticky and lost chromosomes in the root apex cells of seedlings from seeds of tolerant and sensitive tomato cultivars, *Solanum lycopersicum* cvs. Yoshimatsu (YST) and Tropic Two Orders (TTO), respectively, which were grown in control and contaminated (+Cd) soils (0.04 and 3.77 mg kg^{-1} of available Cd, respectively).

Bunch	Treatments			
	YST	YST + Cd	TTO	TTO + Cd
		Stick^{ns}		
3	0.63 (0.129)	0.93 (0.136)	0.80 (0.170)	0.30 (0.047)
4	0.52 (0.495)	0.94 (0.103)	0.50 (0.094)	0.67 (0.119)
		Lost^{ns}		
3	0.60 (0.047)	0.73 (0.098)	0.53 (0.098)	0.50 (0.141)
4	0.56 (0.148)	0.53 (0.154)	0.47 (0.072)	0.37 (0.098)

^{ns}: significant and non-significant differences among treatments by the F test ($P \leq 0.05$). Values inside parentheses are the standard errors of the means. $n = 3$ with 1000 cells.

5.4. Discussion

5.4.1. Cd is translocated to the seeds, but its concentration depends on tomato genotype

Contamination of agricultural land by heavy metals has increased since the first industrial revolution and a scenario for reductions of their release in the environment is uncertain. Currently, heavy metals are the most frequently occurring contaminant in soils (Science Communication Unit, 2013), from where plants can uptake and distribute from roots to shoots (Gallego et al., 2012; Teklić et al., 2013). In tomato, previous studies reported that Cd is translocated to stems, leaves, flowers and even fruits of several cultivars (Hédiji et al., 2010, 2015; Gratão et al., 2012; Hartke et al., 2013), revealing that tomato fruits are a pathway for Cd entry into the food chain. Such studies did not involve a transgenerational approach, which revealed that (i) Cd reaches the seeds of tomato plants subjected to exposure to this metal, and (ii) a genotype-specific mechanism drives the Cd accumulation to the seeds, since tolerant and sensitive cultivars exhibited different Cd accumulation patterns (Fig. 1).

Data indicated that Yoshimatsu tomato cultivar has a natural, increased ability to accumulate Cd in seeds in comparison to the sensitive cultivar, Tropical Two Orders, as shown when plants were cultivated in control soil (Fig. 1). This result is in line with the findings that tolerant varieties/cultivars/ecotypes/ populations of diverse plant species have an increased Cd concentration in shoots when compared to the sensitive genotypes (Uraguchi et al., 2009; Fernández et al., 2013), probably due to a high root-to-shoot translocation rate (Zhu et al., 2004; Puschenreiter et al., 2010; Rui et al., 2016), that may provide a great accumulation of this metal in tomato seeds. Another approach that corroborates this hypothesis is the use of ectopic and non-ectopic overexpression of genes that are related to the concurrent increases in the Cd accumulation and plant tolerance, as observed in transformed A. thaliana when compared to non-transformed wild-type (Park et al., 2012; Rui et al., 2016). Differences in the seed Cd concentration were also observed in wheat, rice and barley cultivars (Yan et al., 2010; Kubo et al., 2016; Lin et al., 2017). For wheat, variations in the direct transport of Cd from the roots to the shoots were closely related to the Cd level in the seeds (Kubo et al., 2016). However, Cd redistribution, in addition to its root-shoot translocation, provided the differences in the Cd concentration in rice under exposure to this heavy metal (Yan et al., 2010).

5.4.2. Cd accumulation decreases Mn status in tomato seeds

Within the plant, Cd is translocated by the same transporters that are employed by some essential elements (Thomine et al., 2000; Sasaki et al., 2012; Wu et al., 2016). It is especially consistent for Mn transporters, which are enrolled in both absorption and translocation of Cd in several species (Thomine et al., 2000; Sasaki et al., 2012; Wu et al., 2016). Thus, reductions in the seed Mn status can be coupled to the increases in Cd accumulation (Fig. 1, Fig. 2a). This association is supported by the inverse, significant relation between these elements in both cultivars, Yoshimatsu ($r = -0.92$, $P = 0.0012$) and Tropic Two Orders ($r = -0.84$, $P = 0.0088$). This result also evidenced that the differential tolerance degree to Cd toxicity between the two cultivars was not enough to avoid the Cd-side effects on the seed Mn status, indicating that the antagonist behavior between these elements can be extended to the seeds. Hédiji et al. (2015) also reported decreases in the seed Mn status of tomato under exposure to 10 μM $CdCl_2$, however, there were no statistical differences among control and treated plants, suggesting that the magnitude of reduction in the Mn status depends on Cd concentration in the growth media.

In addition to the above hypothesis (Cd-induced disturbances in the Mn uptake and root-to-seed transportation) the data suggested that Cd drives, through an unknown mechanism, the Mn redistribution in tomato in a way that leaves act as sink of Mn that are stored in roots, stems, fruits (data obtained by our group but not yet published) and probably seeds (Fig. 2a). On the other hand, the concentration of other nutrients in the seeds was not affected by the plant-mother exposure to Cd (Table 1), but there were changes in the K/Mg ratio (Fig. 2d). Nutrients such as K^+ and Mg^{2+} are needed for cell expansion and root development (Marschner, 2012; Niu et al., 2014), so a balanced proportion between these ions may be necessary for the appropriate root protrusion and subsequent seedling development. A suitable K/Mg ratio is also necessary to decrease the incidence of plant diseases (Huber and Jones, 2013), but there was no correlation between K/Mg ratio and the percentage of infested seedlings (Table 2), which represents the highest proportion of the non-normal seedlings in the tolerant tomato (Yoshimatsu) that was grown in control soil.

5.4.3. A genotypic, Cd-concentration-dependent relation regulates the physiological potential of tomato seeds

Surprisingly, the percentage of diseased seedlings (Table 2) was strongly related to the Cd concentration in seeds of Yoshimatsu tomato (Fig. 1; $r = -0.89$, $P = 0.0024$), indicating a possible role of this heavy metal in the reduction of disease susceptibility in seedlings of Yoshimatsu. Early studies reported a protective effect of the Cd application against the incidence of diseases caused by Drechslera oryzae and Phytophora infestans in rice seedlings and potato leaves and tubers, respectively (Giri and Sinha, 1983; Stroinski et al., 1997). In the research that was carried out by Giri and Sinha (1983), a solution containing CdCl2 was sprayed on (i) rice seedlings, (ii) used for seed soaking before sowing (iii) or as root-dip treatment to young seedlings prior to transplanting into field plots. Seed treatment by soaking provided the most effective and durable protection to seedlings, probably through an induced resistance that may limit the number of infections and restrict the lesion size (Giri and Sinha 1983). Such results may be related to the Cd-induced selection of endophytic communities in Arabidopsis seeds harvested from plants exposed to Cd (Truyens et al., 2013, 2014, 2016).

The data further indicated that an increased Cd concentration in seeds of the tolerant cultivar (Fig. 1) could diminish the occurrence of diseases (Table 2) and, at same time, it was not enough to cause any visual toxicity symptoms and other damages in the seedlings. The vigor of Yoshimatsu tomato seeds (Table 2) was also enhanced by the plant-mother exposure to Cd, but this result can be related to the reductions in the percentage of abnormal seedlings. We also consider the existence of transgenerational Cd-mediated effects that were not evaluated in this study, such as alterations in the DNA sequence, epigenetics modifications and maternal effects, which include seed provisioning with nutritive resources (e.g. carbohydrate, lipid, and protein), regulatory elements (mRNAs, small RNAs) and hormones (Herman and Sultan, 2011; Donohue et al., 2009). However, with the current data, the mechanism by which Cd triggers these positive effects in the tolerant cultivar cannot be clearly established, so further research involving the analysis of seed composition, hormonal profile, epigenetics and microorganism community should be carried out.

5.4.4. Bunch position also affects the seed physiological potential and chromosome aberrations

The physiological potential of the seeds from both tomato cultivars were also affected by the bunch position, indicating advantageous features for those from the 2nd bunch in comparison to the 1st bunch (Table 2), probably due to differential source–sink relation during seed development (Herman and Sultan, 2011; Marcos-Filho, 2016). In pepper (*Capsicum frutescens*), seeds of mature red fruits collected from distinct canopy and branch regions exhibited significant variations in the germination, speed of germination rate and seedling development (*e.g.* root length and dry weight) (Mengarda and Lopes, 2012). Curiously, the bunch position seems to be related to the type of chromosome aberrations because increases in the C-metaphase in the seeds from the 3rd bunch after exposure of the tolerant cultivar to Cd were observed (Table 4). C-mitosis are the most common chromosome abnormality in onion (*Allium cepa*), barley (*Hordeum vulgare*) and *Wedelia trilobata* L. root tips under Cd exposure especially at relatively low concentrations, *i.e.* from 10 to 50 μM (Seth et al., 2008; Shi et al., 2014, 2016). In *Vicia faba*, time and dose-dependent effect on the chromosomal abnormality occurred, being C-metaphase the most frequent aberration in seedlings under 1, 5, 10 μM Cd after 24 and 48 hours of seedling exposure (Zhang et al., 2009). Curiously, under the maximum concentration tested (50 μM) and/or extended time of exposure (72 hours), chromosome stickiness was the abnormality most frequent (Zhang et al., 2009). Taken together, these data indicate that the type of chromosomal aberration depends on (i) Cd level in the culture media, (ii) period of exposure, (iii) plant species and its cultivar/varieties and, for seeds, their position in the mother-plant that was grown in Cd-containing media.

Although data suggested C-metaphase as one of the most frequent chromosome abnormality in plants under Cd exposure, it is possible that variations in the Cd-induced chromosomal abnormalities are coupled to the differential procedures used of plant exposure, *i.e.* usually researchers place the seeds or seedlings in direct contact with Cd, but in our study, we evaluated the effects on the seeds from adult plans that were grown in Cd-containing soil. According to Leme and Marin-Morales (2009), chromosomal abnormalities are often associated to DNA breakage, inhibition of DNA synthesis and replication of altered DNA, evidencing the high genotoxic effect of Cd. This heavy metal also decreased the mitotic index in the sensitive tomato cultivar due to reductions in the percentage of cells in prophase (Table 3). In root tips of barley under Cd stress concurrent decreases in mitotic index and root growth due to increases in the Cd concentration occurred, suggesting that Cd prevents root growth due to cell division inhibition (Shi et al., 2016). It is possible that reductions in the mitotic index be a result of the direct influence of Cd in the tubulin

polymerization because microtubules disorganization happened in the root region where Cd preferentially was accumulated (Shi et al., 2016).

5.5. Conclusions

Overall, this study showed that tomato cultivars with contrasting tolerance degree were able to translocate a high amount of Cd to the seeds. The maternal plant exposure to Cd can affect tomato offspring by improving the seed physiological potential of the tolerant cultivar, while not altering the performance of the sensitive cultivar. Since the tolerant cultivar accumulated more Cd than the sensitive cultivar, tolerance mechanisms may involve the neutralization of most of the Cd effects within the cell system, but not by controlling the uptake of this metal by the plant. Although the beneficial roles of changes in the seed-derived nutrients and even the presence of certain Cd concentration inside seeds were considered, the mechanism by which Cd triggers these transgenerational effects cannot still be established, so further research involving the analysis of seed composition, hormonal profile, epigenetics and microorganism community should be carried out. In addition, both seed physiological potential and type of chromosome abnormality can also be affected by the bunch position of the fruits that were used to obtain the seeds. Summarizing, this study provided new insights about the transgenerational effects caused by Cd exposure on tomato.

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6. FINAL CONSIDERATIONS

The use of tomato accessions with contrasting tolerance degree to Cd exposure proofed to be a powerful approach to identify strategies employed by plants to cope with Cd-induced challenges, as well as the mode of action of Cd toxicity inside plant. The main information that were observed here are:

The avoidance of high Mg concentration in roots was identified as a possible plant strategy to mitigate Cd toxicity by preventing formation of root hairs in tolerant accessions after a short-term exposure to Cd. Further studies showed that plant capacity to decrease Mg status is an ability shared by both tolerant and sensitive genotypes, so tolerant cultivars are probably able to activate this mechanism either early or faster than sensitive genotypes.

Regarding the mode of action of Cd toxicity, this metal causes drastic misbalances in the plant mineral profile by changing the nutrient uptake and remobilization. For instance, the leaf damages are enhanced by the Cd-induced Mn excess in tomato leaves, in addition to the high Cd concentration *per se*. Moreover, such damages are further amplified by increases in the Zn and B concentration in photosynthetic tissues.

Tomato that were grown in soil presents lower Cd accumulation and, consequently, suffers less Cd-induced damages than hydroponic-cultivated plants. Even so, a high Cd concentration was observed in peel and pulp of fruits from the first to the fourth bunches after plant growing in Cd-containing soil. Therefore, Agricultural and Health Organizations should run field experiments in order to check the actual Cd concentration in foodstuffs that are grown in contaminated soil with certain Cd concentrations that are allowed for arable lands..

Probably the floral receptacle and its related-structures act as a barrier to the Cd transportation to the fruits, but it was not enough to avoid Cd reaching the fruits. Even with a high Cd concentration, tomato yield was not affected by this heavy metal, regardless genotype. Moreover, it was showed that the mild Cd-induced stress can positively influence the height, diameter and weight of fruits, depended on genotype.

In addition, plant-mother exposure to Cd triggers genotype-specific transgenerational effects on tomato offspring, improving seed germination and vigor, despite of the increased chromosomal abnormalities in the tolerant genotype. Such effects also can include changes in the seed-derived nutrients, such as reduced Mn concentration in both genotypes, or even indirect effect from the Cd accumulation, since this heavy metal has antimicrobial activity.

This work reports new insights about the effects of Cd exposure on tomato development, tolerance mechanisms, fruit quality and yield of tomato, as well as Cd distribution in the plants. Such knowledges can be potentially used in biotechnological programs to improve fruit yield and quality in crops that were cultivated in contaminated fields.