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Relationship between Mg, B and Mn status and tomato tolerance against Cd toxicity

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A B S T R A C T

Distinct tomato genotypes possess different tolerance degree to cadmium (Cd), but the mechanisms behind this phenomenon are scarcely understood. To this end, the physiological, biochemical, anatomical, nutritional and molecular mechanisms associated to the plant tolerance against Cd toxicity were investigated in five tomato accessions with contrasting sensitivity to Cd exposure. Firstly, the data revealed that larger biomass loss was not always coupled to higher Cd concentration, indicating that other events, in addition to the internal Cd accumulation, impact tomato performance at early stages of Cd exposure. Secondly, the results indicated that the fine regulation of nutrient status, particularly magnesium (Mg), boron (B) and manganese (Mn), is associated to the mitigation of Cd toxicity. Magnesium status was coupled to the modulation of root development, resulting in changes in root hair formation and biomass allocation. Boron accumulation in leaves was linked to Cd toxicity, suggesting that tolerance mechanisms involved strategies to decrease or even avoid B excess in photosynthetic tissues. Disturbances in Mn status, i.e. Mn excess in leaves and Mn deficiency in roots, were also related to tomato sensitivity to Cd exposure. Thirdly, plant capacity to maintain leaf blade expansion is a relevant strategy for a better tomato development after short-term Cd exposure. Fourthly, tomato tolerance to Cd-induced stress does not depend on CAT activity enhancements in such conditions. In conclusion, tomato ability to quickly manage its nutritional status is necessary for alleviation of the Cd effects at early stages of exposure to this metal. The better understanding about tolerance mechanisms and mode of action of Cd toxicity in plants can help in the establishment of strategies to mitigate its impacts on crops.

Keywords: Boron excess, Cadmium, Manganese toxicity, Magnesium status, Root hair, *Solanum lycopersicum*

1. Introduction

Several vegetables are commonly grown near urban and industrial areas where anthropogenic activities have increased the concentration of different contaminants (Kabata-Pendias, 2011; Carvalho, 2017). In such areas, the use of wastewater for plant cultivation is an alternative to minimize economic, environmental, and social impacts on cities (Hernández-Chover et al., 2018; Rehman et al., 2018), but this strategy can further enhance the concentration of pollutants, such as heavy metals, in the growth media (Fu and Wang, 2011). Since crop cultivation in metal-contaminated regions is generally not prohibited, farmers may grow them in such polluted areas unless plant growth and yield are impacted. This is the case of maize plants, which exhibited no signs of cadmium (Cd) side-effects and produced grains with low Cd concentration after a long-term exposure to this metal (Kato et al., 2019). However, Cd toxicity and concentration varies among organs, plant species and their genotypes (Kovacevic and Vragolovic, 2011; Carvalho et al., 2018a). In this context, the selection of appropriate plant materials is a key aspect to allow a suitable crop development, yield and food quality when plants are grown in non-optimum conditions.

Variations in the Cd toxicity degree were previously observed in different tomato genotypes (Hussain et al., 2015; Carvalho et al., 2018a, b, c; Piotto et al., 2018), but the mechanisms behind the differential tolerance against Cd toxicity are scarcely understood. However, a better understanding about the plant defense mechanisms is crucial for the establishment of strategies to mitigate Cd-induced impacts on crops. In general, the prevention of oxidative stress is the most studied aspect in plants under Cd exposure (Fidalgo et al., 2011; Gallego et al., 2012; Cuypers et al., 2016; Bayçu et al., 2017ab; Borges et al., 2018), but it is not always the first protective strategy to be used (Weber et al., 2006). It has 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). Accordingly, increased nitrogen (N) and sulfur (S) uptakes were associated to protective mechanisms against Cd toxicity through the production of cysteine-rich compounds, namely glutathione, phytochelatin and metallothionein (Khan et al., 2016; Yamaguchi et al., 2016; Gielen et al., 2017).

In leaves, high manganese (Mn) or zinc (Zn) accumulations alleviated Cd-induced damages by supporting chloroplast integrity and photosynthetic activity (Cherif et al., 2012; Rahman et al., 2016).

However, 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 (Souza et al., 2018). The positive outcome of differential Mg status management against Cd toxicity is emerging, but information about this phenomenon is highly contradictory. For instance, low Mg status was coupled to a better development of rice (Chou et al., 2011) and tomato plants under Cd exposure (Carvalho et al., 2018a, b, c). By contrast, high Mg accumulation was associated to the alleviation of Cd-induced damages in Japanese mustard (Kashem and Kaway, 2007) and barley (Kudo et al., 2015). Since modifications in the plant mineral profile have been coupled to tolerance mechanisms against Cd toxicity (Hermans et al., 2011; Borišev et al., 2016; Sebastian and Prasad, 2016), in this study the nutrient composition in hydroponic solution was not varied (except by the CdCl₂ addition) in order to avoid extra outcomes from nutrient excess or starvation. Moreover, tomato accessions with contrasting sensitivity to Cd exposure were used to investigate plant tolerance against Cd toxicity.

The present study aimed to identify the mechanisms that are associated to the differential tolerance degree of tomato accessions to Cd exposure. We show that the ability of tomato plants to quickly manage its nutritional status, especially Mg, B and Mn, is important and necessary for alleviation of the Cd effects at early stages of exposure to this metal. The better understanding of both tolerance mechanisms and mode of action of Cd toxicity in plants can help in the establishment of strategies to mitigate its impacts on crop yield. In addition, both the identification and confirmation of genotypes with higher tolerance to Cd toxicity is an important step for programs of tomato breeding.

2. Materials and methods

2.1. Plant material and growth conditions

Five tomato accessions with contrasting tolerance to Cd exposure were selected according to the work of Piotto et al. (2018). The group of tolerant plants comprised the tomato (*S. lycopersicum*) cultivars Indigo Rose and Yoshimatsu. The group of sensitive plants comprised *S. lycopersicum* var. *cerasiforme* (CNPH0920), the tomato cultivar Tropic Two Orders, and the tomato relative *S. pimpinellifolium* (LA0122). These genotypes showed varied biomass production when grown in Cd-free hydroponic solution, exhibiting from 176.67 to 243.33 g plant⁻¹ dry weight (DW) (Piotto et al., 2018). Under Cd exposure for seven days, distinct impacts on the biomass production were observed; the tolerance index for both tolerant cultivars was 0.91, but varied from 0.23 to 0.07 for the sensitive accessions (Piotto et al., 2018).

During the entire trial, seeds/plants were grown in a greenhouse with natural light, temperature and air humidity conditions. Before the sowing of tomato seeds, they were chemically scarified in 2% HCl (v:v) for 15 min, under agitation, in order to synchronize germination. Subsequently, seeds were sown in polystyrene trays with thin exfoliated vermiculite and watered 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 $78.9 \pm 5.22\%$, respectively. After seedling emergence, daily application of macro- and micronutrients (Peters Professional 20-20-20 at 1 g L^{-1}) was applied in order to maintain adequate seedling development. After one week, the concentration was increased to 1.5 g L^{-1} , which was maintained until the 18-days-old seedlings were transplanted to the hydroponics system.

Seedlings were removed from the trays, the roots were rinsed and then transferred to a hydroponic system (tanks) containing nutrient solution (Hoagland and Arnon, 1950) at 10% ionic strength which is used for adult tomato plants. Seedlings were placed 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 the 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 the increased concentration of salts in solution.

Twenty-four-day-old plants (three/four-leaf stage) were then subjected to Cd exposure by adding $35 \text{ }\mu\text{M CdCl}_2$ to the nutrient solution, which was monitored through electrical conductivity and pH at 1.2 mS cm^{-2} and 6.54 ± 0.07 average values, respectively. The Cd concentration chosen and used in this study was based on the work of Piotto et al. (2018), who indicated $35 \text{ }\mu\text{M CdCl}_2$ as a suitable metal concentration to be employed in studies related to the tomato tolerance/sensitivity to Cd toxicity after a short-time period of plant exposure to this metal. Seedlings were grown under control (Cd free) and Cd-containing hydroponic solution for 6 days. This period was chosen since it is sufficient to detect the onset of the most frequently toxicity symptoms (i.e. chlorosis, necrosis and decreased height) in tolerant accessions, but avoiding severe damages to sensitive genotypes. During the experiment, distilled-deionized water was added to the tanks daily, in order to replace water lost through evapotranspiration. Homogeneous distribution of nutrient solution and suitable oxygenation level were maintained by air pump systems in each tank.

2.2. *Plant biometry*

The length (cm) of stems and the longest roots were measured with a ruler. The stem diameter (mm) was measured in the region immediately above the cotyledons (or their scars) using a digital caliper. For leaf area (cm²) determination, 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 °C) until a constant weight was achieved for dry mass determination. The specific leaf area [leaf area divided by leaf dry weight (cm² g⁻¹)] was also calculated. All growth analyses and related variables were obtained from nine plants, which were used to calculate the average value for each of the three replicates.

2.3. *Chlorophyll content*

Chlorophyll content was indirectly calculated by using a chlorophyll meter SPAD equipment (Konica Minolta, SPAD-502 model). Two measurements were obtained from the middle third of the largest terminal leaflet of two youngest and fully expanded leaves of three plants, which composed each replicate (i.e. 12 evaluations per experimental unit).

2.4. *Lipid peroxidation, H₂O₂ production and antioxidant enzyme activities*

In order to evaluate the oxidative stress triggered by Cd exposure, malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) 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 six plants that composed each of the three replications. In the greenhouse, leaves were collected, immediately frozen in liquid nitrogen, and stored in an ultra-freezer (-80 °C) for further analyses.

Samples were then ground to a fine powder in liquid nitrogen. Lipid peroxidation was measured as MDA content as described by [Heath and Packer \(1968\)](#), and hydrogen peroxide content was determined as described by [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. \(2015\)](#).

2.5. *Cd, Mg, Mn, Fe and B concentrations*

The oven-dried samples were milled to determine Cd, magnesium (Mg), iron (Fe), manganese (Mn), and boron (B) concentrations through inductively coupled plasma optical emission spectrometry (ICP-OES), which was preceded by nitro-perchloric digestion of samples. Three replicates, each composed of three plants, were used. All procedures were carried out at the Instituto Agronômico de Campinas (IAC, Campinas, Brazil).

2.6. Tolerance index

Tolerance index (TI) calculation was based on [Piotto et al. \(2018\)](#), according to the following formula:

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

Where $DWfCdi$ = dry weight of plants of i accession under exposure 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 indicates the maximum tolerance.

2.7. RNA extraction, primer design, cDNA synthesis and RT-qPCR analyses

Samples (three biological replicates from each treatment) stored in the ultrafreezer were ground in liquid nitrogen to a fine powder and total RNA was extracted from roots and leaves (~ 100 mg each) by using TRIzol (Invitrogen). RNA quantity and quality were quantified spectrophotometrically (NanoDrop) and analyzed by electrophoresis in denaturing agarose (1%) gels in TAE (1x) buffer. Total RNA sample (1 µg) was treated with DNAase I and rRNasin RNase Inhibitor (Promega) following the manufacturer's instructions. cDNA synthesis (1 µg) was performed using SuperScript III First-Strand Synthesis System kit according to manufacturer's instructions. cDNA quality was verified by amplification through conventional PCR using primers specific for tomato tubulin gene (forward – AACCTCCATTCAGGAGATGTTT, and reverse – TCTGCTGTAGCATCCTGGTATT). Primers were designed through Primer 3 Input v4.0.0 software ([Untergasser et al., 2012](#)) based on sequences found in SGN database ([Fernandez-Pozo et al., 2015](#)). Primer specificity was checked through basic local alignment search tool – BLAST ([NCBI, 2017](#)). RT-qPCR was used to estimate gene expression analyses that were performed, using two technical replicates, in root samples (*S. lycopersicum* cvs. Yoshimatsu and Indigo Rose, and *S. pimpinellifolium* LA0122).

The 10- μ L RT-qPCRs contained 2 μ L cDNA (10x diluted), 0.3 μ L gene-specific primers (so, totalizing 0.6 μ L - 300 nM, based on the final volume, of forward or reverse primer), 5 μ L Fast SYBR™ Green Master Mix (Thermo Fisher Scientific) and 2.4 μ L RNase free water. The amplification reactions were conducted in a StepOne™ System thermocycler (Thermo Fisher Scientific), programmed for an initial step at 95 °C for 20 s, followed by 40 cycles at 95 ° for 3 s and 60 °C for 30 s. Among the evaluated reference genes, *Glyceraldehyde 3-Phosphate Dehydrogenase* (*GAPDH*; GenBank U97257; forward – GATGTCTCCGTTGTCGATCTT, and reverse – CAAGATACCCTTCAATTTACCCTCT), *Actin2* (*ACT2*; Solyc11g005330; forward – TGAGTCACACTGTCCCTATTTACG, and reverse – GAGGATCTTCATCAGGTTATCAGTTAAA) were selected for data normalization in roots (*GAPDH* + *ACT2*), as indicated by GrayNorm software (Remans et al., 2014). Cq (quantification cycle) was used to determine the expression of Mg transporter gene (*MGT*; Solyc09g008140.2; forward – TACTGAGGAGGAGTTTTCTGGAC, and reverse – CAAAGCGACCACCCTGTTTT) according to procedures of Remans et al. (2014).

2.8. Root anatomy

Anatomical analyses were performed in roots of 30-day-old seedlings (i.e. after six days under Cd exposure) from the tolerant tomato cvs. Indigo Rose and Yoshimatsu, as well as from the sensitive accessions LA0122 and Tropic Two Orders. Samples were obtained from the root tips (1-2 mm). They were then 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, samples were dehydrated by an increasing ethanol series (from 35 to 100%) followed by 100% butanol.

The infiltration was performed slowly in butanol: infiltration medium (3:1, 1:1, 1:2 – Histo-resin kit, hydroxymethacrylate, Leica, Heidelberg, Germany) at 4 °C, 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 μ m) were obtained in a rotary microtome (Leica RM 2155 rotary microtome, Leica, Nussloch, Germany), stained with 1% acid fuchsin in water and 0.05% toluidine blue (Feder and O'Brien, 1968), and covered with slip and Entellan®. The images were recorded in a light microscope (Leica LMD 7000, Leica, Wetzlar, Germany).

2.9. Statistical analysis

A completely randomized design was used in the experiment, which employed a factorial scheme 5 x 2 (accessions vs Cd concentrations, respectively) with 3 replications, totalizing 30 experimental units that were composed by 12 plants used for growth (three), anatomical (three), biochemical and molecular analyses (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). If ANOVA presented significant p -values, the data were subjected to Tukey test ($p \leq 0.05$) for comparison of means among treatments. In addition, some variables were transformed when indicated by the “Guided data analysis” tool to meet the ANOVA assumptions (SAS Institute, 2011). This tool also indicated some outliers or influential data, which were removed before ANOVA (SAS Institute, 2011).

3. Results

3.1. Cd accumulation in vegetative organs

Twenty-four-day-old tomato accessions with contrasting tolerance degrees to Cd toxicity were grown in hydroponic solution containing 0 or 35 μM CdCl₂ for six days. Roots of tomato genotypes Tropic Two Orders (TTO) and LA0122 (LA0) exhibited the maximum (and similar) Cd concentrations, which were higher than those observed in Indigo Rose (IND), Yoshimatsu (YSH), and CNPH0920 (CNP) (Fig. 1a). Cadmium concentration was variable in stems of tomato genotypes: IND exhibited the highest value, while YSH and CNP presented the lowest ones (Fig. 1a). TTO and LA0 had intermediary Cd concentration when compared to the others tomato accessions (Fig. 1a). In leaves, the highest Cd concentration was observed in LA0, which exhibited similar value in comparison to TTO, but significantly higher than the values presented by the other tomato genotypes (Fig. 1a). YSH presented the lowest value for the leaf Cd concentration, but it did not differ from that detected in IND and CNP.

3.2. Tolerance index and plant biomass

YSH presented the highest tolerance index (Fig. 2a), which differed from genotypes TTO and LA0 due to major Cd-induced impacts on their biomass (Figs. 1b and 2b).

3.3. Leaf area and chlorophyll content

After Cd exposure, leaf area was only reduced in TTO and LA0 (Fig. 3a). The chlorophyll content exhibited a decrease trend in Cd-treated plants, but sensitive accessions had major reductions (Fig. 3a).

3.4. Indicators of oxidative stress in leaves

Increased lipid peroxidation was observed in TTO, LA0 and IND under Cd exposure (Fig. 3b). Hydrogen peroxide generation was stimulated in Cd-treated plants of LA0, IND and YSH (Fig. 3b).

3.5. Activity of SOD, CAT and GR in leaves

SOD activity was enhanced in LA0, TTO and IND, and CAT activity was either decreased (TTO and YSH) or unchanged under Cd exposure (Fig. 3c). GR activity was unaffected by Cd (not shown).

3.6. Mg, B, Mn and Fe status

Among all nutrients analyzed in tomato accessions, Mg concentration in roots was the only element that exhibited significant differences between contrasting genotypes groups, since sensitive genotypes (CNP, LA0, and TTO) exhibited increased Mg concentration, while tolerant accessions were able to maintain it to the levels observed in control plants (Fig. 2c). In this context, further investigation in the expression of gene *MGT* (a Mg transporter) was performed, and data revealed that its expression tended to increase in LA0122 while decreasing in tolerant accessions (Fig. 2d). Manganese concentration was reduced in roots of all tomato accessions under Cd exposure (Fig. 4). Except for YSH, B concentration was increased in roots of Cd-treated tomato (Fig. 4). After Cd exposure, Fe concentration varied according to tomato genotypes, decreasing (IND), increasing (CNP and LA0) or maintaining (YSH and TTO) its concentration in relation to the control plants (Fig. 4).

In leaves of Cd-treated plants, Mg concentration was increased in LA0 and TTO, but decreased in IND in comparison to the control plants (Fig. 4). Boron concentration in leaf tissues was only increased in CNP, LA0, and TTO under Cd exposure, while other accessions exhibited similar values to that observed in control plants (Fig. 4). An elevated Mn concentration was detected in CNP, LA0, TTO and YSH after Cd exposure (Fig. 4). In addition, the leaf Fe concentration was reduced in all tomato genotypes after plant growth in Cd-containing media (Fig. 4).

3.7. Alterations in root anatomy

After six days of plant exposure to 35 μM CdCl_2 , a high number of intercellular spaces (asterisks) was observed in the cortical region of tomato roots after Cd exposure, regardless genotype (Fig. 5). In addition, both sensitive accessions, TTO and LA0, presented an increased formation of root hair bulges (arrows) in the Cd-stressed plants when compared to the control plants (Fig. 5).

3.8 MGT expression

In spite of *MGT1* expression tended to increase in *S. pimpinelifolium* but to decrease in tolerant accessions under Cd exposure, no differences among the genotypes were detected (Fig. 2c).

4. Discussion

Tomato plants can be grown in different cultivation systems, such as hydroponics and aquaponics (Schmautz et al., 2016; Suhl et al., 2016), enabling its farming in urban centers and its surrounding areas. Using such soilless systems potentially allows the achievement of two great goals: reduction of environment impacts by the use of wastewaters, and decreases in the nutrient inputs due to the existence of high levels of elements essential for plant growth (Hernández-Chover et al., 2018; Rehman et al., 2018). Wastewaters, however, can contain elevated concentrations of heavy metals like Cd (Fu and Wang, 2011), which is highly toxic to living organism (Kabata-Pendias, 2011). In this context, the selection of appropriate genotypes is a key aspect to allow suitable crop development and management, and also to support breeding programs.

Tomato accessions tolerant to Cd exposure were previously identified (Piotto, 2012; Piotto et al., 2018), but the mechanism behind such tolerance are unknown. To this end, tomato accessions with contrasting tolerance to Cd toxicity were used to investigate the plant strategies to cope with Cd-induced impacts. The selection of genotypes was based on previous research that pointed out tomato YSH and IND as one of the most tolerant plants to short-term Cd exposure (seven days), whereas TTO, CNP and LA0 were classified as sensitive plants (Piotto, 2012; Piotto et al. 2018). The results revealed that variations in Cd accumulation, per se, did not explain totally the level of plant sensitivity to Cd toxicity. Tolerance mechanisms are particularly associated to the fine regulation of Mg, B and Mn status, as well as to the plant capacity to maintain leaf blade expansion under Cd exposure. All these aspects are discussed in the next sections.

4.1. Larger biomass loss, at organ level, is not always coupled to higher Cd concentration

Although the plant tolerance to non-essential elements is, in essence, directly related to the reduction in their internal accumulation, the behavior of tomato accessions with contrasting sensitivity to short Cd exposure is hardly explained by this statement at organ level (Carvalho et al., 2018c). For instance, tomato cv. Indigo Rose, which exhibited the highest Cd concentration in stems, presented just minor variations in stem biomass after Cd exposure (Fig. 1). However, genotypes with intermediary Cd concentration in stems exhibited significant biomass reductions in such organ (Fig. 1). Furthermore, despite CNPH classification as sensitive tomato (Piotto, 2012; Piotto et al., 2018), it exhibited a low Cd concentration in all vegetative organs (Fig. 1). Therefore, the data indicated that higher Cd concentrations are not always related to major biomass losses at organ level. This phenomenon can be observed in several tomato accessions under either short- or long-term Cd exposure (Piotto, 2012; Hussain et al., 2015; Alves et al., 2017; Carvalho et al. 2018a, b, c). Special cases involve even a better plant performance in the presence of “mild” Cd-induced stress, when compared to the plants cultivated in control conditions (i.e. non-contaminated growing media). Such phenomenon, named “hormetic effect”, was detected in *Lonicera japonica* (Jia et al., 2015), *Brassica napus* (Durenne et al., 2018) and tomato (Piotto et al., 2018). In other words, this also can clarify why seeds from the sensitive genotype presented no changes in their vigor, even after Cd accumulation (Carvalho et al. 2018b).

4.2. Reduced sensitivity to Cd toxicity is linked to B accumulation in photosynthetic tissues

Therefore, we can infer that additional events influence the magnitude of Cd toxicity, which generally triggers leaf chlorosis and necrosis (Carvalho et al., 2018c; Piotto et al., 2018), as observed in Cd-treated plants of all tomato genotypes (data not shown). Cadmium toxicity were previously related to B excess in leaves, in addition to the own Cd accumulation, at early stages of tomato exposure to this non-essential metal (Borges et al., 2019; Carvalho et al., 2018c). In line with these findings, only sensitive tomato accessions exhibited increased leaf B concentration (Fig. 4). According to Kaya et al. (2009), the visual toxicity symptoms of B excess in tomato leaves start as a yellow–green interveinal chlorosis followed by small patches of necrotic tissues, which developed first in the oldest leaves and progressed to the youngest. All these features are similar to the Cd-induced effects on tomato leaves (Piotto et al., 2018). Interestingly, different tomato genotypes seem to employ distinct mechanisms to mitigate B accumulation in leaves. For instance, tomato cv. Indigo Rose may decrease the root-to-leave B translocation (Fig. 4) by accumulating this micronutrient in stems (data not shown). By contrast, tomato cv. Yoshimatsu may avoid B excess by limiting its uptake (Fig. 4). Interestingly, an increased B accumulation in shoots was previously associated with elevations in the Mn concentration in the aerial

parts of plants under Mn-induced toxicity (Santos et al., 2017). Accordingly, Carvalho et al. (2018c) and Borges et al. (2019) provided evidences that Mn accumulation in leaves may enhance Cd-induced toxicity especially at high B concentrations.

4.3. High root-to-leaf Mn translocation can be both an enhancer of Cd toxicity or its alleviator

The tomato genotype with the lowest tolerance index also exhibited the highest Mn concentration in leaves simultaneously to increased B concentration (Fig. 2, and Fig. 4). Per se, the leaf Mn concentration in Cd-treated tomato seedlings can be a potential source of problems to the plants, since the threshold for Mn toxicity in leaves of tomato adult plants is 250 mg kg⁻¹ DW (Alvarenga, 2013), but the young tomato accessions under Cd exposure exhibited Mn concentrations that ranged from 308.4 to 401.1 mg kg⁻¹ DW (Fig. 4). Mn excess in leaves is mainly stored in chloroplasts (Ramos et al., 2002) where it can disorganize their lamellae (Lavres Junior et al., 2010), causing effects that resemble those reported in tomato grown in Cd-containing media (Gratão et al., 2009; Pompeu et al., 2017). Cadmium-treated tomato should also cope with root Mn deficiency, which can be related to reductions in the Mn uptake due to Cd and Mn competition for the same transporters (Sasaki et al., 2012; Wu et al., 2016) and/or Cd-induced changes in the root plasma membrane antiporter system (Migocka and Klobus, 2007). This decreased root Mn concentration is probably enhanced by Mn remobilization from roots to shoots, as corroborated by the increased root-to-leaf Mn translocation under Cd exposure (not shown). Interestingly, previous studies using different plant species evidenced that the increased shoot Mn accumulation is a protective mechanism to reduce Cd toxicity (Ramos et al., 2002; Zornoza et al., 2010; Liu et al., 2013; Rahman et al., 2016). We do consider this hypothesis, but this action of Mn may be limited because of the toxicity of high Mn concentrations (Carvalho et al., 2018c).

4.4. Increased tolerance depends on control of Mg status, which adjusts the root development

Therefore, the association of protective mechanisms to the regulation of macronutrients status, rather than micronutrients, can be a good plant strategy. Curiously, only sensitive plants presented increased Mg concentration in roots (TTO, LA01, CNPH), while only tolerant accessions were able to maintain the root Mg concentration (Fig. 2c). High Mg supply triggers reduction in root growth while enhancing trichoblast initiation (Niu et al., 2014), as corroborated by the increased formation of root hair bulges in the sensitive tomato accessions (Fig. 5). Maybe this phenomenon can be related to the deregulation of nutrient acquisition. Elevated Mg concentration seems to enhance Cd toxicity degree, as shown by reductions in the plant biomass in barley under Cd exposure (Kudo et al., 2015). By

contrast, the low Mg status was associated to positive outcomes in rice (Chou et al., 2011), *Arabidopsis* (Hermans et al., 2011), and willow development under Cd exposure (Borišev et al., 2016). In addition, tomato capacity to mitigate the toxicity from short Cd exposure (Borges et al., 2019), and also to acclimate to long-term Cd exposure (Carvalho et al., 2018a) was probably associated with decreased Mg status in roots. Considering this information, Mg transporters were investigated in root tissues. Data provided evidences that *MGT1*, an orthologue of genes that encode transporters with high affinity for Mg^{2+} (Li et al., 2001; Chen et al., 2012), is a potential molecular target for the regulation of Mg absorption, since its expression tends to increase in *S. pimpinelifolium*, while decreasing in tolerant accessions. The lack of strong differences among tomato genotypes and growth conditions can be related to late evaluation (after six days of plant exposure).

4.5. Alleviation of reductions in the leaf area and chlorophyll content provide better performance

In comparison to roots, tomato leaves are more sensitive to heavy metal toxicity (Djebali et al., 2010; Branco-Neves et al., 2017; Borges et al., 2018). The problem is that leaves are the main source of photosynthates for tomato growth so that reduced leaf area and chlorophyll content, which are usual Cd side-effects, may negatively impact plant development. Accordingly, all sensitive tomato accessions exhibited major reductions in chlorophyll content in comparison to the tolerant plants (Fig. 3a). Regarding leaf area, although a lower transpiration surface can be a strategy to avoid both water losses and Cd translocation to leaves (Perfus-Barbeoch et al., 2002; Delpérée and Lutts, 2008; Lux et al., 2011; Gratão et al., 2015), a reduced dimension of leaves also means a decreased potential to harvest light for photosynthesis. This can explain why LA0 and TTO exhibited major biomass losses, while CNP, a sensitive accession that maintained the leaf area, presented a tolerance index that did not differ from the most tolerant genotype (Fig. 2). In addition, damages in the cell membranes may take part of the events that decrease the plant tolerance to Cd toxicity, since elevated lipid peroxidation was detected in leaves of the most sensitive accessions under Cd exposure (Fig. 3b). The overproduction of superoxide anions ($O_2^{\bullet-}$) was probably linked to the increase in MDA levels in tomato accessions under Cd exposure because of a high SOD activity was detected in all plants with increased lipid peroxidation. Since heavy metal-stressed plants are able to modulate enzymes of the antioxidant machinery to mitigate potential damages from oxidative challenges (Branco-Neves et al., 2017; Souza et al. 2018; Soares et al., 2018), the roles of CAT and GR were also evaluated.

4.6. *Enhancements in the CAT activity are not crucial for tomato tolerance to short Cd exposure*

Catalase activity was either preserved or even reduced at the sixth day of Cd exposure (Fig. 3), supporting previous reports that CAT does not play a crucial role in protecting plants against Cd toxicity, as shown in leaves of tobacco (Iannone et al., 2015) and other tomato accessions (Carvalho et al., 2018c). Possible explanations are either the overproduction of compounds that can reduce CAT activity, such as $O_2^{\cdot -}$ (Kono and Fridovich, 1982) that is frequently induced by Cd exposure (Iannone et al., 2015), or the existence of mechanisms that are dependent on intermediary even high H_2O_2 concentrations to enhance CAT activity (Kar, 2018). The last assumption meets the fact that CAT activity is only efficient when high H_2O_2 concentrations are present because CAT affinity for this ROS is relatively lower than that of other enzymes, such as ascorbate peroxidase – APX (see articles revised by Soares et al., 2018). Regarding GR, 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). The direct function of GR is to convert oxidized glutathione (GSSG) to reduced glutathione (GSH), which is a disulphide reductant that participates directly and indirectly in H_2O_2 detoxification through glutathione peroxidase (GPX) and APX activation (GSH is used for ascorbate regeneration), also reacting with another ROS (Soares et al., 2018). However, the leaf tissues of all tomato accessions did not exhibit modifications in GR activity after six days of plant exposure to Cd (Fig. 3), so potentially indicating the action of peroxidases that do not use GSH as a cofactor to combat oxidative challenges in leaves of tomato under short-term Cd-induced stress.

5. Conclusions

The use of tomato accessions with different tolerance degrees to Cd toxicity was a valuable approach to unveil tolerance and toxicity mechanisms in plants under short-term Cd exposure. It was shown that larger biomass losses, at organ level, are not always coupled to higher Cd concentration. We also provided evidences that plant capacity to regulate the Mg uptake supports a better tomato development by modulating root architecture and biomass partitioning. In shoots, tolerance mechanisms are associated to avoidance of B excess in leaves. Moreover, mitigation of Cd-induced disturbances in Mn status, i.e. Mn excess in leaves and Mn starvation in roots, may decrease Cd toxicity in tomato at the early stage of plant exposure. In addition, tolerance degree is associated with the plant capacity to maintain the leaf blade expansion when subjected to the short Cd exposure. However, the tolerance level to Cd exposure does not strictly depends on enhancements in CAT activity. Summarizing, this study showed that the ability of tomato plant to quickly manage its

nutritional status is necessary for the alleviation of Cd toxicity at early stages of exposure to this metal. The better understanding on the mode of action of Cd toxicity in plants can help in the establishment of strategies to mitigate its impacts on crops. Additionally, the identification and confirmation of genotypes with higher tolerance to Cd toxicity is an important step for programs of tomato breeding.

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Figure captions

Fig. 1. (a) Cadmium concentrations (mg kg^{-1} DW) in leaves, stems and roots, and (b) variations (%) in dry weight of organs from contrasting tomato accessions under exposure to $35 \mu\text{M CdCl}_2$ for six days (black columns). Distinct uppercase and lowercase letters denote significantly different means by Tukey test ($p \leq 0.05$) for comparisons of each accession in control and Cd-containing solutions, and for comparisons among accessions in the same growth condition, respectively. $n = 3$. Bars represent the standard errors of the means. Asterisks denote differences in relation to plants grown in Cd-free hydroponics.

Fig. 2. (a) Tolerance index and seedling dry weight, (b) variations in Mg concentration, and (c) expression of Mg transporter gene (*MGT*) in contrasting tomato accessions grown in hydroponic solution containing 0 or $35 \mu\text{M CdCl}_2$ for six days. Distinct letters denote significantly different means by Tukey test ($p \leq 0.05$). $n = 3$. Bars represent the standard errors of the means. Asterisks denote differences in relation to plants grown in Cd-free hydroponics (white columns). *GAPDH + ACT2* were used as internal standard. Tomato cv. Indigo Rose cultivated in Cd-free hydroponics was used as control for estimation of relative gene expression, and present values equal 1.0; so bars are not visible but standard errors are.

Fig. 3. (a) Variations (%) in chlorophyll content and leaf area, (b) lipid peroxidation - malondialdehyde (MDA) content (nmol g^{-1} FW) and H_2O_2 – hydrogen peroxide content ($\mu\text{mol g}^{-1}$ FW, b), and (c) SOD – superoxide activity (U SOD g^{-1} FW) and catalase - CAT activity ($\mu\text{mol min}^{-1} \text{g}^{-1}$ FW) in leaves of contrasting tomato accessions grown in hydroponic solution with 0 or $35 \mu\text{M CdCl}_2$ 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. $n = 3$. Bars represent the standard errors of the means. Asterisks denote differences in relation to plants grown in Cd-free hydroponics (white columns).

Fig. 4. Variations (%) in B, Mn and Fe concentrations (mg kg^{-1} DW) in leaves and roots of contrasting tomato accessions grown in Cd-containing hydroponic ($35 \mu\text{M CdCl}_2$) for six days, when compared to plants cultivated in Cd-free hydroponics. $n = 3$. Bars represent the standard errors of the means. Asterisks denote differences in relation to plants grown in Cd-free hydroponics.

Fig. 5. Longitudinal and transversal cross sections from root tips of tomato *Solanum pimpinellifolium* and *S. lycopersicum* cvs. Tropic Two Orders (both sensitive accessions), Indigo Rose and Yoshimatsu (both tolerant accessions) grown in hydroponic solution containing 0 or $35 \mu\text{M CdCl}_2$ for six days. Legends: ep: epidermis; co: cortex; pe: pericycle; vc: vascular cylinder; asterisk: intercellular spaces; arrows: root hairs. Bars: $100 \mu\text{m}$.

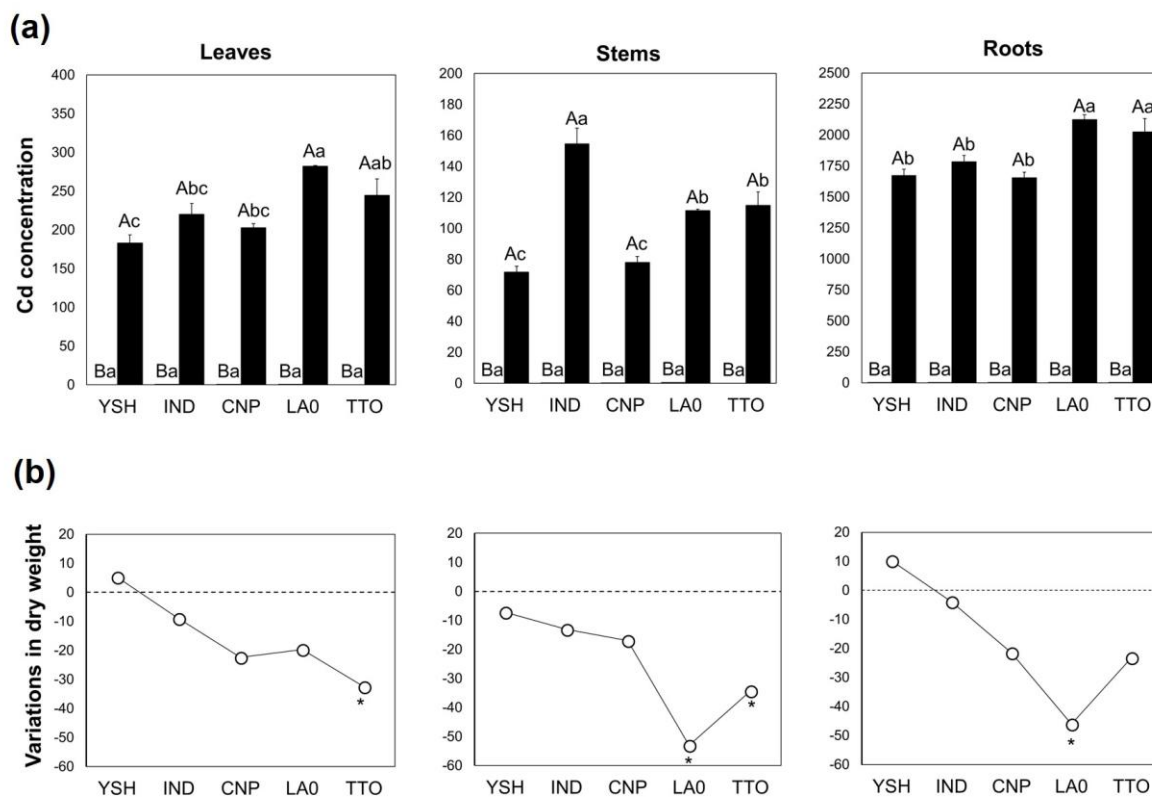


Fig. 1.

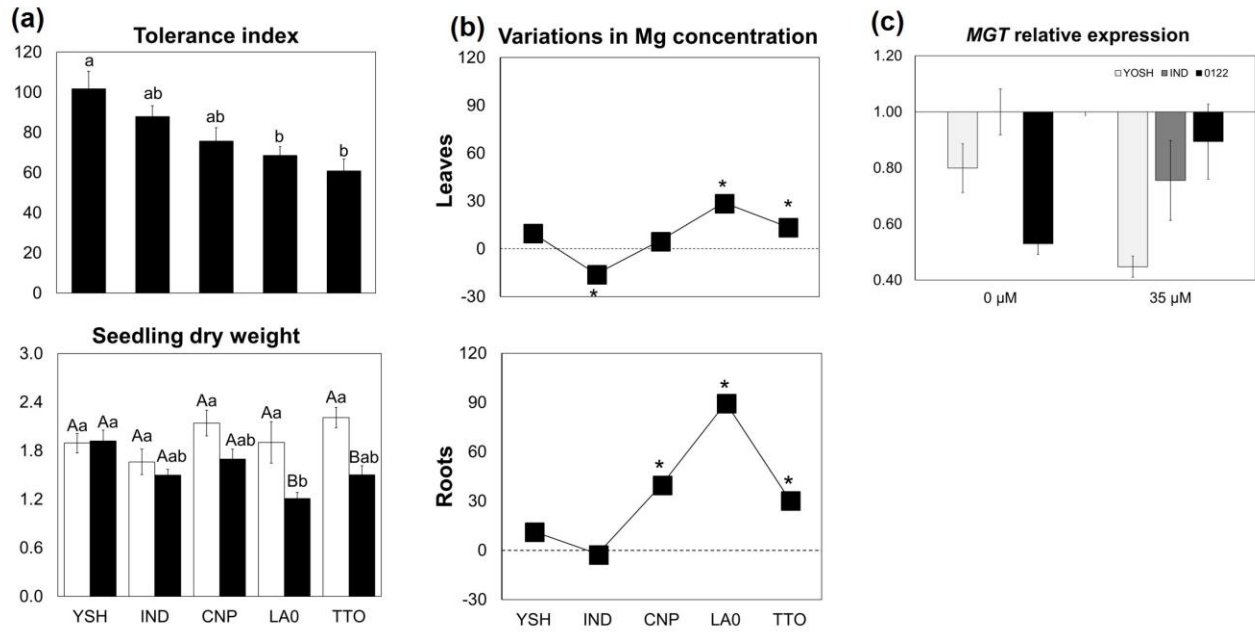


Fig. 2.

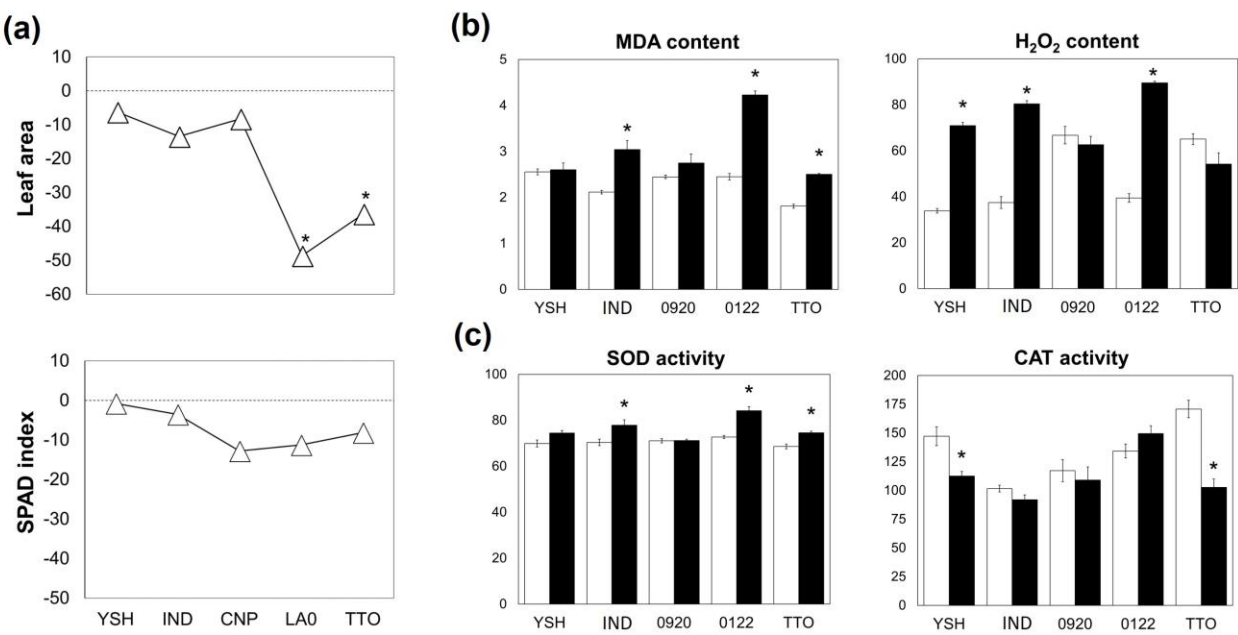


Fig. 3.

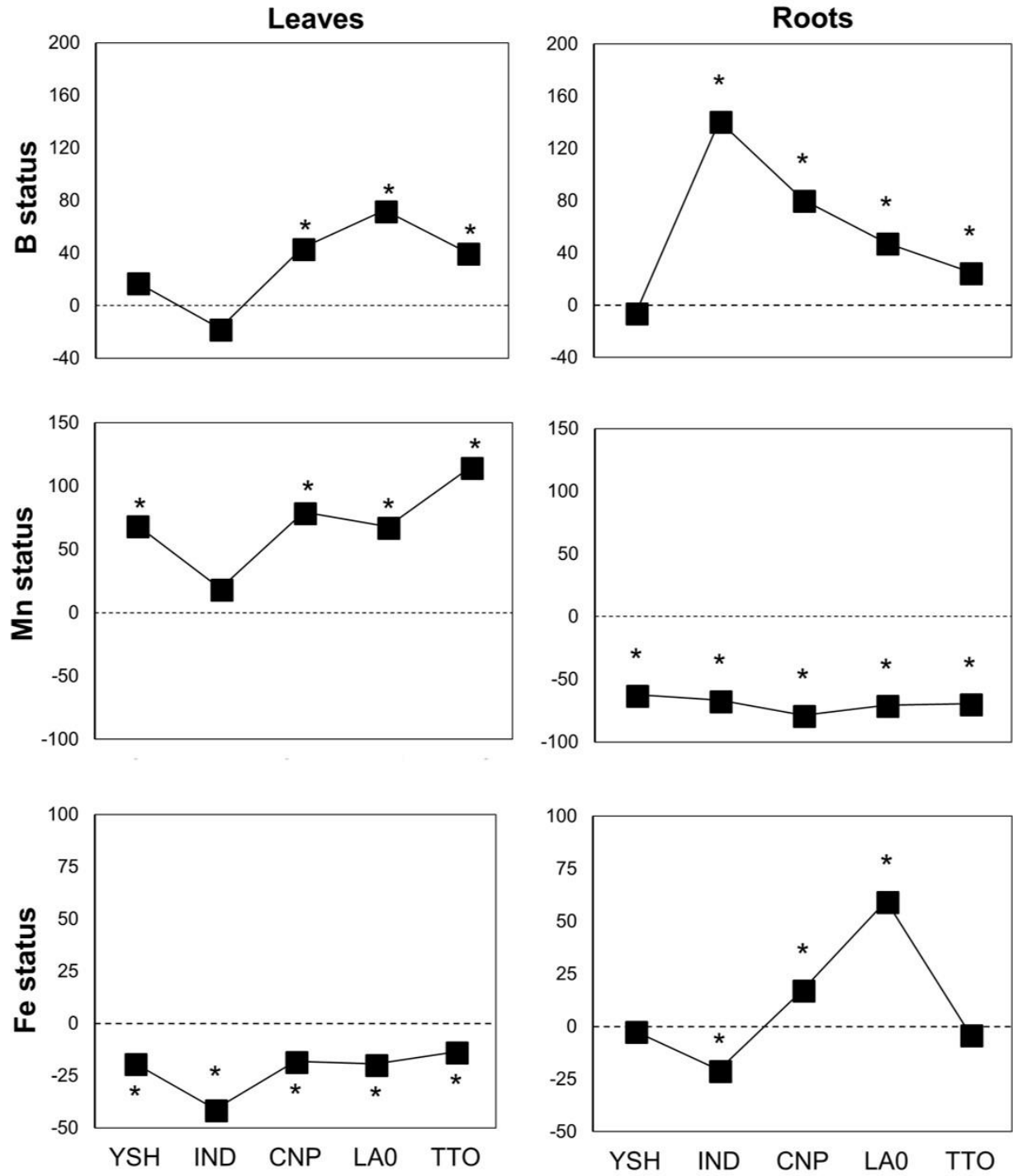


Fig. 4.

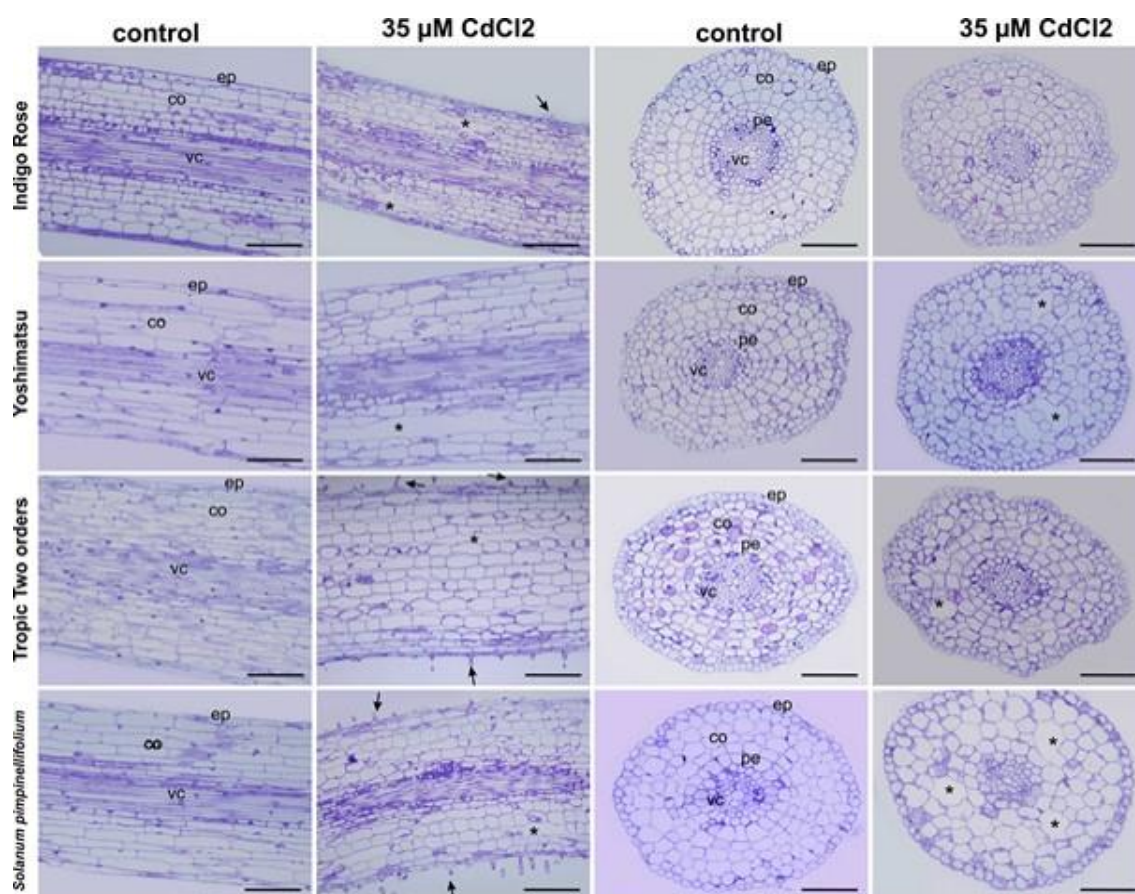


Fig. 5.