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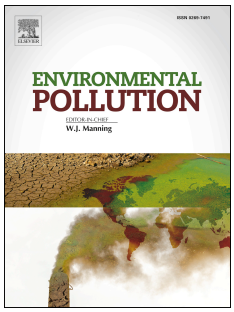

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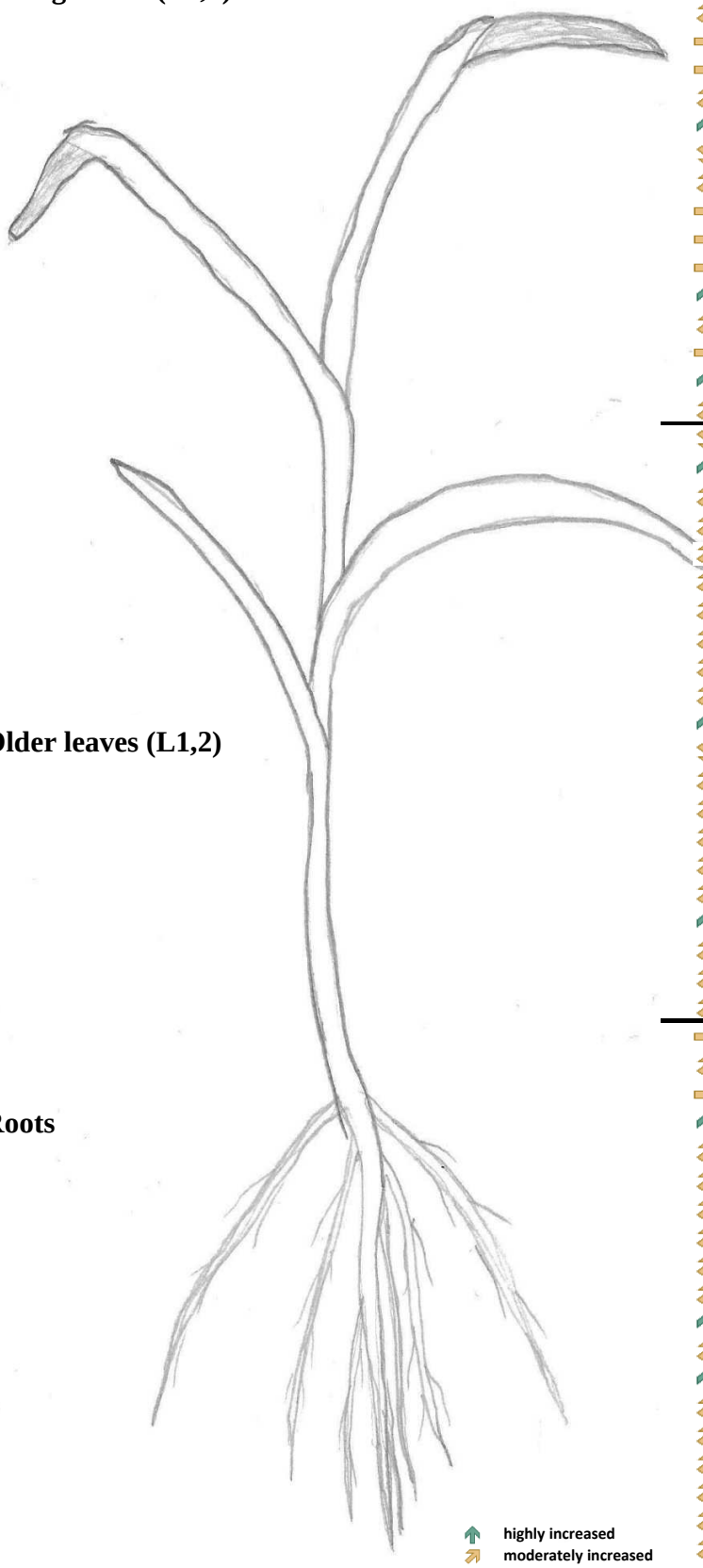

Zn	Ni	Cd	Cu	
↘	↘	↓	↘	DW
↑	→	↑	↗	Accumulation
↗	↑	↑	↑	H_2O_2
–	–	↑	↑	NADPH ox
↗	↗	↗	↗	EL
–	↗	↑	↑	MDA
↗	↗	–	↗	TAC
–	↑	↗	↑	Phenolics
–	–	–	–	Flavonoids
↗	–	–	–	Tocopherols
↑	↗	↑	↑	ASC
↘	↘	↘	↘	ASC redox
↗	↑	↑	–	APX
–	–	↗	↗	GSH
–	–	–	–	GSH redox
–	–	↗	↗	DHAR
↑	↗	↗	↗	MDHAR
↗	↗	↗	↗	GR
–	–	↗	↑	SOD
↑	–	↗	↗	POX
↗	↗	↗	↑	CAT
↘	↘	↘	–	DW
↑	↗	↗	↗	Accumulation
↗	↑	↗	↗	H_2O_2
↗	↗	↑	↑	NADPH ox
↗	↗	↗	↗	EL
↗	↗	↗	↗	MDA
↗	↗	↑	↗	TAC
↗	↗	↑	↑	Phenolics
↗	↗	↗	↗	Flavonoids
↗	↗	↗	↗	Tocopherols
↑	↗	↗	↗	ASC
↘	↘	↗	↘	ASC redox
↗	↑	↑	↑	APX
↗	↗	↗	↗	GSH
↗	↗	↗	↗	GSH redox
↗	↗	↗	↗	DHAR
↗	↗	↗	↗	MDHAR
↑	↗	↑	↗	GR
↗	↗	↗	↗	SOD
↗	↗	↗	↗	POX
↗	↗	↗	↗	CAT
–	–	–	–	DW
↗	–	–	↗	Accumulation
–	–	↗	–	H_2O_2
↑	↗	↗	↑	NADPH ox
↗	↗	↗	↘	EL
↗	↑	↗	↘	MDA
↗	↗	↗	↗	TAC
↗	↗	↗	↗	Phenolics
↗	↗	↗	↗	Flavonoids
↗	↗	↗	↘	Tocopherols
↑	↗	↗	↗	ASC
↗	↗	↘	↓	ASC redox
↑	↗	↗	↘	APX
↗	↗	↗	↘	GSH
↗	↗	↗	↘	GSH redox
↗	↗	↗	↗	DHAR
↗	↗	↗	↗	MDHAR
↗	↗	↗	↗	GR
↗	↗	↗	↘	SOD
↗	↗	↗	↗	POX
↑	↗	↗	↗	CAT

Maize roots and shoots show distinct profiles of oxidative stress and antioxidant defense under heavy metal toxicity

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ABSTRACT

Heavy metal accumulation in agricultural land causes crop production losses worldwide. Metal homeostasis within cells is tightly regulated. However, homeostasis breakdown leads to accumulation of reactive oxygen species (ROS). Overall plant fitness under stressful environment is determined by coordination between roots and shoots. But little is known about organ specific responses to heavy metals, whether it depends on the metal category (redox or non-redox reactive) and if these responses are associated with heavy metal accumulation in each organ or there are driven by other signals. Maize seedlings were subjected to sub-lethal concentrations of four metals (Zn, Ni, Cd and Cu) individually, and were quantified for growth, ABA level, and redox alterations in roots, mature leaves (L1,2) and young leaves (L3,4) at 14 and 21 days after sowing (DAS). The treatments caused significant increase in endogenous metal levels in all organs but to different degrees, where roots showed the highest levels. Biomass was significantly reduced under heavy metal stress. Although old leaves accumulated less heavy metal content than root, the reduction in their biomass (FW) was more pronounced. Metal exposure triggered ABA accumulation and stomatal closure mainly in older leaves, which consequently reduced photosynthesis. Heavy metals induced oxidative stress in the maize organs, but to different degrees. Tocopherols, polyphenols and flavonoids increased specifically in the shoot under Zn, Ni and Cu, while under Cd treatment they played a minor role. Under Cu and Cd stress, superoxide dismutase (SOD) and dehydroascorbate reductase (DHAR) activities were induced in the roots, however ascorbate peroxidase (APX) activity was only increased in the older leaves. Overall, it can be concluded that root and shoot organs specific responses to heavy metal toxicity are not only associated with heavy metal accumulation and they are specialized at the level of antioxidants to cope with.

Key words: Heavy metals; environmental pollution; maize organs; antioxidants; oxidative stress

63 INTRODUCTION
64
65 Heavy metal pollution is a serious problem to environment and human health. Heavy
66 metals are present naturally in soils, but anthropogenic activities such as urbanization,
67 industrialization and land-use change have significantly increased their prevalence to
68 toxic levels. Also, withering of rocks, mining and application of agrochemicals contribute
69 to increase of heavy metals in soil (Gambuś and Wieczorek, 2012; Mohammed et al.,
70 2011; Shahzad et al., 2018). Soil health is fundamental for producing food crops and
71 over-accumulation of heavy metals hinders plant productivity, and risks human health
72 via bio-magnification in food chains (Jaishankar et al., 2014; Mohammed et al., 2011).
73
74 Some of the heavy metals are required in minute quantities and have physiological
75 functions, while others are highly toxic even at low levels. Zn is an essential
76 microelement that acts as a cofactor of various metalloproteins such as anhydrases,
77 dehydrogenases and oxidases (Rout and Das, 2009). It takes part in the regulation of
78 nitrogen metabolism, photosynthesis and auxin biosynthesis (Broadley et al., 2007).
79 However, excessive levels of Zn cause growth inhibition, leaf curling, chlorosis, and leaf
80 tip necrosis (Broadley et al., 2007; Rout and Das, 2009). Similarly, Ni is an essential
81 component of the urease enzyme and is activator of nitrogen-fixing enzymes (Shahzad
82 et al., 2018). But high Ni concentrations cause chlorosis, necrosis and impairment of
83 nitrogen metabolism (Mishra and Kar, 1974; Nagajyoti et al., 2010). Cu is a key
84 structural component of plastocyanin protein of photosystem II (Cook et al., 1998), and
85 also acts as an activator of Cu/Zn superoxide dismutase (SOD) (Burzyński and Żurek,
86 2007; Yruela, 2005). However, excess Cu can bind to sulfhydryl groups of proteins
87 rendering them inactive (Yruela, 2005). As a result, Cu toxicity causes stunted plant
88 growth, leaf chlorosis and necrosis. Cd is a non-essential element and have no
89 physiological function. It interferes with other essential elements present in soil, reduce
90 growth of beneficial soil microflora and is extremely toxic to plants (Benavides et al.,
91 2005). Also, it retards plant growth by affecting plant-water relations via altering
92 stomatal movement and transpiration (Anjum et al., 2016a; Benavides et al., 2005).
93

Heavy metal uptake, translocation, and sequestration are key aspects of plant's life to cope with heavy metal toxicity. The physiochemical properties of heavy metals resemble with essential microelements, so their uptake is facilitated by plasma membrane transporters present in roots. The root-to-shoot transport of metals is carried out via xylem vessel with the help of transport proteins (DalCorso et al., 2013). Metallochaperones bind to metal ions and traffic them to metal-requiring proteins and cell organelles. Chelators such as phytochelatins, metallothioneins, amino acids and organic acids sequester extra metals into vacuoles (Callahan et al., 2007; Cobbett and Goldsbrough, 2002; Sharma and Dietz, 2006). Over-accumulation of heavy metals impairs the electron transport chain, functioning of mitochondria and chloroplast and disturbs redox homeostasis eventually leading to overproduction of reactive oxygen species (ROS) (Anjum et al., 2014; Chibuike and Obiora, 2014; Cuypers et al., 2010; Schützendübel and Polle, 2002). Noteworthy, redox-active heavy metals such as Cu, Fe and Ni directly catalyze the formation of ROS via Fenton reactions, while redox-inactive metals such as Cd, Zn, and Pb induce oxidative stress by depleting antioxidants (Valko et al., 2016). Cells deal with overwhelming ROS accumulation by executing antioxidant defense system. Antioxidant molecules (e.g. ascorbate, glutathione, tocopherols) and enzymes (superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR) participate to alleviate the deleterious effects of ROS (Hossain et al., 2012; Mishra et al., 2013). Depending on stress type and developmental stage plants execute organ-specific components of antioxidant arsenal to confront oxidative stress (AbdElgawad et al., 2016; Mishra et al., 2013).

Roots and shoots are spatially, physiologically and functionally distinct organs. Overall plant fitness is determined by coordination between root and shoot organs. Roots are the first organs to come in contact with metal contaminated soils. Roots restrict the entrance of heavy metals by undergoing several modifications including lignification and callose deposition that serve as an apoplastic and plasmodesmatal barrier, respectively (Cuypers et al. (2002). Maize is a widely grown and economically important cereal crop that is severely affected by different kinds of heavy metals, resulting in productivity

losses globally (Gu et al., 2019; Lu et al., 2015). Although it is well established that different types of stresses, exposure time, organs and developmental stages generate specific responses in plants (AbdElgawad et al., 2016; Kravchik and Bernstein, 2013; Lazof and Bernstein, 1999). However, systematic analysis comparing different types of heavy metals (redox and non-redox reactive) and responses triggered by them in the roots and shoots of crop plants were not performed.

The main questions of this study were first to set a direct comparison between the responses of different maize organs to the same heavy metal in terms of growth, physiological and biochemical parameters, second to reveal whether redox and non-redox reactive metals impose different responses in the different maize seedlings' organs and third whether the observed responses of the different maize organs are related to the accumulation of heavy metals within their tissues.

MATERIALS AND METHODS

Plant material and growth conditions

The maize (*Zea mays* L.) variety Giza 117 was used in this study. It's a commercial high yielding hybrid variety grown in large areas of Egypt, moreover, this variety showed moderate sensitivity to the tested heavy metals, so it has been used as a model. Seeds were sown on a filter paper saturated with distilled water and incubated at 26℃ in the dark. After three days, seedlings with uniform growth were selected and transplanted into soil potting mix (Tref EGO substrates, Moerdijk, The Netherlands, 20×20 cm pots) mixed with either Cadmium chloride ($CdCl_2$), Zinc chloride ($ZnCl_2$), Copper sulphate ($CuSO_4$) or Nickel sulphate ($NiSO_4$) to obtain concentrations of 16, 200, 400, and 50 mg salts kg^{-1} dry soil, respectively. These heavy metal concentrations were derived from a preliminary dose-response study targeting a 25% reduction of whole plant biomass reduction in maize seedlings (Supplementary Figure 1). Plants grown on the non-treated soil served as controls. Pots were moved to a growth cabinet maintained at

26/22℃ (day/night), 80/70% relative humidity, 16/8 h photoperiod. The roots, basal mature leaf-pair (L1,2) and distal young leaf-pair (L3,4) were harvested after 14 and 21 DAS (days after sowing), and immediately stored in liquid nitrogen for further biochemical analyses. Fresh weight (FW) was determined after quick gentle washing followed by water soaking between the folds of Whatman filter paper. The dry weight (DW) was determined after drying of samples in an oven at 70^{o}C for three days.

Photosynthesis and stomatal conductance

Light-saturated photosynthetic rates (µmol CO_2 m^{-2} s^{-1}) were measured (LI-COR LI-6400, LI-COR Inc., Lincoln, NE, USA) (AbdElgawad et al., 2015). Stomatal conductance (gs) was measured in situ on leaves (L1,2 and L3,4) with a Leaf Porometer (Model SC-1, Decagon Devices, Inc., Hopkins, Pullman, WA USA) at 14 and 21 DAS.

Abscisic acid content

ABA was extracted and quantified from the maize tissues as described previously (Qi et al., 1998). Briefly, the extracts were dried and methylated by adding diazomethane. Analyses were done using a GC-MS SIM (6890N network GC system) and 5973 network mass selective detector (Agilent Technologies, Palo Alto, CA, USA). The Lab-Base (ThermoQuset, Manchester, UK) data software was used for the ABA signal quantification.

Tissue and soil metal content

Leaves and roots were washed with $CaCl_2$ and deionized water to remove any apoplastic accumulated metal ions. One hundred mg dry weight (DW) of washed leaves and roots were digested in a 5:1 ratio of HNO_3/H_2O_2 in a microwave oven, and heavy metals were determined by mass spectrometry (ICP-MS, Finnigan Element XR, Scientific, Bremen, Germany). A mixture of standards was prepared in 1% (v/v) nitric acid (Hamad et al., 2015). Similarly, heavy metal content was determined in soil samples before sowing of seeds and after harvesting of plants and heavy metal content was expressed as µg/ g DW of soil.

H_2O_2 concentration

Fifty mg FW tissue were homogenized in 0.5 ml cold 0.1% TCA. After centrifugation for 30 min at 4 ^oC, 50 µl extract was used to measure hydrogen peroxide (H_2O_2) concentration by mixing with the xylenol orange dye reagent (Jiang et al., 1990), based on the peroxide-mediated oxidation of Fe^{2+}. After 45 min incubation, the Fe^{3+}-xylenol orange complex was measured at 595 nm. The standard curve was obtained by diluting 30% H_2O_2. Specificity for H_2O_2 was ascertained by comparing samples with CAT treated samples.

NADPH Oxidase

NADPH oxidase (ROS generating enzyme) was extracted from 100 mg fresh tissues in 1 ml ice-cold potassium phosphate buffer (50mM, pH 7.0), containing 10% polyvinyl pyrrolidone (PVP), 0.25% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). The crude extract was precipitated with acetone (9:1 acetone:homogenate) at 20℃ for 15 min. Precipitated proteins were recovered by centrifugation at 14,000 g for 10 min at 4℃. Protein pellets were resuspended in Tris buff er (50 mM Tris-Cl, 0.1 mM MgCl2, 0.25 M Sucrose, 0.1% Triton-X-100, pH 8.0) and used to assay for NADPH-oxidases. Oxidase activities were calculated from the difference in NBT reduction using an extinction coefficient of 12.8 mM^{-1} cm^{-1} in the absence or presence of 50 U ml^{-1} superoxide dismutase (Sarath et al., 2007).

Electrolyte leakage

Electrolyte leakage (EL) was determined in roots and leaves as described previously (Lutts et al., 1995). Briefly, 1 cm^2 discs of leaf tissue and 1 cm long root segments were prepared, and rinsed three times with deionized water to remove solutes from surfaces. These discs and segments were incubated in 20 ml of deionized water at room

218 temperature for 18 h, and then boiled for 30 min to measure the maximum level of
219 leakage. Conductivity was measured at these time points with the help of conductivity
220 meter (WTW GmbH, Weilheim, Germany), and relative EL was calculated.
221
222 **Malondialdehyde**
223
224 Fifty mg tissue fresh weight (FW) was homogenized in 1 ml of 80% (v/v) ethanol using a
225 MagNALyser (Roche, Vilvoorde, Belgium). The extract was clarified by centrifugation
226 and the supernatant incubated with thiobarbituric acid (TBA), to produce the pinkish-red
227 chromogen, thiobarbituric acid-malondialdehyde (TBA-MDA). Absorbance was
228 measured at 440, 532 and 600 nm (micro-plate reader, Synergy Mx, Biotek Instruments
229 Inc., Vermont, VT, USA). MDA content was calculated and expressed as $nmol.g^{-1}$ FW
230 tissue (Hodges et al., 1999).
231
232 **Total antioxidant capacity**
233
234 Two hundred mg FW powdered frozen plant tissue was extracted in 2 ml ice-cold 80%
235 (w/w) ethanol. Total antioxidant capacity was measured by using a FRAP (ferric
236 reducing antioxidant power) assay with tripirydylo-S-triazine (TPTZ), 300 mM acetate
237 buffer (pH 3.6), 0.01 mM 2,4,6-tripirydylo-S-triazine (TPTZ) in 0.04 mM HCl, and 20 mM
238 $FeCl_3.6H_2O$ by measuring an OD at 600 nm using a microplate reader (Benzie and
239 Strain, 1999). Trolox was used as a standard.
240
241 **Polyphenols and flavonoids**
242
243 Polyphenols and flavonoids were extracted by homogenizing 50 mg FW plant tissue
244 with 80% ethanol (v/v). After centrifugation, the total phenolic content was determined in
245 the clear supernatant by using a Folin-Ciocalteu assay (Zhang et al., 2006), with gallic
246 acid as a standard. Flavonoid content was estimated using the modified aluminum
247 chloride method with quercetin as a standard (Chang et al., 2002).
248

Ascorbate and glutathione

One hundred mg FW plant tissue was ground in a MagNALyser (Roche, Vilvoorde, Belgium), and extracted in ice-cold 6% (v/v) phosphoric acid. Reduced ascorbate (ASC) and glutathione (GSH) contents were determined by HPLC (SPD-M10AVP, Shimadzu), and the identity of peaks was confirmed by using an in-line diode array detector (DAD) (Potters et al., 2004). Total ascorbate (tASC) and glutathione (tGSH) levels were determined after reducing samples with 40 mM DTT and the ascorbate and glutathione redox status were calculated as the ratio between reduced and total amounts (ASC/tASC, GSH/tGSH).

Tocopherols

Tocopherols were extracted in hexane (100 mg FW in 6 ml hexane), and centrifuged at 14,000g for 15 min. Extracts were dried (CentriVap concentrator, Labconco, Kansas, USA) and resuspended in hexane. Tocopherols were separated and quantified by HPLC (Shimadzu, Hertogenbosch, The Netherlands), normal phase conditions, Particil Pac 5 mm column material, length 250 mm, i.d. 4.6 mm, with dimethyl tocol (DMT) as internal standard (5 ppm). Data were analyzed with Shimadzu Class VP 6.14 software.

Enzyme activities

100 mg FW frozen plant tissue were homogenized by a MagNALyser (Roche, Vilvoorde, Belgium) in 1 ml buffer (50 mM potassium phosphate, pH 7.0), 10% (w/v) polyvinyl pyrrolidone (PVP), 0.25% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ASC. After centrifugation for 10 min at 13200 rpm at 4^{o}C, the supernatant was used to estimate the activities of superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). SOD activity was determined according to Dhindsa et al. (1982) by measuring the inhibition of nitroblue tetrazolium (NBT)

reduction at 560 nm. POX activity was determined by the oxidation of pyrogallol (ε_{430} = 2.47 $mM^{-1}.cm^{-1}$) (Kumar and Khan, 1982). CAT activity was assayed according to Aebi (1984) by monitoring the decomposition of H_2O_2 at 240 nm (ε_{240}= 0.0436 $mM^{-1}.cm^{-1}$). APX, MDHAR, DHAR and GR activities were measured as suggested previously (Murshed et al., 2008). GPX activity was assayed by measuring the decrease in NADPH absorbance at 340 nm (ε_{340}= 6.22 $mM^{-1}.cm^{-1}$) (Drotar et al., 1985). NADPH-oxidase was assayed according to (Sarath et al., 2007), where NADPH-dependent superoxide generation was measured by the reduction rate of nitroblue tetrazolium into monoformazan at 530 nm. Assays were scaled down to semi-high throughput using a micro-plate reader (Synergy Mx, Biotek Instruments Inc., Vermont, USA), and optimized to obtain linear time and protein concentration dependence.

Protein concentration

The soluble protein content of tissues was estimated in the enzymes extract according to the method of Lowry et al. (1951).

Statistical analysis

Results were analyzed by one way ANOVA (SPSS 16.0 software), and significant differences between means of parameters (n=8) when comparing the treatments with their respective controls were determined using the Duncan test ($P < 0.05$). Heat maps were generated with Multi Experiment Viewer (MeV) TM4 software (Dana-Farber Cancer Institute, Boston, USA) by using Euclidian distance metric. Principal component analysis (PCA) was performed with Origin Lab 9 software (Origin Lab, Northampton, MA, USA). The average values of oxidative and antioxidant parameters quantified in the root and shoot organs exposed to four heavy metals for two-time points were used to make biplots. The PCA allowed identification of main associations among variables that are responsible for the total variability of the studied data.

RESULTS

Effects of metal toxicity on growth

A comparative analysis was performed to elucidate the effects of different heavy metals (Zn, Ni, Cd and Cu) on growth of roots, older (L12) and young leaves (L3,4) at 14 and 21 DAS of maize seedlings. All heavy metals caused significant decreases in dry weight and fresh biomass of roots and older leaves (L1,2) at 14 DAS and 21 DAS (Figure 1). Cd had the strongest growth reducing effect on root DW (33 % inhibition at 14 DAS and 47% inhibition at 21 DAS). DW of L1,2 was reduced to the same extent by all metals at 14 DAS and 21 DAS. Heavy metal exposure showed no significant effect on the younger leaves, even after prolonged exposure (21 DAS). Heavy metals significantly reduced FW, particularly the older leaves (1,2) and root FW was also reduced but to a lesser extent either at 14 DAS or 21 DAS. No distinction could be observed between the effect pf redox reactive and non-redox reactive metals, however, roots being the most affected organ at all time points.

Heavy metal content

To illustrate the relationship between the reduction in DW and FW and the accumulation of the heavy metals in the tissues, heavy metal contents in the different organs were estimated. Under heavy metal stress, Zn and Cu accumulated to significantly higher levels in all plant parts after 14 and 21 DAS. Both of Ni and Cd, showed significant accumulation in roots as compared to control plants, but only Cd also accumulated in old leaves (L1,2) after 14 DAS as well as after 21 DAS. The highest fold increase can be observed in Cd accumulation in roots at 21 DAS (Figure 2). Ni is the fasted to move within the maize seedlings, it accumulated in L3,4 early (at 14 DAS) where no other element showed similar accumulation. The concentration of all the tested heavy metals in the soil samples decreased after the seedlings growth as compared to their concentration before sowing the seeds (Supplementary Table 1).

342
343 **ABA content**
344 To test whether the metal-induced stomatal closure (Figure 3B, explained below) is
345 regulated by ABA level and whether the effect of heavy metals is organ and
346 developmental stage specific, we measure ABA level in stressed organs. Our results
347 showed that the content of ABA in roots, L1,2 and L3,4 very much increased (hundreds
348 of times fold) under the effect of any of the tested heavy metals as compared to ABA
349 content in control tissues. This effect was observed early at 14 DAS and ABA content
350 did not increase further at later stage (21 DAS). The increase in ABA was highest under
351 Ni and Cd treatments in all organs (Figure 3A). This indicates that ABA accumulation is
352 a universal response to metal toxicity, and variation in the degree of ABA accumulation
353 suggests its redox-independent mode of action.
354
355 **Photosynthesis and Stomatal conductance**
356 To address the effects of the observed increase in ABA accumulation in leaves of heavy
357 metals-stressed plants, we analyzed the stomatal conductance and photosynthetic rates
358 of the plants. Stomatal conductance of L1,2 and L3,4 was reduced significantly in metal
359 stressed plants as compared to controls both after 14 and 21 DAS. Ni and Cd had the
360 strongest effect on stomatal conductance on both leaf groups at 14 DAS and even
361 stronger reduction at 21 DAS (Figure 3B). Photosynthesis rate, however, was declined
362 by any of the tested heavy metals. A stronger reduction in photosynthesis was observed
363 in L1,2 compared to L3,4 only at FH (Figure 3C). The lower stomatal conductance
364 under Ni and Cd effect is linked to the observed highest levels of ABA in their leaves.
365 This was reflected as low accumulation of these metals in the old leaves, especially for
366 Ni. Moreover, the immobilization of Cd in roots could be also explained by stomatal
367 closure and slow root to shoot transport which in turn is a consequence of ABA
368 accumulation in leaves.
369
370 **Oxidative stress markers**
371 To test whether the observed effects of the heavy metals on organs growth is
372 associated with oxidative stress and to reveal whether redox active and non-redox

active metals have different effects, oxidative stress markers (H_2O_2, NADPH oxidase and MDA contents as well as EL) were assayed. Induction of oxidative stress by heavy metal exposure was monitored at the level of ROS production and cellular damage. H_2O_2 levels increased significantly with all metal treatments and in all organs, at 21 DAS, but less pronounced in the younger leaves (Figure 4A). This suggested that the Zn, Ni, Cd and Cu treatments induced oxidative stress in maize seedling organs.

NADPH oxidase activity constitutes an important source of stress-induced ROS formation. From the four heavy metals tested, only Cd and Cu markedly increased NADPH oxidase activity, in all organs, and at 14 and 21 DAS. Only after prolonged exposure in older leaves, also Zn and Ni increased NADPH oxidase activity (Figure 4B).

Heavy metal exposure increased EL in roots and old leaves, but not younger leaves, at 14 DAS but no further significant change at 21 DAS (Figure 4C). Increases in lipid peroxidation also occurred mostly in roots and older leaves and were more pronounced at 21 DAS.

The membrane damage as measured by MDA level was increased in root tissues under the effect of Ni and Cu at 14 DAS, and under Zn, Ni and Cu at 21 DAS relative to their controls (Figure 4D). MDA also increased in L1,2 with maximum values under Zn stress at 14 DAS and under Zn, Cd and Cu at 21 DAS.

To reveal whether the observed oxidative stress (e.g. lipid peroxides) is countered by the antioxidant systems (enzymatic and non-enzymatic) and to what degree, the content of non-enzymatic antioxidants and activities of antioxidant enzymes were estimated.

Non-enzymatic antioxidants

The overall scavenging activity was assessed as the total antioxidant capacity (TAC, Figure 5A). The TAC was induced in nearly all heavy metal treatments in all organs, and at 14 and 21 DAS. The response to Cd was somewhat distinct, in that there was no induction in roots, but a rather strong induction in older leaves. Also notable was that

403 the TAC increase was not progressive, i.e. induction at 21 DAS was not higher than at
404 14 DAS.
405
406 Total cellular antioxidant capacity results from a diverse group of molecules, with high
407 antioxidant properties. Zooming in on prominent groups, we analyzed polyphenols,
408 flavonoids, tocopherols, ascorbate and glutathione. Polyphenol concentrations
409 increased mostly at 21 DAS, for Ni, Cd and Cu, and all organs (Figure 5B). Zn exposure
410 had the highest effect on polyphenol levels in the roots. Flavonoid levels remained
411 unaltered in roots, but only increased under Ni, Cd and Cu exposure (Figure 5C).
412 Whereas polyphenols and flavonoids are mainly water-soluble antioxidants, tocopherols
413 are lipid radical scavengers, primarily located in chloroplast membranes. Tocopherols
414 showed little or no induction in roots, and were induced in older, but not in younger
415 leaves (Figure 5D).
416
417 Reduced ascorbate (ASC), increased, progressively, in roots under exposure to all
418 metals. In leaves, on the other hand, only Zn induced ASC levels (Figure 6A). A similar
419 pattern was observed in GSH, with increases in the roots (for all metals), and in the
420 leaves predominantly with Zn exposure (Figure 7A). The ascorbate and glutathione
421 redox status changed little or not in old and young leaves but decreased in roots under
422 heavy metal exposure (6B and 7B, respectively).
423
424 **H_2O_2-scavenging activities**
425
426 Heavy metal-induced APX activity, more prominent at 21 DAS, and more in roots and
427 young leaves (Figure 7C). Zn treatment most prominent and consistently increased APX
428 activity. Also, POX and CAT activities were induced by heavy metal treatment (Figure
429 8B & C), in all organs and most treatments, and again most prominently by Zn. SOD
430 activity, was remarkably little affected by the heavy metals, particularly in leaves. Only
431 Cd and Cu treatments increased root SOD activity at 21 DAS (Figure 8A).
432
433 **The ascorbate-glutathione cycle**

The role of ascorbate as an antioxidant is coupled to the cell's capacity to regenerate the reduced molecule (ASC). DHAR activity was induced in all organs at 14 and 21 DAS, but notably only in Cd and Cu exposure, not Zn and Ni (Figure 6D). The MDHAR activity was induced by some metals and in some organs at 14 DAS, and by nearly all treatments and in all organs by 21 DAS (Figure 6E). GR activity increased in nearly all heavy metal treatments and in roots as well as in young and old leaves (Figure 7C).

A summary of the growth, oxidative markers and antioxidants changes in the different organs of maize seedlings after 21 DAS under heavy metals stress is presented (Supplementary Figure 2). Zn and Ni altered growth and biochemical levels in maize seedlings organs. Although Cd and Cu also have effects on the levels of antioxidant metabolites and enzymes in all tested maize organs, they, however, impose more strongest and sharper changes on young leaves (L3,4) as compared to roots or older leaves (L1,2) or as compared to effects of Zn and Ni on L3,4. This also can be noticed in their effect on oxidative stress markers and dry weight reduction (Figures 1 and 4).

Principal component analysis (PCA)

The plots depict standardized scores along the first two components, together explaining 60.6% at 14 DAS (Figure 9A) and 58% at 21 DAS (Figure 9C) of the data variability. Roots are separated along the PCA1 (44.75% and 38.30% for 14 DAS and 21 DAS plants, respectively) while control plants were separated along the PCA2 (15.86% and 19.69%, for 14 DAS and 21 DAS plants, respectively). The pattern of distribution was more concentrated for roots at 21 DAS to the positive side of PC2 indicating more related responses in roots under the different heavy metals. Cd and Ni treatments were more related as compared to Zn and Cu at both 14 DAS and 21 DAS as it is clear from their pattern of distribution along the PC2. Overall PCA indicated that organs effects on plant responses to heavy metal were more separated as compared to the effect of the different heavy metal.

DISCUSSION

We investigated the effects of sub-lethal concentrations of Zn, Ni, Cd and Cu on redox metabolism, photosynthesis and mass accumulation in leaves and roots of maize seedlings, to address the hypothesis that responses depend on plant organs, developmental stage, as well as the category of the heavy metal (redox or non-redox reactive). Moreover, the comparison will reveal whether the effect of heavy metal is directly depending on its accumulation within the tissues. Therefore, the novelty of this work is that at the same time a direct comparison between the effects of different heavy metals of two categories on growth, photosynthesis and redox metabolism on two developmental stages of different plant organs.

The reduction in dry matter under the effect of heavy metals is obvious in roots and L1,2 leaves at the early stage (14 DAS) and becomes more pronounced at 21 DAS. This reflects the progressive accumulation of all studied heavy metals in roots first and later in the leaves especially the older ones (L1,2). This pattern is similar for all tested heavy metals; notably for Ni and Cd. However, the underlying mechanism is different with different metals as differences in biochemical parameters show and is discussed later. The reduction in dry mass could be due to a competition of these heavy metals with essential nutrients, thus limiting their uptake, and/or by inhibition of key metabolic enzymes. The immobilization of essential nutrients and competition between them and heavy metals has been reviewed (Bolan et al., 2003; DalCorso et al., 2013). Consistently, tomato plants under Ni treatment and two maize varieties under Cd treatment showed decreased shoot and root biomass mainly due to disturbance of essential elements absorption (Anjum et al., 2016b; Palacios et al., 1998). Fresh weight of roots was reduced under the effect of the tested heavy metals, this effect was more pronounced in old than young leaves. This indicates that the different heavy metals affected the water relations of the seedlings and resulted in reduced water uptake and transport to the shoot system. The reduction in fresh weight of roots and leaves triggered the accumulation of ABA in all seedling's parts. The accumulation of ABA in leaves resulted in stomatal closure which in turn negatively affected photosynthesis and

accumulation of dry matter as it is clear for roots and L1,2 leaves. The negative effect of heavy metals on water balance in plants is well documented (Barceló and Poschenrieder, 1990; Schat et al., 1997). ABA is a well-studied stress hormone that acts as long-distance signal (Mehrotra et al., 2014) that triggers a response in distal organs. Although ABA accumulated in L3,4 as in L1,2, and L3,4 shows similar reduction in gas exchange as L1,2 which means closure of stomata and lower photosynthetic rate, L3,4 did not show a reduction in dry mass under heavy metals stress. This could be explained by the fact that they are young and act as sink for photosynthates from older leaves. This would also explain why younger leaves (L3,4) did not show oxidative stress as discussed below. Our results show that Ni and Cd accumulated mainly in root tissues and little (Ni) or no accumulation in L3,4 (Cd), however, stomatal closure and reduction in fresh weight are obvious in L3,4 leaves where no accumulation occurs. This can be accounted for by the accumulation of ABA which then triggers protective mechanisms as shown by lower levels of oxidative stress markers in L3,4 leaves compared to roots and L1,2 leaves and discussed below. Moreover, our results show that the effect is not always associated with accumulation of the heavy metal. For example, Cu at 14 DAS is accumulated only in roots, however, reduction in their growth parameters and photosynthesis could be observed. These results indicate signaling of stress from roots and early response and acclimation in above soil organs. ABA could play at least part of this signaling mechanism. ABA might also reversibly reduce the uptake and accumulation of heavy metals by roots. Exogenous ABA reduced the uptake and accumulation of Zn in *Vitis vinifera* and enhanced its tolerance to toxic levels of Zn (Song et al., 2019). The results could not differentiate between redox and non-redox active heavy metals in their effects on growth and accumulation pattern. Zn (non-redox reactive) showed same accumulation pattern over time like that of Cu (redox reactive) and similar effect on dry mass accumulation in roots and leaves at the two studied time points.

No differences in oxidative stress parameters could be observed in organs under different metals at an early stage of growth (14 DAS), however, NADPH oxidase was preferentially induced by Cd and Cu in roots and L1,2 at 14 DAS and 21 DAS. The

induction of NADPH oxidase by Cd and Cu could have been mediated by ABA, which has been shown to enhance NADPH oxidase activity and hence H_2O_2 accumulation and propagation in guard cells (Sridharamurthy et al., 2014), thus resulting in stomatal closure and reducing water loss. Although only Ni accumulated in L3,4 tissues early at 14 DAS, low level of oxidative stress was induced in their tissues as it was indicated by the slight increase in H_2O_2, EL and lipid peroxidation, especially at 21 DAS. Young leaves are distinguished from roots and older leaves by being less exposed to oxidative stress. H_2O_2 could have played the signaling role since it accumulated early in L1,2 leaves and because of its relatively long lifespan and being uncharged hence its ability to cross membranes (Das and Roychoudhury, 2014). Priming or accumulation of H_2O_2 in tissues confers tolerance to plants against heavy metals by provoking antioxidant machinery in their tissues (Chao et al., 2008; Cuypers et al., 2016; Hu et al., 2009). H_2O_2 accumulation in roots could also serve as a protective mechanism under heavy metals stress. It was hypothesized that H_2O_2 accumulation in roots of bean seedlings under Zn and Cu stress and pine under Cu stress could be a protective mechanism through increased lignification of the root cell walls that could act as an apoplastic trap for the heavy metals (Cuypers et al., 2002; Schützendübel et al., 2001). Interestingly, the leaves of scions of non-stressed tomato plant grafted on rootstocks of Cd-stressed plants showed induction of oxidative stress markers. This could indicate oxidative stress signaling between the root and shoots of plants, however, the nature and dynamics of these signals are still to be elucidated (Gratão et al., 2015). The observed oxidative stress in maize organs was countered by increased levels of non-enzymatic antioxidants. Organ-specific responses were observed in response to Ni, Cd and Cu stress, where levels of tocopherols, flavonoids and polyphenols increased only in shoots but not the root system. To our knowledge, no organ-specific antioxidant responses have been reported to heavy metals exposure in plants. however, this result could be related to the time point of measurement and the mobility of the heavy metal in question rather than an organ-specific response. Zn, being of the highest mobility induced increase in non-enzymatic antioxidants in both shoot and root systems (Haslett et al., 2001; Mehes, 2013). Probably the less mobile Ni, Cd and Cu could have induced increase in non-enzymatic antioxidants in roots earlier and could have relieved to

558 normal levels by 14 DAS. Differences between redox reactive and non-redox reactive
559 metals in terms of inducing oxidative stress cannot be observed. This could be due to
560 differences in the active concentration in the tissues and/or differences in sensitivity of
561 organs to the different heavy metals. Antioxidant enzymes, POX, CAT, APX and GR
562 also showed increased levels in all maize seedling organs, emphasizing their role as
563 general enzymatic antioxidants in response to heavy metals-induced oxidative stress.
564 Many reports have shown increased activities of these enzymes under heavy metals
565 stress and abiotic stresses in general in different plant species (Cuypers et al., 2002;
566 Gill and Tuteja, 2010; Hu et al., 2009; Islam et al., 2014) and reviewed by Cuypers et al.
567 (2016). Older maize leaves showed increased APX activity after Ni treatment compared
568 to control plants, however, the activity decreased to normal levels as in control leaves
569 after 21 days of treatment (Kumar et al., 2007). SOD activity was increased only in roots
570 under Cd and Cu stress at 14 DAS. This could be due to the early accumulation of
571 these metals in root tissues, thus inducing oxidative stress and hence SOD activity
572 increased as protective mechanism. Cd does not reduce oxygen directly but rather
573 induces oxidative stress indirectly through enhancing NADPH oxidase activity.
574 Moreover, Cd increases the production of phytochelatins through stimulating
575 phytochelatin synthase activity. Phytochelatins chelate Cd and sequester it in vacuoles,
576 which depletes the GSH pool, negatively affecting the redox balance (Nazar et al.,
577 2012). In two maize cultivars, Cd resulted in oxidative stress and induced the SOD,
578 CAT, POX and APX activities in leaves (Anjum et al., 2015).
579 Overall, cluster analysis of the assayed oxidative stress markers and antioxidants
580 (Supplementary Figure 2) also clearly shows organ-specific response to the heavy
581 metal treatment. Treatments of L3,4 leaves at 14 and 21 DAS with any of the tested
582 metals comprise one cluster that is characterized by increased levels of non-enzymatic
583 antioxidant metabolites. Treated roots at 14 and 21 DAS form another cluster which
584 shows decreased non-enzymatic antioxidants but increased antioxidant enzymes POX,
585 GR and APX. A third cluster containing older leaves (L1,2) shows slight changes in the
586 different antioxidant metabolites and enzymes. PCA analysis also shows a clear
587 separation of response between the roots on one hand and aerial parts of the other
588 hand. Moreover, the response of L1,2 to heavy metals is distinguished from that of L3,4

589 especially at 21 DAS. Our results indicate that ascorbate metabolism in maize seedling
590 exposed to Zn and Ni seems to be independent of the glutathione metabolism since the
591 DHAR activity is not affected. Also, since the ascorbate redox status did not change
592 while reduced ascorbate increased and APX activity increased, then *de novo* synthesis
593 and ascorbate recycling were active, it confirms the independency of ascorbate
594 metabolism from glutathione metabolism under Zn and Ni treatment. The increased GR
595 activity indicates regeneration of reduced glutathione that could have been directly
596 oxidized by ROS (Gill et al., 2013).
597
598 Based on our results, we can conclude a mechanism of how the different metals
599 behaved and how maize seeding's organs responded. Since Cd and Cu accumulate
600 mainly in roots especially at early stages (14 DAS) of maize seedlings growth, they
601 impair water balance and induce ABA accumulation in roots and leaves which in turn
602 stimulates stomatal closure and ensues ROS accumulation. The early accumulation of
603 Ni, Cd and Cu in roots could have also resulted in oxidative stress in roots and
604 consequent H_2O_2 accumulation and transport to leaves where it stimulates ROS
605 detoxifying mechanisms which helped to mitigate the further oxidative stress that could
606 have resulted from the later transport and accumulation of Cd and Cu in leaves. The
607 main ROS detoxifying mechanism in leaves dependent on the non-enzymatic
608 antioxidants and the Cd, Cu did not stimulate specific responses in leaves rather they
609 triggered defense mechanisms at different amplitudes. Zn, being of the highest mobility
610 among the studied heavy metals, and Ni as the second most mobile element,
611 accumulated early in roots and at least older leaves and they impaired the water
612 balance in the seedlings that in turn triggered ABA synthesis and accumulation as well
613 as ABA transport to young leaves. ABA triggered stomatal closure and consequently,
614 imbalance light and dark reactions of photosynthesis and hence ROS accumulated in
615 leaves. Moreover, Zn and Ni induced oxidative stress directly by positively affecting
616 ROS forming enzymes and their negative effect on ROS scavenging systems, notably
617 affecting the balance between ASC and GSH metabolism. The effect of Zn is not
618 qualitatively distinguished from that of Ni, but they differ in their amplitude in the same
619 organ and their individual effect has a different amplitude in different organs.

Conclusion

Our initial hypothesis that redox reactive and non-redox reactive heavy metals would impose quantitative and qualitative differences in redox metabolism could not be validated. However, our results tempted us to group the tested heavy metals into Cd-Cu group and Zn-Ni group with regard to their antioxidant responses in maize seedling organs as indicated by enhancement of activities of NADHP oxidase in roots and L1,2, SOD in roots, DHAR in all organs and APX in older leaves under Cu and Cd stress as compared to those under Zn and Ni stress. In addition, young leaves were less affected by the oxidative stress induced by heavy metals probably because of their higher growth rate and higher cell division which means more metabolic dynamics, while older leaves and roots were most affected, pointing towards a role of ABA and ROS signaling. Contribution of signaling molecules between roots and above ground organs could also explain the uncoupling of accumulation of the heavy metals in an organ and the degree of growth retardation and redox disturbance in this organ. However, further investigations are needed to unfold these observed different weights of the contribution of ABA and ROS signaling in response and alleviation of heavy metals stress, and to identify the molecular mechanism underlying heavy metals stress responses in root and shoot organs in plants.

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Figure Legends

Figure 1: Effect of heavy metals (Zn, Ni, Cd and Cu) on A) dry weight (DW) and B) fresh weight (FW) of root (R), mature leaf pair (L1,2) and young leaf pair (L3,4) at 14 DAS and 21 DAS of maize seedlings. Values were means of at least three replicates and significant differences ($P<0.05$) between treatments and their respective control were indicated by different letters.

Figure 2: Effect of heavy metals (Zn, Ni, Cd and Cu) on A) ABA content of root (R), mature leaf pair (L1,2) and young leaf pair (L3,4), B) stomatal conductance and C) photosynthetic rate of root (R), mature leaf pair (L1,2) and young leaf pair (L3,4) at 14 DAS and 21 DAS of maize seedlings. Values were means of at least three replicates and significant differences ($P<0.05$) between treatments and their respective control were indicated by different letters.

Figure 3: Accumulation of Zn, Ni, Cd and Cu in root (R), mature leaf pair (L1,2) and young leaf pair (L3,4) at 14 DAS and 21 DAS of maize seedlings. Values were means of

881 at least three replicates and significant differences (P<0.05) between treatments and
882 their respective control were indicated by different letters.
883
884 **Figure 4:** Effect of heavy metals (Zn, Ni, Cd and Cu) on A) H_2O_2 , B) NADPH oxidase
885 activity, C) relative electrolyte leakage (EL), and D) lipid peroxidation measured as
886 malondialdehyde content of root (R), mature leaf pair (L1,2) and young leaf pair (L3,4)
887 at 14 DAS and 21 DAS of maize seedlings. Values were means of at least three
888 replicates and significant differences (P<0.05) between treatments and their respective
889 control were indicated by different letters.
890
891
892 **Figure 5:** Effect of heavy metals (Zn, Ni, Cd and Cu) on A) total antioxidant capacity
893 (TAC), B) polyphenols , C) flavonoids, D) tocopherols of root (R), mature leaf pair (L1,2)
894 and young leaf pair (L3,4) at 14 DAS and 21 DAS of maize seedlings. Values were
895 means of at least three replicates and significant differences (P<0.05) between
896 treatments and their respective control were indicated by different letters.
897
898 **Figure 6:** Effect of heavy metals (Zn, Ni, Cd and Cu) on A) reduced ascorbic acid
899 (ASC), B) ascorbate redox status (reduced/total ascorbate), C) ascorbate peroxidase
900 (APX), D) dehydroascorbate reductase (DHAR) and E) monodeydroascrobate
901 reductase (MDHAR) level in root (R), mature leaf pair (L1,2) and young leaf pair (L3,4)
902 at 14 DAS and 21 DAS of maize seedlings. Values were means of at least three
903 replicates and significant differences (P<0.05) between treatments and their respective
904 control were indicated by different letters.
905
906 **Figure 7:** Effect of heavy metals (Zn, Ni, Cd and Cu) on A) reduced glutathione (GSH),
907 B) glutathione redox status (reduced/total glutathione ratio), C) glutathione reductase
908 (GR) level in root (R), mature leaf pair (L1,2) and young leaf pair (L3,4) at 14 DAS and
909 21 DAS of maize seedlings. Values were means of at least three replicates and
910 significant differences (P<0.05) between treatments and their respective control were
911 indicated by different letters.

912
913 **Figure 8**: Effect of heavy metals (Zn, Ni, Cd and Cu) on A) superoxide dismutase
914 (SOD), B) peroxidase (POX), C) and catalase (CAT) activities in root (R), mature leaf
915 pair (L1,2) and young leaf pair (L3,4) at 14 DAS and 21 DAS of maize seedlings. Values
916 were means of at least three replicates and significant differences ($P<0.05$) between
917 treatments and their respective control were indicated by different letters.
918
919 **Figure 9**: Principal component analysis (PCA) of oxidative and antioxidant parameters
920 in root (R), mature leaf pair (L1,2) and young leaf pair (L3,4) at 14 DAS and 21 DAS of
921 maize seedlings. PCA plots separating the samples (A and C) and the measured
922 parameters (B and D).
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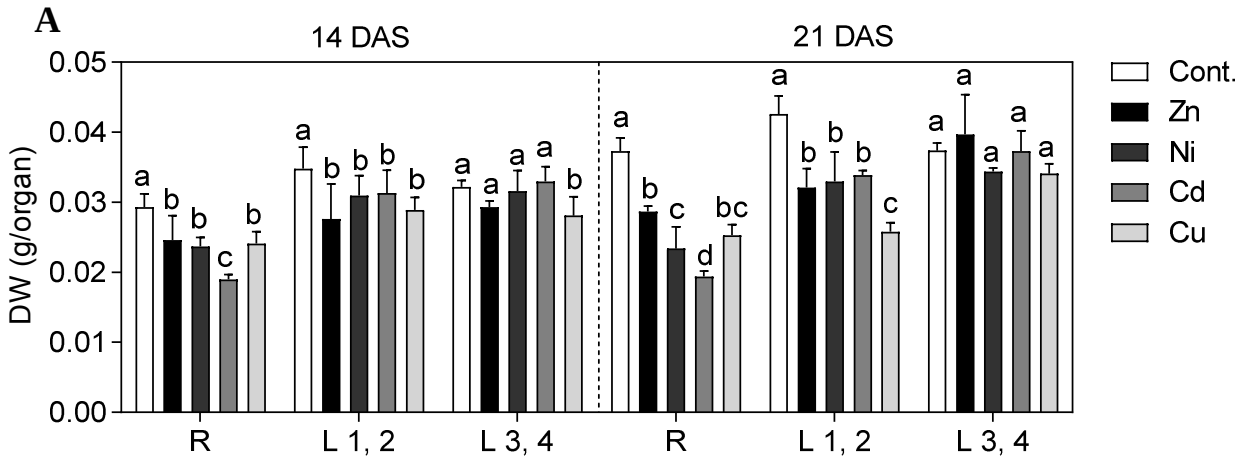
Figure 1


A
14 DAS
21 DAS
DW (g/organ)
0.05
0.04
0.03
0.02
0.01
0.00
R
L 1, 2
L 3, 4
Cont.
Zn
Ni
Cd
Cu

B

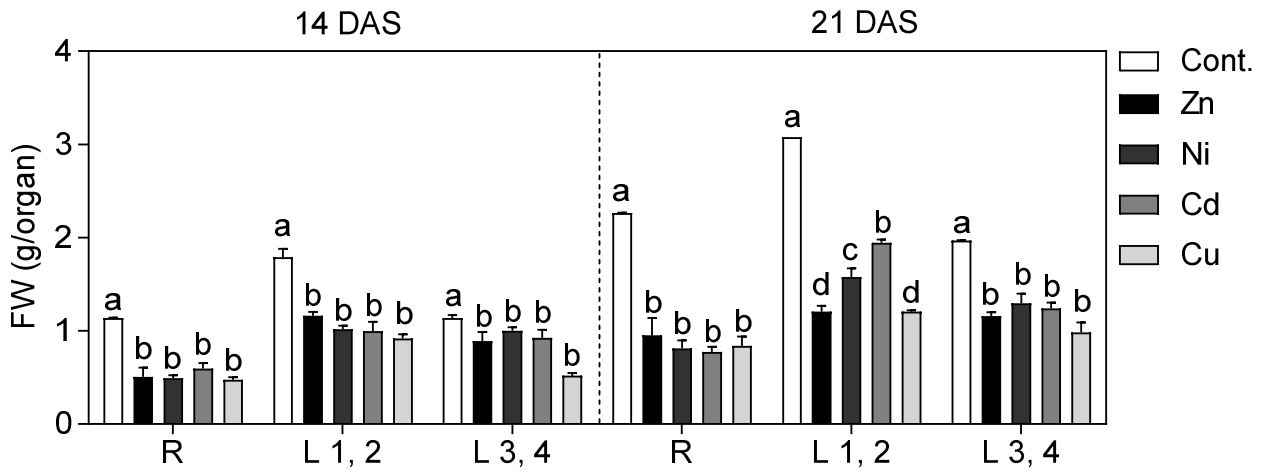
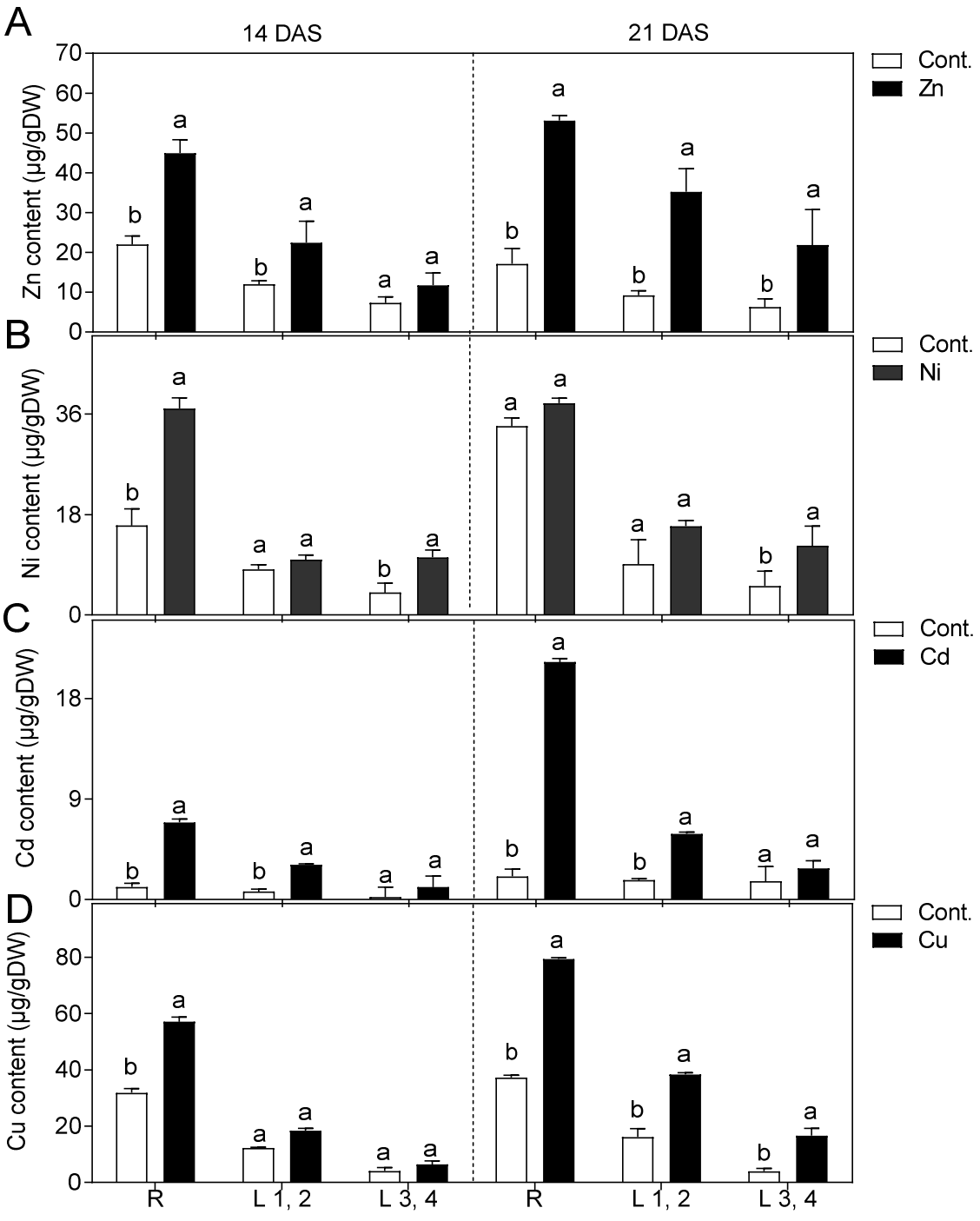


Figure 2



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969 **Figure 3**

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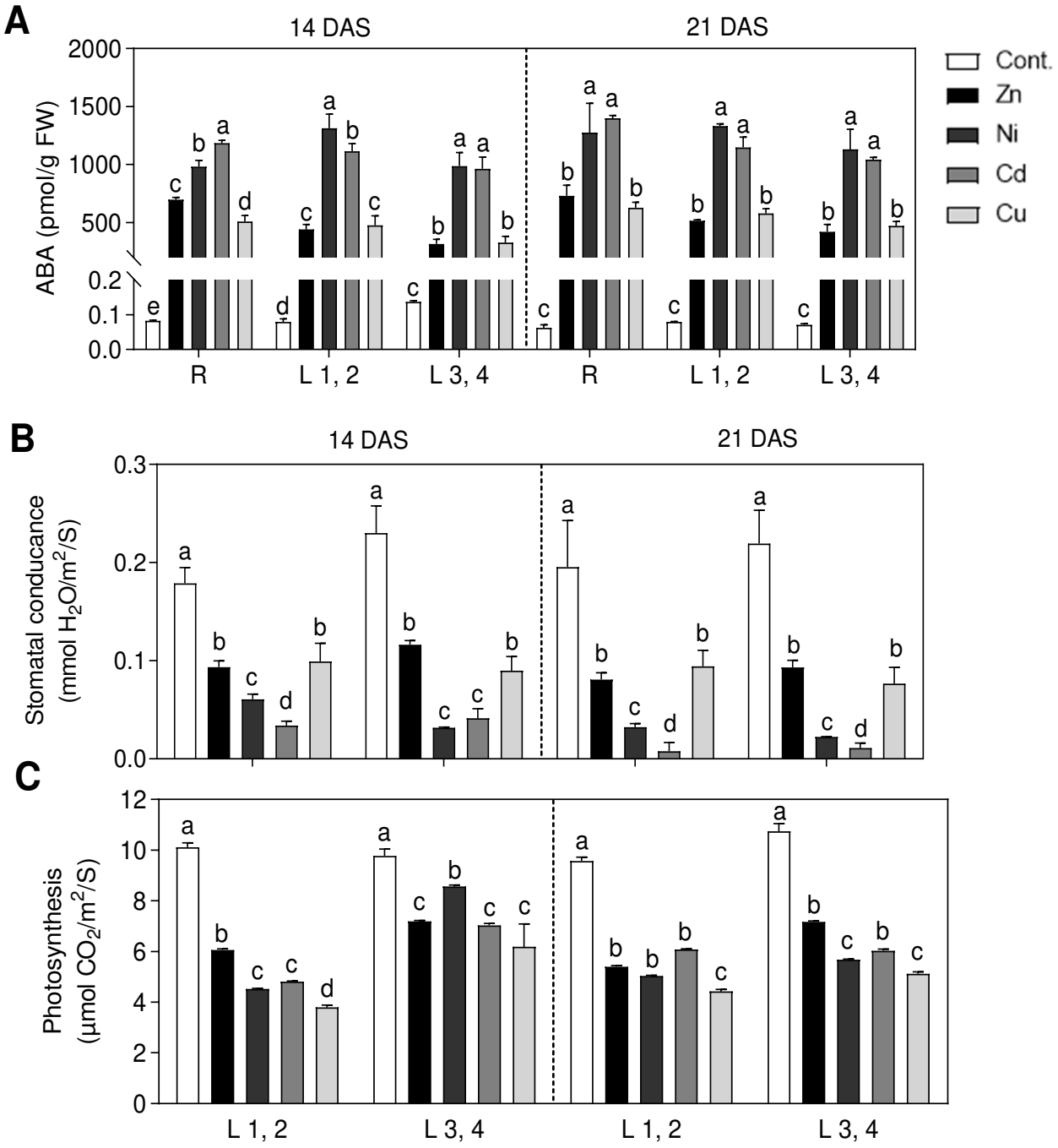

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975 **Figure 4**

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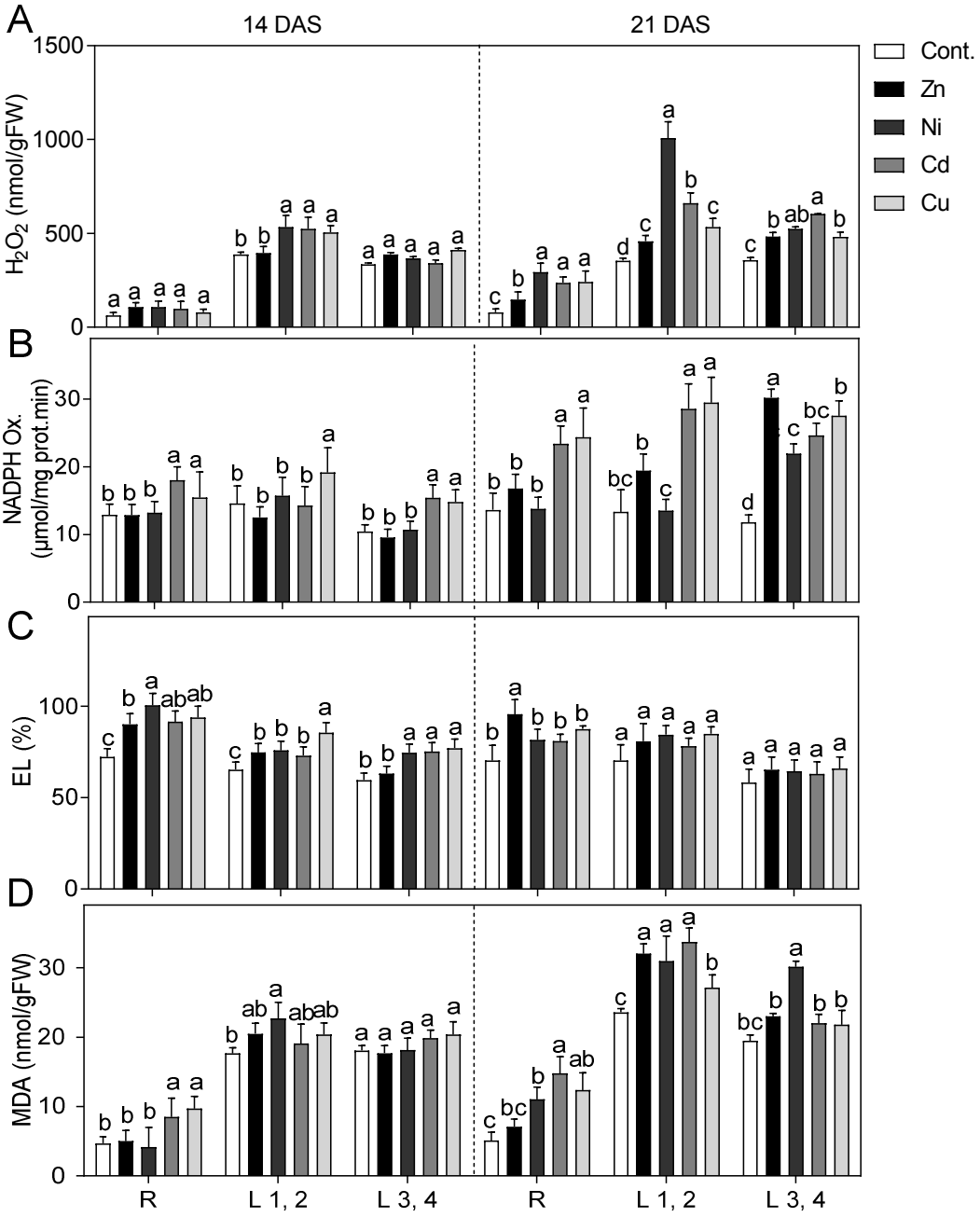

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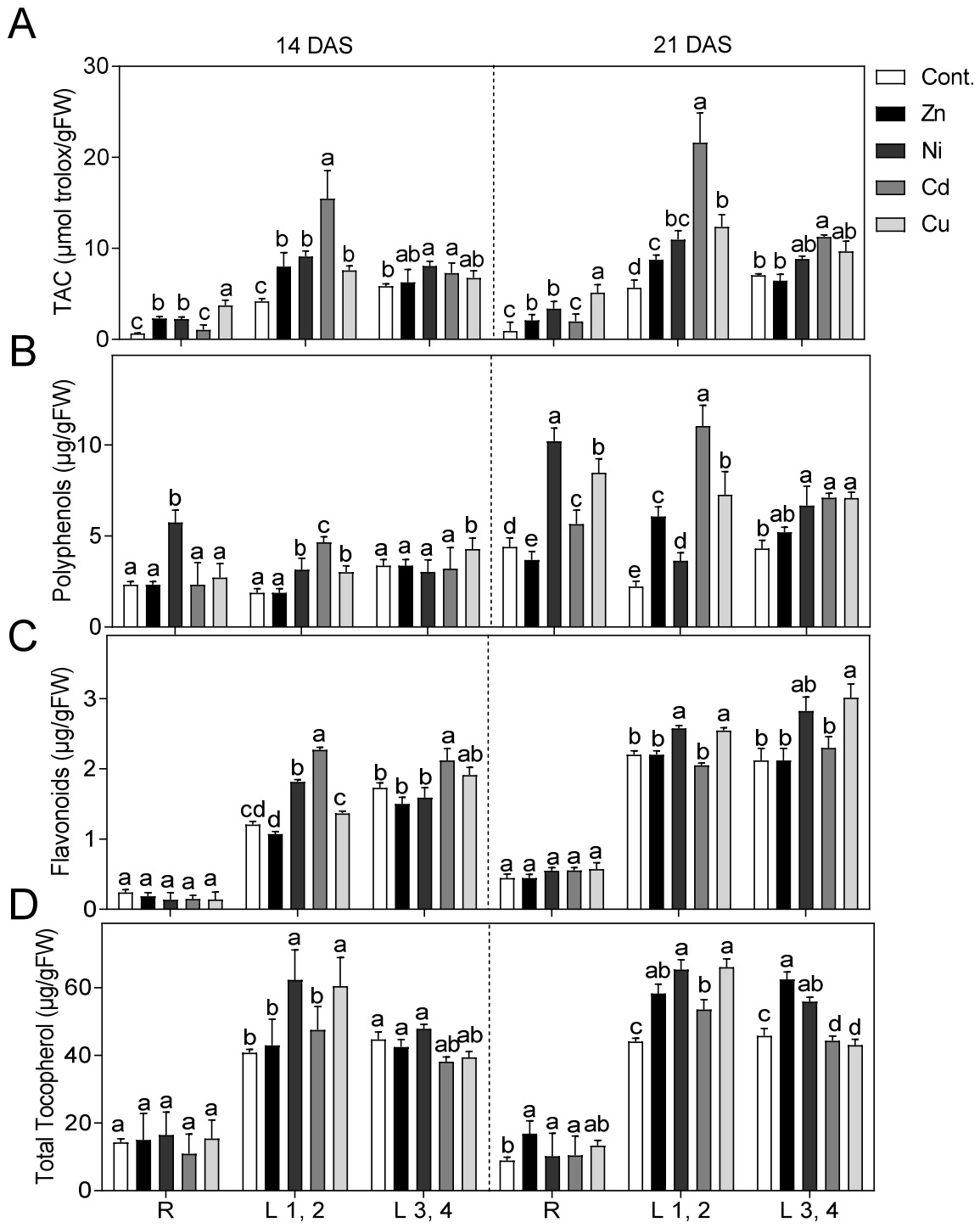
981 **Figure 5**



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985 **Figure 6**

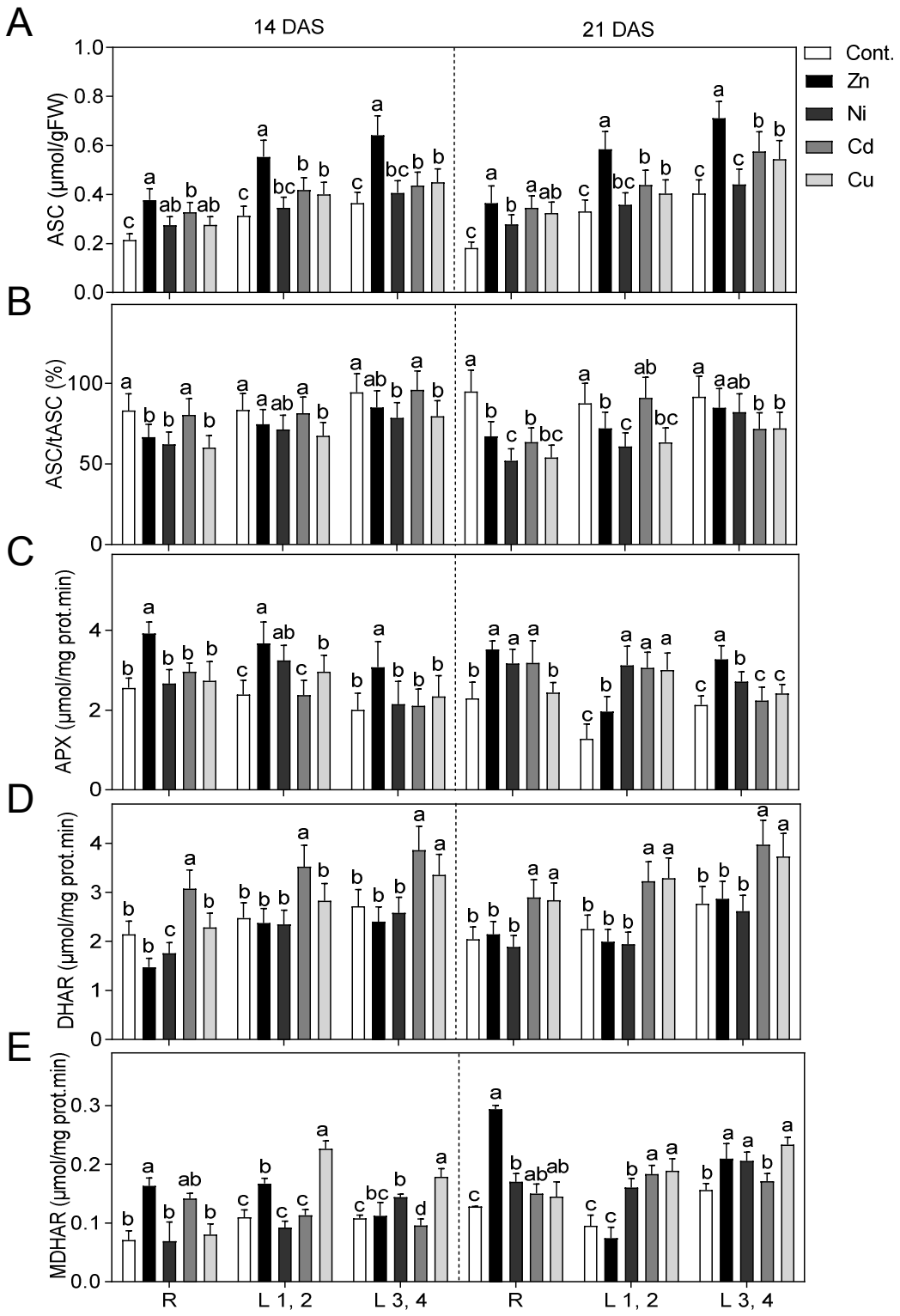

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Figure 7

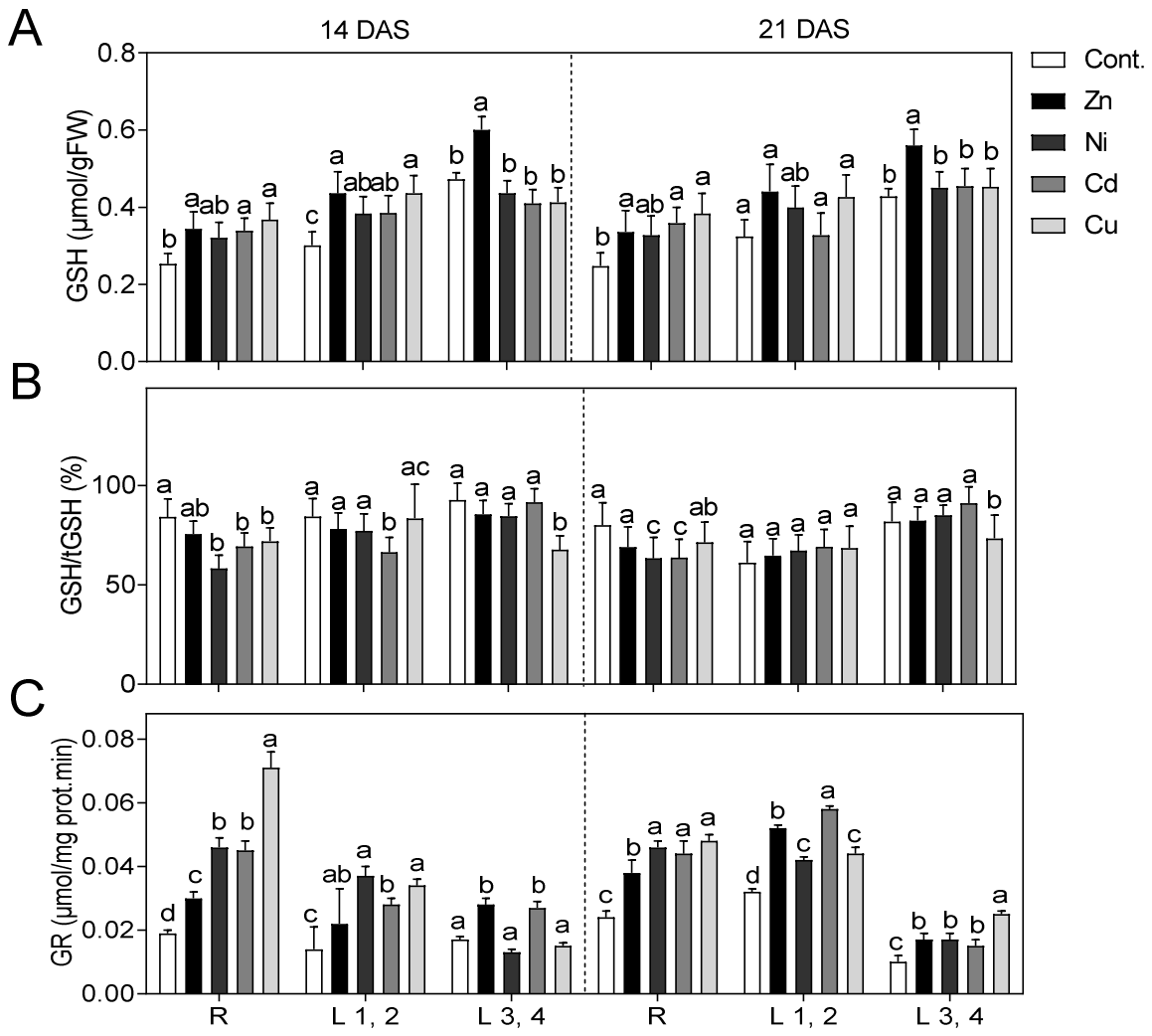

A
14 DAS
21 DAS
Cont.
Zn
Ni
Cd
Cu
GSH (µmol/gFW)
B
GSH/tGSH (%)
C
GR (µmol/mg prot.min)
R
L 1, 2
L 3, 4
R
L 1, 2
L 3, 4

Figure 8

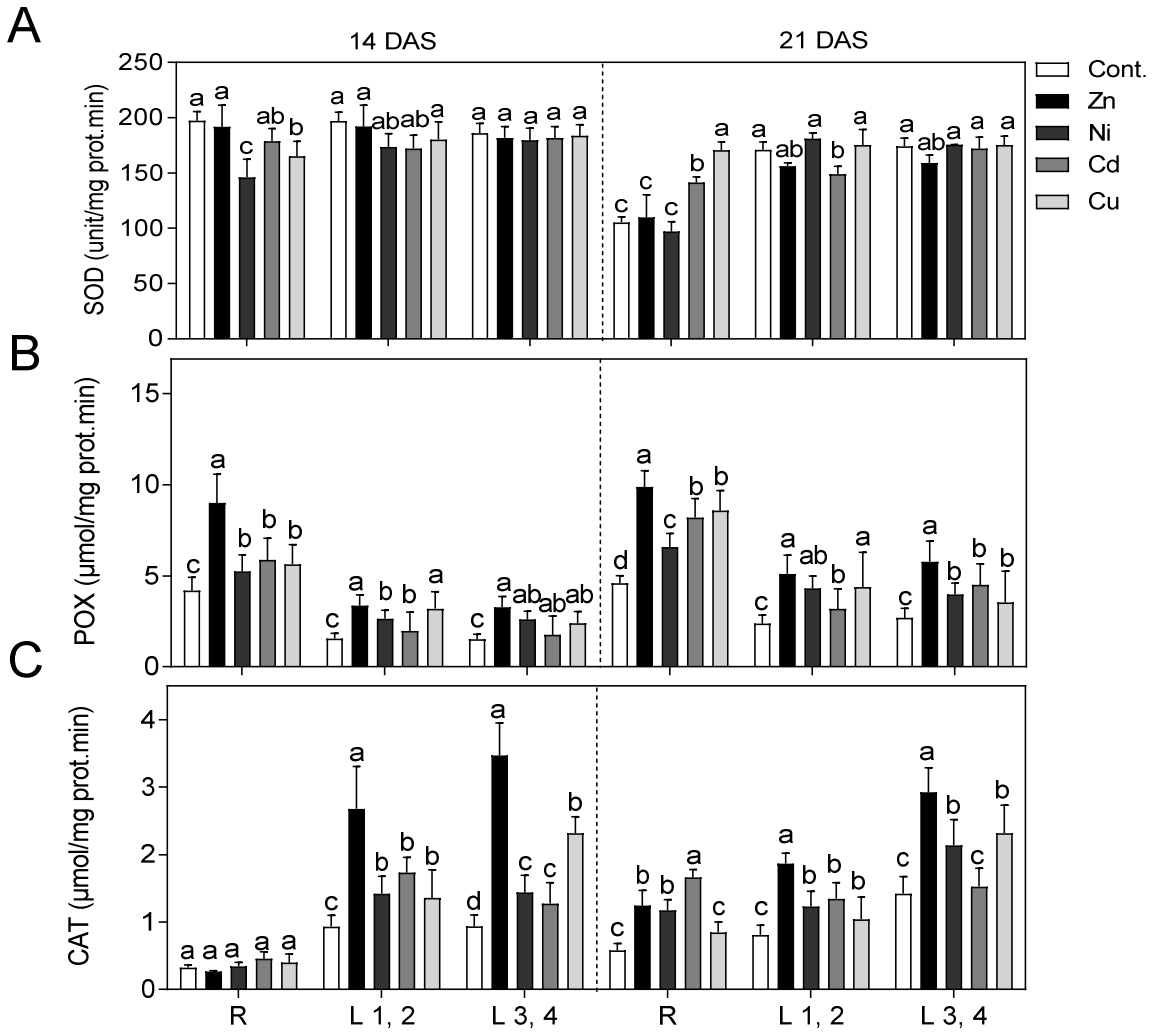
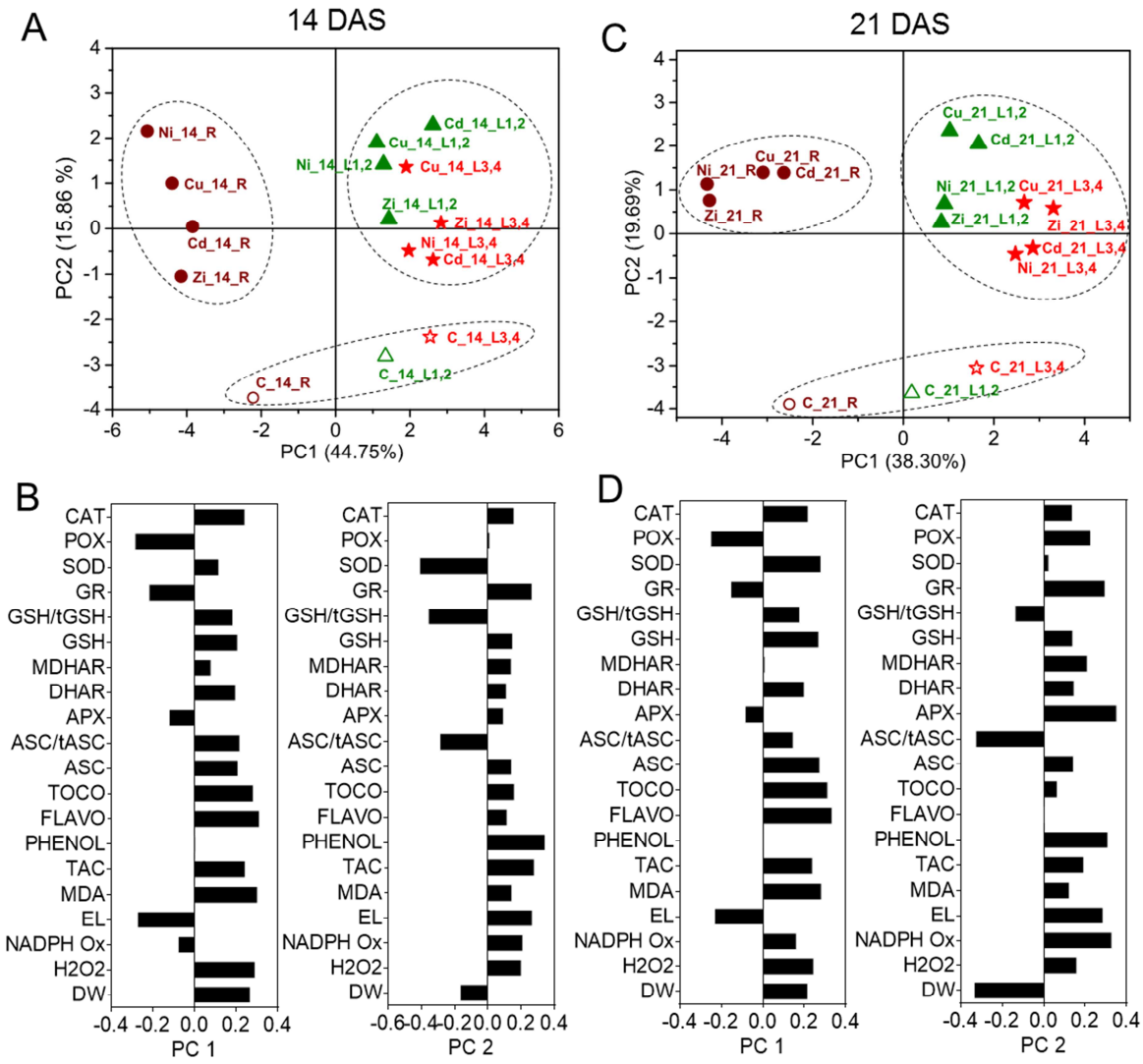

A
B
C
14 DAS
21 DAS
Cont.
Zn
Ni
Cd
Cu
SOD (unit/mg prot.min)
POX (µmol/mg prot.min)
CAT (µmol/mg prot.min)
R
L 1, 2
L 3, 4
R
L 1, 2
L 3, 4

Figure 9


A
14 DAS
PC2 (15.86 %)
PC1 (44.75%)
Ni_14_R
Cu_14_R
Cd_14_R
Zi_14_R
Cd_14_L1,2
Cu_14_L1,2
Ni_14_L1,2
Cu_14_L3,4
Zi_14_L1,2
Zi_14_L3,4
Ni_14_L3,4
Cd_14_L3,4
C_14_L3,4
C_14_L1,2
C_14_R
C
21 DAS
PC2 (19.69%)
PC1 (38.30%)
Cu_21_R
Ni_21_R
Cd_21_R
Zi_21_R
Cu_21_L1,2
Cd_21_L1,2
Ni_21_L1,2
Cu_21_L3,4
Zi_21_L1,2
Zi_21_L3,4
Cd_21_L3,4
Ni_21_L3,4
C_21_L3,4
C_21_L1,2
C_21_R
B
D
CAT
POX
SOD
GR
GSH/tGSH
GSH
MDHAR
DHAR
APX
ASC/tASC
ASC
TOCO
FLAVO
PHENOL
TAC
MDA
EL
NADPH Ox
H2O2
DW
PC 1
PC 2

1020

Highlights:

- Maize organs of different types and ages respond differently to heavy metals
- Cluster analyses results grouped into Cd-Cu and Zn-Ni groups with regard to their antioxidant responses in maize seedling organs.
- Mature leaves are more sensitive to heavy metals compared to younger ones.
- Different antioxidant enzymes are induced in different organs

- Type of contribution:

  Hamada AbdElgawad & Gaurav Zinta: Experimental design and analysis, manuscript writing and reviewing.

  Gaurav Zinta: Experimental design and analysis, manuscript writing and reviewing.

  Badreldin A. Hamed: Experimental design and analysis, manuscript writing and reviewing.

  Samy Selim: : Experimental design and analysis

  Han Asard & Gerrit Beemster: Reviewing the experiments and manuscript

  Wael N. Hozzein: Experimental design and analysis, manuscript editing and reviewing

  Mohammed A.M. Wadaan: analyses

  Walid Abuelsoud: data analysis, manuscript writing and reviewing

Conflict of interest declaration

The authors of the manuscript titled "**Maize Roots and Shoots Show Distinct Profiles of Oxidative Stress and Antioxidant Defense Under Heavy Metal Toxicity**" declare no conflict of interest