Alleviation of Cadmium Toxicity in *Zea Mays* L. through Up-Regulation of Antioxidant Defense System and Organic Osmolytes under Supplemental Calcium

Muhammad Kaleem (kaleemakmal5798@gmail.com)
University of Agriculture Faisalabad https://orcid.org/0000-0003-3237-4886
Iqbal Hussain
Government College University Faisalabad
Mansoor Hameed
University of Agriculture Faisalabad
Muhammad Sajid Aqeel Ahmad
University of Agriculture Faisalabad
Anam Mehmood
Government College University Faisalabad
Muhammad Faisal Maqsood
University of Agriculture Faisalabad
Ummar Iqbal
University of Agriculture Faisalabad
Zunaira Anwar
University of Agriculture Faisalabad
Syed Mohsan Raza Shah
University of Agriculture Faisalabad
Waseem Ashfaq
The University of Melbourne

Research Article

Keywords: Antioxidants, cadmium, calcium, maize, oxidative stress, reactive oxygen species

Posted Date: July 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-611406/v1

Version of Record: A version of this preprint was published at PLOS ONE on June 22nd, 2022. See the published version at https://doi.org/10.1371/journal.pone.0269162.

Abstract

Calcium (Ca) is a macronutrient and work as a modulator to mitigate oxidative stress induced by heavy metals. Present work was conducted to elucidate the role of Ca in modulating growth, physio-biochemical traits, and cellular antioxidant defense system in *Zea mays* L. seedlings under Cd stress. The experiment was designed in a complete randomized design with two levels of Cd (0 and 150 µM) and six levels of Ca (0, 0.5, 1, 2.5, 5 and 10 mM). Maize seedlings exposed to Cd at150 µM concentration showed a notable decrease in growth, biomass, anthocyanins, chlorophylls, and antioxidant enzymes activities. Higher level of Cd (150 µm) also caused an upsurge in oxidative damage observed as higher electrolyte leakage (increased membrane permeability), H_2O_2 production and MDA accumulation. Supplementation of Ca notably improved growth traits, photosynthetic pigments, cellular antioxidants (APX, POD and ascorbic acid), anthocyanins and level of osmolytes. The significant improvement in the osmolytes (proteins and amino acids), and enzymatic antioxidative defense system enhanced the membrane stability and mitigated the damaging effects of Cd. The present results concluded that exogenously applied Ca can potentially improve growth by regulating antioxidants and enable maize plants to withstand the Cd toxicity.

Introduction

While growing in natural environments, plants are exposed to various environmental stresses that limit yield and productivity (Huybrechts et al. 2019). Heavy metal pollution is spreading in cultivated lands and is causing severe environmental hazards to crop plants, human health and ecosystems (Liu et al. 2014). Cd is regarded as the most toxic heavy metal, typically when present in agricultural lands due to its higher mobility and toxicity (Goix et al. 2014). Plants can readily absorb Cd through roots directly from the soil along with essential nutrients (Nogueirol et al. 2016). Like other heavy metals, Cd causes structural changes in plants and adversely affects the morphological, physiological, and biochemical mechanisms which eventually lead to loss of agricultural productivity (Kováčik et al. 2014). Cd is typically non-essential for agricultural crops as no known role is ascribed for Cd in growth and development of crop plants (Huybrechts et al. 2019). Therefore, Cd even in minor concentrations disturb the photosynthesis, change the ultrastructure of the chloroplast, increase lipid peroxidation and enhance production of ROS that leads to oxidative damage (Gallego et al. 2012; Dourado et al. 2015; Gratão et al. 2015). Another direct effect of high Cd is production of excessive ROS (H_2O_2, OH^-, $O_2^{\cdot -}$, $^1O_2$) resulting in lipids peroxidation which ultimately reduces plant growth (Maleki et al. 2017).

Many defensive mechanisms are induced in plants to counteract Cd toxicity mainly by hyper production of antioxidants (non-enzymatic or enzymatic) to control heavily produced ROS (Kováčik et al. 2014; Asgher et al. 2014). These enzymatic antioxidants (like peroxidase, superoxide dismutase, ascorbate peroxidase, and catalases), and non-enzymatic anti-oxidants (such as α-tocopherol and glutathione) have been reported to successfully mitigate Cd-induced oxidative damage in many crop plants (Mishra et al. 2014; Maleki et al. 2017). Other reports show that heavy metals may results in hyper-accumulation of proteins as an effective strategy to mitigate Cd induced toxicity (El-Beltagi and Mohamed 2013).

Calcium (Ca^{2+}) is an essential macromolecule and divalent cation that performs an imperative role in membrane permeability, metabolism and signal transduction (El-Beltagi and Mohamed 2013; Vigani and Costa 2019; Malik et al. 2020). It is a central regulator in the physio-chemical process and regulates plant growth (Siddiqui et al. 2012). Exogenously applied Ca alleviates oxidative stress by chelating with target proteins (for instance calcium-binding proteins) and activating the antioxidant enzymes (Song et al. 2009; Grzybowska 2018). Though, the induction of antioxidative defense system by Ca is not yet elucidated sufficiently, however, some reports support that Ca is involved in the modulation of genes for antioxidant enzymes (Niu and Liao 2016). Ca mitigates Cd toxicity in plants by modifications in morphological and physiological process (Siddiqui et al. 2012; Ahmad et al. 2015). For example, Ca maintains permeability of membranes by a reduction in peroxidation of lipids and solute leakage which ultimately reduces oxidative stress caused by Cd stress (Lwalaba et al. 2017). In other reports, Ca improved growth and photosynthesis by restricting Cd translocation and accumulation, scavenging ROS, enhancing antioxidant level, and maintaining Ca-dependent signal transduction (Srivastava et al. 2015; Huang et al. 2017). Still, the ameliorative role of Ca to alleviate heavy metals toxicity remains inconclusive and therefore it is imperative to investigate its specific roles and associated mechanisms in improving growth of *Zea mays* L. seedlings.

Maize is a valuable cereal crop and provides food for humans as well as fodder for the livestock. It contributes to 36 % (782 Mt) in global grain production (Sah et al. 2020). Maize seeds are enriched with energy as 100g seeds contain 365 kilocalories of energy (Nuss and Tanumihardjo 2010). Among worldwide production, 70–80% of maize is used as food and was ranked third in Pakistan for consumption after wheat and rice. Pakistan was ranked 18th in the production with 6130 thousand tons maize produced annually that was cultivated at 1334 thousand hectares (Rani et al. 2015). The requirement for the maize production has significantly increased recently due to excessive usage in the wet milling industry as well as food for poultry (Boomsma and Vyn 2008). This needs not only to increase the cultivation area but also the exploration of new promising techniques to increase crop survival and yield under stressful environments like in soils contaminated with heavy metals (Khan 2020).

Cadmium contamination is gradually increasing in soils and is causing significant crop losses. Therefore, there is a dire need to devise new strategies to combat the problems associated with Cd toxicity. Considering many critical roles played by Ca in plant growth and metabolism, it was hypothesized that Ca supplementation should effectively ameliorate the adverse effects of Cd imposed on germinating seeds of maize. The research questions included to probe into the toxic effects of Cd on the growth, physio-biochemical characteristics and to what extent supplemental Ca can alleviate Cd toxicity in maize. These findings would contribute to explore the mechanism and recommendation of the best levels of Ca in ameliorating Cd toxicity in maize.

Materials And Method

Plant materials and layout

Maize seeds (Sahiwal-2002) were procured from Maize and Millets Research Institute (MMRI), Yousaf Wala Sahiwal, Pakistan. Seeds were immersed in 30 % (v/v) H_2O_2 for 5 min. for sterilization, washed with deionized water for 24 h and dried. Seeds were placed in Petri dishes lined with double layer of What-man # 02 filter papers. The surface of each filter paper was moistened with 15 mL of H_2O and kept in dark condition 25 ± 2 °C for a 48 h. After germinations, six seeds were planted in plastic pots (depth; 40 cm and diameter; 35 cm) in sterilized sand with a particular particle size 0.25 mm. The sand was soaked for 24 h in 30 % (v/v) HCl solution to remove all cations and anions and then thoroughly rinsed with deionized water three time (with 24 h soaking). The pots were treated with 0 and 150 µm Cd^{2+} applied as $CdCl_2$ and 0, 0.5, 1, 2.5, 5 and 10 mM Ca^{2+} levels using $Ca(NO_3)_2$ as source . The ½-strength Hoagland's (Hoagland 1938) nutrient solution was applied to the seedlings throughout the experimentation. The Hoagland solution was applied to the plants to saturate the sand at 3 days interval with draining any past solution left in sand. All pots were arranged as CRD with 3 replications under controlled glasshouse environment with day/night temperature of 24 ± 4°C/14 ± 2°C and RH 58-60 % (Rady et al. 2019).

Plant sampling and measurements

Plants material was sampled at the seedling stage to determine plant growth attributes, physio-biochemical traits, ROS, and enzymes of antioxidants defense system. Harvested seedlings were washed with distilled water and growth attributes were recoded. Leaf samples of maize seedlings were frozen at –80°C for physio-biochemical traits and antioxidants. Sampled seedlings were dried in oven at 70 °C to achieve a constant dry weight for determination of root (RDW) and shoot (SDW) dry weight.

Growth attributes

The shoot length (SL) of plants from each treatment was measured from sand level to the topmost leaf of the plant. The roots of seedlings were carefully removed from the sand for recording root length (RL). Root (RFW) and shoot fresh weight (SFW) of seedlings were measured immediately after excision. The leaf area (LA) was estimated by measuring length × width × 0.68.

Physiological attributes

Chlorophyll contents

Chlorophyll contents were assessed as described by Arnon (1949) and carotenoids following the method of Davis (1976). For the appraisal of chlorophyll contents, 0.1 g of leaf sample was grounded in 5 mL of acetone (80 %). Extract was filtered through a Whatman # 02 filter paper (GE Healthcare, UK) and absorbance was recorded through a spectrophotometer (Hitachi U-2910, Tokyo, Japan) at 645, 663, and 480 nm. The values of photosynthetic pigments were calculated by using the following formula.

$$\text{Chl. } a \text{ (mg/g of leaf FW)} = [12.7(OD663)-2.69(OD645)] \times V/1000 \times W$$

$$\text{Chl. } b \text{ (mg/g of leaf FW)} = [22.9(OD645) - 4.68(OD663)] \times V/1000 \times W$$

$$\text{Total Chl. (mg/g of leaf FW)} = [20.2(OD645) + 8.02\ (OD663)] \times V/1000 \times W$$

$$\text{Carotenoids (g/ ml of FW)} = \{[(OD480) +0.114\ (OD663) – 0.638\ (OD645)]/2500\}$$

Here, V characterizes the volume of acetone and (FW) showed the leaf fresh weight.

Determination of relative membrane permeability

The fresh leaf samples were collected and washed thoroughly with 4 changes of water to eradicate any adhered electrolytes on the surface. The leaves were cut into small discs with a borer and placed in the small glass test tube containing deionized water (10 mL), The EC_o was measured by the help of Cond/Salinity meter (TPS AQUA-CPA). The test tubes were incubated for 24 h at 4 °C and EC_1 was measured. The tubes were then wrapped with aluminum foil, autoclaved for 10 min. at 100 kPa and (EC_2) was recorded. The ratio of % ion leakage was computed as designated by Yang et al. (1996).

$$RMP\ (\%) = (EC_1\text{-}EC_0) / (EC_2\text{-}EC_0) \times 100$$

Assessment of biochemical traits

Anthocyanin contents

Anthocyanin content was appraised according to the method of Giusti and Wrolstad (2001). The 0.1 g of leaf was pulverized in trichloroacetic acid (TCA) by using pestle and mortar. The homogenized material was transferred to test tubes and shifted to water bath at 80 °C for 20 min. Homogenized material was centrifuged at 12,000 *xg* for 10 min. in the absorbance was noted at 516 and 700 nm using a spectrophotometer (Hitachi U-2910, Tokyo, Japan). Acetone was run as blank and amount of monomeric anthocyanin contents was calculated as follows.

$$\text{Chl. } a \text{ (mg/g of leaf FW)} = [12.7(OD663)-2.69(OD645)] \times V/1000 \times W$$

$$\text{Chl. } b \text{ (mg/g of leaf FW)} = [22.9(OD645) - 4.68(OD663)] \times V/1000 \times W$$

$$\text{Total Chl. (mg/g of leaf FW)} = [20.2(OD645) + 8.02\ (OD663)] \times V/1000 \times W$$

$$\text{Carotenoids (g/ ml of FW)} = \{[(OD480) +0.114\ (OD663) – 0.638\ (OD645)]/2500\}$$

Here, A= (A510-A700) MW= 449.2 and ε= 26900 [ε is the molar absorptivity measured the amount of cyanidin-3-glucoside pigment and DF is dilution factor].

Oxidative stress markers (MDA and H_2O_2)

Lipids peroxidation (LPX) was quantified by means of malondialdehyde (MDA contents) by following Heath and Packer (1968). LPX content was determined by the reaction of thiobarbituric acid-TCA with trichloroacetic acid-TCA. The 0.25 g leaf sample was grinded in 500 µL of TCA (0.1 %) and then centrifuged at 15,000 *xg*. An aliquot (1 mL) was taken and mixed with 2 mL of 0.5 % of TBA and 20% TCA. Test tubes containing reactants were incubated at 85 °C for 20 min. and reaction was terminated in an icebox. Absorption was recorded at 532 and 600 nm by spectrophotometer (Hitachi U2910, Tokyo, Japan). All absorption ODs (at 532nm) were subtracted from 600 nm. LPX concentration was calculated by using 155 mM cm^{-1} as an extinction coefficient.

Amount of H_2O_2 was quantified by measuring the oxidation of ferrous ions medicated by peroxidase and ferric ions react with the xylenol (Bellincampi et al. 2000). Leaf sample 0.5 g was grounded in 5 mL of 10 mM sodium phosphate buffer (SPB). Centrifugation of homogenized material was done at 15,000 *xg*. A 2 mL of aliquot was reacted with the assay reagent containing 200 mM sorbitol, 200 µM xylenol, 50 mm H_2SO_4, and 500 µM ammonium ferrous sulphate. The reactant material was incubated at 24 °C for a 1 h and absorption was recorded at 560 nm by using a spectrophotometer (Hitachi U-2910, Tokyo, Japan).

Cellular antioxidants (APX and POD)

The maize seedlings' shoot were grounded in liquid nitrogen and extracted with 1 mM L^{-1} of 5% polyvinylpyrrolidone, and, sodium phosphate buffer (SPB) having pH 7.8. Extracted material was centrifuged at 15,000 *xg*. Enzyme crude extract was stored at 4 °C for 36 h till analysis.

Ascorbate peroxidase activity (APX)

Activity of APX was quantified by oxidation of ascorbate (Chen and Asada 1989). Reaction was started by adding 10 µL of crude enzyme extract to 2 mL of assay reagent (30 % H_2O_2, 0.5 mM $C_6H_8O_6$, and sodium phosphate buffer (SPB) having pH 7.2,). After 30 s of reaction initiation, a shift in absorption was noted at 290 nm for 4 min. on a spectrophotometer (Hitachi U-2910, Tokyo, Japan). Activity of enzyme was estimated through extinction coefficient (2.8 mM cm^{-1}), while the specific activity of the enzyme was calculated on the basis of protein contents and expressed as an mg^{-1} $min.^{-1}$ FW.

Peroxidase (POD) activity

POD activity was appraised spectrophotometrically by using the method of Goliber (1989) based on oxidized of guaiacol in the presence of H_2O_2 and expressed as a Units mg^{-1} proteins. A 20 µL of the enzyme extract was added to assay reagent (20 mM guaiacol, 10 mM H_2O_2, and 0.1 M phosphate buffer) and volume was maintained up to 3 mL. Enzyme activity was measured at 460 nm after 60 s interval

through a spectrophotometer (Hitachi U-2910, Tokyo, Japan). Enzyme specific activity was expressed on the base of proteins.

Ascorbic acid

Ascorbic acid was determined as described by Nino and Shah (1986). Plant tissues (100 mg) were pulverized in thiobarbituric acid (TCA) and centrifuged 10,000 *xg* for 10 min. An aliquot (500 µL) was taken with 500 µL of dthiocarbamate (DTC) in glass tubes. Reactants were left for ½ h at 37 °C. Test tubes containing reactant material was transferred to the ice-bath to terminate the reaction. After that, 2 mL of diluted H_2SO_4 was mixed slowly and left over for ½ h at 37° C in incubator. Extracted material was centrifuged at 12,000 *xg*. The shift in absorption was measured at 520 nm with the help of a spectrophotometer (Hitachi U-2910, Tokyo, Japan).

Organic osmolytes

Total amino acids

Free amino acid was quantified followed by Hamilton and Van-Slyke (1943) method. The 0.1 g of the leaf sample was grinded and immersed in a potassium phosphate buffer (SPB) overnight. After incubation, 1 mL of plant extract was transferred to 25 mL test tubes after adding 1 mL each of 10 % ninhydrin and 2% of pyridine solution. The test tubes containing reactants were placed in boiling water bath for 1 h. The final volume of samples was made to 25 mL by using deionized H_2O. Absorbance was recorded at 570 nm spectrophotometrically (Hitachi U-2910, Tokyo, Japan) and resulting absorbance were compared with the standard curve plotted for leucine.

Soluble proteins

Soluble proteins were appraised following Lowry *et al* (1951). Plant sample (0.1 g) was grounded in 50 mM sodium phosphate buffer (SPB) having pH 6.8. The extracted aliquot (500 µL) was mixed in 0.3 mL of deionized H_2O and 3 mL of Bio-Rad protein assay dye and vortexed for 15 s. The absorbance was measured spectrophotometrically at 595 nm (Hitachi U-2910, Tokyo, Japan). Soluble proteins were estimated by comparing absorbance of samples with bovine serum albumin (BSA) using a standard value.

Statistical analysis

Minitab-19, software e (Minitab, LLC, State College, PA, USA) was used to analyze the data. The means values were compared by using the Tukey pairwise comparison test at $P \leq 0.05$ following an analysis of variance (ANOVA). The effect of Ca and Cd treatments was assessed by using a multivariate analysis (PCA by ggbiplot), correlation matrix (ggbiplot2) and heatmaps were plotted by customized code (pheatmap) by using R statistical software (R Core Team, 2019). Response curves under cadmium and calcium stress treatments were constructed by fitting a generalized linear model (GLM) in CONACO version 5 for windows.

Results

Plant growth traits

Growth traits such as SL, RL, SFW, SDW, RFW, RDW and LA significantly decreased at Cd applied at150 µm concentration ($P \leq 0.05$). However, different levels of Ca significantly alleviated Cd toxicity and enhanced all growth traits. The increase in growth traits was more obvious in response to higher level of Ca applied at 10 mM under Cd stress (Table 1).

Table 1
Morphological characteristics of maize seedlings under Ca and Cd treatments

Treatments		SL (cm)	RL (cm)	SFW (g $plant^{-1}$)	SDW (g $plant^{-1}$)	RFW (g $plant^{-1}$)	RDW (g $plant^{-1}$)	LA (Cm^2)
Cd	**Ca**							
Cd-0 µm	0 mM	57.44 ± 5.71^{d}	18.66 ± 1.32^{d}	32.65 ± 1.66^{d}	3.12 ± 0.13^{d}	2.43 ± 0.08^{d}	0.26 ± 0.02^{c}	49.51 ± 2.23^{d}
	0.5 mM	66.22 ± 1.92^{c}	23.61 ± 1.28^{d}	40.72 ± 1.82^{c}	3.55 ± 0.09^{d}	3.09 ± 0.16^{c}	0.36 ± 0.04^{c}	57.79 ± 0.86^{c}
	1 mM	66.22 ± 1.34^{c}	30.94 ± 0.80^{c}	47.14 ± 0.95^{c}	4.46 ± 0.15^{c}	3.29 ± 0.04^{c}	0.45 ± 0.03^{b}	60.99 ± 4.06^{c}
	2.5 mM	73.00 ± 0.84^{b}	40.66 ± 2.47^{b}	48.31 ± 1.85^{c}	4.54 ± 0.36^{c}	3.73 ± 0.06^{b}	0.56 ± 0.02^{b}	73.04 ± 3.07^{b}
	5 mM	93.66 ± 2.95^{b}	45.61 ± 3.11^{b}	59.00 ± 1.51^{b}	5.87 ± 0.22^{b}	4.63 ± 0.12^{a}	0.62 ± 0.02^{a}	75.91 ± 3.60^{b}
	10 mM	105.7 ± 2.01^{a}	65.94 ± 2.84^{a}	81.91 ± 3.19^{a}	7.75 ± 0.10^{a}	5.60 ± 0.11^{a}	0.66 ± 0.03^{a}	95.87 ± 2.34^{a}
Cd-150 µm	0 mM	24.64 ± 2.38^{d}	11.61 ± 2.22^{d}	22.17 ± 1.74^{d}	1.40 ± 0.22^{c}	1.35 ± 0.17^{c}	0.14 ± 0.02^{c}	31.96 ± 1.06^{d}
	0.5 mM	52.44 ± 3.75^{c}	18.95 ± 0.80^{c}	28.45 ± 1.63^{d}	1.61 ± 0.03^{c}	1.99 ± 0.11^{c}	0.16 ± 0.01^{c}	39.30 ± 0.94^{d}
	1 mM	63.22 ± 3.74^{b}	20.34 ± 2.35^{c}	56.50 ± 2.39^{c}	1.91 ± 0.39^{c}	2.11 ± 0.15^{b}	0.21 ± 0.01^{b}	53.40 ± 5.49^{c}
	2.5 mM	72.44 ± 2.92^{b}	30.60 ± 1.45^{b}	73.11 ± 2.11^{b}	2.81 ± 0.28^{b}	2.94 ± 0.26^{a}	0.23 ± 0.01^{b}	54.09 ± 5.11^{c}
	5 mM	82.77 ± 4.29^{a}	35.10 ± 2.69^{b}	77.96 ± 5.19^{b}	3.26 ± 0.11^{b}	3.10 ± 0.18^{a}	0.32 ± 0.03^{a}	62.96 ± 1.46^{b}
	10mM	91.55 ± 3.83^{a}	51.14 ± 3.16^{a}	90.64 ± 1.52^{a}	4.97 ± 0.20^{a}	3.11 ± 0.09^{a}	0.30 ± 0.04^{a}	76.99 ± 2.57^{a}

Means provided with error bars; in columns different letter indicates significance ($P \leq 0.05$) between treatments

Abbreviation: Shoot length **(SL)**; Root length **(RL)**; Shoot fresh weight **(SFW)**; Shoot dry weight **(SDW)**; Root fresh weight **(RFW)**; Root dry weight **(RDW)**; Leaf area **(LA)**

Photosynthetic Pigments

Under Cd stress, a significant ($P \leq 0.05$) reduction occurred in the concentration of photosynthetic pigments of maize seedlings. Exogenously supplied Ca significantly increased photosynthetic pigments both in Cd stressed and non-stressed seedlings. Calcium applied at 10 mM level was more beneficial in increasing chlorophyll and carotenoids contents of maize seedlings (Table 2).

Table 2
Physiological traits of maize seedlings under various levels of Ca and Cd treatments

Treatments		Chl *a* (mg g^{-1} FW)	Chl *b* (mg g^{-1} FW)	Caro. (mg g^{-1} FW)	T. Chl (mg g^{-1} FW)	APX (Units mg^{-1} Pro)	POD (Units mg^{-1} Pro)
Cd	**Ca**						
Cd-0 µm	0mM	9.76 ± 0.25^{c}	2.34 ± 0.09^{c}	0.35 ± 0.01^{d}	10.69 ± 0.26^{c}	0.92 ± 0.05^{c}	0.55 ± 0.11^{c}
	0.5mM	12.98 ± 0.54^{c}	2.84 ± 0.23^{c}	0.43 ± 0.01^{c}	11.43 ± 0.75^{c}	1.02 ± 0.04^{c}	0.60 ± 0.12^{c}
	1mM	16.56 ± 0.84^{b}	4.44 ± 0.15^{b}	0.48 ± 0.02^{c}	14.02 ± 0.64^{b}	1.98 ± 0.02^{b}	0.73 ± 0.04^{b}
	2.5mM	18.32 ± 0.62^{a}	4.57 ± 0.19^{b}	0.51 ± 0.02^{b}	14.93 ± 0.83^{b}	1.93 ± 0.02^{b}	0.78 ± 0.03^{b}
	5mM	18.60 ± 0.38^{a}	5.48 ± 0.18^{a}	0.52 ± 0.03^{b}	16.00 ± 1.06^{a}	1.98 ± 0.06^{b}	0.87 ± 0.07^{a}
	10mM	19.26 ± 0.76^{a}	6.49 ± 0.10^{a}	0.70 ± 0.02^{a}	16.02 ± 0.25^{a}	2.17 ± 0.03^{a}	0.91 ± 0.06^{a}
Cd-150 µm	0 mM	3.63 ± 0.22^{d}	1.41 ± 0.03^{c}	0.13 ± 0.01^{c}	5.02 ± 0.23^{d}	1.27 ± 0.03^{c}	0.45 ± 0.09^{d}
	0.5mM	5.33 ± 0.19^{c}	2.85 ± 0.12^{c}	0.17 ± 0.01^{c}	6.35 ± 0.46^{c}	1.39 ± 0.19^{b}	0.72 ± 0.02^{c}
	1mM	6.50 ± 0.82^{c}	3.08 ± 0.08^{b}	0.22 ± 0.01^{b}	7.25 ± 0.45^{c}	2.01 ± 0.04^{b}	0.85 ± 0.06^{b}
	2.5mM	10.72 ± 0.47^{b}	3.47 ± 0.14^{b}	0.27 ± 0.01^{b}	8.22 ± 0.22^{b}	2.17 ± 0.03^{a}	0.82 ± 0.06^{b}
	5mM	12.42 ± 1.11^{b}	4.13 ± 0.13^{a}	0.32 ± 0.03^{a}	9.18 ± 0.37^{b}	2.10 ± 0.02^{a}	1.10 ± 0.01^{a}
	10mM	14.02 ± 0.53^{a}	5.16 ± 0.20^{a}	0.41 ± 0.03^{a}	12.09 ± 0.52^{a}	2.22 ± 0.01^{a}	1.12 ± 0.06^{a}

Means provided with error bars; in columns different letter indicates significance (P≤0.05) between treatments

Abbreviations: Chlorophyll *a* **(Chl *a*)**; Chlorophyll *b* **(Chl *b*)**; Carotenoids **(Caro)**; Total chlorophyll **(T. Chl)**; Ascorbate per oxidase **(APX)**; Peroxidase **(POD)**; Protein **(Pro)**

Antioxidative enzyme activities

Mean values for antioxidant activity was higher in Cd stressed (150 µM) as compared to non-stressed plants (0 µM). However, the activity of APX significantly enhanced as levels of Ca increased both in non-stressed (0 µM) and stressed plants (150 µM). This increase was the maximum in response to Ca applied at 10 mM concentration. Peroxidase activity showed the same trend as noted for APX under Ca and Cd treatments (Table 2).

Anthocyanin and relative membrane permeability (RMP)

Under Cd stress, maximum RMP values were observed indicating a high level of electrolyte leakage due to membrane damage. A significant ($P \leq 0.05$) reduction was observed as the level of Ca increased (Fig 1). Anthocyanin contents under both treatments of Cd significantly increased as levels of Ca increased. Maximum anthocyanin contents were noticed at 10 mM Ca concentration (Fig. 1). Cadmium applied at 150 µm level and without any Ca supplementation had the most toxic effects as the highest electrolyte leakage was observed at this treatment level.

Lipid peroxidation (LPX) and ROS

The accumulation of H_2O_2 and MDA significantly increased in maize seedlings under Cd stress. However, the elevated levels of Ca significantly reduced the generation of H_2O_2 and LPX. The LPX in terms of MDA contents significantly decreased as the level of Ca increased in growth medium of the seedlings. The maximum decrease was observed under 10 mM concentration of Ca (Fig. 1).

Osmolytes

Osmolyte (proteins and amino acids) production was significantly increased in maize seedling in stressed and non-stressed maize seedlings. Soluble proteins were significantly higher in non-stressed maize seedlings as the level of Ca increased (Fig 2). In Cd stressed seedlings (150 µM), the concentration of soluble proteins significantly increased and the maximum was observed under 10 mM Ca concentration (Fig 2). Applications of Ca substantially increased the concentration of amino acids in both stressed and non-stressed seedlings and almost parallel results were observed as noted for soluble proteins (Fig 2).

Ascorbic acid contents

Ascorbic acid contents were substantially improved as Ca levels increased in maize seedlings under normal and stress conditions. Maximum values of ascorbic contents were observed under 10 mM concentration of Ca in both stressed and non-stressed condition (Fig 2).

Multivariate analysis

Principal component analysis (PCAs)

PCAs results demonstrated high variations on the effects of Cd and Ca treatments among different growth and physio-biochemical traits of maize seedlings (Fig. 3). The first and second PCAs explained 75.8% and 17.2 % (total 93%) variation among treatments and seedlings characteristics. The major contributors to 150 µM Cd level were amino acids (A-Ac), peroxidase (POD), H_2O_2, and RMP with high positive eigenvalues. The activity of antioxidative enzymes (POD, APX, ASC), photosynthetic pigments (Chl *a*), and growth traits significantly increased under Cd stress (150 µM) with a higher concentration of Ca (C5-C6). Under lower levels of Ca *i.e.* C1 and C2, the ROS and RMP increased under Cd stress. The major principal components to control plants (0 µM Cd) were RFW, RDW, anthocyanin contents, Chl *b*, T. Chl, carotenoids, and LPX with negative eigenvalues (Fig. 3). Cd stress significantly increased level of reactive oxygen species, while supplemented Ca significantly increased the antioxidative enzymes activity and growth parameters (Fig. 3).

Correlation matrix

In control plants, anthocyanin contents (Antho-C) was positively correlated with RFW, RDW, SL, LA, Caro, Chl *b* and TSP. The RMP, H2O2, and RMP were negatively correlated with RFW, RDW, Chl a, b, RL, LA, A-AC and APX (Fig. 4a). Under Cd stress, a highly positive correlation was assessed between POD, SFW, and ASC.A, APX, Chl *a*, SL, and RL. However, a strong negative correlation was assessed between H_2O_2, RMP, and antioxidant enzymes under Cd-150 µM stress (Fig. 4b).

Clustered heatmap

A clustered heatmap was constructed to evaluate the effect of Cd and Ca treatments on the different traits as shown in Fig. 5. Under higher concentration of Ca (10 mM), RMP, H_2O_2 and MDA showed significant reduction in response to 0 and 150 µM concentration of Cd indicating a parallel response in both treatments. A noteworthy influence of 10 mM level of Ca in non-stressed seedlings (0 µM Cd) was recorded with a greater increase in growth traits (RFW, RDW, SFW, SDW, SL, RL, LA), chlorophyll (Chl *a* & *b*, T. Chl), organic osmolytes (TSP), anthocyanin contents (Antho.C) and ascorbic acid (ASC.A). All these traits were tightly grouped together and indicated high performance of 10 mM level of Ca under non-stressed conditions. In Cd stressed (150 µM) seedlings, 10 mM level of Ca contributed to a significant increase in amino acids (A.AC), peroxidase (POD), ascorbic acid (ASC.A) and shoot fresh weight (SFW). Shoot length (SL), root length (RL), leaf area (LA), activity of ascorbate peroxidase (APX) and chlorophyll showed a strong and clear similarity and strongly clustered together. Antioxidants (APX and POD), ascorbic acid (ASC.A), anthocyanin contents (Antho.C) reduce the RMP, H_2O_2 and MDA and clustered together in same group. At highest level of Ca (10 mM), clustering and similarity indicated a high performance and a possible relationship between different traits under stress treatments.

Response of different traits under stressed and non-stressed conditions

In non-stressed conditions (0 µM), a conspicuous positive response was observed for the growth traits (RL, SL, SFW, SDW, and LA) and chlorophyll (Chl *a*, Chl *b* and T. Chl) as Ca levels increased (Fig 6a).

Organic osmolytes (TAA, TSP), anthocyanin contents (AC) and ascorbic acid (A,ASc) showed a sharp positive response with increasing Ca regimes (Fig 6b). H_2O_2, MDA and RMP exhibits a strong negative response with an increase in Ca levels, however APX and POD exhibits increasing pattern in curve with elevated Ca gradients (Fig 6c). In Cd stressed conditions (150µM), growth traits (RL, SL, SFW, and SDW, LA) and chlorophyll (Chl *a*, Chl *b* and T.Chl) displayed a strong positive response and in response to Ca levels (Fig 6d). Concentration of TAA, TSP, AC and AA were the maximum with positive response (Fig 6e). A strong positive response was noted in activity of APX and POD along increasing Ca levels. In contrast, a strong negative response was assessed for H_2O_2, MDA and RMP with increase in Ca regimes (Fig 6f).

Discussion

Calcium plays an essential role in mitigation of abiotic stresses and protection from drastic impacts (Liang et al. 2005; Ahmad et al. 2015). It interacts with proteins like calmodulin to up-regulate gene expression and regulate movement of metal ions across membranes (Niu et al. 2017). The present work demonstrated that Ca significantly alleviated the toxic effect of Cd in maize by improving all growth traits. Furthermore, the alleviation of Cd toxicity was more obvious at higher treatment levels of Ca in stressed and non-stress plants. Previous studies revealed that Ca applications regulate the uptake of heavy metal ions as it competes for transporter sites on plasma membrane (Ahmad et al. 2015). Supplemented Ca^{2+} reduced Cd toxicity by enhancing growth traits as reported in other crops like in mustered (Ahmad et al. 2015) and rice (Farzadfar et al. 2013). Additionally, Ca reduced the toxic effect of nickel in rice seedlings (Aziz et al. 2015).

Reduction in growth traits under Cd toxicity is directly linked to the reduction of photosynthetic contents. As anticipated, the photosynthetic pigments significantly declined under 150 µM Cd treatment level. However, higher levels of Ca significantly improved carotenoids, total Chlorophyll (Chl), Chl *a*, *b* pigments in maize seedlings under Cd stress (Table 2). Previously, the interactive effect between Ca and heavy metal was reported in some studies where exogenously applied Ca significantly prevented the damaging effects of Cd on photosynthetic pigments (Wu and Hendershot 2010; Aziz et al. 2015). Calcium is a divalent cation and shares many parallel physical properties (like pH) with divalent heavy metals like Cd, Ni, and Co (Tian et al. 2011). Therefore, exogenously applied Ca ions through rooting medium can successfully restrict the uptake of Cd metal ions through competition for uptake and transport in plants (Tian et al. 2011). In current work, the enhanced amount of photosynthetic pigments in Cd treated maize seedlings seemed to be a direct effect of enhanced activities of anti-oxidative enzymes, and other protective molecules that reduced membrane damage (Zouari et al. 2016)

The improvement in the antioxidant defense system enables plants to alleviate the heavy metals toxicity (Aziz et al. 2015). In current study, APX and POD activities significantly improved in Ca treated plants under Cd toxicity. These results suggest that applied Ca can effectively alleviate Cd induced oxidative stress (Rodriguez-Serrano et al. 2009). Under heavy metal stress, Ca activates diverse protein kinases and strengthens antioxidant defense system (Siddiqui et al. 2012; Sudha and Ravishankar 2003). Tolerant plants had evolved an efficient antioxidant system to balance the concentration of reactive oxygen

species (Mittler 2002; Huang et al. 2017). Enzymes like APX and POD also take part in the detoxification of free radicles and lead to sequestering of H_2O_2 (Sharma and Dietz 2006). APX is mainly localized in chloroplast, apoplast, cytosol, mitochondria, and peroxisome and POD in cell walls, cytosol, and vacuoles, Both APX and POD are mainly implicated to the scavenging of H_2O_2 (Mittler 2002). Their efficiency enhanced during Cd stresses and that greatly imparts stress tolerance and modulates the physiological process in maize seedlings in this study (Ahmad et al. 2010; Siddiqui et al. 2011).

Plants exposed to metal stress showed alterations in cell membrane permeability (RMP) and consequently cell loses membranes integrity (O'Lexy et al. 2018). Cell membrane integrity is considered as a tool to regulate the ionic movements and use as a selection criteria to quantify damage magnitude. In current results, the relative RMP markedly increased under Cd stress. However, the RMP significantly was markedly reduced by the Ca treatments that alleviated the damaging consequences of Cd. In plants exposed to Cd stress, relative membrane permeability (RMP) substantially increased and caused membrane impairments (Mishra et al. 2019). Under Cd stress, supplemented Ca decrease relative membrane permeability (electrolyte leakage) showing the role of Ca played in membrane stability (Javed et al. 2017). Ca mainly stabilizes the membrane integrity and also controls the movement of divalent cations and prevent solute leakage by reducing peroxidation of lipids (Antosiewicz and Hennig 2004; Farzadfar et al. 2013).

The excessive accumulation of both MDA and H_2O_2 in under metal stresses damages biomolecules by excessive lipid peroxidation, degrades membranes, decreases photosynthesis and hampered the activity of other essential enzymes (Mittler 2002). Plants enhance antioxidant system to deplete the ROS which ultimately reduce oxidative stress generated by high metal concentrations (Karuppanapandian and Manoharan 2008). The Ca applications as observed in this study, improved activities of various antioxidants (enzymatic or non-enzymatic) and reduced the level of H_2O_2 and lipid peroxidation (Candan and Tarhan 2005; Siddiqui et al. 2012). Calcium also up-regulates genes which are responsible to encode the antioxidant under oxidative stress (Jiang and Huang 2001). In present work, the level of ROS increased under Cd stress, however, the addition of Ca considerably reduced the production of ROS in maize seedlings (Fig. 1).

The anthocyanin contents remarkably increased in present study that was more pronounced in highest levels of Ca (Fig. 1)that is are parallel to many previous findings (Sudha and Ravishankar 2003; Yamdech et al. 2012). High level of anthocyanin regulates heavy metal transport toward the vacuole and sequestration (Amiri et al. 2012). Exogenously applied calcium is reported to reduced Cd toxicity by stimulating the synthesis of glutathione-S-transferase (GST) enzyme to increase anthocyanin contents that in turn ameliorates the oxidative stress by scavenging the free radicals (Amiri et al. 2012).

Heavy metal stress causes determinal changes in cellular structures and cause osmotic stress (Rucińska-Sobkowiak 2016)Plants mitigate with osmotic stress by accumulating the lower or higher weight osmolytes that do not hinder the functioning of important metabolites (Rhodes et al. 2002). Osmolytes primarily reduces water potential and ensure the water balance (Wang et al. 2003), protects subcellular

structures, and reduces oxidative damage (Slama et al. 2008). Amino acids act as organic osmolytes and participate in osmotic adjustments, stabilize proteins in membranes (Lee et al. 2008), ion homeostasis (Gleeson et al. 2005), scavenges the ROS and neutralize the redox potential during oxidative stress caused by noxious heavy metals (Lee et al. 2008). In the present studies, the seedlings showed more accumulation of osmolytes under application of Ca (Fig. 2). Ascorbic acids (ASc) are non-enzymatic antioxidant enzymes, acts as a cofactor for many important enzymes and accumulate in leaves (Ahmad et al. 2010). It serves a defensive role during oxidative stress and reduces the H_2O_2 and detoxifies the free radicals (Türkan et al. 2005). In present study, ascorbic acid in maize seedlings was significantly enhanced by the addition of Ca (Fig. 2).

Conclusions

In conclusion, Cd induced oxidative stress caused negative influences on growth and physio-biochemical traits of plants. The addition of Ca significantly enhanced the growth and physio-biochemical traits. Exogenously applied Ca ameliorated the oxidative stress by increasing the APX and POD activities, and ascorbic acid contents to withstand Cd toxicity and increased tolerance. Calcium treatments significantly ($P \leq 0.05$) reduced the ROS by enhancing the antioxidant enzymes. The amounts of osmolytes (amino acids and proteins) improved significantly for osmotic adjustments. In conclusion, exogenous applications of Ca can mitigate the adverse effects caused by Cd and enabled maize seedlings to thrive as evident by increased osmolytes, antioxidants, and growth traits.

Abbreviations

Melanoaldehyde **(MDA)**; Relative membrane permeability **(RMP)**; Hydrogen per oxide **(H_2O_2)**; Root fresh weight **(RFW)**; Root length **(RL)**; Total chlorophyll **(T. Chl)**; Shoot dry weight **(SDW)**; Ascorbic acid **(ASC.A)**; Carotenoids **(Caro)**; Anthocyanin contents **(Antho-C, ACC)**; Chlorophyll *b***(Chl *b*)**; Chlorophyll *a***(Chl *a*)**; Total soluble proteins **(TSP)**; Leaf area **(LA)**; Root dry weight **(RDW)**; Peroxidase **(POD)**; Shoot fresh weight **(SFW)**; Ascorbate per oxidase **(APX);** Amino Acids **(A-Ac)**; Sodium phosphate buffer **(SPB)**

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: The authors confirm that the final version of the manuscript has been reviewed, approved, and agreed for publication by all authors**.**

Data availability: Authors confirm that all relevant data are included in the article and would be ready to share the raw data upon request.

Funding statement: Project is not funded by any organization.

Author contribution

Muhammad Kaleem: This manuscript was part of his MSc Thesis research work submitted to Government College University, Faisalabad

Iqbal Hussain: acted as major research supervisor of the first author

Mansoor Hameed, Muhammad Sajid Aqeel Ahmad: Research planning and conceptualization

Anam Mehmood, Muhammad Faisal Maqsood: Data interpretation and manuscript compilation

Ummar Iqbal, Syed Mohsan Raza Shah: Biochemical analysis and data collection

Waseem Ashfaq, Zunaira Anwar: Data analysis and visualization

Declaration of interest: No conflicts of interest

Acknowledgment: This manuscript was part of his MSc Thesis research work submitted to Government College University, Faisalabad (GCUF). The authors acknowledge the support provided by the GCUF for planning, execution and analysis of this research work.

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Figures

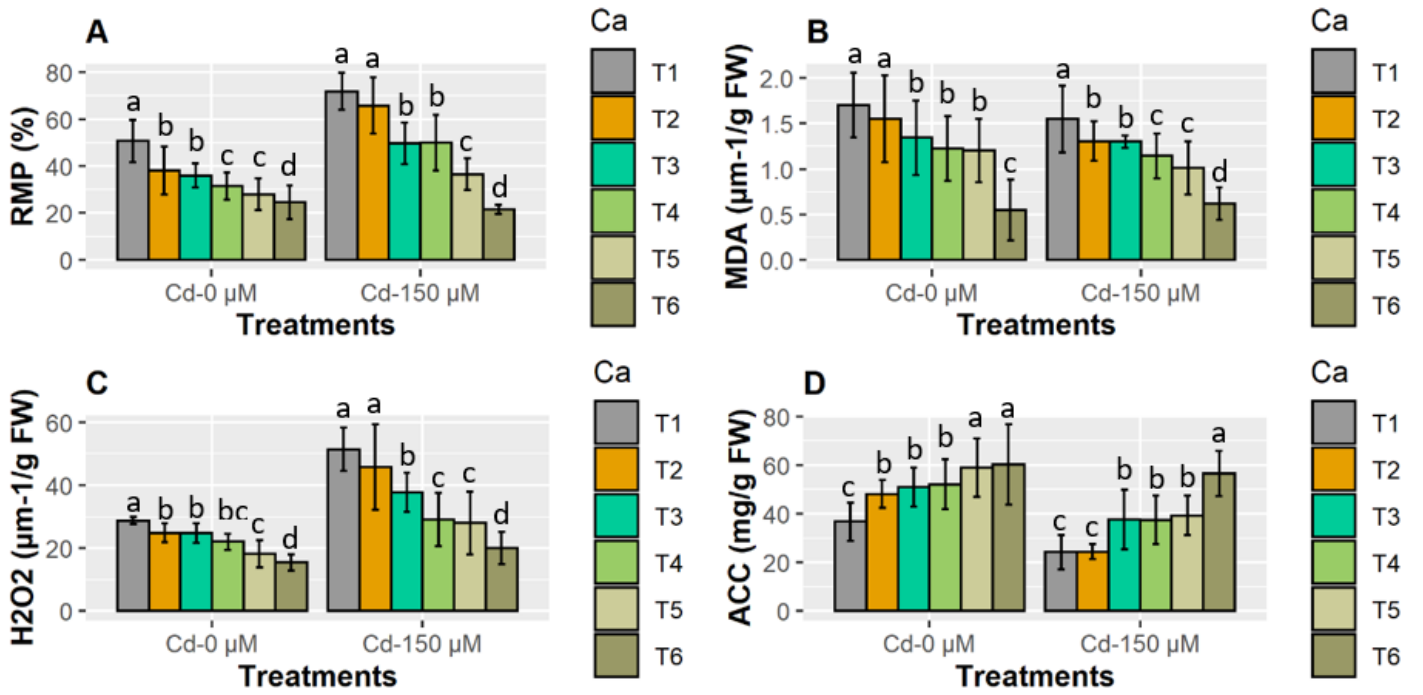

Figure 1

Effect of calcium (Ca; T1-0 mM, T2-0.5 mM, T3-1 mM, T4-2.5 mM, T5-5 mM, T6-10 mM) and cadmium (Cd) treatments on the A) relative membrane permeability (RMP), B) melanoaldehyde contents, C) H2O2, and D) anthocyanine contents (ACC) of maize seedlings. Means ± SE provided with error bars; different letter indicates significance (P≤0.05) between Ca and Cd treatments.

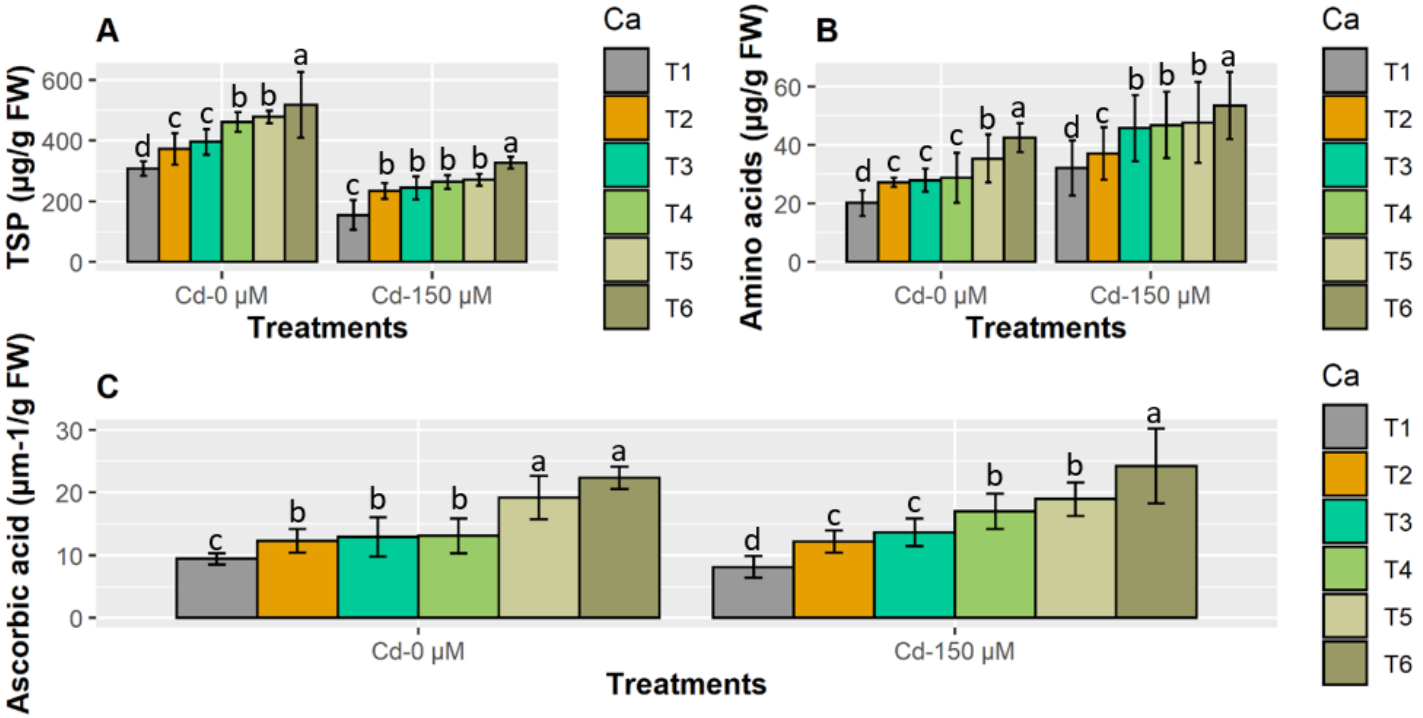

Figure 2

Effect of calcium (Ca; T1-0 mM, T2-0.5 mM, T3-1 mM, T4-2.5 mM, T5-5 mM, T6-10 mM) and cadmium (Cd) treatments on the A) toatl soluble protiens, B) amino acids and C) ascorbic acid contents of maize seedlings. Means ± SE provided with error bars; different letter indicates significance (P≤0.05) between Ca and Cd treatments.

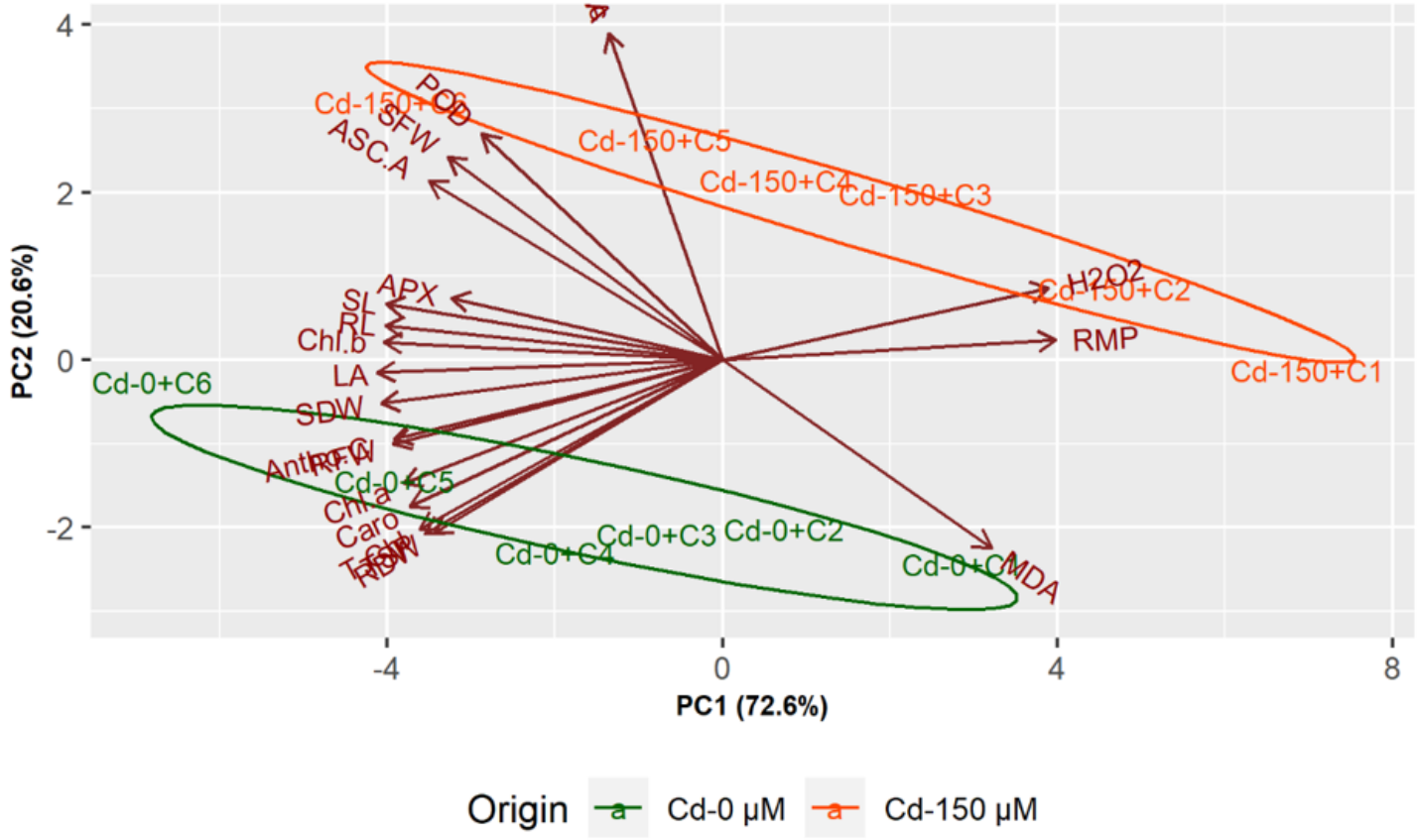

Figure 3

PCA biplot for growth and physio-biochemical traits under Cd and calcium treatments Abbreviations: Shoot length (SL); Root length (RL); Shoot fresh weight (SFW); Shoot dry weight (SDW); Root fresh weight (RFW); Root dry weight (RDW); Leaf area (LA); Chlorophyll a (Chl a); Chlorophyll b (Chl b); Carotenoids (Caro); Total chlorophyll (T. Chl); Ascorbate per oxidase (APX); Peroxidase (POD); relative membrane permeability (RMP); hydrogen per oxide (H2O2) and ascorbic acid (ASC.A).

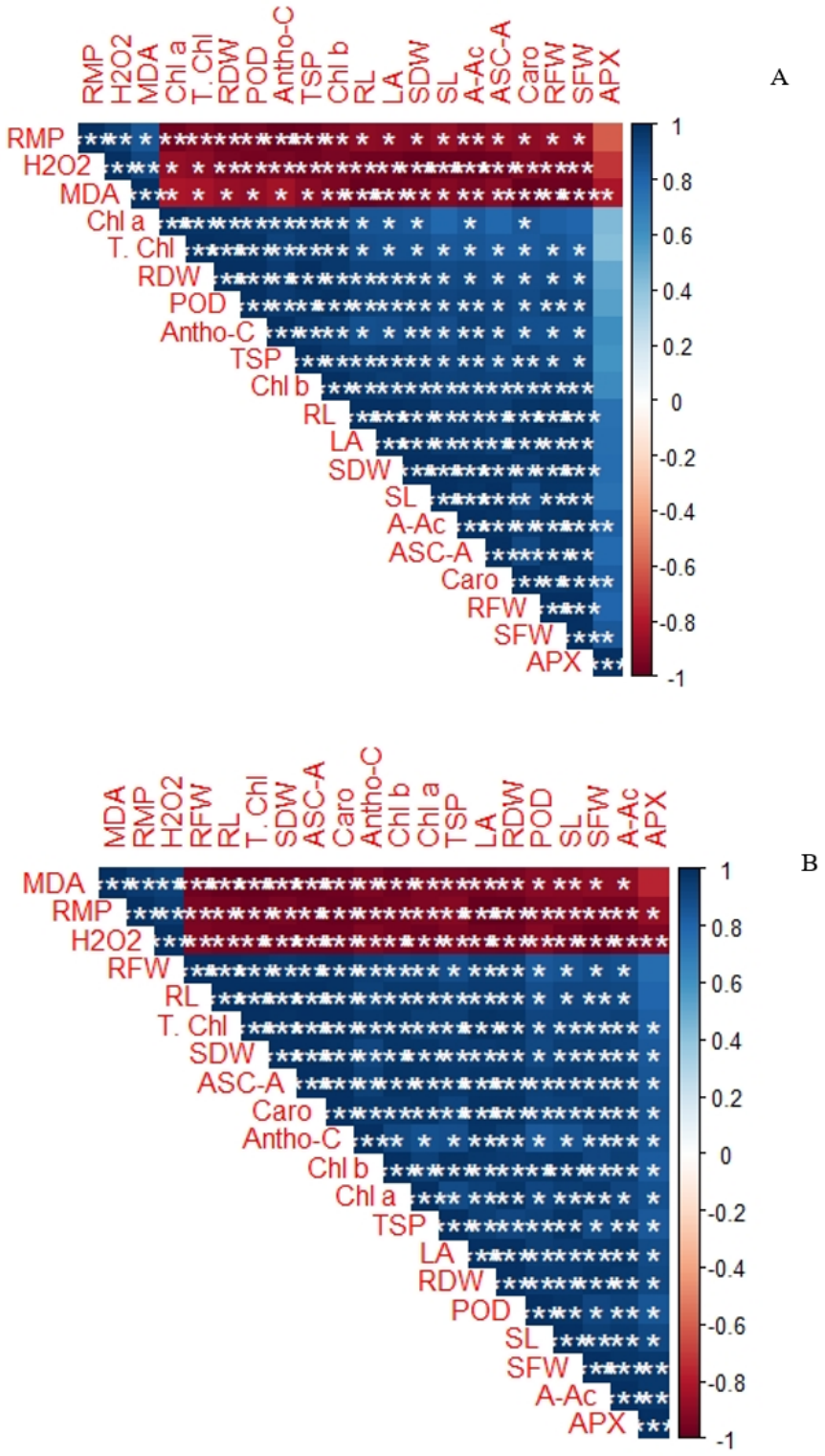

Figure 4

Correlation among morphological and physiochemical traits of maize seedlings under (A) under control and (B) Cd stress condition. Abbreviations: Melanoaldehyde (MDA); Relative membrane permeability (RMP); Hydrogen per oxide (H2O2); Root fresh weight (RFW); Root length (RL); Total chlorophyll (T.Chl); Shoot dry weight (SDW); Ascorbic acid (ASC-A); Carotenoids (Caro); Anthocyanin contents (Antho-C);

Chlorophyll b (Chl b); Chlorophyll a (Chl a); Total soluble proteins (TSP); Leaf area (LA); Root dry weight (RDW); Peroxidase (POD); Shoot fresh weight (SFW); Ascorbate per oxidase (APX)

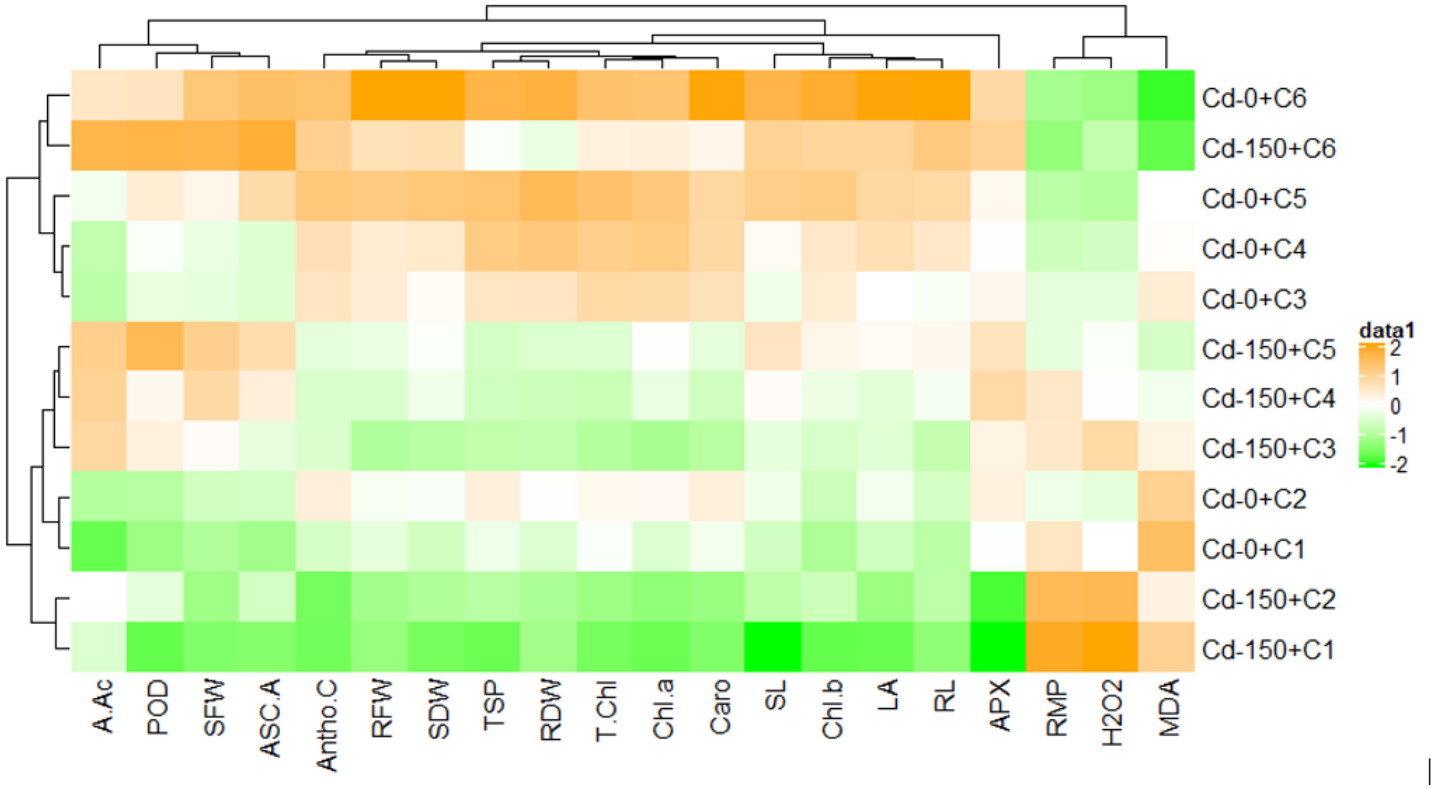

Figure 5

Clustered heatmap representing the effect of Cd and Ca (C1- 0 mM, C2- 0.5 mM, C3- 1 mM, C4- 2.5 mM, C5- 5 mM, C6- 10 mM) treatments on different studied traits Figure abbreviations: Melanoaldehyde (MDA); Relative membrane permeability (RMP); Hydrogen per oxide (H2O2); Root fresh weight (RFW); Root length (RL); Total chlorophyll (T.Chl); Shoot dry weight (SDW); Ascorbic acid (ASC-A); Carotenoids (Caro); Anthocyanin contents (Antho-C); Chlorophyll b (Chl b); Chlorophyll a (Chl a); Total soluble proteins (TSP); Leaf area (LA); Root dry weight (RDW); Peroxidase (POD); Shoot fresh weight (SFW); Ascorbate per oxidase (APX)

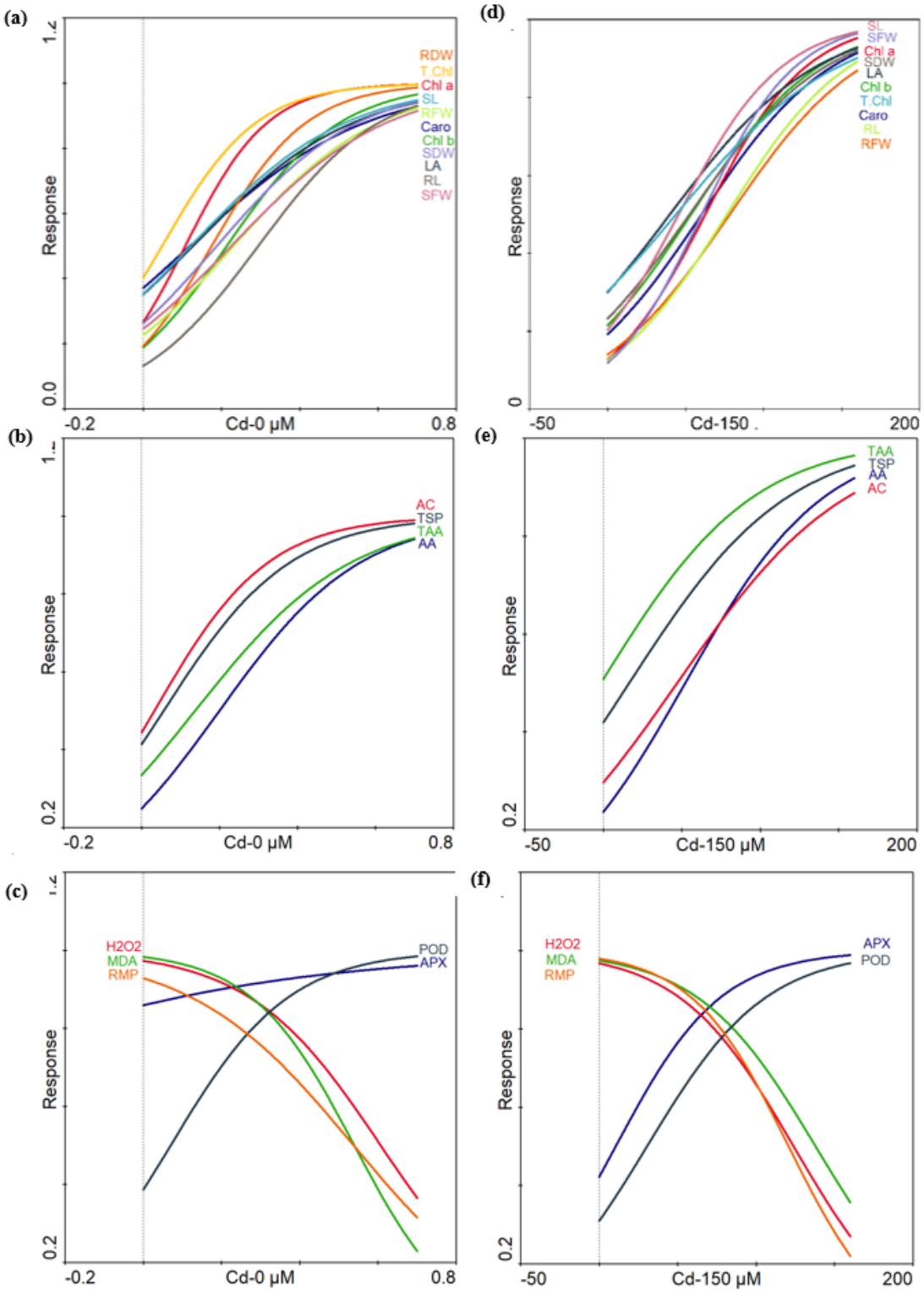

Figure 6

Generalized linear model showing response curve of traits under Cd and Ca treatments Cd- 0 µM stress: (a) growth and chlorophyll (b) organic osmolytes, ascorbic acid and anthocynin contents (c) hydrogen peroxide, relative membrane permeability, lipidperoxidation and antioxdants. Cd- 150 µM stress; (d) growth and chlorophyll (e) organic osmolytes, ascorbic acid and anthocynin contents (f) hydrogen peroxide, relative membrane permeability, lipidperoxidation and antioxdants Abbreviations:

Melanoaldehyde (MDA); Relative membrane permeability (RMP); Hydrogen per oxide (H2O2); Root fresh weight (RFW); Root length (RL); Total chlorophyll (T.Chl); Shoot dry weight (SDW); Ascorbic acid (ASC-A); Carotenoids (Caro); Anthocyanin contents (Antho-C); Chlorophyll b (Chl b); Chlorophyll a (Chl a); Total soluble proteins (TSP); Leaf area (LA); Root dry weight (RDW); Peroxidase (POD); Shoot fresh weight (SFW); Ascorbate per oxidase (APX).